On the Intracellular Trafficking of CC Chemokine Receptor 5

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University College London
2008
DECLARATION OF WORK

I, Tom Kershaw, declare that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

TOM KERSHAW
ABSTRACT

CC chemokine receptor 5 (CCR5) is a G protein-coupled receptor (GPCR) involved in the recruitment of a subset of leukocytes to sites of inflammation. In addition, CCR5 functions as a co-receptor for several strains of the human immunodeficiency virus (HIV). The finding that agonist-induced internalisation of CCR5 can protect susceptible cells from infection in vitro has stimulated research demonstrating that activated receptors are phosphorylated on C-tail serine residues and bind β-arrestins, which couple them to the clathrin-mediated endocytic machinery. After internalisation, receptors are recycled to the cell surface via a perinuclear recycling compartment, identified as the recycling endosome. Here, I extend these findings, demonstrating that CCR5 also traffics through the trans-Golgi network (TGN), and thus, the perinuclear recycling compartment can be considered to comprise both recycling endosome and TGN elements. Moreover, I show that Rab11 regulates trafficking through this compartment and that dynamin inhibition blocks the recycling of CCR5. In combination with other electron microscopy studies, my data support the notion that clathrin and dynamin are involved in the recycling of CCR5 from the recycling compartment. Through morphological and biochemical analysis, I also show that β-arrestins maintain an interaction with CCR5 into the recycling compartment. Kinetic analysis of receptor recycling suggests that β-arrestin2 acts a negative regulator of CCR5 recycling but my data do not discount the possibility that β-arrestins couple CCR5 to clathrin to effect recycling. In keeping with the sustained β-arrestin interaction, CCR5 molecules remain phosphorylated as they traffic to the recycling compartment, but contrary to previous reports showing that receptor C-tail phosphorylation is required for high-affinity β-arrestin binding, I show that a phosphorylation-deficient CCR5 mutant undergoes β-arrestin-dependent agonist-induced internalisation. In addition to its relevance for HIV biology and inflammation, this study contributes to an understanding of GPCR trafficking in general.
ACKNOWLEDGEMENTS

Although duty-bound to give thanks to my supervisor, Mark Marsh, I view it as no chore to acknowledge not only his guidance over the past 3 years but also the role he has played in my development as an intellectual being. Indeed, probably the most enjoyable aspect of the PhD has been our discussions, where I, and I believe he also, were free to explore off-piste ideas of how nature controls those silly little serpentine sausages!

A big shout goes out to the members of the Marsh lab, past and present, for being such friendly companions along my journey. In particular, I owe a great many thanks to Team CCR5 - those of the lab more interested in cell biology than 'non-living' entities that subvert it for their own means - including Nathalie and Silène, on whose strong foundations I have attempted to build, and Lars (Larzipan), whose bench-side banter made lab work enjoyable. I also owe Lars, and more recently Kristina, gratitude for teaching me that the Germans and English are not actually that different! On a related point, it has been a culturally enlightening experience to work with people from so many different nationalities: from the French and Swiss to the Poles and Spaniards!

I am also grateful to Julie Pitcher, Dan Cutler and Sara Mole for being a supportive committee. Julie, especially, has given some very useful β-arrestin-related chat and I was able to pick up several key techniques from her in a short rotation project in her lab before commencing my PhD project.

I am also very grateful for all the people who have been charitable enough to donate reagents to my cause; their largesse has been acknowledged where relevant.

Lastly, I am thankful to my partner, Karen, who has listened over the past 3 years to my scientific thinking-out-loud and has put up with my venting of cumulative frustrations that have ultimately driven me from academia. I am forever indebted - until she has written up her thesis, that is!
# Table of Contents

**Declaration of Work** ..................................................... 2

**Abstract** .......................................................................... 3

**Acknowledgements** .......................................................... 4

**Table of Contents** ............................................................. 5

**List of Figures** ................................................................. 10

**List of Tables** ..................................................................... 13

**Abbreviations** ................................................................. 14

**Chapter 1: Introduction** ....................................................... 19

- Chemokines and chemokine receptors ........................................... 20
  - Classification of chemokines and their receptors .......................... 20
  - Role of chemokines in recruitment of leukocytes to sites of inflammation 22
- CC chemokine receptor 5 (CCR5) .................................................. 23
  - Expression pattern and function ................................................. 23
  - CCR5 structure ..................................................................... 26
  - Signalling through CCR5 .......................................................... 30
  - Control of CCR5 signalling activity .......................................... 32
    - Desensitisation and internalisation ....................................... 32
    - Resensitisation .................................................................. 39
- The regulation of endocytic recycling ........................................... 45
  - Transferrin receptor recycling .................................................. 46
  - GPCR intracellular trafficking: sequence-directed recycling ........... 48
  - β-arrestins and GPCR intracellular trafficking ............................ 51
  - Regulation of membrane traffic by Rab GTPases ......................... 55
  - Sorting to the biosynthetic pathway ......................................... 57
  - Heterologous receptor regulation: implications for CCR5 trafficking 59

Outline of this investigation ....................................................... 60

**Chapter 2: Materials and Methods** ........................................ 62

- General reagents .................................................................. 62
CCRS down-modulation ................................................................. 84
The effect of various drugs on CCR5 internalisation ............................. 85
CCRS recycling ........................................................................ 85
The effect of various drugs on CCR5 recycling ..................................... 85
The effect of BFA on γ-adaptin distribution ......................................... 86
Lysosomal degradation assay .......................................................... 86

FACS analysis ............................................................................ 86
Basic procedure for CCR5 trafficking assay ........................................ 86
Details of specific assays ................................................................ 88
The effect of β-arrestin2 and β-arrestin2 mutants on CCR5 trafficking ... 88
The effect of various drugs on CCR5 recycling ................................. 88
FACS data analysis ..................................................................... 89
Graphical representation of FACS data ............................................. 89
Calculation of CCR5 trafficking rates ................................................ 89

CHAPTER 3: MORPHOLOGICAL OUTLINE OF CCR5 TRAFFICKING 91

CHO CCR5 cells as a system for studying CCR5 trafficking .................. 91
Internalised, agonist-activated CCR5 passes through early endosomes ...... 94
In the constant presence of agonist, CCR5 accumulates in a perinuclear, Tfr-positive compartment .................................................. 96
There is no agonist-induced degradation of CCR5 .............................. 99
Rab11 regulates CCR5 trafficking through the recycling endosome ....... 105
Down-modulated CCR5 partially colocalises with endogenous Rab11 .... 109
Tfr and Rab11 show only a limited overlap in different cell-lines .......... 111
Some down-modulated CCR5 accumulates in AP-1-positive structures ... 114
CCRS traffics through the trans-Golgi network .................................... 117
CCRS traffics through Rab11-positive recycling endosomes and the TGN .. 119
CCRS traffics through the TGN in HOS cells ..................................... 122

Discussion .................................................................................. 124
Rab11 as a regulator of CCR5 trafficking ........................................... 124
Rab11 and recycling endosomes ...................................................... 126
Model for the trafficking itinerary of agonist-activated CCR5 ............... 127
Influence of antibody binding on CCR5 trafficking ............................ 130
CCRS trafficking in other cell-lines ............................................... 131

CHAPTER 4: ASSOCIATION OF CCR5 WITH β-ARRESTINS 133
\( \beta \)-arrestins remain bound to CCR5 as it internalises and traffics to early endosomes \hspace{1cm} 134

\( \beta \)-arrestins remain bound to internalised CCR5 as it traffics to a perinuclear, TFR-positive compartment \hspace{1cm} 137

Endogenous \( \beta \)-arrestins are stably recruited to CCR5 in RBL cells \hspace{1cm} 145

Stable recruitment of \( \beta \)-arrestins to CCR5 is not agonist-specific \hspace{1cm} 146

Biochemical evidence for a stable interaction between CCR5 and \( \beta \)-arrestins \hspace{1cm} 148

CCR5 C-tail phosphorylation correlates with \( \beta \)-arrestin association \hspace{1cm} 153

C-tail phosphorylation is not required for CCR5 internalisation or recycling \hspace{1cm} 157

The internalisation of CCR5 4S\( \rightarrow \)A is \( \beta \)-arrestin-dependent \hspace{1cm} 158

\( \beta \)-arrestin recruitment to agonist-activated CCR5 4S\( \rightarrow \)A \hspace{1cm} 161

Discussion \hspace{1cm} 164

Association of CCR5 with \( \beta \)-arrestins \hspace{1cm} 165

\( \beta \)-arrestin association with CCR5 in the recycling compartment \hspace{1cm} 165

CCR5 as a Class C receptor \hspace{1cm} 166

\( \beta \)-arrestins as regulators of GPCR intracellular trafficking \hspace{1cm} 168

\( \beta \)-arrestin ubiquitination and association with GPCRs \hspace{1cm} 169

Studying a regulatory role for \( \beta \)-arrestins in CCR5 trafficking in \( \beta \)-arrestin knock-out MEFs \hspace{1cm} 170

CCR5 phosphorylation and recycling \hspace{1cm} 170

Phosphorylation-independent \( \beta \)-arrestin binding \hspace{1cm} 170

Phosphorylation, resensitisation and recycling \hspace{1cm} 174

CCR5 recycling and sequence-directed recycling \hspace{1cm} 176

Accessibility of phospho-sites and implications for control of CCR5 trafficking \hspace{1cm} 177

Summary of CCR5 C-tail phosphorylation and trafficking with \( \beta \)-arrestins \hspace{1cm} 179

**CHAPTER 5: THE CONTROL OF CCR5 RECYCLING** \hspace{1cm} 181

Down-modulated CCR5 accumulates in compartments decorated with clathrin \hspace{1cm} 181

Down-modulated CCR5 accumulates in intracellular compartments positive for AP-1 \hspace{1cm} 187

Brefeldin A partially inhibits CCR5 recycling \hspace{1cm} 190

AP-1 knock-down does not inhibit CCR5 recycling \hspace{1cm} 197

Dynasore blocks CCR5 recycling \hspace{1cm} 203

Dyngo-4a, but not Bis-T-23, inhibits the recycling of CCR5 \hspace{1cm} 206
LIST OF FIGURES

Figure 1—I Leukocyte recruitment during inflammation-----------------------------23
Figure 1—II Two dimensional topology diagram of the human CCR5 sequence
-----------------------------------------------------------------------------26
Figure 1—III Signalling through CCR5 --------------------------------------------31
Figure 1—IV Arrestin engagement with an activated, phosphorylated GPCR 36
Figure 1—V Model for CCR5 trafficking -------------------------------------------44
Figure 2—I Specificity and binding characteristics of MC-5 and MC-5 conjugates
--------------------------------------------------------------------------------------------------72
Figure 2—II Heating cell lysates to 95°C leads to loss of CCR5 -------------------79
Figure 3—I Agonist-activated CCR5 traffics through early endosomes --------------95
Figure 3—II In the constant presence of agonist, CCR5 accumulates in a
perinuclear, TfR-positive compartment---------------------------------------------98
Figure 3—III CCR5 turnover is not affected by RANTES-treatment ---------------100
Figure 3—IV Agonist stimulation does not target CCR5 to lysosomes ---------------103
Figure 3—V Rab11 regulates CCR5 trafficking --------------------------------------108
Figure 3—VI Agonist-activated, internalised CCR5 partially colocalises with
endogenous Rab11 -------------------------------------------------------------------110
Figure 3—VII Perinuclear, steady-state TfR shows only a partial colocalisation
with Rab11 in different cell types -----------------------------------------------113
Figure 3—VIII In the constant presence of agonist, some CCR5 accumulates in
a perinuclear compartment positive for AP-1 but negative for steady-state
TfR -----------------------------------------------------------------------------115
Figure 3—IX Agonist-activated CCR5 traffics through the TGN ---------------------118
Figure 3—X In the constant presence of agonist, CCR5 accumulates in both
Rab11- and p230-positive structures ---------------------------------------------122
Figure 3—XI In the constant presence of agonist, some CCR5 accumulates in
the TGN in HOS cells -------------------------------------------------------------123
Figure 3—XII Model for CCR5 intracellular trafficking in CHO CCR5 cells ----------129
Figure 4—I β-arrestins remain bound to CCR5 as it internalises and traffics to
early endosomes ----------------------------------------------------------------136
Figure 4-11 β-arrestin1-YFP remains bound to agonist-activated CCR5 as it traffics to a perinuclear, TfR-containing compartment

Figure 4-III β-arrestin2-GFP remains bound to agonist-activated CCR5 as it traffics to a perinuclear, TfR-containing compartment

Figure 4-IV Endogenous β-arrestins are stably recruited to agonist-activated CCR5 in RBL CCR5 cells

Figure 4-V β-arrestins are stably recruited to MIP-1α- and MIP-1β-activated CCR5

Figure 4-VI The stable interaction between CCR5 and β-arrestins can be followed by co-immunoprecipitation

Figure 4-VII CCR5 phosphorylation correlates with β-arrestin1-YFP association

Figure 4-VIII CCR5 phosphorylation correlates with β-arrestin2-GFP association

Figure 4-IX CCR5 C-tail phosphorylation is not required for internalisation or recycling

Figure 4-X Agonist-induced internalisation of CCR5 wt and CCR5 4S→A is β-arrestin-dependent

Figure 4-XI β-arrestin recruitment to CCR5 wt and CCR5 4S→A

Figure 4-XII Cartoon illustrating the association of β-arrestins with CCR5 throughout intracellular trafficking

Figure 5-I In the constant presence of agonist, down-modulated CCR5 accumulates in intracellular compartments decorated with clathrin

Figure 5-II In the constant presence of agonist, down-modulated CCR5 accumulates in intracellular compartments positive for AP-1

Figure 5-III BFA treatment inhibits CCR5 recycling

Figure 5-IV Knock-down of AP-1 does not affect CCR5 internalisation or recycling

Figure 5-V Dynasore inhibits CCR5 internalisation and recycling

Figure 5-VI The effect of the dynamin inhibitors, Dyngo-4a and Bis-T-23, on CCR5 internalisation

Figure 5-VII The effect of various dynamin inhibitor drugs on CCR5 recycling

Figure 5-VIII Possible sites of dynamin-dependent clathrin-coated vesicle formation at the recycling compartment
Figure 6-I Diagram of the C-termini of wild-type β-arrestin2 and constitutively active β-arrestin2 mutants 224
Figure 6-II The effect of over-expression of wild-type and constitutively active mutants of β-arrestin2 in CHO CCR5 cells 229
Figure 6-III The effect of constitutively active β-arrestin2 mutants on the distribution of down-modulated CCR5 232
Figure 6-IV Diagram of the C-termini of wild-type β-arrestin2 and mutants defective for clathrin and AP-1/A-2 binding 234
Figure 6-V Trafficking of CCR5 in cells expressing β-arrestin2 mutants defective in clathrin or AP-2 binding 237
Figure 6-VI The effect of β-arrestin2 mutants defective for clathrin and AP-2 binding on the distribution of down-modulated CCR5 239
Figure 6-VII Possible roles for β-arrestins in regulating CCR5 trafficking 248
LIST OF TABLES

Table 1-1 Summary of recycling sequences identified in GPCR C-tails and known interacting partners ................................................................. 50
Table 2-I Details of cell-lines ........................................................................ 63
Table 2-II Details of antibodies ........................................................................ 65
Table 2-III Details of DNA constructs ............................................................... 73
Table 6-I The effect of over-expression of wild-type and constitutively active mutants of β-arrestin2 on CCR5 trafficking ................................. 231
Table 6-II The effect of β-arrestin2 mutants defective for clathrin or AP-2 binding on CCR5 trafficking ................................................................. 238
ABBREVIATIONS

ACAP1 ARF GAP with coiled coil, ANK repeat, and pleckstrin homology domains
AOP Aminooxypentane
AP Adaptor protein
ARF ADP-ribosylation factor
AT₁R Type 1a angiotensin II receptor
β₁AR β1-adrenergic receptor
β₂AR β2-adrenergic receptor
B₂R Bradykinin type 2 receptor
βarr β-arrestin
BERP Brain-expressed RING-finger protein
BFA Brefeldin A
BM Binding medium
BRET Bioluminescent resonance energy transfer
BSA Bovine serum albumin
C₅a Complement factor 5a
C₅aR C₅a receptor
CAL Cystic fibrosis transmembrane conductance regulator-associated ligand
CART Cytoskeleton-associated recycling or transport
CCL CC chemokine
CCP Clathrin-coated pit
CCR CC chemokine receptor
CCV Clathrin-coated vesicle
CD4 Cluster of differentiation 4
CH-C Clathrin heavy chain
CHO Chinese hamster ovary
CHX Cycloheximide
Cl-MPR Cation-independent mannose 6-phosphate receptor
CLCa Clathrin light chain a
CXCL CXC chemokine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DSP</td>
<td>Dithiobissuccinimidylpropionate</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
</tr>
<tr>
<td>ECL</td>
<td>Extracellular loop</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early endosome antigen 1</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor (EGF) receptor</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-regulated kinase</td>
</tr>
<tr>
<td>ESCRRT</td>
<td>Endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>ETA</td>
<td>Endothelin A</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>fl</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>fl\textsubscript{max}</td>
<td>Maximum cell surface fluorescence reached after recycling</td>
</tr>
<tr>
<td>FPR</td>
<td>N-formyl peptide receptor</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>FYVE</td>
<td>Fablp, YOTB, Vaclp, EEA1</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GAM</td>
<td>Goat anti-mouse</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GAR</td>
<td>Goat anti-rabbit</td>
</tr>
<tr>
<td>GDF</td>
<td>GDI displacement factor</td>
</tr>
<tr>
<td>GDI</td>
<td>GDP dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>GTP exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GIPC</td>
<td>GAIP-interacting protein, carboxyl terminus</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>gp120</td>
<td>Glycoprotein 120</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein–coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>GPCR kinase</td>
</tr>
<tr>
<td>GRP</td>
<td>GPCR phosphatase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine–N’-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HRS</td>
<td>Hepatocyte growth factor–regulated tyrosine kinase substrate</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HOS</td>
<td>Human osteosarcoma</td>
</tr>
<tr>
<td>hScrib</td>
<td>Human homologue of the <em>Drosophila</em> protein</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>ICL</td>
<td>Intracellular loop</td>
</tr>
<tr>
<td>IB</td>
<td>Immunoblot</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus kinase/signal transducers and activators of transcription</td>
</tr>
<tr>
<td>JNK</td>
<td>c–jun N–terminal kinase</td>
</tr>
<tr>
<td>k_rec</td>
<td>Rate constant for recycling</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosome–associated membrane protein</td>
</tr>
<tr>
<td>LB</td>
<td>Liquid broth</td>
</tr>
<tr>
<td>LBPA</td>
<td>Lysobisphosphatidic acid</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low density lipoprotein receptor</td>
</tr>
<tr>
<td>lgp</td>
<td>Lysosomal glycoprotein</td>
</tr>
<tr>
<td>MAGI</td>
<td>Membrane–associated guanylate kinase inverted</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen–activated protein kinase</td>
</tr>
<tr>
<td>MCP–2</td>
<td>Monocyte chemoattractant protein–2</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin–Darby canine kidney</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MIP–1α</td>
<td>Macrophage inflammatory protein–1α (CCL3)</td>
</tr>
<tr>
<td>MIP–1β</td>
<td>Macrophage inflammatory protein–1β (CCL4)</td>
</tr>
<tr>
<td>MOR</td>
<td>μ–opioid receptor</td>
</tr>
</tbody>
</table>
MTOC  Microtubule-organising centre  
MVB  Multi-vesicular body  
NES  Nuclear export signal  
NHERF  Na⁺/H⁺ exchanger regulatory factor (EBP50  
[ezrin/radixin/moesin-binding phosphoprotein of 50 kDa])  
NHS  N-hydroxysuccinimde  
NSF  N-ethylmaleimide-sensitive fusion protein  
PAF  Platelet-activating factor  
PAGE  Polyacrylamide gel electrophoresis  
PAR2  Protease activated receptor 2  
PBS  Phosphate-buffered saline  
PDZ  Post-synaptic density 95/disc large/zonula occludens-1  
PDZK1  PDZ domain containing 1  
PFA  Paraformaldehyde  
PI3K  Phosphatidylinositol 3-kinase  
PI(3)P  Phosphatidylinositol 3-phosphate  
PIP₂  Phosphatidylinositol (4,5)-bisphosphate  
PKC  Protein kinase C  
PLC  Phospholipase C  
PMA  Phorbol myristate acetate  
PMSF  Phenylmethanesulphonylfluoride  
PP  Protein phosphatase  
PVR  Platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) receptor  
Pyk2  Proline-rich tyrosine kinase 2  
Rab  Ras-like protein from brain  
RANTES  Regulated on activation normal T-cell expressed and secreted (CCL5)  
RBL  Rat basophilic leukaemia  
RH  Regulator of G protein signalling (RGS) homology  
Rho  Ras homologue  
RNAi  RNA interference  
\( r_{int} \)  Initial rate of internalisation  
rpm  revolutions per minute  
RPMI  Roswell Park Memorial Institute
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tr>
<td>$r_{rec}$</td>
<td>Initial rate of recycling</td>
</tr>
<tr>
<td>PKD</td>
<td>Protein kinase D</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Post-synaptic density 95</td>
</tr>
<tr>
<td>SAP97</td>
<td>Synapse-associated protein 97</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>SNX4</td>
<td>Sorting nexin-4</td>
</tr>
<tr>
<td>Tac</td>
<td>Interleukin-receptor-α-chain domain</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TEMED</td>
<td>$N,N,N',N'$-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>TFP</td>
<td>Tetrafluorophenyl</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TfR</td>
<td>Tf receptor</td>
</tr>
<tr>
<td>TIR–FM</td>
<td>Total internal reflection fluorescence microscopy</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris[hydroxymethyl]aminomethane</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyrotropin-stimulating hormone</td>
</tr>
<tr>
<td>V2R</td>
<td>Vasopressin type 2 receptor</td>
</tr>
<tr>
<td>wt</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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</table>
Chapter 1:
INTRODUCTION

"The point of philosophy is to start with something so simple as not to seem worth stating, and to end with something so paradoxical that no-one will believe it."
Bertrand Russell, Philosopher (1872-1970)

CC chemokine receptor 5 (CCR5) is a G protein-coupled receptor (GPCR) that is principally involved in the recruitment of leukocytes to sites of inflammation. The human gene for CCR5 was cloned in late 1995 and the protein product functionally assigned to a growing family of receptors mediating the recruitment of cells involved in immune and inflammatory processes (Samson, Labbe et al. 1996). But within a few months, CCR5 was catapulted into the limelight with the discovery of its role as a co-receptor for the human immunodeficiency virus-type 1 (HIV-1) (Alkhatib, Combadiere et al. 1996; Choe, Farzan et al. 1996; Deng, Liu et al. 1996; Dragic, Litwin et al. 1996). Around the same time, it was found that individuals homozygous for a 32 base-pair deletion in the CCR5 gene, which results in a truncated protein that, unlike the wild-type receptor, is not expressed on the cell surface, displayed significant resistance to HIV infection (Dean, Carrington et al. 1996; Liu, Paxton et al. 1996). In addition, it had previously been reported that certain chemokines - later assigned as CCR5 agonists - were able to inhibit HIV infection of susceptible cells in vitro (Cocchi, DeVico et al. 1995). With the subsequent demonstration that chemokine-induced internalisation of CCR5 provides the major component of such resistance (Mack, Luckow et al. 1998), the notion was born that down-regulating CCR5 cell surface expression could be an effective antiviral strategy. This has fostered an interest in understanding the post-
translational mechanisms controlling CCR5 cell surface expression, which are intimately related to the control of its signalling activity. Indeed, desensitisation of agonist-activated GPCRs is often followed by their endocytosis. Internalised receptors are then either targeted to lysosomes for long-term down-regulation or reprogrammed and recycling back to the cell surface in a state competent to respond to further agonist stimulation (resensitisation) (Drake, Shenoy et al. 2006; Hanyaloglu and von Zastrow 2007). Previous studies have shown that agonist-activated CCR5 undergoes clathrin-mediated endocytosis and that after internalisation, CCR5 molecules are rapidly recycled to the cell surface (Signoret, Pelchen-Matthews et al. 2000; Signoret, Christophe et al. 2004; Signoret, Hewlett et al. 2005). However, little is currently known about the regulation of the post-endocytic trafficking of CCR5. In addition to its relevance to HIV biology, a clear description of this process is needed to understand how CCR5 contributes to leukocyte trafficking. Moreover, such a description should add to our understanding of the trafficking of GPCRs in general, of significant import, given their prevalence — there are approximately 1000 genes coding for GPCRs in the human genome.

**Chemokines and chemokine receptors**

Chemokine (chemoattractant cytokine) activation of chemokine receptors coordinates leukocyte traffic throughout the body during immune surveillance, inflammation and in the establishment of immunity. Binding of chemokines to their cognate receptors initiates a series of cellular events that include cytoskeletal rearrangements, increased cellular adhesion and directed cell movement along a chemoattractant gradient (chemotaxis). Additional roles of chemokines and their receptors in angiogenesis, haematopoiesis, organogenesis and metastasis have also been described (Gerard and Rollins 2001; Yoshie, Imai et al. 2001).

**Classification of chemokines and their receptors**

Chemokines are small, highly basic proteins of 70–125 amino acids (8–10 kDa). There are currently 45 known human chemokines, which are structurally classified into 4 subfamilies (CC, CXC, CX3C and XC) based on
the arrangement of conserved amino-terminal cysteine residues. The 2 major groups are the CC and CXC chemokines, which have either adjacent amino-terminal cysteine residues (CC) or amino-terminal cysteines separated by a single amino acid (CXC). Additional related molecules, fractalkine and two lymphotactins (splice variants of the same gene), represent the CX3C and XC families, respectively (Bacon, Baggiolini et al. 2002).

Eighteen chemokine receptors have thus far been identified and their nomenclature follows that of their ligands, giving the chemokine receptors CCR1 to 10, CXCR1 to 6, CXC3CR1 and XCR1.

Chemokines have also been historically divided into inflammatory and homeostatic groups based on their proposed functions (Muller, Hopken et al. 2002; Moser, Wolf et al. 2004). The inflammatory chemokines, involved in leukocyte accumulation at inflammatory sites, were the first discovered, after the realisation in the late 1980s that they are induced by major inflammatory cytokines, which themselves lack the ability to directly induce leukocyte chemotaxis. Homeostatic chemokines, on the other hand, are produced under normal conditions and are responsible for leukocyte recirculation, homing into the lymphoid organs and organisation of functional lymphoid microenvironments under steady-state conditions and during lymph organogenesis (Cyster 2003; Ohl, Bernhardt et al. 2003). However, this definition is somewhat artificial, since some so-called homeostatic chemokines are produced in inflammatory lesions and contribute to leukocyte recruitment (Chiu, Shang et al. 2002; Eddleston, Christiansen et al. 2002; Odemis, Moepps et al. 2002; Obermeier, Schwarz et al. 2003; Kallinich, Schmidt et al. 2004; Ohl, Mohaupt et al. 2004) and some so-called inflammatory chemokines are produced under normal conditions with undefined homeostatic functions (Jones, Webb et al. 1995; Glatzel, Wesch et al. 2002; Sallusto, Geginat et al. 2004; Zhang, Shimoya et al. 2004).

The greater number of chemokines compared with their receptors suggests that multiple chemokines may be able to engage single chemokine receptors and indeed this is the case, although the promiscuity is bidirectional, with particular chemokines also having the ability to bind different receptors. Some monogamous chemokine–chemokine receptor
interactions are found amongst the homeostatic group members, whereas overlapping specificities are more characteristic of the inflammatory group (Bacon, Baggioioli et al. 2002).

Although most chemokines are secreted proteins, it must be appreciated that in order to function in vivo to recruit leukocytes from the bloodstream, for instance during inflammation, there must be a mechanism for their cell surface retention, since in the absence of such a mechanism, they would be washed away from their site of production by blood flow and unable to maintain a chemoattractant gradient. The retention of chemokines is achieved by interaction with surface glycosaminoglycans (GAGs), which are linear, heterogeneous polysaccharides with an overall negative charge conferred by the presence of carboxylate and sulphate groups (Middleton, Patterson et al. 2002). They therefore bind positively charged chemokines primarily through electrostatic interactions. The main GAGs expressed by endothelial cells are heparan sulphate proteoglycans (Ihrcke, Wrenshall et al. 1993).

**Role of chemokines in recruitment of leukocytes to sites of inflammation**

One of the best described roles - yet by no means a complete description - of chemokine/chemokine receptor function is in the recruitment of leukocytes circulating in the bloodstream to sites of inflammation (Figure 1-1). The specificity of leukocyte recruitment is achieved by the combinatorial action of leukocyte and endothelial cell adhesion molecules and their counter-ligands, as well as the chemokines presented on the luminal endothelial surface (Ley, Laudanna et al. 2007). Recruitment begins with the capture of free-flowing leukocytes to the vessel wall, typically of inflamed postcapillary venules, followed by rolling along the endothelium, both processes mediated by selectins binding to their counter-receptors. While rolling, leukocytes come into close contact with the inflamed endothelium, enabling them to interact with immobilised chemokines. The binding of chemokines to their cognate receptors leads to the activation of integrins, resulting in firm leukocyte arrest and eventual transmigration.
However, to date, only a few chemokines have been demonstrated to be capable of arresting rolling leukocytes in vitro or in vivo (Ley 2003).

Figure 1-1 Leukocyte recruitment during inflammation

Intravital microscopy with superimposed cartoon features illustrating the process of leukocyte recruitment at sites of inflammation. See main text for details.

Chemokines released at the site of injury within the tissue are then responsible for guiding leukocyte chemotaxis to this site, with chemokine receptors able to translate a chemokine gradient into directed cell migration in a process that is poorly understood. Presumably, within the inflamed tissue, a GAG immobilisation mechanism for chemokines also restricts the radius of chemokine activities and is probably a part of the mechanism for establishing a chemokine gradient.

**CC chemokine receptor 5 (CCR5)**

Expression pattern and function

CCR5 is expressed on a subset of leukocytes, namely monocytes, macrophages, immature dendritic cells and memory T-lymphocytes, which
are recruited to sites of inflammation by the CC chemokines and CCR5 agonists, CCL3 (macrophage inflammatory protein [MIP]-1α), CCL4 (MIP-1β), CCL5 (regulated on activation normal T-cell expressed and secreted [RANTES]) and CCL8 (monocyte chemoattractant protein [MCP]-2) (Murphy, Baggiolini et al. 2000). CCR5 knock-out mice attest to its role as an inflammatory chemokine receptor (Floess, Rot et al. 2005). These mice show a defect in macrophage recruitment, with reduced efficiency of *Listeria monocyctogenes* clearance, decreased protection against LPS-induced endotoxemia and an inability to eliminate cryptococcal microorganisms, leading to an increased mortality rate of infected mice (Zhou, Kurihara et al. 1998; Huffnagle, McNeil et al. 1999; Huffnagle and McNeil 1999). CCR5 knock-out mice also present with an inability to regulate T cell responses, leading to an enhanced delayed-type hypersensitivity (DTH) response and an increased response to T cell-dependent antigen challenge. Moreover, after *Mycobacterium bovis* infection, higher numbers of primed T cells were observed in CCR5 knock-out mice, attributed to an increase in activated dendritic cells (Zhou, Kurihara et al. 1998; Algood, Lin et al. 2004).

As previously mentioned, in addition to its role as a chemokine receptor, human CCR5, in concert with CD4, has been shown to function pathologically as a co-receptor for R5-tropic (macrophage-tropic) strains of HIV-1 and HIV-2, as well as for simian immunodeficiency viruses (SIV) (Berger, Murphy et al. 1999). R5-tropic strains of HIV-1 appear to be responsible for virus transmission and are the prevalent virus type isolated from asymptomatic individuals (Roos, Lange et al. 1992; Schuitemaker, Koot et al. 1992; Connor, Mohri et al. 1993). This role for CCR5 in virus transmission is highlighted by the resistance to HIV-1 infection of individuals homozygous for a 32 base-pair deletion mutant of the CCR5 gene, CCR5Δ32, which results in a truncated protein that is not expressed on the cell surface (Dean, Carrington et al. 1996; Liu, Paxton et al. 1996; Samson, Libert et al. 1996). Disease progression in HIV-infected CCR5Δ32 heterozygotes is also slower than normal, most probably due to reduced cell surface levels of CCR5 (Benkirane, Jin et al. 1997). Interestingly, 1% of Caucasians are homozygous for the CCR5Δ32 allele and, despite some of the defects reported in mice lacking CCR5, there appear to be no major deleterious effects of lacking a functional version of CCR5 in humans (Liu,
Indeed, homozygosity may confer some advantages to individuals, rendering them less susceptible to rheumatoid arthritis (Pokorny, McQueen et al. 2005) and childhood asthma (Hall, Wheatley et al. 1999), and is also associated with a reduced likelihood of renal transplant rejection (Fischereider, Luckow et al. 2001). Moreover, the high prevalence of the CCR5Δ32 allele in some populations – up to 18% of individuals are heterozygotes in some North European countries – has led some researchers to suggest that the allele might have become fixed as the result of a selective pressure exerted by an unknown pathogen that utilised CCR5 in the past (Dean, Carrington et al. 1996; Samson, Libert et al. 1996).

Over the course of an HIV infection, typically after several years, X4-tropic (T cell-tropic) strains often emerge in infected individuals that are better able to infect certain CD4-positive cells, correlating with accelerated disease progression (Tersmette, de Goede et al. 1988; Tersmette, Cruters et al. 1989). X4-tropic virus strains use the chemokine receptor, CXCR4, as a co-receptor for entry. As for CCR5, chemokine-induced internalisation of CXCR4 protects susceptible cells from infection in vitro (Signoret, Oldridge et al. 1997). This re-enforces the notion that reducing cell surface expression of chemokine receptors involved in HIV entry could be an effective anti-viral strategy. However, although CCR5Δ32 homozygotes tolerate the loss of functional CCR5, compensatory mechanisms may come into play during immune system development and the loss of cell surface CCR5 may not be so well tolerated in normal individuals whose immune systems have developed with functional CCR5.

Although several chemokine receptors have been shown to function as HIV co-receptors in vitro, only CCR5 and CXCR4 appear to function in vivo (Zhang and Moore 1999; Zhang, Lou et al. 2000).

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CCR5 structure

Chemokine receptors belong to the superfamily of G protein-coupled receptors (GPCRs), a group of 7 transmembrane domain-containing proteins that signal through heterotrimeric G proteins. They can be further structurally classified as family 1b GPCRs, with which they share a common molecular architecture (Bockaert and Pin 1999). All chemokine receptors are between 340 and 370 amino acids in length and, in addition to having 7 transmembrane spanning domains, they have an extracellular N-terminal domain and a cytoplasmic C-tail (Murphy, Baggioni et al. 2000).

Figure 1: Two dimensional topology diagram of the human CCR5 sequence

The grey box marks the approximate position of the membrane bilayer, with the extracellular space at the top and the intracellular space (cytoplasm) at the bottom. The N-terminal domain, extracellular loops (ECLs), intracellular loops (ICLs) and C-
Human CCR5 is composed of 352 amino acids, with a predicted molecular mass of 40.6 kDa. The 3-dimensional structure of CCR5 (or that of any chemokine receptor) has not yet been determined, due to difficulties with crystallisation of integral membrane proteins. However, domain-specific antibodies confirm the general 7 transmembrane-spanning topology of CCR5 (Lee, Sharron et al. 1999; Blanpain, Vanderwinden et al. 2002) and structural models have been produced based on the available structure of rhodopsin (Govaerts, Blanpain et al. 2001; Paterlini 2002). A 2-dimensional topology diagram is shown in Figure 1–II (Oppermann 2004).

A structural hallmark of the GPCR superfamily is the presence of a disulphide bridge between conserved cysteine residues on the second and third extracellular loops. In addition, chemokine receptors contain two extra cysteine residues that are thought to form a disulphide bond between the N-terminal domain and the third extracellular loop. Since mutation of any of the extracellular cysteines in CCR5 resulted in an inability to bind MIP-1α or respond functionally to other CCR5 agonists, these disulphide bonds appear necessary for maintaining a receptor conformation capable of agonist binding and/or signalling (Blanpain, Lee et al. 1999). Other features conserved among members of the chemokine receptor family include a DRYLAVVHA sequence in the second intracellular loop, which has been
implicated in G protein interaction, and an unusually short third intracellular loop, which is enriched in positively charged residues (Murphy, Baggioioli et al. 2000).

The amino-terminal domain of CCR5 undergoes certain post-translational modifications, which are crucial for CCR5 function. One modification is the addition of sulphate residues to several tyrosines (Farzan, Mirzabekov et al. 1999). This sulphation confers negative charge to the amino-terminus and contributes significantly to chemokine binding (Farzan, Mirzabekov et al. 1999; Farzan, Chung et al. 2002), as well as binding to the HIV envelope protein (Farzan, Mirzabekov et al. 1999; Cormier, Persuh et al. 2000; Farzan, Chung et al. 2002; Huang, Lam et al. 2007). It is not known whether all of the tyrosines in the amino-terminal domain are sulphated, but site-directed mutagenesis indicated that Tyr3 almost certainly undergoes sulphation (Farzan, Mirzabekov et al. 1999) and sulphation of Tyr10 and Tyr14 appears to be important for chemokine binding and HIV co-receptor activity, since only sulpho-peptides that contained at least two sulphate moieties at these residues could substitute for the CCR5 amino-terminus in chemokine binding or HIV co-receptor assays (Cormier, Persuh et al. 2000; Farzan, Chung et al. 2002). More recently, structural data has confirmed the importance of sulphation at tyrosines 10 and 14 in co-receptor activity (Huang, Lam et al. 2007). Using a computer simulation, Huang et al. docked the nuclear magnetic resonance (NMR) structure of a 14-residue peptide of CCR5 covering residues 2–15 and sulphated at tyrosines 10 and 14 (full-length CCR5 protein numbering) obtained in the presence of CD4 and the viral envelope protein, gp120, to a crystal structure of CD4 complexed with gp120 crystallised in the presence of an antigen-binding fragment of a functionally tyrosine-sulphated antibody that binds to a CD4-induced epitope that overlaps the site of co-receptor binding on HIV-1 gp120. This analysis showed that sulpho-tyrosine 10 forms a salt bridge with an arginine residue of the gp120 core and, more interestingly, sulpho-tyrosine 14 is completely sequestered in a binding pocket, the formation of which requires the structural rearrangement of gp120, contributing to HIV entry.

The amino-terminal domain of CCR5 is also post-translationally modified by O-linked glycosylation, preferentially on Ser6 (Farzan, Mirzabekov et al. 1999; Bannert, Craig et al. 2001). O-linked glycans significantly contribute
to chemokine binding, with terminal sialic acid moieties being particularly important. However, removal of O-linked oligosaccharides has little effect on HIV co-receptor activity (Bannert, Craig et al. 2001). Although a potential N-glycosylation site exists on the third extracellular loop, the asparagine residue does not appear to be modified, probably due to its proximity to one of the disulphide-bridge-forming cysteines, rendering it inaccessible to modifying enzymes (Farzan, Mirzabekov et al. 1999).

The CCR5 C-tail is also post-translationally modified, through palmitoylation of cysteine residues. Indeed, many chemokine receptors contain one or more cysteine residues in their C-tails compatible with receptor palmitoylation, although CCR5 is the only chemokine receptor for which palmitoylation has been demonstrated (Neel, Schutyser et al. 2005). Palmitoylation of CCR5 occurs on cysteines 321, 323 and 324 and is believed to anchor the C-tail of the receptor to the cytoplasmic leaflet of the membrane, resulting in the creation of a fourth intracellular loop (Blanpain, Wittamer et al. 2001; Kraft, Olbrich et al. 2001; Percherancier, Planchenault et al. 2001). Unlike some other GPCRs, palmitate turnover is not affected by agonist binding. Palmitoylation appears to be required for efficient biosynthetic transport of CCR5 to the plasma membrane (Blanpain, Wittamer et al. 2001; Percherancier, Planchenault et al. 2001). Non-acylated receptors that do reach the cell surface are slightly affected in their ability to couple to certain signalling pathways and are impaired in desensitisation and agonist-induced internalisation (Blanpain, Wittamer et al. 2001; Kraft, Olbrich et al. 2001).

Finally, there is now significant evidence that CCR5 can form homo- or heterodimers, as has been reported for many other GPCRs (Angers, Salahpour et al. 2002). Bioluminescent resonance energy transfer (BRET) experiments have demonstrated that CCR5 homo-oligomers form early during biosynthetic maturation in the endoplasmic reticulum and that there is no additional increase in oligomerisation upon agonist stimulation (Blanpain, Vanderwinden et al. 2002; Issafras, Angers et al. 2002; Huttenrauch, Pollok-Kopp et al. 2005). CCR5 has also been shown to form constitutive oligomers in HEK293 cells by co-immunoprecipitation of differentially-tagged receptors (Chelli and Alizon 2002). Moreover, immunogold electron microscopy has provided some evidence for CCR5
homodimerisation in human macrophages and T cells, in that CCR5 was frequently found in pairs on microvilli and in biosynthetic vesicles originating from the trans-Golgi network (TGN) (Singer, Scott et al. 2001). The evidence for hetero-oligomerisation is discussed below.

**Signalling through CCR5**

Chemokine binding to CCR5 activates a host of intracellular signalling pathways that co-ordinate the cellular processes required for the recruitment of leukocytes to sites of inflammation. Chemokine binding to CCR5 has been proposed to occur via a two-step mechanism, whereby there is an initial interaction between the chemokine core and both the amino-terminal domain and the second extracellular loop of the receptor, the latter interaction conferring agonist specificity. This is followed by the binding of the amino-terminal domain of the chemokine molecule to the transmembrane helix bundle of CCR5, which triggers a conformational change in the receptor that results in its activation (Blanpain, Doranz et al. 2003). In the classical model of chemokine receptor signalling, receptor activation promotes exchange of GDP for GTP on pertussis toxin-sensitive heterotrimeric G proteins that engage the cytoplasmic face of the receptor, leading to the dissociation of Gα and Gβγ subunits, which can then regulate downstream effectors, such as adenylyl cyclase and phospholipase Cβ (PLCβ). Interestingly, it has been shown that for Gαi-coupled receptors, it is release of the Gβγ subunit rather than the Gαi subunit that is important for mediating chemotaxis (Neptune and Bourne 1997). Following CCR5 activation, Gβγ activates PLCβ, leading to the hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP2) to inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3, in turn, leads to the release of calcium ions from intracellular stores, which has multiple cellular effects (Aramori, Ferguson et al. 1997; Zhao, Ma et al. 1998). In addition, DAG production leads to the activation of protein kinase C (PKC), which, inter alia, phosphorylates CCR5 on its C-tail, contributing to its desensitisation and internalisation (see below) (Pollok-Kopp, Schwarze et al. 2003).

RANTES binding to CCR5 also leads to the activation of class I phosphatidylinositol 3-kinase (PI3K), required for RANTES-induced T
lymphocyte migration (Turner, Ward et al. 1995). Moreover, in T cells, CCR5 activates RhoA (Ras homologue A) (Bacon, Schall et al. 1998) and the two focal adhesion kinases, proline-rich tyrosine kinase 2 (Pyk2) and focal adhesion kinase (FAK) (Bacon, Szabo et al. 1996; Ganju, Dutt et al. 1998), which, together with RhoA, play important roles in cell motility.

![Figure 1: Signalling through CCR5](image)

Figure 1: Signalling through CCR5

The cartoon illustrates the signalling pathways known to be activated by CCR5. Solid brown arrows indicate direct regulatory effects; dashed arrows indicate effects mediated through other signalling molecules. All of the pathways are believed to be activated through classical G protein signalling, except JAK/STAT activation, which may proceed via a G protein-independent mechanism. In rat basophilic leukaemia (RBL) cells, CCR5 also stimulates Src-mediated granular enzyme release (not shown) (Kraft, Olbrich et al. 2001).
In addition to signalling pathways linked to cell motility, CCR5 activates the three main members of the mitogen-activated protein kinase (MAPK) family, extracellular-regulated kinase 1/2 (ERK1/2), p38 and c-jun N-terminal kinase (JNK), which have proposed roles in T cell proliferation and the transcriptional activation of cytokine genes (Dairaghi, Franz-Bacon et al. 1998; Kraft, Olbrich et al. 2001) (Del Corno, Liu et al. 2001; Misse, Esteve et al. 2001; Wong, Uddin et al. 2001).

Unlike the signalling pathways described above, which are all mediated through classical G protein-dependent pathways (they can be blocked with pertussis-toxin, which affects \( \alpha_i \)-coupled GPCRs), CCR5 may also signal in a G protein-independent fashion in its activation of the janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. RANTES activation of CCR5 in PM1 T cells leads to the phosphorylation of JAK2/JAK3 in a pertussis-toxin insensitive manner (Wong, Uddin et al. 2001).

**Control of CCR5 signalling activity**

A cell's sensitivity to GPCR agonists depends, in part, on the number of receptors present at the cell surface that are capable of binding agonist and transmitting a signal. After agonist exposure, a series of mechanisms, operating on different time-scales, act to regulate the number of functional receptors at the cell surface. The first of these follows shortly after agonist stimulation (seconds to minutes), whereby cell surface receptors are uncoupled from further G protein activation, in a process termed desensitisation. After this, trafficking mechanisms result in the clearance of desensitised receptors from the cell surface and either lead to receptor degradation or to resensitisation and return to the cell surface (within minutes to hours) (Hanyaloglu and von Zastrow 2007).

**Desensitisation and internalisation**

Desensitisation is initiated by phosphorylation of serine and threonine residues in the intracellular loops and carboxyl tails of activated GPCRs (Ferguson 2001). The principal mediators of this phosphorylation are a family of serine/threonine kinases, known as the G protein-coupled receptor kinases (GRKs). GRKs carry out what is termed homologous
receptor phosphorylation, since they are specifically recruited to agonist-activated receptors. Seven mammalian GRKs have so far been identified (GRKs 1–7), which all share a highly homologous kinase domain as well as an amino-terminal regulator of G protein signalling (RGS) homology (RH) domain. Based on other structural features, they are divided into subfamilies consisting of GRKs 1 and 7, GRKs 2 and 3 and GRKs 4–6. Aside from GRKs 1 and 7 and GRK4, which are restricted to the retina and testes, respectively, the GRKs are ubiquitously expressed (Krupnick and Benovic 1998; Ferguson 2001). Second messenger-activated kinases, such as PKC, can also mediate receptor phosphorylation. These kinases can be activated downstream of the receptor that is their substrate for phosphorylation or they can contribute to heterologous receptor desensitisation if activated through a different means, for example, another GPCR.

Alanine scanning mutagenesis of CCR5 identified four C-tail serine residues at positions 336, 337, 342 and 349, which are phosphorylated in a non-hierarchical manner upon receptor activation. Moreover, agonist-activated CCR5 has been shown to be a substrate for both GRKs and PKC, which is activated downstream of the receptor (see above). Blocking of GRKs 2 and 3, but not of GRKs 4 and 6 with inhibitory antibodies led to a reduction in CCR5 phosphorylation, suggesting that GRKs 2 and 3, which are highly expressed in human leukocytes, are responsible for the GRK-mediated phosphorylation (Oppermann, Mack et al. 1999). Additionally, using phospho-site specific antibodies, Ser337 has been identified as a PKC substrate and Ser349 as a GRK substrate (Pollok-Kopp, Schwarze et al. 2003). GRK-mediated phosphorylation of Ser349 proceeds with a $t_{1/2} \approx 2$ min, whereas PKC very rapidly phosphorylates Ser337 after receptor activation, with a $t_{1/2} \approx 20$ s.

For some GPCRs, phosphorylation itself results in the uncoupling of the receptor from G protein activation (Krupnick and Benovic 1998). In addition, the phosphorylation of many agonist-activated GPCRs facilitates the binding of an arrestin molecule to the cytoplasmic face of the receptor, which sterically hinders the binding and activation of G proteins (Moore, Milano et al. 2007). Indeed, for the $\beta_2$-adrenergic receptor ($\beta_2$AR), the prototypical GPCR, phosphorylation of the agonist-activated receptor itself causes only minor impairment of its capacity to activate heterotrimeric G proteins, and
full desensitisation is only achieved with subsequent arrestin binding (Lohse, Benovic et al. 1990). Arrestins comprise a family of four members: visual arrestin, cone arrestin, β-arrestin1 (arrestin2) and β-arrestin2 (arrestin3). The expression of visual and cone arrestin is restricted to the visual system, whereas β-arrestins 1 and 2 are ubiquitously expressed, although with variable expression levels (Lohse, Benovic et al. 1990; Attramadal, Arriza et al. 1992; Krupnick and Benovic 1998).

A sequential multi-site binding model explaining arrestin selectivity for agonist-activated, phosphorylated receptors was first proposed in 1993, based primarily on biochemical data from studies of the visual arrestin–rhodopsin system (Gurevich and Benovic 1993). This model, which appears to hold true for the non–visual arrestin proteins as well, posits that the arrestin molecule has 2 sensor sites: an activation recognition site that binds receptor elements that change conformation upon activation and a phosphorylation recognition site that detects phospho-residues on the receptor. The sensors, by themselves, mediate low affinity binding to either activated or phosphorylated receptors, allowing arrestins to probe the functional state of receptor molecules and rapidly dissociate from receptors that do not meet the two conditions of being in the active conformation and in a phosphorylated state. Simultaneous engagement of the 2 sensor sites promotes a transition into an active, high affinity receptor–binding state, resulting in a semi-irreversible interaction. In this model, receptor deactivation is required for arrestin dissociation.

Several X-ray crystal structures of arrestin proteins in the basal conformation have now been solved and the data have contributed to a better molecular understanding of the mechanism of receptor interaction (Granzin, Wilden et al. 1998; Hirsch, Schubert et al. 1999; Han, Gurevich et al. 2001; Milano, Pace et al. 2002; Sutton, Vishnivetskiy et al. 2005). In the basal conformation, the arrestins have an elongated shape and are composed almost exclusively of β-sheets and connecting loops, which form two major globular domains, the N– and C–domains, connected by a short hinge. These domains are held together by several intramolecular interactions, including a polar core in the centre of the molecule that must be destabilised to promote high affinity receptor binding. In addition, there is a C–tail, connected by a flexible linker to the C–domain, which locks back
onto the globular domains through hydrophobic interactions with β-strand I and α-helix I in the N-domain — the so-called 3-element interaction. The C-tail also contributes an arginine residue to the polar core. Based on the available biophysical and biochemical data, a model for arrestin–receptor interaction has been proposed (Gurevich and Gurevich 2004). Arrestin binding to an activated, phosphorylated receptor via its activation recognition site localised within its N-domain, is thought to weaken the interaction between its 2 globular domains. At the same time, receptor-attached phosphates interact with lysine residues in the arrestin N-terminus and indirectly destabilise the 3-element interaction, releasing the C-tail and, in the process, removing its arginine residue from the polar core. Moreover, the lysine residues steer the phosphates towards the primary phosphorylation recognition site deep in the polar core, where they neutralise the charge of the so-called phosphate-sensitive residue Arg169 (β-arrestin1 numbering), which initiates arrestin activation. With the interactions holding arrestin in the basal state now disrupted, the mutual repulsion of the 3 remaining negatively charged asparagine residues in the polar core facilitates a gross structural rearrangement of the 2 globular domains, transforming the arrestin molecule into an ‘open’ or active conformation and promoting additional interactions that result in high affinity receptor binding. The structural rearrangements in the arrestin molecule upon receptor binding are summarised in Figure 1-IV.

As well as contributing to receptor desensitisation, β-arrestin binding links many activated GPCRs to the endocytic machinery. The release of the C-tail of β-arrestin1/2 upon binding to activated GPCRs is believed to unmask binding sites located in the C-tail for clathrin heavy chain and the β2-

The adaptin subunit of the heterotetrameric adaptor complex, AP-2, which couple activated receptors to the clathrin-mediated endocytic machinery (Lohse, Benovic et al. 1990; Pippig, Andexinger et al. 1993; Goodman, Krupnick et al. 1996; Laporte, Oakley et al. 1999). Indeed, it has recently been shown that an isoleucine-valine-phenylalanine (IVF) motif in the C-tail of the β-arrestin molecule, which is involved in the docking of the C-tail to the N-domain in the basal conformation, also mediates binding to the β2-adaptin subunit of AP-2 (Burtey, Schmid et al. 2007). Thus, the activation of the β-arrestin molecule, where the C-tail is released, is coupled to its role as an endocytic adaptor.

**Figure 1-IV Arrestin engagement with an activated, phosphorylated GPCR**

The figure shows a model for arrestin interaction with an activated, phosphorylated GPCR. The arrestin molecule is orientated with its C-domain on the left and its N-domain on the right. Arrestin engagement with the receptor results in the destabilisation of the basal, ‘closed’ conformation of the arrestin molecule, with disruption of the polar core, rearrangements of the N- and C-domains and transition into an ‘open’, active conformation that binds the receptor with high affinity. In the active conformation, the arrestin C-tail is released, exposing binding sites in β-arrestins 1 and 2 for β2-adaptin and clathrin heavy chain.

Figure from Moore et al. (2007). “Regulation of receptor trafficking by GRKs and arrestins.” *Annual Review of Physiology* 69:451-482.
Both β-arrestin1 and β-arrestin2 have been shown to interact with agonist-activated CCR5, and intact C-tail phosphorylation sites have been shown to be required for high-affinity β-arrestin binding (Kraft, Olbrich et al. 2001). Moreover, using various serine to alanine replacement mutants of CCR5, Huttenrauch et al. (2002) found that in vivo, β-arrestin recruitment to activated CCR5 required at least two intact phosphorylation sites, although the exact position of these sites did not appear to be critical. This flexibility in choice of phosphorylation sites with respect to β-arrestin binding has also been observed for the N-formyl peptide receptor (FPR) (Bennett, Foutz et al. 2001). An additional phosphorylation-independent β-arrestin binding site, encompassing the DRY motif in the second intracellular motif (see Figure 1-II) has also been reported, consistent with the model of sequential multi-site arrestin binding. This motif is conserved among chemokine receptors (and all GPCRs of the rhodopsin family) and is involved in the activation of CCR5 (Gosling, Monteclaro et al. 1997).

Like the majority of GPCRs, CCR5 is internalised shortly after agonist-activation and β-arrestins are required for this process, since the receptor does not internalise in β-arrestin 1 and 2 knock-out mouse embryonic fibroblasts (MEFs), whereas it does internalise in wild-type MEFs (Fraile-Ramos, Kohout et al. 2003). Moreover, it has been convincingly demonstrated in Chinese hamster ovary (CHO CCR5) and mink lung endothelial cells stably expressing CCR5, that the receptor internalises in a clathrin-dependent fashion, possibly involving the initial lateral redistribution of activated receptors into flat clathrin lattices at the plasma membrane (Signoret, Hewlett et al. 2005). Thus, it seems likely that β-arrestin binding links activated CCR5 molecules to the clathrin-mediated endocytic pathway.

There have been some reports suggesting that CCR5 is internalised through a clathrin-independent mechanism that depends on lipid rafts and caveolae (Mueller, Kelly et al. 2002; Venkatesan, Rose et al. 2003). However, Signoret et al. (2005) saw no evidence for CCR5 association with caveolae or caveolin-positive structures before or after agonist treatment and expression of a dominant-negative form of GFP-tagged caveolin-1 did not interfere with agonist-induced internalisation. Moreover, the evidence in favour of a raft-dependent mechanism was based on the finding that CCR5
endocytosis was inhibited by interfering with or removing cholesterol, a component of lipid rafts. Significantly, results obtained using several different compounds that interfere with membrane cholesterol suggest that cholesterol is required to maintain CCR5 (Nguyen and Taub 2002; Nguyen and Taub 2003; Nguyen and Taub 2003) and other GPCRs (Gimpl, Burger et al. 1997) in a conformation that supports agonist binding, so the major effect of cholesterol perturbation may be to abrogate chemokine binding. Also, cholesterol extraction can interfere with the formation of clathrin-coated vesicles (CCVs) (Rodal, Skretting et al. 1999; Subtil, Gaidarov et al. 1999), and the cholesterol sequestering compound, filipin, which inhibited CCR5 internalisation, also inhibited transferrin (Tf) uptake, which occurs via a clathrin-dependent mechanism (Signoret, Hewlett et al. 2005). Despite this, CCR5 has been seen to accumulate at the leading edge of leukocytes undergoing RANTES-induced chemotaxis, where it colocalises with markers for raft domains (Nieto, Frade et al. 1997; Gómez-Moutón, Lacalle et al. 2004). It has also been suggested that raft localisation may be required for this polarisation (Mañes, Mira et al. 1999; Gómez-Moutón, Lacalle et al. 2004). Palmitoylation of CCR5 may drive this association with lipid rafts.

Although the role of β-arrestins in CCR5 internalisation is clear, their role in desensitisation is less clear. CCR5 mutants lacking 3 or 4 phosphorylation sites are reported to be defective in their ability to recruit β-arrestins and to undergo desensitisation. Although CCR5 mutants that lack any two phosphorylation sites retain their ability to bind β-arrestins and internalise, RANTES stimulation of a S336A/S349A mutant triggered a sustained calcium response and enhanced granular enzyme release in RBL cells stably expressing the receptor. This suggests that CCR5 internalisation largely depends on a β-arrestin-mediated mechanism that requires the presence of any two phosphorylation sites, whereas receptor desensitisation is independently regulated by the phosphorylation of distinct serine residues (Huttenrauch, Nitzki et al. 2002).

In addition to β-arrestin-sponsored clathrin-mediated endocytosis, other chemokine receptors may effect internalisation by directly interacting with AP-2, through C-tail di-leucine sequences (Signoret, Rosenkilde et al. 1998; Fan, Yang et al. 2001). Although CXCR2 undergoes agonist-induced phosphorylation and β-arrestin binding, it also directly interacts with AP-2.
mutation of an LLKIL motif shown to mediate AP-2 binding resulted in a severe impairment of receptor internalisation. In contrast, a C-terminally truncated mutant receptor lacking phosphorylation sites in its C-tail and the ability to support robust β-arrestin recruitment, but with an intact LLKIL motif, did undergo agonist-induced internalisation, underscoring the importance of the direct interaction with AP-2 in receptor internalisation (Fan, Yang et al. 2001). For CXCR4, in addition to a β-arrestin-mediated mode of internalisation that results from agonist binding, internalisation can be effected by treatment with phorbol esters that results in the activation of PKC and presumed phosphorylation of CXCR4 on (a) serine residue(s) within an SSSLKIL sequence that is assumed to interact with AP-2 (Signoret, Rosenkilde et al. 1998). Although there is a di-leucine motif in the C-tail of CCR5, it is not in the context of upstream serine residues and CCR5 surface expression is not affected by phorbol ester treatment (Signoret, Rosenkilde et al. 1998).

Resensitisation

After internalisation, GPCRs can either be targeted for degradation or recycled back to the plasma membrane. Degradation results in long-term down-regulation of receptor signalling by decreasing the number of functional receptors at the cell surface and thus reducing the cell’s responsiveness to subsequent agonist challenge. Sorting for degradation involves mono-ubiquitination, and is well described for the chemokine receptor, CXCR4 (Marchese, Raiborg et al. 2003). Agonist-activated CXCR4 is ubiquitinated at the plasma membrane by a Nedd4-like E3 ubiquitin ligase, AIP4. After internalisation, CXCR4 localises to endosomal microdomains that are positive for AIP4 and hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), a mono-ubiquitin binding protein that is responsible for recognising proteins destined for degradation. Here, CXCR4 is sorted away from the recycling pathway into luminal vesicles during early endosome maturation into multi-vesicular bodies (MVBs), a process that is dependent on the action of the ESCRT (endosomal sorting complex required for transport) machinery. Interestingly, AIP4 also mediates HRS ubiquitination in a CXCR4-dependent manner, suggesting that ubiquitination of HRS is important for CXCR4 sorting. In addition, a more
recent study has shown that AIP4 interacts directly with the amino-terminal half of β-arrestin1 via its WW domains and siRNA depletion of β-arrestin1 blocked agonist-promoted degradation of CXCR4 by preventing CXCR4 trafficking from early endosomes to lysosomes (Bhandari, Trejo et al. 2007). CCR5 contains cytoplasmic domain lysine residues that could potentially be ubiquitinated, but there is no evidence to suggest that they are. Indeed, it has been shown that there is no agonist-induced degradation of CCR5 (Signoret, Pelchen-Matthews et al. 2000; Delhaye, Gravot et al. 2007) and CCR5 recycling after agonist-induced internalisation has been demonstrated in lymphocytes and monocytes (Mack, Luckow et al. 1998). Moreover, recycling is faithfully recapitulated with similar kinetics in a CHO cell system (Signoret, Pelchen-Matthews et al. 2000; Signoret, Christophe et al. 2004), which has been used extensively to map out the post-endocytic trafficking pathway taken by CCR5 molecules. In these cells it has been shown that after internalisation, receptors are delivered to early endosomes, often located in the periphery of the cell, from where they are sorted to a perinuclear recycling compartment, whence they recycle back to the cell surface. In the constant presence of agonist in the extracellular medium, receptors accumulate in this recycling compartment (receptors that recycle to the cell surface are re-internalised due to the presence of agonist), and show significant overlap with steady-state transferrin receptor (TfR) staining, which predominantly localises to recycling endosomes. Recycling endosomes are a distinct population of perinuclear endosomes often found close to Golgi stacks that were first characterised in CHO cells (Yamashiro, Tycko et al. 1984). They have a more tubular morphology than early endosomes, a higher pH (~ 6.5, similar to the TGN; compare pH of early endosomes of 5.9–6.0) and are enriched for recycling receptors but have low levels of fluid phase material destined for the lysosome (Yamashiro, Tycko et al. 1984; Hopkins, Gibson et al. 1994; Marsh, Leopold et al. 1995). Observations of immunolabelled cryosections showing the accumulation of down-modulated CCR5 in small membrane-bound vesicles and tubules in the perinuclear region of the cell and colocalisation of TfR in the same clusters confirmed the trafficking of CCR5 through recycling endosomes and provided the first evidence for a GPCR taking such a route (Signoret, Pelchen-Matthews et al. 2000).
As alluded to above, internalisation and recycling are generally considered to be necessary for GPCR resensitisation, which involves agonist dissociation, conformational change back to an inactive conformation, dephosphorylation of C-tail Ser/Thr residues and recycling back to the plasma membrane in a state competent to respond to further agonist stimulation (Hanyaloglu and von Zastrow 2007). For several GPCRs, vesicular acidification has been shown to regulate their recycling (Grady, Garland et al. 1995; Pippig, Andexinger et al. 1995; Hsieh, Brown et al. 1999). The low pH of the early endosome is assumed to induce agonist dissociation, as has been demonstrated for other receptors (Maxfield 1982; Stein, Bensch et al. 1984), which presumably involves a conformational change, that in the case of the β2AR, the prototypical GPCR, results in a direct interaction with a protein phosphatase 2A (PP2A)-like GPCR phosphatase (GRP). Raising endosomal pH blocks the association of β2AR with GRP and inhibits both receptor dephosphorylation and recycling to the cell surface (Pippig, Andexinger et al. 1995; Krueger, Daaka et al. 1997; Signoret, Christophe et al. 2004). This has led to the suggestion that dephosphorylation is required for recycling, which would seem to be a reasonable check-point as this may be the last step in resensitising a receptor before its return to the plasma membrane, although subsequent conformational changes may take place after receptor dephosphorylation to achieve the resting state.

Unlike the β2AR, recycling of CCR5 is not affected by raising endosomal pH and a pH-dependent agonist-dissociation mechanism does not operate for CCR5 agonists, since CCR5-bound chemokines only exhibit pH-dependent dissociation below pH 4 (Signoret, Christophe et al. 2004). Furthermore, it has been shown that CCR5 molecules can recycle to the plasma membrane in an agonist-bound form (by ligand displacement assays), which results in their re-internalisation (Signoret, Pelchen-Matthews et al. 2000). Dephosphorylation of CCR5 is also unaffected by raising endosomal pH (Signoret, Christophe et al. 2004) and may not be required for recycling either, since CCR5 bound to an N-terminally modified form of RANTES, AOP-RANTES (aminooxypentane-RANTES), which mediates enhanced association of CCR5 with GRKs and increased phosphorylation of C-tail serine residues (Oppermann, Mack et al. 1999; Vila-Coro, Mellado et al. 1999), recycles with similar kinetics to RANTES-treated CCR5 (Signoret,
Pelchen-Matthews et al. 2000). Resensitised receptors that can respond to further agonist stimulation do re-appear on the cell surface after agonist wash-out (Signoret, Pelchen-Matthews et al. 2000) but it may take several cycles of internalisation and recycling for an activated CCR5 molecule to be fully resensitised and to be retained on the cell surface in a state competent to respond to further agonist stimulation. Agonist dissociation (and subsequent resensitisation steps) could result from iterative cycling and could simply be a function of the off-rate of the agonist. Alternatively, agonist dissociation could be dependent on proteolysis of the bound chemokine, which may take place in recycling endosomes. The pH of recycling endosomes is around 6.5, so if a protease were acting there then its activity would probably be unaffected by weak bases, which inhibit the recycling of the β2AR.

There is currently no explanation for why CCR5 should exhibit such seemingly odd behaviour when expressed in CHO cells; however, it must be pointed out that this does not seem to be peculiar to CHO cells or to be a function of CCR5 over-expression. CCR5 stimulated with AOP-RANTES, which has a higher affinity for CCR5 than RANTES and results in a higher proportion of receptors returning to the cell surface being agonist-occupied in CHO CCR5 cells (Signoret, Pelchen-Matthews et al. 2000), takes longer to recover to the cell surface after agonist wash-out in lymphocytes than RANTES-stimulated CCR5 (Mack, Luckow et al. 1998). Given that CCR5 down-modulated with AOP-RANTES has been shown to recycle with similar kinetics to CCR5 down-modulated with RANTES in CHO CCR5 cells (Signoret, Pelchen-Matthews et al. 2000), this result is explained by more efficient re-internalisation of AOP-RANTES occupied receptors upon recycling, and, hence, suggests that CCR5 can recycle in an agonist-bound state in primary cells.

Some details regarding CCR5 dephosphorylation are known but these have been obtained under relatively artificial conditions and shed little light on the timing of the dephosphorylation event(s) in vivo. Significantly, though, the dephosphorylation of serines 337 and 349, phosphorylation substrates for PKC and GRK2/3, respectively, appears to be mediated by different phosphatases. After stimulation of rat basophilic leukaemia cells stably expressing CCR5 (RBL CCR5 cells) with RANTES for 2 min, followed by
incubation with the CCR5 antagonist, TAK-779, Ser337 was rapidly and completely dephosphorylated within 3 min ($t_{1/2} \sim 90$ s), whereas dephosphorylation of Ser349 proceeded much slower ($t_{1/2} \sim 12$ min). Moreover, in an *in vitro* assay, okadaic acid, a potent inhibitor of protein phosphatases 1 (PP1) and 2A (PP2A), significantly inhibited dephosphorylation of Ser337 but had little effect on the dephosphorylation of Ser349. The okadaic-acid sensitive protein phosphatase(s) was shown to be different from GRP, because whereas GRP dephosphorylates receptors only in the acidic milieu of early endosomes and, thus, requires receptor endocytosis (Pitcher, Payne et al. 1995), the phosphatase(s) that dephosphorylated Ser337 was active at neutral pH and efficiently dephosphorylated receptors at the cell surface that were phosphorylated by a heterologous mechanism (phorbol myristate acetate [PMA] treatment). In contrast to the ability of Ser337 dephosphorylation to proceed at neutral pH *in vitro*, Ser349 was much less efficiently dephosphorylated by phosphatase(s) present in RBL cells lysates at neutral pH than Ser337 (Pollok-Kopp, Schwarze et al. 2003). It may be that resting cell surface CCR5 molecules are subject to some level of background phosphorylation by PKC activated by other means in the cell and that a phosphatase that is active at the cell surface is required to maintain resting receptors in a dephosphorylated state. This cell surface dephosphorylation may or may not be relevant for activated receptors. Such a mechanism need not operate for the GRK phosphorylation site(s), since GRKs only recognise and phosphorylate activated receptors.

Despite the ability to undergo dephosphorylation at the cell surface, S337, as well as S349, has been shown by immunofluorescence to maintain its phosphorylated status, on at least some receptors, as they accumulate in the perinuclear region of RBL CCR5 cells in the constant presence of agonist (Pollok-Kopp, Schwarze et al. 2003). Such conditions, where agonist is constantly present in the medium better reflect the situation in which CCR5 would function in a physiological setting than a pulse of chemokine exposure. For instance, a cell undergoing chemotaxis would be constantly exposed to agonist (of increasing extracellular concentration) as it migrates towards a site of inflammation.
A model integrating the current data on CCR5 trafficking, as discussed above, is shown in Figure 1-V.

![Model for CCR5 trafficking](image)

**Figure 1-V Model for CCR5 trafficking**

The cartoon presents a model for the agonist-induced trafficking of CCR5 as described in the main text. Most of the details of the intracellular trafficking have been derived from studies using CHO cells stably expressing CCR5. Briefly, agonist binding leads to the activation of CCR5 (illustrated by a colour change from black to red) and the initiation of signalling pathways summarised in Figure 1-III. Receptors are rapidly desensitised, a process that is initiated through phosphorylation of C-tail serine residues by PKC and GRKs 2 and 3. This leads to the binding of β-arrestins, which target receptors for clathrin-mediated endocytosis. Internalised receptors are delivered to early endosomes from which they are sorted to perinuclear recycling endosomes; there is no evidence for agonist-induced lysosomal targeting. From recycling endosomes, receptors can recycle to the cell surface. Receptors can return to the cell surface in a resensitised form or in an agonist-bound, and presumably phosphorylated, state, in which case, they can re-engage the endocytic machinery and undergo a further round of internalisation and recycling. It may take several cycles of internalisation and recycling for the average CCR5 molecule to be returned...
to the surface in a resensitised form. The sites of agonist dissociation and receptor dephosphorylation are currently undetermined. The microtubule-organising centre (MTOC) is shown in the bottom right corner; dashed grey lines represent microtubules emanating from the MTOC.

It must be remembered that CCR5 functions in relaying extracellular cues in the form of chemokines into a cellular response and that the trafficking pathways described above are subordinate to this role, responsible for regulating the response to chemokine challenge. One of the major physiological roles of chemokine receptors is to mediate chemotaxis in response to a chemokine gradient. However, whether chemokine receptor internalisation and recycling are necessary for optimal chemotaxis as a general rule for chemokine receptors is controversial (Neel, Schutyser et al. 2005). For CCR5, C-terminal truncation mutants lacking the serine phosphorylation sites supported a higher chemotactic response than the wild-type receptor in RBL cells and a CCR5 mutant with all 4 phosphorylatable C-tail serines mutated to alanines had a similar ability to mediate chemotaxis towards RANTES as wild-type CCR5 (Kraft, Olbrich et al. 2001). In this study, chemotaxis was assessed by measuring the number of cells moving through a membrane into a chamber containing a certain concentration of chemokine in a given amount of time. The results suggest that in RBL cells, a cell-line of haematopoietic lineage, cell migration does not depend on C-tail phosphorylation or β-arrestin association and can presumably occur without receptor desensitisation or internalisation. Although interesting, the limitations of such a study and its relevance to chemotaxis in vivo are obvious and it would be dangerous to extrapolate these results to conclude that desensitisation and internalisation of CCR5 are not required for the chemotactic activity of cells in a physiological setting.

The regulation of endocytic recycling

Although the route that CCR5 takes through the cell after its internalisation has largely been mapped, the key molecules involved in regulating this pathway have not been identified. The basis of our knowledge on the
recycling of endocytosed receptors has largely been drawn from studies on the transferrin receptor (TfR). More recently, studies on numerous GPCRs have identified diverse cytoplasmic domain sequences involved in recycling and there is also a growing appreciation of the role of β-arrestins in the regulation of GPCR intracellular trafficking. The key regulatory roles of members of the Rab family of small GTPases are also becoming clearer.

**Transferrin receptor recycling**

The TfR is constitutively endocytosed by clathrin-mediated endocytosis and delivered to early endosomes. From here it can either undergo rapid recycling to the plasma membrane ($t_{1/2} \sim 2$ min) or transfer to recycling endosomes, from where it recycles to the cell surface more slowly ($t_{1/2} \sim 10$ min) (Dunn, McGraw et al. 1989; Mayor, Presley et al. 1993).

Sorting of recycling membrane proteins in the early endosome is believed to be achieved by a geometric process, although specific mechanisms for cargo sorting via sorting motifs may be superimposed on this geometric sorting mechanism. Early endosomes accept incoming material for about 5 to 10 min before maturing into MVBs/late endosomes, which involves centripetal translocation along microtubules, acidification and acquisition of acid hydrolases. Before endosome translocation, most membrane proteins that are to be recycled are removed by the extrusion and scission of narrow-diameter tubules (Dunn, McGraw et al. 1989; Mayor, Presley et al. 1993). Because the surface area:volume ratio of the tubules is greater than that of the vesicular portion of the early endosome, recycled membrane is preferentially sorted from soluble material, without the need necessarily for a specific mechanism for cargo recognition. This is in contrast to membrane receptors that are to be degraded, which requires ubiquitination that leads to recognition and sorting to intraluminal vesicles of the maturing early endosome/MVB by the ESCRT machinery, as already discussed for CXCR4 (see above).

A recent study has implicated sorting nexin-4 (SNX4) in the early endosome to recycling endosome trafficking of the TfR. SNX4 associates with tubular and vesicular elements of early endosomes and also associates with the minus end-directed microtubule motor protein, dynein. By driving
membrane tubulation at the early endosome, it is proposed that SNX4 coordinates iterative, geometric-based sorting of the TfR with the long-range transport of carriers from early endosomes to recycling endosomes (Traer, Rutherford et al. 2007).

It was initially thought that the recycling of the TfR was signal-independent because a truncated TfR lacking the whole of its cytoplasmic tail recycles at the same rate as the wild-type receptor (Johnson, Dunn et al. 1993). However, more recent studies have identified *bona fide* recycling determinants in the TfR cytoplasmic tail, in the form of two phenylalanine-based signals that interact with ACAP1 (ARFGAP with coiled coil, ANK repeat, and pleckstrin homology domains), which is a GTPase-activating protein for ADP-ribosylation factor 6 (ARF6). Disruption of ACAP1 binding to TfR slows recycling from the recycling endosome to the plasma membrane (Dai, Li et al. 2004). More recently, there has also been a report that ACAP1 acts as a clathrin adaptor protein in stimulation-dependent integrin recycling and insulin-stimulated recycling of glucose transporter type 4 (GLUT4) from recycling endosomes (Li, Peters et al. 2007). Endosomal clathrin-coated buds that label positively for TfR have also been observed in A431 and HeLa cells (Stoorvogel, Oorschot et al. 1996; van Dam and Stoorvogel 2002), and TfR recycling from recycling endosomes has been shown to be clathrin- and dynamin-dependent (van Dam and Stoorvogel 2002). However, although likely, a role for ACAP1 as a clathrin adaptor for TfR has not been formally demonstrated. In polarised cells, another clathrin adaptor protein has been implicated in clathrin-dependent TfR exit from recycling endosomes towards the basolateral membrane. A variant of the heterotetrameric adaptor complex 1 (AP-1) containing the μ1B subunit, AP-1B (Fölsch, Ohno et al. 1999), has been shown to mediate TfR recycling from the recycling endosome to the basolateral membrane (Rodriguez-Boulan, Müsch et al. 2004) and has been suggested to function in the basolateral delivery of newly synthesised proteins in epithelial cells (Fölsch 2005).

Interestingly, Signoret et al. (2000) noted, from their observations of immunolabelled cryosections, prominent coats on many of the vesicles containing down-modulated CCR5 in the perinuclear region. Although no immunolabelling was carried out at the time to confirm the identity of the coat, clathrin would be the most likely candidate; thus, CCR5 may access a
clathrin-dependent recycling mechanism (Signoret, Pelchen-Matthews et al. 2000).

The discovery of recycling signals in the TfR C–tail and the finding that a complex named CART (cytoskeleton–associated recycling or transport), consisting of HRS, actinin–4, brain–expressed RING–finger protein (BERP) and myosin–V, is involved in sorting TfR into the fast recycling pathway, are difficult to reconcile with the efficient recycling of the C–terminally truncated TfR. The simplest explanation is that the C–tail also contains a retention signal. Such signals have been identified in insulin–regulated aminopeptidase, a slowly recycling membrane protein, one consisting of an acidic cluster and one consisting of a di-leucine motif (Johnson, Lampson et al. 2001).

**GPCR intracellular trafficking: sequence–directed recycling**

Although the presence of endocytic recycling signals in the cytoplasmic tail of the TfR were only demonstrated in 2004, such signals had previously been identified in several GPCRs (Sorkin and von Zastrow 2002) and many more have now been discovered (Marchese, Paing et al. 2007; Hanyaloglu and von Zastrow 2008). A summary of some of the known recycling sequences and proteins that have been demonstrated to interact with these sequences is shown in Table 1–1 (Hanyaloglu and von Zastrow 2008).

Despite the diversity of the identified sequences, a number correspond to conventional PDZ (post–synaptic density 95/disc large/zonula occludens–1) ligands, including sequences present in the β1AR, β2AR and thyrotropin–stimulating hormone (TSH) receptors (Cao, Deacon et al. 1999; Hu, Tang et al. 2000; Lahuna, Quellari et al. 2005). PDZ ligands, which are bound by PDZ domain–containing proteins, are short, linear sequences, classified as either type I (X–[S/T]–X–φ), type II (X–φ–X–φ), or type III (X–[D/E]–φ, where X is any amino acid and φ is a hydrophobic amino acid), typically located at the receptors’ C–termini. This distal location is necessary for PDZ–domain interaction, as the free carboxylate group forms an ionic bond with the ligand–binding groove of the PDZ domain. However, internal PDZ ligands, which fold into the β–finger structure required for PDZ domain interaction, have been identified in several GPCRs, one of which, in the endothelin A
(ETA) receptor, has been shown to be necessary for recycling (Paasche, Attramadal et al. 2005). The distal recycling sequence of the κ-opioid receptor, despite not conforming to a typical PDZ ligand sequence, is thought to bind to PDZ domain-containing proteins (Li, Chen et al. 2002; Huang, Steplock et al. 2004). Several such proteins, which mediate PDZ-ligand-directed recycling, have now been identified. For instance, the C-terminal DSLL sequence in the β2AR is bound by NHERF/EBP50 (Na+/H+ exchanger regulatory factor / ezrin/radixin/moesin-binding phosphoprotein of 50 kDa), which promotes recycling of the receptor. Ser411 in the -2 position of the PDZ ligand is a substrate for GRK5 phosphorylation and over-expression of GRK5 inhibits recycling. Moreover, replacement of Ser411 with a phospho-mimetic aspartic acid residue blocked the interaction of the receptor with NHERF and also inhibited recycling, leading to the degradation of internalised receptors (Cao, Deacon et al. 1999). Thus, for the β2AR, dephosphorylation of Ser411 may be required for interaction with NHERF. Consistent with this notion, crystallographic and mutational studies indicate that PDZ-mediated interactions with type 1 PDZ ligands involve hydrogen bonding with the free hydroxyl group at the -2 position (Doyle, Lee et al. 1996; Songyang, Fanning et al. 1997). Phosphorylation of proximal serine residues has also been shown to disrupt the binding of PDZ domain-containing proteins to the β2AR and serotonin 5-HT2C receptor (Hu, Chen et al. 2002; Parker, Backstrom et al. 2003).

The last four amino acids of CCR5, SVCL, which contain Ser349, conform to a type II PDZ ligand and a recent study showed that they were crucial for receptor recycling, in that their removal resulted in the re-routing of internalised receptors for lysosomal degradation (Delhaye, Gravot et al. 2007). Despite the fact that Ser349 undergoes GRK-mediated phosphorylation after receptor activation, Delhaye et al. found that CCR5 recycling was independent of the phosphorylation state of this residue: in Jurkat cells, a CCR5 phospho-mimetic mutant in which Ser349 had been mutated to Glu, recycled just as well as the wild-type receptor and the recycling of a Ser349→Ala mutant was only partially inhibited in its ability to recycle.
Table 1-1 Summary of recycling sequences identified in GPCR C-tails and known interacting partners


<table>
<thead>
<tr>
<th>Receptor</th>
<th>Recycling sequence (amino acid number)</th>
<th>Protein(s) interacting with recycling sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_1$-adrenergic</td>
<td>S (312) and ESKV (474-477)</td>
<td>PSD-95, SAP97, GIPC, CAL, MAGI-2, MAGI-3</td>
<td>(Hu, Tang et al. 2000; Xu, Paquet et al. 2001; Hu, Chen et al. 2002; Gardner, Santos et al. 2004; Gage, Matveeva et al. 2005; He, Bellini et al. 2005; Gardner, Tavlal et al. 2006; Gardner, Naren et al. 2007)</td>
</tr>
<tr>
<td>$\beta_2$-adrenergic</td>
<td>DSLL (410-413)</td>
<td>NHERF-1, NHERF-2, PDZK1, NSF</td>
<td>(Cao, Deacon et al. 1999; Cong, Perry et al. 2001; Gage, Kim et al. 2001; He, Bellini et al. 2005)</td>
</tr>
<tr>
<td>$\mu$-opioid</td>
<td>LENLEAE</td>
<td>Unknown</td>
<td>(Tanowitz and von Zastrow 2003)</td>
</tr>
<tr>
<td>$\kappa$-opioid</td>
<td>NKPV</td>
<td>NHERF-1</td>
<td>(Li, Chen et al. 2002; Huang, Steplock et al. 2004)</td>
</tr>
<tr>
<td>D1 dopamine</td>
<td>IETVSINNGAAMFSSHH (360-382)</td>
<td>Unknown</td>
<td>(Vargas and von Zastrow 2004)</td>
</tr>
<tr>
<td>Lutenising hormone (LH)</td>
<td>L---GT---------C (683-699)</td>
<td>GIPC</td>
<td>(Galet, Min et al. 2003; Hirakawa, Galet et al. 2003; Galet, Hirakawa et al. 2004)</td>
</tr>
<tr>
<td>Follicle-stimulating hormone (FSH)</td>
<td>PL-H--QN</td>
<td>Unknown</td>
<td>(Krishnamurthy, Kishi et al. 2003)</td>
</tr>
<tr>
<td>Thyrotropin-stimulating hormone (TSH)</td>
<td>TVL (762-764)</td>
<td>hScrib</td>
<td>(Lahuna, Quellari et al. 2005)</td>
</tr>
<tr>
<td>ETA endothelin</td>
<td>S---L-T-V---GT----K (390-408)</td>
<td>Unknown</td>
<td>(Paasche, Attramadal et al. 2005)</td>
</tr>
</tbody>
</table>

CAL, cystic fibrosis transmembrane conductance regulator-associated ligand; ETA, endothelin A; GIPC, GAIP-interacting protein, carboxyl terminus; hScrib, human homologue of the *Drosophila* protein, Scribble; MAGI, membrane-associated guanylate kinase inverted; NHERF, Na$^+$/H$^+$ exchanger regulatory factor; NSF, N-ethylmaleimide-sensitive fusion protein; PDZK1, PDZ domain containing 1; PSD-95, post-synaptic density 95; SAP97, synapse-associated protein 97; TSH, thyrotropin-stimulating hormone.
β-arrestins and GPCR intracellular trafficking

β-arrestins couple numerous agonist-activated GPCRs to the clathrin-mediated endocytic machinery but their role in receptor trafficking may not be limited to simply facilitating internalisation. There is now various evidence to suggest that the nature of the interaction of a GPCR with β-arrestin may, in part, regulate the subsequent trafficking itinerary of the receptor. The first realisation of this potential role for β-arrestins came from work by Laporte and co-workers. By following the recruitment of fluorescently-tagged β-arrestins to various activated GPCRs by live cell microscopy, they identified 2 major classes of GPCRs, which differed in the nature of their interaction with β-arrestins (Oakley, Laporte et al. 2000).

Class A receptors, including the β2AR, μ-opioid receptor (MOR), and endothelin type A receptor, have a higher affinity for β-arrestin2 compared with β-arrestin1 and they dissociate from the β-arrestin during, or immediately after, endocytosis. Class B receptors, including the angiotensin II type 1 receptor, vasopressin type 2 receptor (V2R) and neurotensin receptor, bind both β-arrestin1 and β-arrestin2 with equally high affinity and maintain an interaction with β-arrestins into endosomes. Whereas Class A receptors recycle rapidly to the plasma membrane after internalisation, Class B receptors recycle very slowly, if at all, presumably undergoing eventual degradation (Oakley, Laporte et al. 1999; Anborgh, Seachrist et al. 2000). Receptor mutagenesis performed on the C-tails of a variety of Class B receptors showed that specific clusters of phosphorylated serine/threonine residues (Ser/Thr residues occupying 3/3, 3/4, or 3/5 consecutive positions), which are conserved in their position relative to an NPXXY motif in the 7th transmembrane domain, are responsible for the sustained β-arrestin association (Oakley, Laporte et al. 1999; Oakley, Laporte et al. 2001). These clusters are absent from Class A receptors, although potential phosphate acceptor sites are present. Mutation of the serine/threonine residues in these clusters to alanines converts many Class B receptors into Class A receptors in terms of the nature of their association with β-arrestins (Oakley, Laporte et al. 1999; Oakley, Laporte et al. 2001; Vilardaga, Krasel et al. 2002). Interestingly, swapping the C-tails of the Class A receptor, β2AR, and the Class B receptor, V2R, not only reversed the
stability of the receptor–β-arrestin complex but also the corresponding rates of receptor dephosphorylation, recycling and resensitisation (Oakley, Laporte et al. 1999). Hence, it was suggested that the stability of the receptor–β-arrestin interaction regulates the rate of receptor dephosphorylation, recycling and resensitisation. The simplest mechanism through which β-arrestins were suggested to operate in this capacity was by restricting access to the receptor of phosphatases involved in receptor resensitisation. In addition, it has been shown more recently that β-arrestin binding facilitates the ubiquitination of the β2AR and V2R, targeting the receptors for degradation (Shenoy, McDonald et al. 2001; Martin, Lefkowitz et al. 2003). Importantly, for the β2AR, a Class A GPCR that recycles rapidly after agonist–induced internalisation and only undergoes appreciable degradation after prolonged agonist stimulation, β-arrestin mutations that render β2AR–β-arrestin complexes more stable enhance degradation (Pan, Gurevich et al. 2003), whereas β-arrestin mutations that make the complex even less stable prevent receptor degradation (Shenoy and Lefkowitz 2003). Although early data linked sustained β-arrestin interaction with very slow receptor recycling/degradation (Oakley, Laporte et al. 1999; Anborgh, Seachrist et al. 2000), more recent studies have identified receptors that despite maintaining an interaction with β-arrestins in endosomes, nevertheless recycle rapidly to the cell surface. These include members of the kinin family, such as the bradykinin type 2 receptor (B2R) (Simaan, Bédard–Goulet et al. 2005) and the neurokinin 1 receptor (Garland, Grady et al. 1996; McConalogue, Dery et al. 1999), as well as the SST2A somatostatin receptor (Tulipano, Stumm et al. 2004). The activity of these receptors has led Laporte and colleagues to suggest an extension of their initial classification system to include a third group, the so-called Class C receptors (Simaan, Bédard–Goulet et al. 2005). These GPCRs internalise with β-arrestins into endosomes but the β-arrestin can dissociate from the receptor in endosomes and the receptor can recycle rapidly to the cell surface. Interestingly, expression of a β-arrestin2 mutant that has an increased affinity for agonist–activated receptors, prevented recycling of the B2R, which remained colocalised with the mutant β-arrestin2 on endosomes after agonist wash–out. From this, it was concluded that dissociation of β–
arrestins from the B2R is necessary for its recycling (Simaan, Bédard-Goulet et al. 2005), which is in keeping with the initial proposal that β-arrestin association impedes receptor recycling. However, there is evidence for β-arrestins acting as facilitators of GPCR recycling. The first of this evidence came from work on the trafficking of the A2B adenosine receptor, where it was found that the expression of β-arrestin anti-sense constructs in HEK293 cells led to a significant reduction in receptor recycling, which was restored by either β-arrestin1 or β-arrestin2 reconstitution, although to a greater extent with β-arrestin2 than β-arrestin1 (Mundell, Matharu et al. 2000). More recently, a role for β-arrestins in the recycling of the FPR, which can also be considered a Class C receptor, has also been demonstrated (Vines, Revankar et al. 2003). Although β-arrestins are not required for the internalisation of the FPR, it nevertheless co-internalises with β-arrestins and maintains this interaction into a Rab11-positive compartment. From here, the FPR recycles efficiently to the plasma membrane. Recycling of the FPR is completely blocked in β-arrestin 1 and 2 knock-out MEFs, but can be rescued by co-expression of either β-arrestin1 or β-arrestin2, suggesting that β-arrestins are required for the recycling of the FPR. In support of this notion, the authors also found a greater concentration of the FPR in the perinuclear recycling compartment in β-arrestin 1 and 2 knock-out cells compared with wild-type MEFs. However, a subsequent study from the same group also concluded that dissociation of β-arrestin from the FPR is required for its recycling (Key, Vines et al. 2005).

What is clear from all of these studies is that a full description of the role of β-arrestins in the intracellular trafficking of GPCRs is lacking. It is hard to reconcile data suggesting that they impede receptor recycling/promote GPCR degradation with that suggesting that they act as facilitators of recycling. It is thus likely that β-arrestins regulate the intracellular trafficking of specific GPCRs through multiple mechanisms and β-arrestin1 and β-arrestin2 may function differently in this respect.

Interestingly, Mueller et al. have observed the colocalisation of GFP-β-arrestin1 with CCR5 on vesicular compartments by immunofluorescence after 60 min MIP-1α treatment in CHO cells, suggesting that at least β-arrestin1 may remain associated with CCR5 after internalisation (Mueller, Kelly et al. 2005).
2002). However, this study was limited in the extent to which it characterised this interaction, providing no information on the compartment in which the two proteins colocalised, whether the interaction was maintained throughout trafficking to the compartment, or whether β-arrestin 2 exhibited similar behaviour.

A potential involvement of β-arrestins in CCR5 function is particularly interesting given the increasing importance that is ascribed to these proteins as central regulators of chemotaxis. A requirement for β-arrestins in this process was first observed with CXCR4-mediated chemotaxis, which was defective in lymphocytes isolated from β-arrestin 2, but not β-arrestin 1, knock-out mice (Fong, Premont et al. 2002) or after siRNA knock-down of β-arrestin 2 in HEK293 cells (Cheng, Zhao et al. 2000). Subsequently, β-arrestins have been shown to play a role in CXCR1- and CXCR2-mediated chemotaxis (Barlic, Khandaker et al. 1999; Richardson, Marjoram et al. 2003; Su, Raghuwanshi et al. 2005). Their involvement in chemotaxis was initially thought to reflect their role as terminators of receptor signalling and mediators of receptor internalisation, fitting into a model that desensitisation and recycling of chemokine receptors is required for leukocytes to maintain an ability to sense a chemokine gradient. However, more recently it has been appreciated that β-arrestins scaffold and activate a variety of signalling molecules, which contribute to a second wave of signalling after G protein signalling is terminated (Lefkowitz and Shenoy 2005), and, based on many lines of evidence, a second theory has emerged that β-arrestins scaffold signalling molecules involved in cytoskeletal reorganisation to promote localised actin assembly events leading to the formation of a leading edge (Defea 2006). For instance, β-arrestins scaffold non-receptor tyrosine kinases and in the case of the neutrophil platelet-activating factor (PAF) receptor, β-arrestin dependent p38 activation induces actin bundle formation and cell polarisation (McLaughlin, Banerjee et al. 2006). Another study showed that in migrating NIH3T3 cells, the protease activated receptor 2 (PAR2) uses a β-arrestin-dependent mechanism to sequester activated ERK1/2 in pseudopodia at the leading edge, required for actin cytoskeleton reorganisation and polarised pseudopod extension (Ge, Ly et al. 2003). In addition, β-arrestins can activate the small GTPase RhoA (Barnes, Reiter et al. 2005) and have been shown to bind to the actin-
scaffolding protein, filamin A (Scott, Pierotti et al. 2006), and thus regulate changes in cell shape and motility. Chemokine receptor trafficking, perhaps regulated by β-arrestins, has the potential to influence the localisation of signals involved in chemotaxis.

Regulation of membrane traffic by Rab GTPases

The Rab (Ras-like protein from brain) family of small GTPases comprises over 60 members that regulate virtually all membrane trafficking steps within the secretory and endocytic pathways. They co-ordinate sequential stages in trafficking: the formation of transport vesicles, their transport along cytoskeletal filaments and their docking and fusion with target membranes (Pfeffer 2001; Zerial and McBride 2001). Rab proteins are post-translationally modified by geranylgeranyl addition, which enables them to associate with membranes (Andres, Seabra et al. 1993; Alexandrov, Horiuchi et al. 1994). Each Rab protein is characterised by a specific intracellular distribution, with individual family members associating with particular membrane-bound compartments or domains within a compartment membrane (Zerial and McBride 2001; Pfeffer and Aivazian 2004). They cycle between an inactive, GDP-bound state and an active, GTP-bound state, in which they can interact with effector proteins that mediate downstream processes, such as vesicle budding, motility or fusion (Pfeffer 2001; Zerial and McBride 2001). They undergo cycles of activation and inactivation (GTP binding and subsequent hydrolysis), controlled through catalysis by GTP exchange factors (GEFs) and GTPase activating proteins (GAPs) (Seabra and Wasmeier 2004). In addition, their membrane binding is subject to regulation by GDP dissociation inhibitors (GDIs), which bind to Rabs in their inactive state and chaperone their shuttling between the cytosol and their target membranes, and GDI displacement factors (GDFs), involved in membrane delivery (Sasaki, Kikuchi et al. 1990; Sivars, Aivazian et al. 2003). One of the best described Rab proteins is Rab5, which regulates early endocytic events (Bucci, Parton et al. 1992). Rab5 on early endosomes binds to type I PI3K, which leads to the formation of phosphatidylinositol 3-phosphate (PI(3)P) (Miaczynska and Zerial 2002). Rab5 and PI(3)P recruit various effector proteins, such as early endosome antigen 1 (EEA1), which
binds to PI(3)P via a FYVE (Fablp, YOTB, Vac1p, EEA1) domain, and also to Rab5. These effector proteins then stimulate early endosome fusion (Nielsen, Christoforidis et al. 2000). Internalisation of CCR5, the chemokine receptors, CXCR2 and CXCR4, and the TfR, is inhibited by expression of a dominant negative form of Rab5 (Stenmark, Parton et al. 1994; Fan, Lapierre et al. 2003; Venkatesan, Rose et al. 2003).

Two Rab proteins have been implicated in controlling the recycling of membrane proteins from endosomes. Rab4 appears to mediate the fast recycling of membrane proteins from early endosomes (Sheff, Daro et al. 1999), whereas Rab11, which is mainly found on recycling endosomes, mediates traffic through that compartment (Ullrich, Reinsch et al. 1996; Ren, Xu et al. 1998). Both Rab4 and Rab11 have been shown to control TfR recycling (Ullrich, Reinsch et al. 1996; Ren, Xu et al. 1998; Sheff, Daro et al. 1999). Rab11 has also been implicated in the recycling of CXCR2, where the expression of a dominant negative Rab11 mutant significantly impairs CXCR2 recycling. Moreover, myosin Vb and Rab11-family interacting protein 2 (FIP2), proteins that interact with Rab11, have been shown to play a role in CXCR2 recycling (and resensitisation). In addition, expression of the myosin Vb tail, which acts as a dominant negative since it lacks the motor domain, impairs CXCR2- and CXCR4-mediated chemotaxis in HEK293 cells, which highlights the potential importance of chemokine receptor recycling in chemotaxis (Fan, Lapierre et al. 2004). Interestingly, recycling through recycling endosomes in particular, not just recycling in itself, may be necessary for chemotaxis. The small GTPase RhoB, which localises to endosomes and is activated after CXCL8 stimulation of CXCR2, appears to play a role in the sorting decision between recycling and degradation of this receptor, facilitating sorting of CXCR2 to the degradative pathway after prolonged agonist stimulation. Interestingly, although expression of constitutively active RhoB appeared to block the passage of CXCR2 to the recycling endosome, it did not impair receptor recycling, which was probably because the receptor was routed to alternative recycling pathways, including the Rab4-dependent fast recycling pathway. Despite CXCR2 recycling being unaffected by constitutively active RhoB expression, CXCR2-mediated chemotaxis of HEK293 cells was impaired, suggesting that trafficking through the recycling endosome is important for chemotaxis.
(Neel, Lapierre et al. 2007). This may be a reflection of a resensitisation step that occurs in the recycling endosome or may have to do with targeted recycling of CXCR2 to the leading edge of migrating cells. Some chemotactic receptors have been found to accumulate at the leading edge of migrating cells (Nieto, Frade et al. 1997; Gómez-Moutón, Lacalle et al. 2004) and there is some evidence for polarised delivery of recycling vesicles from recycling endosomes to the leading edge during cell migration (Hopkins, Gibson et al. 1994). However, concentration of chemotactic receptors at the leading edge of migrating cells is a controversial issue, with several studies suggesting that chemotactic receptors are evenly distributed across the surface of migrating cells (Xiao, Zhang et al. 1997; Servant, Weiner et al. 1999; Jin, Zhang et al. 2000; Janetopoulos, Jin et al. 2001).

Sorting to the biosynthetic pathway

Although the intracellular trafficking of many proteins that cycle over the cell surface is restricted to the endosomal system, several proteins exhibit more complex recycling itineraries that involve passage through the TGN. Two such proteins are TGN38 and the cation-independent mannose 6-phosphate receptor (CI-MPR).

The trafficking itinerary of TGN38 was deduced from a kinetic analysis of the trafficking of a hybrid construct consisting of the cytoplasmic domain of TGN38 and an extracellular interleukin-receptor-α-chain domain (Tac) (Ghosh, Mallet et al. 1998). Tac-TGN38, like TGN38, was predominantly found in the TGN, with around 10% of the total pool found at the plasma membrane at steady-state. By labelling surface receptors with a high affinity anti-Tac antibody, it was shown that after internalisation, Tac-TGN38 trafficked to recycling endosomes from where around 80% of receptors returned to the cell surface with a t_{1/2} ~ 10 min, similar to the kinetics of TfR recycling, with the remainder trafficking to the TGN. When longer filling pulses and chases were used to load anti-Tac into the TGN (steady-state distribution achieved), it returned to the cell surface with a t_{1/2} ~ 45 min. Thus, on average, a TGN38 molecule initially at the plasma membrane will recycle several times before it reaches the TGN. A steady-state distribution
where TGN38 is predominantly localised to the TGN is achieved because TGN exit is far slower than recycling from recycling endosomes.

The trafficking itinerary of the CI-MPR further highlights the complexity of the trafficking pathways that integral membrane proteins can take within the cell and also illustrates the bidirectionality between the biosynthetic and endocytic pathways. CI-MPR delivers lysosomal enzymes to late endosomes and lysosomes and traffics between the TGN and late endosomes, and over the plasma membrane. TGN to late endosome trafficking delivers newly synthesised lysosomal enzymes to late endosomes and trafficking over the plasma membrane allows the capture of enzymes that have been secreted (Ghosh, Dahms et al. 2003). It has been shown in CHO cells that rather than be delivered to late endosomes through retention in early endosomes as they mature into late endosomes, internalised CI-MPRs traffic from early endosomes to recycling endosomes. From there, most of the receptors recycle to the surface, but during each cycle of internalisation a small fraction of receptors are delivered to the TGN and then onto late endosomes (Lin, Mallet et al. 2004).

Currently, there is no evidence for a GPCR recycling through the TGN. However, it should be noted that Neel et al. (2007) have reported data that they say suggests that in the presence of constitutively active RhoB, CXCR2 may be forced to recycle through the TGN. As partly described above, the authors showed that expression of RhoB blocked passage of CXCR2 to the recycling endosome (through which it usually recycles) and led to an increased colocalisation of the receptor with Rab7, a marker of late endosomes. However, subsequent delivery to lysosomes was impaired and receptor recycling was still able to occur. The authors also noted that there was a much-increased colocalisation of CXCR2 with both Rab4, and the CI-MPR, the latter, they suggest, indicating that under these conditions, CXCR2 may be forced through a TGN recycling route. However, CI-MPR colocalisation is probably more reflective of an increased late endosomal localisation, which would correlate well with the increased colocalisation of CXCR2 with Rab7 in the presence of constitutively active RhoB. Moreover, the authors found no evidence for such a recycling pathway operating in cells expressing endogenous levels of wild-type RhoB and if the receptor did traffic through the TGN under these conditions, this was probably a
reflection of aberrant trafficking events occurring in the presence of blockades to multiple trafficking pathways. Lastly, chemotaxis was defective in cells expressing constitutively active RhoB, suggesting that alternative recycling routes taken by CXCR2 in the presence of constitutively active RhoB are not physiologically relevant.

Heterologous receptor regulation: implications for CCR5 trafficking

Leukocytes express CCR5 in the context of many other GPCRs, including many $G_{\alpha_i}$-coupled chemoattractant receptors, which may be simultaneously activated during a response to inflammation. Activation of other GPCRs can lead to heterologous desensitisation of CCR5 via PKC-mediated phosphorylation of C-tail serines (Mashikian, Ryan et al. 1999; Grimm, Newman et al. 2003; Szabo, Wetzel et al. 2003). Since PMA treatment, which results in PKC activation, does not lead to CCR5 internalisation by itself (Pollok-Kopp, Schwarze et al. 2003), it is interesting that the activation of CXCR1 and FPR have been shown to not only lead to cross-phosphorylation and desensitisation of CCR5 but also cross-internalisation of the receptor (Shen, Li et al. 2000; Li, Wetzel et al. 2001; Richardson, Tokunaga et al. 2003). A recent study has shed light on the possible mechanism of cross-internalisation, suggesting that it may involve receptor hetero-oligomerisation (Huttenrauch, Pollok-Kopp et al. 2005). In this study, CCR5 was found to form hetero-dimers with the complement factor 5a receptor (C5aR) when both were expressed in RBL cells, whereas CCR5 failed to show an association with the type 1a angiotensin II receptor (AT$_{1a}$R). Whereas C5aR stimulation led to the PKC- and GRK-mediated cross-phosphorylation of CCR5 in a heterologous manner in cells expressing CCR5 and C5aR, in cells expressing CCR5 and AT$_{1a}$R, angiotensin II treatment resulted only in PKC-mediated CCR5 phosphorylation. Moreover, co-expression of C5aR, but not of AT$_{1a}$R, promoted CCR5 co-internalisation upon agonist stimulation; however, CCR5 cross-phosphorylation was not required for this co-internalisation. Co-internalisation of phosphorylated CCR5 was also observed in C5a-stimulated macrophages, demonstrating that such a co-
internalisation mechanism operates in cells where CCR5 is endogenously expressed.

How such hetero-oligomerisation affects the subsequent intracellular trafficking of CCR5 has not been addressed, but it must be borne in mind that the trafficking of CCR5 in vivo may be very different from when it is expressed in cell-lines, where other chemotactic receptors are not expressed/expressed at low levels and high CCR5 expression levels probably favour homo-oligomerisation and reduce the confounding effects of hetero-dimerisation with other GPCRs.

Outline of this investigation

As described above, although the basic outline of CCR5 post-endocytic trafficking has been elucidated, many of the details regarding the control of this trafficking are currently unknown. In this thesis I further characterise the intracellular trafficking of CCR5, with a particular focus on the involvement of the clathrin adaptor proteins, β-arrestins 1 and 2, and the molecular machinery regulating the progression of the receptor through the recycling compartment.

I begin in Chapter 3 by describing a morphological investigation into the receptor's trafficking itinerary, with the finding that in addition to trafficking through recycling endosomes, CCR5 also passes through the TGN - a very similar membrane system. To my knowledge, this is the first real demonstration of an agonist-activated GPCR trafficking through the TGN and changes our perception of the perinuclear recycling compartment, which must now be considered to comprise both recycling endosome and TGN elements. Here, I also describe a role for Rab11 as a regulator of CCR5 trafficking through the recycling compartment.

Given the ever-growing appreciation of the role of β-arrestins in controlling the intracellular trafficking of GPCRs, and the observation by Mueller et al. (2002) of a sustained interaction between GFP-β-arrestin1 and CCR5, much of this thesis describes an investigation into the role of β-arrestins in CCR5 trafficking. I begin in Chapter 4, where I detail a characterisation of the nature of the interaction between CCR5 and β-arrestins, showing that CCR5 exhibits behaviour compatible with membership of the so-called Class C
group of GPCRs, which maintain an interaction with β-arrestins on endosomes, but nevertheless recycle rapidly to the cell surface. As CCR5 phosphorylation has been shown to mediate high affinity β-arrestin binding, I assess the role of C-tail phosphorylation in supporting this sustained association, describing data that suggest that although receptors remain phosphorylated during trafficking with β-arrestins, this phosphorylation is not required for β-arrestin interaction.

After the observation that β-arrestins accompany CCR5 to the perinuclear recycling compartment, in Chapter 5, I show that this compartment is extensively coated with clathrin, which raises the tantalising possibility that β-arrestins may function to couple CCR5 to clathrin in a step required for CCR5 recycling, akin to the role they perform for CCR5 at the cell surface. I also detail an investigation into a role for dynamin in the scission of nascent clathrin-coated vesicles formed at the recycling compartment and largely discount the possible involvement in CCR5 recycling of the clathrin adaptor, AP-1, which also localises to this compartment and has recently been shown to interact with β-arrestins.

Finally, in Chapter 6, I return to the CCR5–β-arrestin association, where I recount my search for a functional role for this interaction in CCR5 intracellular trafficking through the acquisition of kinetic data on the recycling of CCR5 in cells over-expressing β-arrestin2 or expressing constitutively active β-arrestin2 mutants.
Chapter 2: MATERIALS AND METHODS

“When you know the notes to sing, you can sing ‘most anything.”’

Maria, The Sound of Music

General reagents

All chemicals were purchased from Sigma–Aldrich Company Ltd. (Poole, Dorset, UK) unless otherwise stated. Recombinant CCL3 (MIP–1α), CCL4 (MIP–1β) and CCL5 (RANTES) were provided by A. E. I. Proudfoot (Serono Pharamceuticals Research Institute, Geneva, Switzerland). TAK–779 was purchased from the NIH AIDS Research and Reference Reagent Programme (Germantown, Maryland, USA). Bis–T–23 and Dyngo–4a were gifts from P. Robinson (Children’s Medical Research Institute, Westmead, Australia). Formaldehyde fixative was purchased from TAAB Laboratories (Aldermaston, Berks, UK).

General solutions

Phosphate-buffered saline (PBS)

137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, adjusted to pH 7.4

Binding medium (BM)

RPMI–1640 without bicarbonate, 0.01 M HEPES (pH 7), 0.2% BSA, adjusted to pH 7

Tris–buffered saline (TBS)

137 mM NaCl, 2.7 mM KCl, 25 mM Tris base, adjusted to pH 7.4
Cell culture

Reagents

Tissue culture medium and supplements were purchased from Gibco/Invitrogen (Paisley, UK).

Cell-lines and maintenance

The cell-lines used in this thesis are detailed in Table 2-1. Adherent cells were grown in 10 ml of growth medium in 9 cm dishes (Falcon, Becton Dickinson, Oxford, UK) at 37°C in an atmosphere containing 5% CO₂. All cell-lines were passaged twice weekly. Here, medium was removed by aspiration and cells were detached by the addition of 2 ml of trypsin/EDTA solution (0.05% trypsin and 0.7 mM EDTA in PBS) at 37°C. Eight millilitres of growth medium pre-warmed to 37°C was added to the detached cells before re-plating into 9 cm dishes at an appropriate dilution in a final volume of 10 ml. In addition to this, rat basophilic leukaemia CCR5 (RBL CCR5) cells were washed with 10 ml of PBS at 37°C before the addition of trypsin/EDTA and detached cells were pelleted by centrifugation (800 rpm for 3 min) and resuspended in fresh medium before being plated. Both steps were performed to remove cellular secretions/debris before re-plating. All cell-lines were regularly screened for Mycoplasma infection using the VenorGem® PCR assay (Cambio Ltd., Cambridge, UK).

Table 2-1 Details of cell-lines

<table>
<thead>
<tr>
<th>Cell-line</th>
<th>Genetic manipulation</th>
<th>Growth medium</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>Chinese hamster ovary (CHO) cells</td>
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<tr>
<td>CHO (DHFR)</td>
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<tr>
<td>CCR5</td>
<td>Stably expressing human CCR5</td>
<td>MEMα containing Glutamax, 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin</td>
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<tr>
<td>CHO (DHFR)</td>
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<tr>
<td>CCR5 βarr1-YFP</td>
<td>Stably expressing human CCR5 and bovine βarr1-YFP or βarr2-GFP</td>
<td>MEMα containing Glutamax, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mg/ml G418.</td>
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<tr>
<td>CHO (DHFR)</td>
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<tr>
<td>CCR5 βarr2-GFP</td>
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<tr>
<td>Cell-line</td>
<td>Genetic manipulation</td>
<td>Growth medium</td>
<td>Source</td>
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<tr>
<td><strong>CHO (DHFR)</strong></td>
<td></td>
<td></td>
<td>C. Merrifield, MRC LMB, UK. Generated by transfection of CHO (DHFR) CCR5 cells from M. Mack A. Trkola, University Hospital, Zurich, Switzerland (Trkola, Gordon et al. 1999)</td>
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<td>CCR5 clathrin light chain a-</td>
<td>Stably expressing human CCR5 and clathrin light chain a-dsRed</td>
<td>MEMα containing Glutamax, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml G418</td>
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<tr>
<td>dsRed</td>
<td></td>
<td>DMEM/F12 containing 2 mM glutamine, 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin</td>
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<td><strong>CHO-K1</strong></td>
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<td>Mouse embryonic fibroblasts (MEFs)</td>
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<td>MEF wt</td>
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<td>DMEM containing 2 mM glutamine, 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin</td>
<td>R. Lefkowitz, Duke University Medical Center, Durham, USA (Kohout, Lin et al. 2001)</td>
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<td>MEF βarr1 KO</td>
<td>Embryonic fibroblasts obtained from wild-type or β-arrestin knock-out mice</td>
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<tr>
<td>MEF βarr2 KO</td>
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<tr>
<td>MEF βarr1+2 KO</td>
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<td>Human osteosarcoma (HOS) cells</td>
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<td>Centre for AIDS Reagents (EU Programme EVA/AVIP). Original source: Littman and Kewalramni, courtesy of NIH AIDS Research and Reference Reagents Programme</td>
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<td>HOS CD4 CCR5 (ARP078)</td>
<td>Stably expressing human CD4 and CCR5 proteins</td>
<td>DMEM containing 2 mM glutamine, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 mg/ml G418, 100 μg/ml hygromycin and 1 μg/ml puromycin</td>
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<td><strong>Other cell-lines</strong></td>
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<td>Rat basophilic leukaemia CCR5</td>
<td>Stably expressing human CCR5</td>
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<td>M. Oppermann, Georg August University, Goettingen, Germany (Huttenrauch, Nitzki et al. 2002)</td>
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<td>(RBL CCR5)</td>
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<td>DMEM containing 2 mM glutamine, 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin</td>
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<tr>
<td>HeLa Kyoto</td>
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<td>RPMI Glutamax containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, HT (hypoxanthine and thymidine) supplement and 1 mM sodium pyruvate</td>
<td>L. Pelkmanns, ETH Zurich, Switzerland (L. Pelkmanns, ETH Zurich, Switzerland)</td>
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<td>MC-5-producing hybridoma (from</td>
<td>Hybridoma producing MC-5 antibody</td>
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<td>M. Mack, University of Munich, Germany (Segerer, Mack et al. 1999)</td>
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<td>BALB/c mice)</td>
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</tr>
</tbody>
</table>
Cell freezing and thawing

For freezing, adherent cells attached to a 9 cm dish were detached in the same manner as for passaging and resuspended in a total of 10 ml of growth medium pre-warmed to 37°C. Cells were then pelleted by centrifugation (800 rpm, 3 min) and resuspended in 5 ml of growth medium containing 50% foetal calf serum (FCS). 1 ml aliquots were frozen at -80°C in cryovials (Nunc, Gibco/Invitrogen, Paisley, UK) for 1 week before being transferred to a liquid nitrogen tank.

One millilitre aliquots of frozen cells were thawed rapidly in a 37°C water-bath and diluted with 9 ml of growth medium pre-warmed to 37°C. Cells were then pelleted by centrifugation (800 rpm, 3 min), resuspended into 10 ml of medium at 37°C and plated into a 9 cm dish.

Antibodies

The various antibodies used in this thesis are detailed in Table 2-11.

Table 2-11 Details of antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Species/isotype</th>
<th>Conc. used*</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antibodies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR5 N-terminus</td>
<td>MC-5 (hybridoma supernatant)</td>
<td>Mouse IgG2a</td>
<td>1:500</td>
<td>Produced in-house from hybridoma cells¹</td>
</tr>
<tr>
<td>CCR5 N-terminus</td>
<td>MC-5 (purified)</td>
<td>Mouse IgG2a</td>
<td>1 µg/ml (IF, IB), 7.5 µg/ml (IP)</td>
<td>from hybridoma cells¹</td>
</tr>
<tr>
<td>CCR5 P-S349</td>
<td>Alexa Fluor® 488-MC-5</td>
<td>Mouse IgG2a</td>
<td>1 µg/ml</td>
<td>Alexa fluor 488 coupled to purified MC-5 in-house¹</td>
</tr>
<tr>
<td>CCR5 P-S337</td>
<td>E11/19</td>
<td>Mouse IgG1</td>
<td>5 µg/ml</td>
<td>M. Oppermann, Georg August University, Goettingen, Germany</td>
</tr>
<tr>
<td>Clathrin heavy chain</td>
<td>V14/2</td>
<td>Mouse IgG2b</td>
<td>5 µg/ml</td>
<td>Affinity Bioreagents, Nottingham, UK</td>
</tr>
<tr>
<td>Clathrin heavy chain</td>
<td>X22</td>
<td>Mouse IgG1</td>
<td>6 µg/ml</td>
<td>Transduction Laboratories, BD Bioscience, Oxford, UK</td>
</tr>
<tr>
<td>Transferrin receptor</td>
<td>23</td>
<td>Mouse IgG1</td>
<td>0.25 µg/ml</td>
<td>Zymed Laboratories/Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td></td>
<td>H68.4</td>
<td>Mouse IgG1</td>
<td>2 µg/ml</td>
<td></td>
</tr>
<tr>
<td>Antigen</td>
<td>Antibody</td>
<td>Species/isotype</td>
<td>Conc.</td>
<td>Source</td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>----------------</td>
<td>-------</td>
<td>--------</td>
</tr>
<tr>
<td>γ-adaptin (AP-1)</td>
<td>Mary</td>
<td>Rabbit polyclonal</td>
<td>1:500</td>
<td>M. S. Robinson, University of Cambridge, UK</td>
</tr>
<tr>
<td>γ-adaptin (AP-1)</td>
<td>Adaptin γ</td>
<td>Mouse IgG2a</td>
<td>0.25 μg/ml</td>
<td>Ira Mellman, Yale University School of Medicine, New Haven, USA</td>
</tr>
<tr>
<td>μ1A-adaptin (AP-1)</td>
<td>μ1A</td>
<td>Rabbit polyclonal</td>
<td>1:1000</td>
<td>J. Benovic, Thomas Jefferson University, Philadelphia, USA</td>
</tr>
<tr>
<td>β-arrestin1</td>
<td>178</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
<td>Affinity Bioreagents, Nottingham, UK</td>
</tr>
<tr>
<td>β-arrestin2</td>
<td>182-4</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
<td>M. Oppermann, Georg August University, Goettingen, Germany</td>
</tr>
<tr>
<td>Arrestin</td>
<td>Arrestin (pan)</td>
<td>Rabbit polyclonal</td>
<td>1 μg/ml</td>
<td>Transduction Laboratories, BD Bioscience, Oxford, UK</td>
</tr>
<tr>
<td>β-arrestin1+2</td>
<td>21-B1</td>
<td>Mouse IgG1</td>
<td>1.1 μg/ml</td>
<td>Transduction Laboratories, BD Bioscience, Oxford, UK</td>
</tr>
<tr>
<td>c-myc</td>
<td>9E10</td>
<td>Mouse IgG1</td>
<td>1 μg/ml</td>
<td>Roche Diagnostics, West Sussex, UK</td>
</tr>
<tr>
<td>EEA1</td>
<td>EEA1 MAb</td>
<td>Mouse IgG1</td>
<td>1.25 μg/ml</td>
<td>AbD Serotec, Kidlington, UK</td>
</tr>
<tr>
<td>GFP</td>
<td>Anti-GFP Mab</td>
<td>Mouse IgG1</td>
<td>0.4 μg/ml</td>
<td>Zymed Laboratories/Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>TGN46</td>
<td>Sheep anti-human TGN46</td>
<td>polyclonal</td>
<td>5 μg/ml</td>
<td>S. Schmid, California Institute of Technology, Pasadena, USA</td>
</tr>
<tr>
<td>Rab11a</td>
<td>Anti-Rab11</td>
<td>Rabbit polyclonal</td>
<td>0.25 mg/ml</td>
<td>(originally from B. Granger, University of Montana, USA)</td>
</tr>
<tr>
<td>Lgp-B (LAMP2-)</td>
<td>3E9 (hybridoma supernatent)</td>
<td>Mouse IgG1</td>
<td>1:2</td>
<td>Transduction Laboratories, BD Bioscience, Oxford, UK</td>
</tr>
<tr>
<td>p230</td>
<td>p230 trans Golgi</td>
<td>Mouse IgG1</td>
<td>0.5 μg/ml</td>
<td>Sigma-Aldrich, Poole, UK</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>Anti-α-tubulin</td>
<td>Mouse IgG1</td>
<td>1:500</td>
<td>Sigma-Aldrich, Poole, UK</td>
</tr>
</tbody>
</table>

**Secondary antibodies**

- **Rabbit IgG (H+L)** | IRDye® 680 GAR | Goat polyclonal | 0.2 μg/ml | Li-cor Biosciences, Lincoln, Nebraska, USA |
- **Mouse IgG (H+L)** | IRDye® 800 GAM | Goat polyclonal | 0.2 μg/ml | Molecular Probes/Invitrogen, Paisley, UK |
- **Mouse IgG (H+L)** | GAM Alexa Fluor® 488 | Goat polyclonal | 4 μg/ml |
- **Mouse IgG (H+L)** | GAM Alexa Fluor® 594 | Goat polyclonal | 4 μg/ml |
### Materials and Methods

#### Production of MC-5 from hybridoma cells

The mouse monoclonal antibody, MC-5, was harvested from the growth medium of hybridoma cells producing the antibody (Segerer, Mack et al. 1999; Blanpain, Vanderwinden et al. 2002). The cells were grown in suspension in an INTEGRA CELLline 350 system (INTEGRA Biosciences AG, Switzerland). This system houses the cells in a small volume of growth medium separated from a larger pool of nutrient medium (growth medium minus FCS) by a size-exclusion membrane, which allows the passage of nutrients into the cell compartment but prevents antibody exchange. Thus,

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Species/isotype</th>
<th>Conc. used*</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IgG (H+L)</td>
<td>GAM Alexa Fluor® 647</td>
<td>Goat polyclonal</td>
<td>4 μg/ml</td>
<td>Pierce, Perbio Science UK Ltd., Cramlington, UK</td>
</tr>
<tr>
<td>Mouse IgG IgG1</td>
<td>GAM IgG1 Alexa Fluor® 488</td>
<td>Goat polyclonal</td>
<td>4 μg/ml</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG IgG1</td>
<td>GAM IgG1 Alexa Fluor® 594</td>
<td>Goat polyclonal</td>
<td>4 μg/ml</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG IgG1</td>
<td>GAM IgG1 Alexa Fluor® 647</td>
<td>Goat polyclonal</td>
<td>4 μg/ml</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG IgG1</td>
<td>GAM IgG1 Alexa Fluor® 488</td>
<td>Goat polyclonal</td>
<td>4 μg/ml</td>
<td></td>
</tr>
<tr>
<td>Rabbit IgG (H+L)</td>
<td>GAR Alexa Fluor® 594</td>
<td>Goat polyclonal</td>
<td>4 μg/ml</td>
<td></td>
</tr>
<tr>
<td>Rabbit IgG (H+L)</td>
<td>GAR Alexa Fluor® 647</td>
<td>Goat polyclonal</td>
<td>4 μg/ml</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG (H+L)</td>
<td>Anti-mouse HRP</td>
<td>Goat polyclonal</td>
<td>2 μg/ml</td>
<td>Pierce, Perbio Science UK Ltd., Cramlington, UK</td>
</tr>
<tr>
<td>Rabbit IgG (H+L)</td>
<td>Anti-rabbit HRP</td>
<td>Donkey polyclonal</td>
<td>2 μg/ml</td>
<td></td>
</tr>
</tbody>
</table>

IB, immunoblot; IP, immunoprecipitation; IF, immunofluorescence; GAM, goat anti-mouse; GAR, goat anti-rabbit; RAM, rabbit anti-mouse; RAG, rabbit anti-goat; HRP, horse radish peroxidase; H+L, heavy and light antibody chains.

* Concentrations of antibodies used for experiments in this thesis are listed; where different concentrations have been used for specific applications, they are indicated separately. Concentrations are given in mass per volume units; where a concentration has not been determined, a dilution at which the antibody solution has been used is indicated.

1 See Production of MC-5 from hybridoma cells
2 See Coupling of MC-5 to Alexa Fluor® 488
a high cell density and high concentration of antibody can build up within the cell compartment. Cells were grown according to the manufacturer’s instructions, with an aliquot of medium from the cell compartment collected every 7 d for a period of a month. Cells were pelleted by centrifugation (800 rpm, 3 min) and the antibody-containing supernatants stored at 4°C.

Purification of MC-5

Before purification, the concentration of antibody in the supernatants was estimated by running aliquots on a non-reducing sodium dodecyl sulphate (SDS)–polyacrylamide gel and comparing antibody band intensities with a dilution series of a human IgG antibody (Sigma–Aldrich, Poole, UK) of known concentration (protocol described in Preparation of samples for protein electrophoresis and Protein electrophoresis).

Antibody purification from the hybridoma supernatant was achieved using the ImmunoPure® (A/G) IgG Purification Kit from Pierce (Perbio Science UK Ltd., Cramlington, UK), where the antibody is captured on a Protein A/G column, washed and then eluted at low pH. Following the manufacturer’s instructions, for each purification, 14 mg of antibody diluted in binding buffer (pH 8) was loaded onto a 2 ml Protein A/G column (binding capacity of 7 mg of antibody per ml of gel matrix) and washed with 10 ml of binding buffer. The antibody was eluted with elution buffer (pH 2.8) and collected in 0.5 ml fractions. Fifty microlitres of 1 M Tris, pH 7.5, was added to each fraction to raise the pH of the elution buffer towards neutral. The antibody-containing fractions were identified by SDS polyacrylamide gel electrophoresis (PAGE) and by protein assay (DC Protein Assay, Bio–Rad Laboratories Ltd., Hemel Hemstead, UK).

In order to make the purified antibody preparation compatible with chemical coupling reactions involving primary amine groups on the antibody molecule, the amine-containing buffer was exchanged for PBS using a D-Salt™ Dextran Desalting Column (Pierce, Perbio Science UK Ltd., Cramlington, UK) with a molecular mass exclusion limit of 5 kDa. The antibody-containing solution was allowed to enter the PBS-equilibrated column before the addition of further PBS to wash the antibody through. Half millilitre fractions were collected and the presence of antibody
determined by protein assay. The purity of the antibody-containing fractions was assessed by non-reducing SDS PAGE. Figure 2-1 A shows a gel where fractions 4–8 from a representative antibody purification were loaded. The fractions contained a single predominant band of approximately 150 kDa, demonstrating the purity of the preparation. Antibody-containing fractions were snap frozen in liquid nitrogen and stored at −80°C.

**Coupling of MC-5 to Alexa Fluor® 488**

MC-5 was coupled to Alexa Fluor® 488 using a protein labelling kit (A-10235) from Molecular Probes (Paisley, UK). The Alexa Fluor® 488 reactive dye has a tetrafluorophenyl (TFP) ester moiety that reacts with primary amines, i.e., lysine side-chains of the antibody molecule. The coupling reaction was performed according to the manufacturer’s instructions. Briefly, 50 µl of a 1 M sodium bicarbonate solution was added to 0.5 ml of a 2 mg/ml MC-5 solution. This raised the pH of the solution, in order to increase the rate of the coupling reaction. The protein solution was then transferred to a reaction vial containing the reactive dye in solid form. The reaction mixture was stirred at ambient temperature for 1 h, before separation of the unreacted dye from the antibody by passing the reaction mixture through a size-exclusion matrix (Bio–rad BioGel P-30 Fine size exclusion purification resin, 40 kDa molecular mass exclusion limit, Bio–Rad Laboratories Ltd., Hemel Hemstead, UK). A PBS elution buffer (pH 7.2, containing 2 mM sodium azide) was used to elute the labelled antibody, named 488MC-5. The passage of 488MC-5 through the column was followed as a coloured band and collected as a single fraction.

The concentration of antibody and the degree of coupling were calculated from measurements using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Loughborough, UK), according to the following equations, based on the Beer–Lambert law:

\[
\text{protein concentration (M)} = \frac{[A_{280} - (A_{494} \times 0.11)] \times \text{dilution factor}}{203,000}
\]
, where 203,000 cm\(^{-1}\)M\(^{-1}\) is the molar extinction coefficient of a typical IgG and 0.11 is a correction factor to account for absorption of the dye at 280 nm.

\[
\text{molar dye:antibody ratio} = \frac{A_{\text{dye}} \times \text{dilution factor}}{71,000 \times \text{concentration (M)}}
\]

, where 71,000 cm\(^{-1}\)M\(^{-1}\) is the approximate molar extinction coefficient of the Alexa Fluor® 488 dye at 494 nm.

This gave an antibody concentration of 1 mg/ml and a coupling ratio of 5.5 dye molecules to 1 antibody molecule.

**Specificity and binding characteristics of MC-5 and \(^{488}\)MC-5**

Several experiments were performed to assess the specificity and binding characteristics of MC-5 and \(^{488}\)MC-5 to CCR5, using CHO CCR5 cells, which stably express high levels of the receptor on their surface.

Firstly, the binding of purified MC-5 to CCR5 on the surface of CHO CCR5 cells was assessed by immunofluorescence; representative images from one purification are shown in Figure 2-1 B. MC-5 labelled the surface of the CHO CCR5 cells in a concentration-dependent manner, with maximum staining intensity reached around 1 \(\mu\)g/ml. The secondary antibody alone gave no visible staining using the same microscope acquisition settings. The binding of MC-5 was specific to CCR5 because CHO-K1 cells, which do not express CCR5, gave no fluorescence signal when stained with 10 \(\mu\)g/ml MC-5 - the highest concentration tested.

Next, the CCR5-binding characteristics of MC-5, \(^{488}\)MC-5 and \(^{594}\)MC-5\(^3\) were determined by fluorescence-activated cell sorting (FACS). Figure 2-1 C is a graph showing mean fluorescence intensity against concentration of antibody. The data points represented by squares (solid trend lines) show the mean fluorescence intensity for CHO CCR5 cells, corrected for non-specific antibody binding to CHO-K1 cells, relative to the mean fluorescence

\(^3\) Alexa Fluor® 594 was also coupled to CCR5 to produce \(^{594}\)MC-5 but the antibody conjugate generated was not used for experiments described in this thesis.
intensity measured with 100 μg/ml of antibody, which was arbitrarily set to a value of 100. The data points represented by triangles (dotted trend lines) represent the non-specific binding of antibody on CHO-K1 cells relative to the specific binding of the antibody on CHO CCR5 cells at 100 μg/ml, also set to a value of 100.

MC-5 demonstrated a typical sigmoidal binding curve, plateauing at around 3 μg/ml (20 nM), with an apparent K_d of ~ 0.2 μg/ml (~ 1.3 nM). This compares well with the K_d of 0.35 ± 0.21 μg/ml calculated by Blanpain et al. (2002). 488MC-5 also showed a sigmoidal binding curve, but the curve was slightly right-shifted compared with that of MC-5, indicative of a loss of affinity due to coupling to Alexa Fluor® 488, and accordingly, the K_d for 488MC-5 was increased to ~ 0.7 μg/ml (~ 4.7 nM). Although 594MC-5 also showed a concentration-dependent increase in binding to CCR5, saturation binding was not observed at 100 μg/ml and the curve was significantly right-shifted relative to MC-5 and 488MC-5, demonstrating a significant loss of affinity for the receptor due to coupling to Alexa Fluor® 594. The coupling ratio for 594MC-5 was 3:1, lower than that of 488MC-5 (5.5:1), so the reduced affinity for CCR5 is likely to result from the larger size of the Alexa Fluor® 594 molecule relative to the Alexa Fluor® 488 molecule (see Figure 2-I D), which presumably is coupled to at least one lysine residue near to the CCR5 binding interface in at least some of the antibodies within the preparation.

From the graph, it can be seen that for all of the antibodies, non-specific binding, as assessed on CHO-K1 cells, was minimal, and was still negligible at high concentrations of antibody relative to binding to CHO CCR5 cells.

Lastly, the binding of 488MC-5 and 594MC-5 to CHO CCR5 cells was assessed by immunofluorescence. As can be seen in Figure 2-I D, 488MC-5 displayed concentration-dependent binding over the range tested (0.5–10 μg/ml), with a maximum fluorescence signal observed at 5 μg/ml, which agreed well with the FACS data. 594MC-5 showed concentration-dependent binding over the whole range of concentrations tested, with a larger difference in fluorescence intensity between 0.5 μg/ml and 10 μg/ml than 488MC-5, also agreeing well with the FACS data.
Figure 2-1 Specificity and binding characteristics of MC-5 and MC-5 conjugates

A. Coomassie-stained, non-reducing polyacrylamide gel showing fractions 4-8 collected from a desalting column from a representative sample of purified MC-5
antibody. Also shown is a dilution series of human IgG (hlgG), which has approximately the same mobility as MC-5. Molecular mass standards were run in the left-most lane (M); masses are indicated to the left of the gel. B. CHO CCR5 cells grown on coverslips were fixed and stained with various concentrations of purified MC-5 and 4 µg/ml GAM Alexa 594 secondary antibody. The secondary antibody control represents CHO CCR5 cells incubated with 4 µg/ml GAM Alexa 594 only. In a control to assess non-specific binding of MC-5 to CHO cells, CHO-K1 cells were incubated with 10 µg/ml MC-5 and 4 µg/ml GAM Alexa 594 secondary antibody. Coverslips were observed by epifluorescence microscopy (bright-field view also shown for controls). Bar, 50 µm. C. Equal numbers of CHO CCR5 or CHO-K1 cells were incubated with various concentrations of MC-5, 488MC-5 and 594MC-5 in 96-well plates for 1 h at 4°C. Cells were washed and then MC-5- and 594MC-5-labelled cells incubated for a further hour with a GAM Alexa 488 antibody at 4°C. Cell-associated fluorescence was measured by FACS. Details of the data points plotted are given in the text. Data from one representative experiment are shown; error bars represent one standard deviation of the mean of triplicate samples. D. Cells grown on coverslips were fixed and stained with various concentrations of either 488MC-5 or 594MC-5. Coverslips were observed by epifluorescence. Bar, 20 µm. The chemical structures of Alexa Fluor® 488 and Alexa Fluor® 594 are shown above the figure. The unconnected amide bond represents where the primary amine group of a lysine side chain would couple to the Alexa Fluor® moiety.

**Expression of exogenous proteins in cell-lines**

**DNA constructs**

The table below details the DNA constructs used in this thesis.

**Table 2-III Details of DNA constructs**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Expression vector</th>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR5 wt</td>
<td>pEF-BOS</td>
<td>Human</td>
<td>M. Oppermann, Georg August University, Goettingen, Germany</td>
</tr>
<tr>
<td>CCR5 4S→A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-arrestins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFP-βarr2 wt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFP-βarr2(1-380)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Amplification of plasmids

Chemically competent *E. coli* (One Shot® TOP10, Invitrogen, Paisley, UK) were transformed using the heat-shock method. Five hundred nanograms of DNA was added to 50 μl of bacterial suspension and incubated on ice for 30 min. The mixture was then incubated for 30 s in a 42°C water-bath before cooling on ice for 2 min. Two hundred and fifty microlitres of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose; Invitrogen, Paisley, UK) pre-warmed to 37°C was added and the bacteria incubated on a shaker at 37°C for 1 h. One hundred microlitres of transformed bacterial suspension was then plated on a 10 cm LB Agar plate (15 g/l agar in Luria Broth [LB; 10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 10 g/NaCl]) containing 50 μg/ml kanamycin and grown overnight in a 37°C incubator. Single colonies were picked and grown in 2 ml of LB containing 50 μg/ml kanamycin at 37°C with shaking for 8 h. One hundred microlitres of bacterial suspension was then transferred to 250 ml of LB containing 50 μg/ml kanamycin and the bacterial suspension incubated at 37°C overnight, with vigorous shaking. The bacteria were pelleted by centrifugation (4,200 rpm, 30 min) and plasmid DNA was purified using the Qiagen QIAfilter plasmid purification kit (Qiagen, Crawley,
West Sussex, UK) according to the manufacturer's instructions. Purified DNA was resuspended in TE buffer (10 mM Tris, 1 mM EDTA; Qiagen, Crawley, West Sussex, UK) and the DNA concentration determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Loughborough, UK).

**Transfections**

CHO CCR5 cells, CHO-K1 cells and MEFs were transfected using FuGENE® HD (Roche Diagnostics, West Sussex, UK), according to the manufacturer's instructions, with optimisations of cell seeding density and DNA/FuGENE ratio. Three hundred thousand cells were seeded into a well of a 6-well plate (Nunc, Gibco/Invitrogen, Paisley, UK) and grown for 24 h before transfection. For each transfection, 2 μg of DNA was diluted in 100 μl of Opti-MEM (Invitrogen, Paisley, UK). Six microlitres of FuGENE HD transfection reaction was added to the DNA solution and mixed by flicking vigorously. The mixture was incubated at ambient temperature for 15 min to allow the DNA:FuGENE complexes to form. The medium on the cells was replaced with 2 ml of growth medium minus antibiotics and the DNA:FuGENE complexes added drop-wise. Cells were then incubated at 37°C for 24 h hours, after which the cells were detached with trypsin/EDTA and replated into fresh dishes appropriate to the experiment in which the cells were to be used.

**siRNA knock-downs**

**Details of siRNAs**

The target sequences used to create siRNA oligonucleotides were as follows:

AP-1 m1A (Dharma1)

TT CG TTT C AT G G A T T A A

AP-1 m1A (Dharma2)

G T T C G T T T C AT G G A T T A
siRNA oligonucleotides targeting the adaptor protein 1 (AP-1) μ1A subunit were designed using the siDESIGN® Center (Dharma 1 and Dharma 2; Dharmaco RNAi Technologies, www.dharmacon.com) or the BLOCK-iT™ RNAi Designer (Inv; Invitrogen, www.invitrogen.com) using the mouse AP-1 μ1A subunit DNA sequence (NM_007456) as a target. All designed siRNAs were purchased from Dharmaco RNAi Technologies (Chicago, USA). AllStars Negative Control siRNA was purchased from Invitrogen (Paisley, UK).

**Knock-down protocol**

CHO CCR5 cells were transfected with siRNA oligonucleotides using Oligofectamine (Invitrogen, Paisley, UK). Cells were detached from a confluent 9 cm dish with trypsin/EDTA and re-seeded at a dilution of 1:30 into wells of a 6-well plate (Nunc, Gibco/Invitrogen, Paisley, UK) and grown for 5 h. siRNA:Oligofectamine complexes were prepared by incubating for 20 min at ambient temperature a mixture of a dilution of 10 μl of a 20 μM siRNA solution (200 pmol) in 175 μl of Opti-MEM (Invitrogen, Paisley, UK) with 3 μl of Oligofectamine reagent diluted in 12 μl of Opti-MEM (pre-incubated at ambient temperature for 5 min). Growth medium was removed from the cells and replaced with 800 μl of medium without antibiotics and FCS and siRNA:Oligofectamine complexes were added drop-wise to the cells, giving a final concentration of 200 nM siRNA. Cells were incubated for 3 h at 37°C before the addition of 3 ml of complete growth medium to each well and re-incubation at 37°C. Sixteen hours later, another knock-down transfection was performed as above without detaching cells. After a further 24 h, cells were detached with trypsin/EDTA and split at an appropriate dilution onto coverslips for an immunofluorescence assay, or several wells combined appropriately in 9 cm dishes for a FACS assay or for a cell lysate 24 h later. The extent of knock-down was determined from quantification of
immunoblots of cell lysates using the Odyssey infra-red detection system, as described below.

**Preparation of cell lysates and immunoprecipitations**

**Preparation of lysates**

RIPA buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 5 mM EDTA [pH 8.0], 1% v/v NP-40, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS, adjusted to pH 8.0) was used as the standard lysis buffer for preparation of cell lysates. In general, 80% confluent 9 cm dishes of cells were cooled on ice and washed twice with ice-cold PBS. Protease inhibitors (Complete Protease inhibitor cocktail, Roche Diagnostics, West Sussex) and phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail, Pierce, Perbio Science UK Ltd., Cramlington, UK) were added fresh to the lysis buffer according to the manufacturer’s instructions. Immediately before lysing cells, phenylmethanesulphonylfluoride (PMSF, final concentration 1 mM) was also added to the lysis buffer to inhibit serine proteases. PBS was removed from the dish by aspiration and cells were lysed by addition of 1 ml of ice-cold lysis buffer. Cells were scraped into pre-chilled 1.5 ml microfuge tubes and sonicated twice for 10 s to shear DNA and fragment large cellular debris. Insoluble debris was removed by centrifugation at 13,000 rpm for 10 min at 4°C. Lysates were either snap-frozen in liquid nitrogen or used appropriately for the particular experiment. Frozen lysates were thawed quickly at 37°C for subsequent use.

**Immunoprecipitations**

Protein concentrations of cell lysates were determined by protein assay (DC Protein Assay, Bio-Rad Laboratories Ltd., Hemel Hemstead, UK) and equal amounts of protein were used for each immunoprecipitation. Lysates were pre-cleared in 1.5 ml microfuge tubes with Protein A sepharose beads (Sigma-Aldrich Company Ltd., Poole, Dorset, UK) or Protein G sepharose beads (Zymed/Invitrogen, Paisley, UK) for 30 min on a rotating wheel at 4°C. Protein A sepharose was used for immunoprecipitations with MC-5 (mouse IgG2a) and Protein G sepharose was used for immunoprecipitations with
anti-GFP (mouse IgG1). After pre-clearing lysates, beads were pelleted by centrifugation (8000 rpm, 3 min, 4°C) and the supernatants transferred to fresh microfuge tubes. The immunoprecipitating antibody was added to the lysates, which were incubated for 30 min at 4°C on a rotating wheel, before being transferred to protein A/G sepharose-containing tubes and further incubated for 1.5 h at 4°C on a rotating wheel. Beads were then pelleted by centrifugation (8000 rpm, 3 min, 4°C) and washed 3 times for 5 min with ice-cold lysis buffer. Finally, beads were re-suspended in 2x reducing sample buffer (2xRSB; 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris HCl, pH ~ 6.8) and heated at 95°C for 5 min.

Preparation of samples for protein electrophoresis

Non-reducing conditions

Prior to loading on a gel, protein samples were mixed 1:1 with 2x non-reducing sample buffer (4% SDS, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl, pH ~ 6.8) and incubated at ambient temperature for 5 min.

Reducing conditions

Prior to loading on a gel, protein samples were mixed 1:1 with 2x reducing sample buffer (2xRSB; 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris HCl, pH ~ 6.8). Standard protocols recommend heating lysates in sample buffer to 95°C for 5 min to ensure complete denaturation of proteins. However, it was discovered that this resulted in a significant loss of CCR5 from cell lysates (see Figure 2–II). This loss of CCR5 was not observed in heated tubes containing CCR5 immunoprecipitated from cell lysates. As CCR5 is a 7 trans-membrane domain protein with considerable hydrophobicity, the most likely explanation for these observations is that heating favours aggregation of CCR5 with other proteins in cell lysates, through non-specific hydrophobic interactions, but that this does not occur in solutions containing the
immunoprecipitation reaction, where most of the cellular proteins have been removed by washing.

Cell lysates mixed with sample buffer that were to be run for the purpose of detecting CCR5 were, therefore, incubated at ambient temperature and not heated to 95°C. However, cell lysates from which cytoplasmic proteins were to be detected by immunoblot were heated to 95°C. Immunoprecipitates were also heated to 95°C as no loss of CCR5 was observed upon heating and heating assisted denaturation of both CCR5 and the immunoprecipitating antibody, resulting in single bands for CCR5 and both the antibody heavy chain and light chain, which were also recognised by the secondary antibody (see Figure 2-I).

Supernatants of CHO CCR5 cells lysed in RIPA buffer were either mixed with an equal volume of 2xRSB (lysates) or lysate containing 0.5 mg of protein was incubated with MC-5 (7.5 μg) and immunocomplexes captured on protein A-sepharose beads (1.5 h, 4°C) before being eluted by resuspension in RSB (IP:CCR5). Samples were either heated at 95°C or incubated at ambient temperature (RT) for 8 min, before equal volumes were loaded on a 10% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose by immunoblotting and probed for either CCR5 or clathrin heavy chain (C-HC). After subsequent incubation with IRDye® 800 GAM,
proteins were visualised using the Odyssey infra-red detection system.
HC, heavy-chain; LC, light-chain.

Protein electrophoresis
The method for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was based on that of Laemmli (1970). Minigels were cast with a 10% polyacrylamide resolving gel (pH 8.8) and a 4% polyacrylamide stacking gel (pH 6.8) (Protogel acrylamide solution [37.5:1 acrylamide:bis-acrylamide] and resolving/stacking gel buffer, Gene Flow, National Diagnostics, Fradley, UK; 0.1% w/v ammonium persulphate; and 0.1% v/v TEMED). Gels were run in a Hoefer SE260 mini-gel system (Hoefer Inc., San Francisco, California, USA) in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at 80 V while proteins migrated through the stacking gel and at 120 V when the proteins had entered the separating gel.

Coomassie staining
Gels were stained with 0.05% w/v Coomassie blue in 40% v/v methanol, 10% v/v acetic acid for 15 min at ambient temperature and destained for 2 h in 50% v/v methanol, 10% v/v acetic acid. Gels were dried on Whatman paper (Whatman, GE Healthcare Ltd., Little Chalfont, UK) using a gel drier (Bio-Rad Laboratories Ltd., Hemel Hemstead, UK).

Western blotting
Proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Whatman, Dassel, Germany) using a semi-dry transfer system (Bio-rad Laboratories Ltd., Hemel Hemstead, UK; transfer buffer consisting of 0.25 M Tris, 1.92 M glycine, 0.1% SDS and 20 v/v methanol) run at 10 V for 40 min. Nitrocellulose membranes were blocked with blocking buffer (TBS containing 5% milk powder [Marvel, Spalding, UK]) for 30 min at ambient temperature before incubation with primary antibodies in blocking buffer containing 0.1% Tween20 overnight at 4°C. Blots were subsequently washed 3 times for 15 min in wash buffer (TBS containing 0.1% Tween20) and then

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4 Primary antibodies and concentrations at which they were used are listed in Table 2-II.
incubated with secondary antibody in wash buffer for 1 h, all at ambient temperature. Blots were either incubated with horseradish peroxidase (HRP)–conjugated secondary antibodies (Pierce, Perbio Science UK Ltd., Cramlington, UK) or fluorophore–conjugated secondary antibodies compatible with the Odyssey infra-red detection system (Li-cor Biosciences, Lincoln, Nebraska, USA). Following this, blots were washed 3 times for 15 min in wash buffer before a final rinse in PBS, all at ambient temperature. Blots incubated with HRP-conjugated secondary antibodies were developed by 5 min incubation with either Super Signal® West Pico Chemiluminescent Substrate or Super Signal® West Dura Extended Duration Substrate (Pierce, Perbio Science UK Ltd., Cramlington, UK). Chemiluminescent films (Amersham Bioscience, Little Chalfont, UK) were exposed to the blot in a film cassette and developed in an X-ograph X-ray film developer (Xograph Imaging Systems, Tetbury, UK).

Blots incubated with Odyssey-compatible secondary antibodies were scanned in an Odyssey infra-red scanner (Li-cor Biosciences, Lincoln, Nebraska, USA) at either 680 nm or 800 nm. Band intensities were quantified using the Odyssey software, by defining a rectangular region around each band and summing the fluorescence intensities of each pixel corrected for the average background pixel intensity value. The average background pixel intensity value was calculated individually for each band from the average pixel intensity of pixels within a distance of 3 pixels above and below the defined region.

Details of specific assays

Co-immunoprecipitation of β-arrestins with CCR5

CHO K1, CHO CCR5, CHO CCR5 βarr1–YFP or CHO CCR5 βarr2–GFP cells were grown in 9 cm dishes for 2 d to 80% confluency. Cells were washed in 5 ml of BM at 37°C and incubated for various time periods with 125 nM RANTES in BM at 37°C. Some dishes containing cells were also subsequently washed 4 times with ice-cold BM and re-incubated with 400 nM TAK-779 in BM at 37°C for 60 min. After the incubation period, the dishes containing

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5 Details of secondary antibodies and concentrations at which they were used are given in Table 2–II.
the cells were transferred to ice and washed twice with ice-cold PBS before adding 5 ml of ice-cold PBS to each dish. Four hundred microlitres of 25 mM dithiobissuccinimidylpropionate (DSP) in DMSO was added to each dish (final concentration 2 mM) and cells were incubated at 4°C for 2 h. The cross-linking reaction was then stopped by adding 100 µl of 1 M Tris, pH 7.5 to each dish and further incubating for 15 min at 4°C. Cells were then washed twice with ice-cold PBS, lysed in RIPA buffer and immunoprecipitations were carried out as described above.

**CCR5 degradation**

Cells from a confluent 9 cm dish were detached with trypsin/EDTA, seeded into wells of a 6-well plate at a 1:30 dilution per well and grown for 24 h. Cells were incubated in 100 µg/ml cycloheximide (CHX) with or without 125 nM RANTES in BM at 37°C for various time-periods, after which plates were placed on ice, washed twice with ice-cold PBS, lysed in 200 µl of RIPA buffer and supernatants obtained as described above. Equal volumes of cell lysates from the different time-points were mixed with 2xRSB, run on a gel and blotted for CCR5.

**Immunofluorescence**

**Basic fixation and staining protocol**

Cells on coverslips were fixed in 3% paraformaldehyde (PFA) for 20 min on ice. All subsequent washes and incubations were carried out at ambient temperature. Cells were washed in PBS and free aldehyde groups quenched by incubation in 50 mM NH₄Cl for 20 min. Cells were again washed with PBS and incubated with blocking buffer (0.2% gelatine, 0.05% saponin in PBS) for 20 min to block non-specific sites and permeabilise cellular membranes. Cells were then incubated in blocking buffer containing primary antibodies for 1 h at ambient temperature, followed by 3 washes of 5 min in blocking buffer, before being incubated with secondary antibodies in blocking buffer.

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6 Details of primary antibodies and the concentrations at which they were used are given in Table 2-II.
7 Details of secondary antibodies and the concentrations at which they were used are given in Table 2-II.
for 45 min. Finally, cells were washed 3 times in blocking buffer and then once in PBS before rinsing in water and mounting in Mowiol (Calbiochem, La Jolla, USA) on a microscope slide.

**Image capture and processing**

**Epifluorescence microscopy**

Mounted cells on coverslips were viewed at ambient temperature using a 63x oil immersion lens on a Zeiss Axioskop microscope (Zeiss, Welwyn Garden City, UK) and 8-bit images captured using a CCD camera operated through the Openlab software (Improveion, Coventry, UK). Images were exported as TIFF files and processed in ImageJ (NIH) before being assembled into figures using Adobe Illustrator.

**Confocal fluorescence microscopy**

Mounted cells on coverslips were imaged at ambient temperature using a 63x oil immersion lens on a Leica TCS SPE confocal microscope (Leica Microsystems (UK) Ltd., Milton Keynes, UK). Eight-bit images were acquired using the Leica software, either taking single confocal sections (1 Airey unit) or stacks of confocal sections (step size 0.5 μm). Images were exported from the software as TIFF files and processed in ImageJ (NIH); here, projection images were formed from stacks of confocal sections. Images were assembled into figures using Adobe Illustrator.

** Trafficking assays**

**Preparation of cells**

Cells were detached from a confluent 9 cm dish using trypsin/EDTA and seeded at a 1:20 dilution onto glass coverslips placed in a new 9 cm dish and grown for 2 d to reach approximately 50% confluence. Cells that had been transfected using the FuGENE HD protocol (see Transfections) were detached from 6-well plates 24 h post-transfection and re-seeded onto glass coverslips in wells of a new 6-well plate at a 1:10 dilution and grown for 1 d to reach approximately 50% confluence.
Pre-labelling of cell surface CCR5
Cells grown on coverslips were transferred to 4–well plates (Nunc, Gibco/Invitrogen, Paisley, UK) and washed twice in BM at ambient temperature and then incubated with 1:500 MC–5 hybridoma supernatent in BM at room temperature for 40 min. Unbound antibody was then removed by 3 washes with BM at ambient temperature before a trafficking assay was performed.

Pre-clearing of biosynthetic CCR5 with cycloheximide
Cells grown on coverslips in 9 cm dishes were incubated with 100 µg/ml cycloheximide (CHX) in their normal growth medium for 2 h at 37°C. Cells on coverslips were then transferred to 4–well plates (Nunc, Gibco/Invitrogen, Paisley, UK) and washed twice in BM at ambient temperature before a trafficking experiment was performed. One hundred micrograms per millilitre CHX was included in the BM used for all subsequent steps throughout the trafficking assay.

Disruption of microtubules with nocodazole
Cells grown on coverslips in 9 cm dishes were incubated with 1 µg/ml nocodazole in their normal growth medium for 3 h at 37°C (control cells were incubated with a 1:1000 dilution of DMSO [the solvent for nocodazole] at all stages instead of nocodazole). Cells on coverslips were then transferred to 4–well plates (Nunc, Gibco/Invitrogen, Paisley, UK) and washed twice in BM at ambient temperature before a trafficking experiment was performed. One microgram per millilitre nocodazole was included in the BM used for all subsequent steps throughout the trafficking assay.

CCR5 down-modulation
Cells grown on coverslips were either untreated or pre-treated as described above. The cells were then incubated in 4–well plates (Nunc, Gibco/Invitrogen, Paisley, UK) for various time periods with 125 nM RANTES, MIP–1α or MIP–1β in BM at 37°C to induce CCR5 internalisation. At the end of the time period, cells were placed on ice and washed twice with ice-cold
BM followed by two washes in ice-cold PBS, before being fixed and stained as described above.

The effect of various drugs on CCR5 internalisation

CHO CCR5 cells grown on coverslips were incubated in BM containing either MC-5 and 100 μM dynasore monohydrate, 100 μM Bis–T-23, 40 μM Dyngo-4a or an appropriate dilution of DMSO for 20 min at 37°C. Alternatively, cells were incubated for 15 min in BM containing MC-5 at 37°C followed by a 5 min incubation in BM containing either 100 μM Bis–T-23, 40 μM Dyngo-4a or an appropriate dilution of DMSO at 37°C. In both cases, cells were then washed and incubated with 125 nM RANTES in BM containing either 100 μM dynasore monohydrate, 100 μM Bis–T-23, 40 μM Dyngo-4a or an appropriate dilution of DMSO for either 30 or 40 min at 37°C. Cells were then placed on ice and washed twice with ice-cold BM followed by two washes in ice-cold PBS, before being fixed and stained as described above.

CCR5 recycling

Cells that had been treated with RANTES for 60 min were transferred onto ice and washed rapidly 4 times with ice-cold BM. Ice-cold BM containing 400 nM TAK-799 was added to the cells before returning them to 37°C and incubating for either 30 or 60 min. After this incubation, the cells were placed on ice and washed twice with ice-cold BM followed by two washes in ice-cold PBS, before being fixed and stained as described above.

The effect of various drugs on CCR5 recycling

Cells were assayed for recycling as described above, with the inclusion in the BM containing 400 nM TAK-779 of either 10 μg/ml BFA, 100 μM dynasore monohydrate, 100 μM Bis–T-23, 40 μM Dyngo-4a or an appropriate dilution of ethanol (BFA) or DMSO (dynasore monohydrate, Bis–T-23 and Dyngo-4a) as a control.
Chapter 2: Materials and Methods

The effect of BFA on γ-adaptin distribution

CHO CCR5 cells grown on coverslips were either untreated or treated with 1, 10 or 100 μg/ml BFA in BM at 37°C for 1 or 5 min. After the incubations, the cells were placed on ice and washed twice with ice-cold BM followed by two washes in ice-cold PBS, before being fixed and stained as described above.

Lysosomal degradation assay

Cells were seeded onto coverslips in 9 cm dishes as described above and grown for 32 h. One hundred micromolar leupeptin, 1 μM pepstatin and 10 μM E64 were then added to the growth medium and the cells incubated for a further 16 h. Cells on coverslips were then transferred to 4-well plates and washed twice with BM at ambient temperature. Some cells were fixed at this stage as described above and stained for total CCR5 and the lysosomal marker, lgp-b. Others were pre-labelled for cell surface CCR5 and then assayed for CCR5 internalisation and recycling, all as described above, except that 100 μM leupeptin, 1 μM pepstatin and 10 μM E64 were added to the BM during CCR5 surface labelling and both RANTES and TAK-779 incubations.

FACS analysis

Basic procedure for CCR5 trafficking assay

CHO CCR5 cells were grown in 9 cm dishes for 2 d to reach a confluency of 80%. Cells were washed with PBS pre-warmed to 37°C and then detached by incubation with 10 mM EDTA in PBS at 37°C (trypsin was omitted from the detachment step to avoid cleavage of extracellular domains of CCR5). Detached cells were diluted in PBS at 37°C, pelleted by centrifugation (3 min, 800 rpm), resuspended in 2 ml of binding medium at 37°C and transferred to a 37°C water-bath.

A 50 μl aliquot of the cell suspension was removed and transferred to a 96-well plate (Nalgene, Loughborough, UK) on ice, prepared with 100 μl of cold binding medium in each well, to serve as a 0 min time-point. 50 μl aliquots of cell suspension were also removed at this stage to later determine non-
specific secondary antibody binding (where the directly-coupled 488MC-5 antibody was used, non-specific antibody binding was assessed on a similar number of CHO-K1 cells). To elicit agonist-stimulated internalisation of CCR5, 0.5 ml aliquots of cell suspension were added to 0.5 ml of binding medium containing 250 nM RANTES in an assay tube (5 ml polystyrene round-bottom tube, Falcon, BD Biosciences, Oxford, UK) to achieve a final RANTES concentration of 125 nM. One hundred microlitre aliquots of RANTES-treated cell suspension were removed at various time-points over the course of 60 min and transferred to the 96-well plate on ice. After 60 min, the assay tube containing RANTES-treated cells was transferred to ice and 4 ml of ice-cold binding medium added to inhibit further trafficking events. Cells were pelleted by centrifugation (5 min, 1200 rpm) and washed in 5 ml of binding medium before being pelleted again, resuspended in ice-cold binding medium containing 400 nM TAK-779, and returned to the 37°C water-bath. 100 μl aliquots of TAK-779-treated cell suspension were transferred to the 96-well plate on ice at various time points up to 120 min. After the final aliquot had been removed, cells in the 96-well plate were pelleted by centrifugation and washed with ice-cold binding medium. Cells were then incubated with 1 μg/ml purified MC-5 or 488MC-5 for 1 h at 4°C on a reciprocal shaker (controls to determine non-specific secondary antibody binding on CHO CCR5 cells were incubated with 1 μg/ml anti-γ-adpatin [mouse IgG2a, BD Biosciences] instead of MC-5 primary antibody). Cells incubated with MC-5 were washed with ice-cold binding medium and further incubated with a GAM Alexa Fluor® secondary antibody in ice-cold FACS wash buffer (1% FCS, 0.05% NaN₃ in PBS) for 1 h at 4°C on a reciprocal shaker. Cells were then washed 3 times with ice-cold FACS wash buffer and then fixed overnight in FACS wash buffer containing 1% PFA at 4°C. Fixed cells were washed once with ice-cold FACS wash buffer before being transferred to mini FACS tubes (Falcon, BD Bioscience, Oxford, UK) for measurement of cell-associated fluorescence with a FACS Caliber flow cytometer (Becton Dickinson UK Ltd., Oxford, UK). Unless otherwise specified, 10,000 events were counted for each condition. Events representing cells were selected using a gate based on the forward- and side-scatter characteristics of the events.
Details of specific assays

The effect of β-arrestin2 and β-arrestin2 mutants on CCR5 trafficking

CHO CCR5 cells were transfected in 6-well plates according to the FuGENE transfection protocol detailed above. One day post-transfection, cells were detached with trypsin/EDTA and the contents of 2 wells re-seeded into one 9 cm dish and grown for 1 d to achieve cells at 80% confluency for the trafficking assay. The trafficking assay was performed as described above, using a GAM Alexa 647 antibody to detect MC–5. 15,000 events were counted and transfected cells were identified based on their GFP fluorescence relative to a sample of cells that had been treated with the FuGENE transfection reagent only. Subsequent analysis of CCR5–associated cell surface fluorescence was performed on the subset of transfected cells. An estimate of transfection efficiency was also obtained by comparison of the GFP fluorescence distribution of transfected cells with cells treated with the FuGENE reagent only.

The effect of various drugs on CCR5 recycling

CHO CCR5 cells were prepared and treated with RANTES as above. After 60 min RANTES treatment, cells in assay tubes were washed twice with ice-cold BM then re-suspended in ice-cold binding medium containing either 10 μg/ml BFA or 100 μM dynasore monohydrate, or as a control, a 1:1000 dilution of either ethanol or DMSO, the solvents for BFA and dynasore monohydrate, respectively. Cells were then transferred to the 37°C water-bath and 100 μl aliquots removed at various time-points up to 60 min. Cells treated with BFA were labelled with 488MC–5; those treated with dynasore were labelled with MC–5 and GAM Alexa 647, as it was found that the dynasore molecule has an intrinsic fluorescence when stimulated with the 488 nm laser.
FACS data analysis

Graphical representation of FACS data

Cell surface fluorescence corrected for non-specific antibody binding was normalised to the cell surface fluorescence for untreated cells, expressed as a percentage, and plotted against time.

Calculation of CCR5 trafficking rates

Initial rates of internalisation \( (r_{\text{int}}) \) and recycling \( (r_{\text{rec}}) \) were calculated on the background-corrected, normalised cell surface fluorescence values, as follows:

\[
\begin{align*}
 r_{\text{int}} (% \text{ min}^{-1}) &= \frac{100 - fl_{10 \text{ min}}}{10} \\
r_{\text{rec}} (% \text{ min}^{-1}) &= \frac{fl_{70 \text{ min}} - fl_{60 \text{ min}}}{10}
\end{align*}
\]

, where \( fl_t \) = cell surface fluorescence at the indicated time-point (t).

A rate constant for recycling \( (k_{\text{rec}}) \) was calculated by fitting the recycling curve (60 min → 180 min) to a first-order rate equation, where the rate of recycling is proportional to a single, first-order rate constant and the difference between the maximum cell surface fluorescence reached after recycling and the cell surface fluorescence at any time-point. Algebraically,  

\[
\frac{dfl}{dt} = k_{\text{rec}}(fl_{\text{max}} - fl)
\]

, where \( fl \) = cell surface fluorescence, \( fl_{\text{max}} \) is the asymptotic value towards which cell-surface fluorescence tends over time, and \( t = \text{time} \).

Rearranging and integrating yields the equation:

\[
-ln|fl_{\text{max}} - fl| = k_{\text{rec}}t + \text{constant}
\]

Where, \( t=0, fl = fl_0 \),

\[
\therefore \text{constant} = -ln|fl_{\text{max}} - fl_0|
\]
Which yields the equation describing the recycling curve:

\[
fl = f_{\text{max}} (1 - \frac{f_{\text{max}} - f_0}{f_{\text{max}}} e^{-kt})
\]

This linearises to give:

\[
\ln \left| \frac{f_{\text{max}} - fl}{f_{\text{max}}} \right| = -k_{\text{rec}} t + \ln \left| \frac{f_{\text{max}} - f_0}{f_{\text{max}}} \right|
\]

This is of the form \( y = mx + c \), and thus, the slope of the straight line from a plot of \( \ln \left( (f_{\text{max}} - fl)/f_{\text{max}} \right) \) against \( t \) gives the rate constant \( k_{\text{rec}} \).
Chapter 3: MORPHOLOGICAL OUTLINE OF CCR5 TRAFFICKING

"Change we can believe in."
Senator Barack Obama, slogan for US Democratic Party Presidential nomination campaign

The trafficking itinerary of CCR5 has been best described in Chinese hamster ovary cells (CHOs), where it has been demonstrated that agonist-activated receptors undergo clathrin-mediated endocytosis and traffic to perinuclear recycling endosomes, whence they can recycle back to the cell surface (Signoret, Pelchen-Matthews et al. 2000; Signoret, Hewlett et al. 2005). Here, principally through morphological techniques, I expand this description of the intracellular trafficking of CCR5 in CHO cells and present a model where CCR5 traffics through a recycling compartment comprising both recycling endosome and trans-Golgi network (TGN) elements. Finally, I extend the analysis of CCR5 intracellular trafficking to a human cell line, which may be more amenable to RNAi studies in the future.

**CHO CCR5 cells as a system for studying CCR5 trafficking**

Since the initial functional expression in 1995 of the newly-cloned human CCR5 gene in CHO-K1 cells by the group of Marc Parmentier (Samson, Labbe et al. 1996), CHO cells have been a popular system for studying CCR5 function. The CHO cells stably expressing human CCR5 (CHO CCR5 cells)
used for experiments described in this thesis have been extensively
employed in studying CCR5 function and in generating our current picture
of agonist-activated CCR5 trafficking (Mack, Luckow et al. 1998; Signoret,
Pelchen-Matthews et al. 2000; Signoret, Christophe et al. 2004; Signoret,
Hewlett et al. 2005).

Although chemokine receptors are not naturally expressed in CHO cells, the
system has several practical advantages over cells that endogenously
express the receptor, e.g. primary cells or leukocyte cell-lines. Apart from
being easy to culture, CHO cells maintain high levels of chemokine receptor
expression, an advantage with respect to detection of CCR5 in commonly
used trafficking assays in comparison with primary cells and leukocyte cell-
lines, where endogenous receptor levels are far lower. Moreover, CCR5 has
been shown to undergo internalisation and recycling in CHO CCR5 cells with
similar kinetics to those exhibited in lymphocytes/monocytes, where the
receptor is endogenously expressed (Mack, Luckow et al. 1998), suggesting
that, at least on a basic level, physiological CCR5 trafficking is faithfully
recapitulated in the CHO cell system. Finally, removing CCR5 from its
physiological background, reduces the complicating effects of interactions
with other chemotactic receptors, such as that demonstrated with the C5a
receptor (C5aR), where the activation of one receptor has been shown to
influence the trafficking of the other through cross-phosphorylation and/or
hetero-oligomerisation (Huttenrauch, Pollok-Kopp et al. 2005). Although
physiologically relevant interactions and cross-regulation by other
chemoattractant receptors that may be engaged simultaneously by multiple
mediators present at inflammatory sites will ultimately have to be
considered when developing a fully integrated model for CCR5 trafficking,
simple systems are necessary initially to understand the fundamental
properties of the receptor.

One distinct disadvantage of the CHO cell system is that the hamster
genome has not yet been sequenced, making RNAi knock-downs, which are
routine in cell-lines from species with sequenced genomes (e.g. human cell-
lines) difficult to perform, and also meaning that reagents generated against
proteins from other species with known sequences, such as antibodies,
must be rigorously tested to ensure specificity.
The CHO cells used in this thesis express an average of 100–200,000 copies of CCR5 per cell, with the majority present on the cell surface in unstimulated cells, although a very small intracellular pool can be observed in the perinuclear region by immunofluorescence in some cells. This intracellular pool can be cleared by treatment with cycloheximide (CHX), an inhibitor of protein translation, and probably represents newly synthesised CCR5 passing through the biosynthetic route towards the cell surface (data not shown). Thus, for some of the experiments described below, cells were incubated with CHX before and during an immunofluorescence-based trafficking assay to ensure that only receptors initially localised to the cell surface were followed.

Another method routinely used for the same purpose was to pre-label cell surface receptors by incubating cells in binding medium containing MC-5, an antibody that recognises the extreme N-terminus of CCR5 (Blanpain, Vanderwinden et al. 2002), for 40 min at room temperature. These conditions were found to result in negligible internalisation of antibody-bound receptors, but resulted in sufficient binding of antibody to be easily detected (data not shown). MC-5 does not interfere with agonist (RANTES, MIP-1α and MIP-1β) binding, or internalisation and recycling of CCR5 (Blanpain, Vanderwinden et al. 2002). MC-5 binds specifically to CCR5 with very little non-specific binding to other cellular epitopes (see Chapter 2) and, importantly, has a high affinity for the receptor ($K_d \approx 1 \text{ nM}$, see Chapter 2), so remains bound to CCR5 as it traffics within the cell, as evidenced by immunofluorescence of cell surface receptors before and after internalisation and recycling (data not shown).

All trafficking assays described in this thesis were performed using cells incubated in an RPMI-based binding medium. For consistency, the CCR5 agonist, RANTES (regulated on activation normal T cell secreted; also known as CCL5) was used for most of the trafficking assays, at a concentration of 125 nM. This is a saturating concentration of RANTES, used to achieve as near a synchronous wave of CCR5 internalisation as possible (Signoret, Hewlett et al. 2005). In all experiments, the binding medium was adjusted to pH 7, since RANTES has been shown to form high-order oligomers above this pH, which may influence binding to CCR5 (Duma, Häussinger et al. 2006). Incubation in BM or pre-labelling CCR5 with MC-5 did not induce
down-modulation of the receptor itself, as shown in Figure 3–I A. Here, CHO CCR5 cells were pre-labelled for surface CCR5 with \(^{125}\text{I} \text{MC-5}\), which, like unconjugated MC-5, has a high affinity for the receptor (K\(_d\) ~ 4.7 nM, see Chapter 2) and incubated in BM at 37°C for the indicated time-periods before being fixed. After 60 min or 120 min of incubation in BM, CCR5 remained principally localised to the cell surface. A small amount of receptor probably underwent constitutive endocytosis by bulk-flow during the incubation, but this was presumably recycled efficiently, since no intracellular pool of CCR5 accumulated during the incubation.

**Internalised, agonist-activated CCR5 passes through early endosomes**

Agonist-activated CCR5 has been shown to colocalise with the early endosome marker, EEA1, shortly after agonist addition, suggesting that internalised receptors are initially targeted to early endosomes (Signoret, Hewlett et al. 2005). I initially sought to confirm this observation in RANTES-treated CHO CCR5 cells. Figure 3–I B shows CHO CCR5 cells that were pre-labelled for cell surface CCR5 with MC-5 and treated with RANTES for either 5 min or 60 min before being fixed and co-stained for EEA1. In unstimulated cells, EEA1 was present on punctate structures throughout the cytoplasm, a pattern expected for early endosome staining. After 5 min RANTES treatment, CCR5 was found distributed diffusely on the cell surface as well as in punctate structures at or just under the cell surface. Some of these puncta at the cell surface presumably represent activated receptors that had clustered in clathrin-coated pits (CCPs) or flat clathrin lattices previously observed in CHO cells by 'rip-off' electron microscopy (Signoret, Hewlett et al. 2005). The CCR5-positive puncta just under the plasma membrane may represent clathrin-coated vesicles (CCVs). CCR5 was also observed in punctate structures that colocalised with EEA1 in the periphery of the cell, suggesting that CCR5 is targeted to early endosomes soon after internalisation.
Chapter 3: Morphological Outline of CCR5 Trafficking

A.

<table>
<thead>
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<th>binding medium</th>
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Figure 3.1 Agonist-activated CCR5 traffics through early endosomes

A. CHO CCR5 cells were pre-labelled for cell surface CCR5 with ³⁵S-MC-5 and then incubated for the indicated time-periods in binding medium (BM) at 37°C before being fixed. B. CHO CCR5 cells were pre-labelled for cell surface CCR5 with MC-5 and then incubated for the indicated time-periods in BM containing RANTES at 37°C. Cells were then fixed and incubated in permeabilising blocking buffer with anti-EEA1. CCR5 and EEA1 were detected with Alexa 488 GAM IgG2a and Alexa 594 GAM IgG1, respectively. Single confocal sections are shown. Bars, 5 µm
It has been previously shown that after 60 min of continuous RANTES stimulation, CCR5 establishes a new steady-state distribution, determined by the rates of internalisation and recycling (receptors that recycle to the plasma membrane re-internalise due to the presence of agonist in the medium) (Mack, Luckow et al. 1998). After 60 min RANTES treatment, CCR5 had accumulated in perinuclear structures that were largely negative for EEA1 staining, suggesting that CCR5 moves through early endosomes and traffics to a distinct perinuclear compartment. However, a small amount of EEA1 staining did colocalise with CCR5 in the perinuclear region, so some of this perinuclear CCR5 may retain an early endosomal character. A small amount of punctate CCR5 staining was still visible in the rest of the cytoplasm after 60 min, much of this staining colocalising with EEA1, suggesting that at steady-state, at least some of the internal receptor population is found in more peripherally-located early endosomes. A small amount of CCR5 was also observed on the cell surface.

In the constant presence of agonist, CCR5 accumulates in a perinuclear, TfR-positive compartment

Under constant agonist treatment, CCR5 was found, by electron microscopy observations of immunolabelled cryosections, to accumulate in a tubulo-vesicular, perinuclear compartment that was positive for steady-state transferrin receptor (TfR) (Signoret, Pelchen-Matthews et al. 2000). Moreover, CCR5 was previously found to show a good overlap with steady-state TfR staining in RANTES-treated CHO cells by immunofluorescence (Mack, Luckow et al. 1998). In CHO cells, it has been shown that the vast majority TfR population is located in recycling endosomes, making steady-state TfR staining a good marker of recycling endosomes (Yamashiro, Tycko et al. 1984). Therefore, these observations suggested that CCR5 accumulated in recycling endosomes under conditions of constant agonist stimulation.

Figure 3-11 shows the results of an immunofluorescence experiment designed to verify these observations, where CHO CCR5 cells were prelabelled for cell surface CCR5 with MC-5. In untreated cells, the TfR population was often clustered in the perinuclear region but there was
significant punctate staining observed throughout the rest of the cytoplasm. CCR5 was present over the whole of the cell surface, including tubular membrane protrusions, presumably microvilli, which can be seen in the maximum intensity projection image of unstimulated cells. CCR5 labelling on microvilli has also been observed in whole-mount electron microscopy studies of CHO CCR5 cells (Signoret, Hewlett et al. 2005) and CCR5 has been shown to preferentially localise to microvilli in human macrophages and T cells, and also when expressed in HeLa cells (Singer, Scott et al. 2001).

In cells treated with RANTES for 60 min, CCR5 accumulated in a perinuclear compartment that almost completely colocalised with steady-state TfR, suggesting that receptors had accumulated in recycling endosomes. It is noteworthy that the TfR distribution appeared to be more focussed around the nucleus after 60 min RANTES treatment. A plausible explanation for this is that a large amount of flux through the recycling endosome modifies its gross morphology. Alternatively, signalling from activated CCR5 molecules may lead to a modification of the compartment.

To confirm the ability of CCR5 to recycle in CHO CCR5 cells, some cells were treated with RANTES for 60 min to induce down-modulation of the receptor and then subsequently incubated in BM containing the CCR5 antagonist, TAK-779. TAK-779 has been reported to inhibit the binding of RANTES to CHO CCR5 cells with an IC₅₀ of 1.4 nM and to inhibit chemokine-induced Ca²⁺ mobilisation (Baba, Nishimura et al. 1999). Moreover, we have demonstrated that TAK-779 is capable of efficiently displacing RANTES bound to CCR5 (N. Signoret, unpublished data). It has previously been demonstrated that CCR5 molecules are capable of recycling back to the cell surface in an agonist-bound state (Signoret, Pelchen-Matthews et al. 2000); in this case, the receptors can engage the endocytic machinery and re-internalise. The addition of TAK-779 in the medium during recycling prevents re-internalisation of receptors that have recycled back to the cell surface by displacing agonists bound to the receptors.

In cells that had been treated with RANTES for 60 min, almost all of the CCR5 returned to the cell surface after 60 min incubation in the presence of TAK-779. This is in contrast to cells that had been treated with RANTES for a total of 120 min, where the CCR5 distribution was very similar to cells that
had been treated with RANTES for 60 min. Thus, apparently, the receptor will continue to cycle over the cell surface in the constant presence of RANTES for at least 120 min without significant alteration of internalisation or recycling rates, or diversion to a different pathway.

Figure 3-II In the constant presence of agonist, CCR5 accumulates in a perinuclear, TfR-positive compartment

CHO CCR5 cells were pre-labelled for cell surface CCR5 with MC-5 and then incubated for the indicated time-periods in BM containing RANTES at 37°C, or incubated with BM containing RANTES for 60 min followed by binding medium containing TAK-779 for 60 min at 37°C. Cells were then fixed and incubated in permeabilising blocking buffer with anti-TfR (H68.4). CCR5 and TfR were detected with Alexa 488 GAM IgG2a and Alexa 594 GAM IgG1, respectively. Maximum intensity projections of stacks of confocal sections are shown in the left panel. A
single confocal section at approximately the same horizontal plane through the cells in each condition is also shown. Bars, 5 μm

Interestingly, after CCR5 had recycled to the plasma membrane, the TfR distribution appeared to revert back to a distribution similar to that in unstimulated cells, where it was less concentrated around the nucleus.

**There is no agonist-induced degradation of CCR5**

Agonist binding targets many GPCRs for lysosomal degradation (Marchese, Paing et al. 2007). The results presented in Figure 3–II suggest that the majority of internalised, agonist-activated CCR5 recycles back to the cell surface. However, they do not exclude the possibility that a small amount of CCR5 is targeted for degradation by RANTES treatment. Several studies have addressed whether CCR5 undergoes agonist-stimulated degradation by following total receptor levels in the presence of CHX, where new protein synthesis is blocked. Signoret et al. determined that CCR5 had a half-life of 6–9 h in CHO CCR5 cells and that treatment of cells with RANTES did not significantly affect the turnover of CCR5 after 6 h (Signoret, Pelchen-Matthews et al. 2000). Similarly, Delhaye et al. found that there was no agonist-induced degradation of CCR5 in HEK293 cells, but the turnover of CCR5 was far slower in this cell-line, with negligible loss of the receptor during 4 h incubation with CHX (Delhaye, Gravot et al. 2007).

To determine if there was any agonist-induced degradation of CCR5 in CHO CCR5 cells under the conditions used in my experiments, I incubated equal numbers of CHO CCR5 cells in BM containing CHX at 37°C for various time periods in the presence or absence of RANTES. Cell lysates were prepared and the proteins separated on a gel, transferred to nitrocellulose and probed for CCR5 (Figure 3–III). CCR5 migrated as a broad band, a characteristic feature of a glycosylated protein, with an apparent molecular mass of 30–35 kDa. The predicted molecular mass of the unprocessed protein is 40.6 kDa; the discrepancy in molecular mass is probably due to post-translational modifications, including tyrosine sulphation (Farzan, Chung et al. 2002) and O-linked glycosylation (Bannert, Craig et al. 2001; Farzan, Chung et al. 2002). The band shown on the blot represents CCR5
protein, since this band is absent from CHO-K1 cell lysates - where there is no CCR5 expressed - probed with MC-5 (data not shown). It was also noted that in the presence of RANTES, there was a decrease in the electrophoretic mobility of CCR5, which is most likely due to C-tail phosphorylation (Oppermann, Mack et al. 1999).

**Figure 3-III CCR5 turnover is not affected by RANTES-treatment**

Equal numbers of CHO CCR5 cells were incubated in BM containing CHX with or without RANTES at 37°C for the indicated time-periods, after which cell lysates were prepared. Proteins from equal volumes of lysates were separated on a gel, transferred to nitrocellulose and probed for CCR5 with MC-5 primary antibody. MC-5 was detected with IRDye® 800 GAM and visualised using the Odyssey infra-red detection system. Band intensities were quantified and the amount of CCR5, expressed as a percentage of the initial amount, plotted against time. Individual data points represent the mean of 6 independent samples from 2 independent experiments. Error bars represent 1 standard deviation of the means. Also shown is a representative blot, cropped for the bands representing CCR5.

As can be seen in Figure 3–III, there was no significant difference in the turnover of CCR5 in the presence of RANTES compared with untreated cells. In contrast to the estimated half-life calculated for CCR5 in CHO CCR5 cells by Signoret et al. (2000), here, CCR5 had a slightly longer estimated half-life of 10–12 h.
Degradation of CCR5 - agonist-stimulated or constitutive - would be expected to occur in lysosomes (Marchese, Paing et al. 2007). Signoret et al. (2000) assessed whether any agonist-activated CCR5 was routed to the lysosome by observing CHO CCR5 cells that had been treated with RANTES for 4 h and stained for CCR5 and the lysosomal markers, LBPA (lysobisphosphatidic acid) and Igp-b (lysosomal glycoprotein b, also known as LAMP-2). CCR5 did not colocalise with either lysosomal marker and, hence, it was suggested that there was no agonist-induced CCR5 degradation. However, in this experiment, cells were not treated with lysosomal protease inhibitors, so it is possible CCR5 could have been delivered to the lysosome but that the antibody epitope was rapidly degraded.

To address the possibility that agonist-activated CCR5 is targeted to lysosomes, I fed the lysosomal protease inhibitors, leupeptin, pepstatin and E64, to CHO CCR5 cells for 16 h to allow uptake of the protease inhibitors by fluid-phase endocytosis and delivery to the lysosome, before performing a trafficking assay. I firstly demonstrated that the CCR5 epitope recognised by the anti-CCR5 antibody, MC-5, was detectable in lysosomes under these conditions by fixing cells after this 16 h incubation and staining for CCR5 and the lysosomal marker, Igp-b in permeabilising conditions (Figure 3-IV A). In cells that were not treated with lysosomal protease inhibitors, CCR5 was predominantly present on the cell surface, whereas Igp-b staining was mainly punctate and cytoplasmic, although some larger ring structures were observed in confocal sections. In cells that had been incubated with the lysosomal protease inhibitors, Igp-b predominantly stained larger structures, suggesting that in the absence of protein degradation, lysosomes had increased in size. Moreover, CCR5 was found to colocalise with Igp-b, demonstrating that under these conditions, CCR5 that accumulates in lysosomes is detectable by immunofluorescence. The CCR5 that accumulated over the period of lysosomal protease inhibitor incubation represents the pool of CCR5 that would have been constitutively degraded in that time period. There did not appear to be any significant decrease in CCR5 surface fluorescence intensity relative to cells that were not treated with lysosomal protease inhibitors, suggesting that CCR5 synthesis proceeded normally in the absence of degradation.
### Chapter 3: Morphological Outline of CCR5 Trafficking

<table>
<thead>
<tr>
<th>60 min RANTES + 60 min TAK-779</th>
<th>60 min RANTES</th>
<th>unstimulated</th>
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<th>+ protease inhibitors</th>
<th>16 h incubation</th>
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<td>+ protease inhibitors</td>
<td>- protease inhibitors</td>
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**Images:**

- **CCR5**
- **Igp-b**
- **Merge**
Figure 3-IV Agonist stimulation does not target CCR5 to lysosomes
A. CHO CCR5 cells were either incubated in normal growth medium or growth medium containing lysosomal protease inhibitors (leupeptin, pepstatin and E64) for 16 h before being fixed. Cells were then permeabilised in blocking buffer and incubated with MC-5 and 3E9 primary antibodies against CCR5 and lgp-b, respectively. Primary antibodies were detected with Alexa 488 GAM IgG2a and Alexa 594 GAM IgG1, respectively. Single confocal sections are shown. Bar, 5 μm. B. CHO CCR5 cells were incubated in growth medium containing lysosomal protease inhibitors (leupeptin, pepstatin and E64) for 16 h. Cells were then labelled for cell surface CCR5 with MC-5. Some cells were then fixed (unstimulated), while others were treated with RANTES in BM for 60 min (60 min RANTES) or treated with RANTES for 60 min followed by a 60 min incubation in BM containing TAK-779 (60 min RANTES + 60 min TAK-779), before being fixed. Lysosomal protease inhibitors were included in all the incubations described. Cells were then permeabilised in blocking buffer and incubated with 3E9 primary antibody against lgp-b. Primary antibodies were detected with Alexa 488 GAM IgG2a (CCR5) and Alexa 594 GAM IgG1 (3E9). Single confocal sections are shown. Bar, 5 μm. C. A series of confocal sections from the 60 min RANTES-treated cells shown in B. The step size between sections is 0.5 μm. Also shown at the top of the montage is a maximum intensity projection of the confocal sections. Bars, 5 μm.

To determine whether any CCR5 was directed to lysosomes in an agonist-dependent fashion, cells were pre-incubated with lysosomal protease inhibitors, labelled for cell surface CCR5, and trafficking of the receptor followed by immunofluorescence with co-staining for lgp-b (Figure 3-IV B). In cells treated with RANTES for 60 min, CCR5 predominantly accumulated in punctate structures in the perinuclear region, often in close proximity, but not overlapping with lgp-b positive structures (Figure 3-IV C shows a stack of confocal sections through the group of 60 min RANTES-treated cells in B, which illustrate this point). Cells treated with RANTES for 60 min followed by a 60 min incubation with TAK-779 showed mainly cell surface CCR5 staining, demonstrating that down-modulated receptors had recycled back to the cell surface. These cells also showed no colocalisation of CCR5 with lgp-b. Altogether, these results demonstrate that there is no agonist-induced degradation of CCR5 in CHO CCR5 cells. Moreover, they show that pre-labelling cell surface CCR5 with MC-5 does not result in lysosomal targeting
of CCR5, which could occur, for instance, if the antibody were to cause cross-linking of receptors.

**Rab11 regulates CCR5 trafficking through the recycling endosome**

The above data support the view that internalised, agonist-activated CCR5 molecules traffic to perinuclear recycling endosomes, from where they recycle back to the cell surface. At present, there are few known markers for this perinuclear compartment, although there is strong evidence that the small GTPase, Rab11, is predominantly localised to recycling endosomes and regulates the traffic of several receptors through the compartment, including the TfR (Ullrich, Reinsch et al. 1996; Ren, Xu et al. 1998) and the chemokine receptor, CXCR2 (Fan, Lapierre et al. 2003; Fan, Lapierre et al. 2004).

To determine whether Rab11 plays a role in regulating CCR5 trafficking, I transiently transfected myc-tagged Rab11 wild-type and mutant constructs into CHO CCR5 cells and observed CCR5 trafficking by immunofluorescence (Figure 3-V). In unstimulated cells (Figure 3-V A), wild-type Rab11 displayed a largely perinuclear distribution, with some more dispersed punctate, cytoplasmic staining, suggesting that most of the Rab11 is membrane-bound. In some cells, wild-type Rab11 staining was seen in tight, perinuclear clusters, characteristic of clustering around the microtubule-organising centre (MTOC). An active-site mutant of Rab11, Q70L, which is incapable of hydrolysing bound GTP, so is constitutively active, was also expressed: this mutant adopted a distribution that was very tightly clustered in perinuclear accumulations, with far less punctate, cytoplasmic staining. In contrast, a Rab11 mutant that is locked in the GDP-bound form, S25N, showed a more disperse staining pattern, which was, nevertheless, punctate. These observations are similar to those of Ullrich et al. (1996), who expressed similar Rab11 mutants in CHO and BHK (baby hamster kidney) cells. The authors made the further observation that although the distribution of the Rab11 mutants differed from wild-type Rab11 by immunofluorescence observation, the morphology of the compartments labelled by the transiently-expressed Rab11 proteins was
indistinguishable by EM, suggesting that both Rab11 mutants localise to recycling endosomes and alter the positioning rather than the morphology of compartment.

After 60 min RANTES treatment (Figure 3-V B), CCR5 down-modulated in cells expressing each of the Rab11 constructs. In cells expressing wild-type Rab11, CCR5 accumulated in the perinuclear region and showed a partial colocalisation with Rab11 staining in this area, suggesting that at least some of the CCR5 was present in Rab11-positive recycling endosomes. In cells expressing Rab11 Q70L, this overlap was increased, with almost all the CCR5 colocalising with Rab11 Q70L in large perinuclear clusters. In contrast, in cells expressing Rab11 S25N, CCR5 largely failed to cluster around the nucleus and did not colocalise with Rab11 S25N, which, like in unstimulated cells, had a punctate staining pattern that was present throughout the cytoplasm.

The effect of Rab11 over-expression and mutant Rab11s on CCR5 recycling was assessed by treating cells in which CCR5 had been down-modulated with RANTES, with TAK-779 for 60 min (Figure 3-V C). Ordinarily, CCR5 recycles back to the plasma membrane under these conditions (Figure 3-II) and in cells that were not expressing the exogenous Rab11 proteins on the same coverslips as transfected cells, CCR5 cell surface staining was recovered. In cells over-expressing wild-type Rab11, CCR5 also fully recycled to the cell surface. In cells expressing Rab11 S25N, most of the CCR5 recycled back to the plasma membrane, although a small amount of internal CCR5 was frequently observed, suggesting that the rate of recycling was slightly slower in these cells. Strikingly, in cells expressing Rab11 Q70L, although some CCR5 returned to the cell surface, a large amount was retained in perinuclear clusters that stained for Rab11 Q70L, demonstrating a significant inhibitory effect of constitutively active Rab11 on CCR5 recycling.

The increased overlap and concentration of internalised CCR5 in the perinuclear region in the presence of increased levels of Rab11:GTP and failure of agonist-activated CCR5 to colocalise with Rab11:GDP (or cluster in the perinuclear region in the presence of high levels of Rab11:GDP), suggest that the GTP-bound form of Rab11 is required for transport of CCR5 to recycling endosomes. The dispersed CCR5 staining pattern in cells
expressing Rab11 S25N treated for 60 min with RANTES, which does not overlap with Rab11 S25N, presumably represents CCR5 that has failed to traffic to the recycling endosome and may be retained in early endosomes, as appears to be the case for the TfR in cells expressing Rab11 S25N (Ullrich, Reinsch et al. 1996). That there is a significant inhibitory effect of Rab11 Q70L expression on CCR5 recycling suggests that hydrolysis of Rab11-bound GTP to GDP is required for trafficking of CCR5 from the recycling endosome to the cell surface, again consistent with the mechanism of Rab11 functioning in TfR trafficking (Ren, Xu et al. 1998).
Figure 3-V Rab11 regulates CCR5 trafficking

CHO CCR5 cells were transfected with myc-tagged Rab11 constructs with FuGENE
HD. Cells were pre-labelled for cell surface CCR5 with MC-5 and then either fixed (A), treated with RANTES in BM for 60 min and then fixed (B), or treated with RANTES in BM for 60 min followed by an incubation in BM containing TAK-779 for 60 min, before being fixed (C). Cells were then permeabilised in blocking buffer and incubated with 9E10 anti-myc primary antibody, followed by incubation with Alexa 488 GAM IgG2a and Alexa 594 GAM IgG1 to detect MC-5 (CCR5) and myc (Rab11), respectively. Single confocal sections are shown. Bars, 5 μm.

**Down-modulated CCR5 partially colocalises with endogenous Rab11**

Although the above results demonstrate that Rab11 affected the passage of CCR5 through the recycling endosome, only a partial overlap of CCR5 with over-expressed wild-type Rab11 was observed after 60 min RANTES treatment. It is possible that this overestimates the colocalisation of CCR5 with Rab11 after RANTES treatment as over-expression of Rab proteins, which are regulators of membrane trafficking, may alter intracellular trafficking on a gross level, with, for instance, effects on the steady-state size of membrane-bound organelles. Therefore, I stained cells for endogenous Rab11 and assessed colocalisation with RANTES-activated CCR5 by immunofluorescence (Figure 3–VI). Here, I pre-treated cells with CHX to clear any internal CCR5 from the biosynthetic pathway, stimulated with RANTES for 60 min (in the continued presence of CHX) and then stained for CCR5 and Rab11 post-fixation. The commercially-available Rab11 antibody used was specific for Rab11, since no staining was observed on Rab11 knock-down cells (R. Prekeris, personal communication).

In unstimulated cells, Rab11 staining was punctate and largely confined to the perinuclear region, with some smaller puncta visible throughout the rest of the cytoplasm. CCR5 was restricted to the cell surface, with no internal staining seen, suggesting that any CCR5 present inside cells had been chased through the biosynthetic export route. Again, CCR5 was found to decor an cell membrane protrusions, highly likely to be microvilli. After 60 min RANTES treatment, CCR5 had accumulated in perinuclear structures, some of which colocalised with endogenous Rab11, whose distribution was unchanged by RANTES treatment, although a lot of the CCR5, despite being
perinuclear, did not colocalise with Rab11. Moreover, Rab11 puncta that were negative for CCR5 were also seen.

Figure 3-VI Agonist-activated, internalised CCR5 partially colocalises with endogenous Rab11

CHO CCR5 cells were treated with CHX to clear newly synthesised CCR5 from the biosynthetic pathway. Cells were then either fixed (unstimulated) or treated with RANTES in BM containing CHX for 60 min (60 min RANTES) and then fixed, before being permeabilised in blocking buffer and incubated with MC-5 and anti-Rab11a primary antibodies to detect CCR5 and Rab11, respectively. MC-5 and anti-Rab11a were detected with Alexa 488 GAM and Alexa 594 GAR, respectively. A single confocal section for each condition is shown in large, with a merged channel montage of confocal sections through the same group of cells (0.5 μm step size) shown in small. Bar for large images, 5 μm.
These data suggest that agonist-activated, internalised CCR5 traffics through a Rab11-positive compartment. That a significant amount of the down-modulated CCR5 that clustered perinuclearly did not colocalise with Rab11 and that Rab11-positive, CCR5-negative structures were observed, suggests that either a significant amount of steady-state down-modulated CCR5 resides in a non-recycling endosome compartment or that only a portion of the recycling endosomes containing CCR5 are Rab11-positive.

**TfR and Rab11 show only a limited overlap in different cell-lines**

Recycling endosomes are often defined as Rab11-positive, perinuclear, tubulo-vesicular compartments. The transferrin receptor has also been used as a marker for the compartment and CCR5 is found primarily in recycling endosomes in CHO cells (Yamashiro, Tycko et al. 1984). However, despite showing a very strong overlap with TfR in the perinuclear region, the CCR5 that accumulates there under constant agonist treatment only showed a partial overlap with Rab11. I therefore investigated the possibility that Rab11 is only present on a subset of perinuclear, TfR-positive structures.

Figure 3—VII A shows a confocal immunofluorescence micrograph of a CHO CCR5 cell stained for both TfR and Rab11. The two proteins showed a typical punctate, perinuclear disposition, but the Rab11 staining was less extensive than the TfR staining, with fewer peripheral puncta observable. There was some overlap between TfR and Rab11 staining in the perinuclear region; however, there were significant numbers of solely TfR-positive and solely Rab11-positive puncta.

I then investigated the distribution of Rab11 and TfR in two other human cell-lines, HeLa cells (Figure 3—VII B) and human osteosarcoma (HOS) CD4 CCR5 cells (Figure 3—VII C). In these two cell-lines, Rab11 and TfR were also concentrated in the perinuclear region, although there were significant amounts of surface TfR staining in the HOS cells. As in the CHO cells, only a partial overlap of TfR and Rab11 was observed in the perinuclear region. In the HeLa cells, a stronger overlap was observed near the tops of the cells, although this may be a reflection of cell shape, as the cytoplasmic volume
becomes smaller towards the top, restricting the space between different membrane-bound compartments.

A

B

montage of sections

bottom  top

merge  TfR  Rab11

TfR  Rab11  merge

max. projection  

single section
Figure 3-VII Perinuclear, steady-state TfR shows only a partial colocalisation with Rab11 in different cell types

CHO CCR5 (A), HeLa (B) or HOS (C) cells were fixed, permeabilised in blocking buffer and incubated with H68.4 and anti-Rab11a primary antibodies to detect TfR and Rab11, respectively. H68.4 and anti-Rab11a were detected with Alexa 488 GAM and Alexa 594 GAR, respectively. A. A single confocal section through a cell is shown. B. and C. Shown at the top is a montage of confocal sections through a group of cells; 0.5 μm step size between sections. Below is shown an enlarged maximum intensity projection of the stack of confocal sections and an enlargement of section 2. Scale bars for enlarged images, 5 μm.
Altogether, these data suggest that only part of the perinuclear, TfR-containing endosomal pool is positive for Rab11.

**Some down-modulated CCR5 accumulates in AP-1-positive structures**

The heterotetrameric clathrin adaptor protein complex, adaptor protein complex 1 (AP-1) has been shown to mediate traffic between endosomes and the TGN. Although initially assumed to function at the TGN (Heilker, Spiess et al. 1999), AP-1 has also been found on TfR-containing tubular endosomes in Madin-Darby kidney (MDCK) cells, where it is involved in basolateral sorting of the TfR (Futter, Gibson et al. 1998), and it has been implicated in retrograde endosome to TGN transport (Meyer, Eskelinen et al. 2001; Valdivia, Baggott et al. 2002). To further characterise the intracellular trafficking route taken by agonist-activated CCR5 and to see if AP-1 would be correctly localised to play a role in CCR5 intracellular trafficking (see Chapter 5), I co-stained cells that had been treated with RANTES for TfR and γ-adaptin, a component of AP-1 (Figure 3–VIII). Here, I pre-treated cells with CHX to clear any internal CCR5 from the biosynthetic pathway. In unstimulated cells, CCR5 was only present on the cell surface and γ-adaptin had a predominantly punctate, perinuclear staining pattern, with some more peripheral, punctate staining. As seen before, TfR also showed some accumulation in the perinuclear region, but was also present on other punctate structures in the cytoplasm. The majority of the staining for the two proteins was separate but some colocalisation was observed in both the perinuclear region and throughout the cytoplasm. That the staining patterns for TfR and γ-adaptin were largely distinct, suggests that in CHO cells, the majority of the AP-1 staining is on the TGN rather than on endosomes, where an overlap with TfR would be expected. Peripheral structures where the two proteins colocalise are likely to be early endosomes and perinuclear colocalisation may represent AP-1 on recycling endosomes.
Figure 3-VIII In the constant presence of agonist, some CCR5 accumulates in a perinuclear compartment positive for AP-1 but negative for steady-state TfR

CHO CCR5 cells were treated with CHX to clear newly synthesised CCR5 from the biosynthetic pathway. Cells were then either fixed (unstimulated) or treated with RANTES in 8M containing CHX for 60 min (60 min RANTES), before being fixed. Cells were then permeabilised in blocking buffer and incubated with MC-5, H68.4 or Mary primary antibodies to detect CCR5, TfR or γ-adaptin, respectively. MC-5, H68.4 and Mary were detected with Alexa 488 GAM IgG2a, Alexa 647 GAM IgG1 and Alexa 594 GAR, respectively. Single confocal sections are shown, with two sections, 0.5 μm apart, shown for the RANTES-treated condition. Dual channel, green-red merges between CCR5 and TfR or γ-adaptin for RANTES-treated cells are also shown. The area of interest referred to the main text is indicated by a white box Bar, 5 μm.
In cells treated with RANTES for 60 min, CCR5 accumulated in the perinuclear region as well as in peripheral structures throughout the cytoplasm. Some triple colocalisation between CCR5, γ-adaptin and TfR was observed in both peripheral and perinuclear structures, suggesting the presence of AP-1 on CCR5-containing early and recycling endosomes, respectively. However, distinct populations of CCR5-positive, γ-adaptin-positive but TfR-negative, and CCR5-positive, TfR-positive but γ-adaptin-negative structures were observed. This is best observed in the dual colour merges, where it can be seen that CCR5 and γ-adaptin displayed a very strong overlap in parts of the perinuclear region, but that some of the γ-adaptin-positive, CCR5-positive structures were negative for TfR, suggesting that these structures were not endosomal (see indicated region of interest). However, CCR5 colocalised almost completely with TfR in the more peripheral structures and some perinuclear structures, which were, therefore, presumably endosomal in nature.

This result is in contrast to Figure 3-II, where practically all of the internal CCR5 was found to colocalise with TfR. The difference has recently been found to be due to the way in which the experiments were performed. In Figure 3-II, cell surface CCR5 was pre-labelled with the anti-CCR5 antibody, MC-5, before inducing down-modulation; in Figure 3-VIII, cells were labelled for CCR5 after fixation. Although CCR5 internalises and recycles similarly whether labelled before agonist stimulation or post-fixation (data not shown), and MC-5 pre-labelling does not target the receptor for lysosomal degradation (Figure 3-IV), it does appear to subtly alter the steady-state distribution of the receptor in the presence of agonist. In cells pre-labelled with MC-5, treated with RANTES for 60 min, and stained for both TfR and γ-adaptin, CCR5 showed a very strong colocalisation with TfR in the perinuclear region and very weak colocalisation with perinuclear γ-adaptin (data not shown). When CCR5 was labelled post-fixation, a pool of perinuclear CCR5 that did not colocalise strongly with TfR, but overlapped well with γ-adaptin was revealed.
**CCR5 traffics through the trans-Golgi network**

The accumulation of CCR5 in TfR-negative, γ-adaptin-positive perinuclear structures suggested that CCR5 may traffic through the TGN. To test this hypothesis, I performed a trafficking assay by immunofluorescence, staining for both γ-adaptin and p230 (Figure 3-IX). p230 is a protein that cycles between the cytosol and the TGN and is involved in the formation of distinct populations of non-clathrin coated vesicles at the TGN (Erlich, Gleeson et al. 1996; Kjer-Nielsen, Teasdale et al. 1999; Kjer-Nielsen, van Vliet et al. 1999). The antibody showed a nearly complete overlap with the classical TGN marker, TGN46, in HeLa cells (data not shown). TGN46 could not be used as a marker of the TGN in CHO cells as the antibody against this protein does not recognise the equivalent protein in hamster cells.

Again, cells were pre-treated with CHX to clear CCR5 from the biosynthetic pathway and CCR5 was found only on the cell surface in unstimulated cells. p230 was found exclusively in the perinuclear region. γ-adaptin showed a very strong overlap with p230 in the perinuclear region, but, as before, γ-adaptin was also observed on more peripheral punctate structures.

After 60 min RANTES-treatment, CCR5 largely accumulated in a perinuclear compartment, although some more peripheral CCR5 staining was also observed as previously described. Much of this perinuclear CCR5 showed a strong overlap with p230 and most of the CCR5-positive, p230-positive structures also colocalised with γ-adaptin, as would be expected. In addition, there were some perinuclear puncta that were only positive for CCR5 and some that were positive for CCR5 and γ-adaptin, but not p230, presumably representing CCR5 in recycling endosomes. Interestingly, most of the p230-associated fluorescence was co-incident with CCR5 staining, suggesting that steady-state, agonist-activated CCR5 fills most of the TGN elements in the CHO cells.
Figure 3-IX Agonist-activated CCR5 traffics through the TGN

CHO CCR5 cells were either treated for 3 h with nocodazole or DMSO in normal growth medium. For the last two hours of this incubation, CHX was added to clear newly synthesised CCR5 from the biosynthetic pathway. Cells were then either fixed (unstimulated) or treated with RANTES in BM containing CHX and either nocodazole or DMSO for 60 min (60 min RANTES), before being fixed. Cells were then permeabilised in blocking buffer and incubated with MC-5, Mary, or anti-p230 primary antibodies to detect CCR5, γ-adaptin or p230, respectively. MC-5, Mary and
anti-p230 were detected with Alexa 488 GAM IgG2a, Alexa 594 GAR and 647 GAM
IgG1, respectively. Single confocal sections are shown. Dual channel, green-red
merges between CCR5 and γ-adaptin or p230 for RANTES-treated cells are also
shown. Bar, 5 μm.

The TGN and recycling endosome are closely apposed organelles, the
maintenance of both in the perinuclear region being dependent on the
organisation of microtubules originating at the MTOC (Rogalski and Singer
1984; Turner and Tartakoff 1989; Sakai, Yamashina et al. 1991; McGraw,
Dunn et al. 1993). To increase the separation of the two organelles and rule
out spurious overlap between CCR5 and the TGN markers cause by close
proximity of the TGN and recycling endosome, cells were treated with
nocodazole before and during stimulation with agonist (Ullrich, Reinsch et
al. 1996). In unstimulated cells, nocodazole treatment led to a dispersal of
p230 and γ-adaptin staining throughout the cytoplasm, although some
staining remained close to the nucleus. The majority of the p230 staining
still overlapped with γ-adaptin, though, confirming the presence of AP–1 on
TGN elements.

In cells treated with RANTES for 60 min, nocodazole treatment also led to a
dispersal of p230 and γ-adaptin staining throughout the cytoplasm. CCR5
was successfully down-modulated from the cell surface and puncta were
observed throughout the cytoplasm. Many of the CCR5 puncta colocalised
with p230 and γ-adaptin, representing CCR5 in the TGN. Also, CCR5–
positive, γ-adaptin-positive, p230-negative puncta were observed,
confirming the presence of AP–1 on some CCR5–containing endosomes.
CCR5 puncta devoid of γ-adaptin and p230 were also observed, presumably
representing CCR5–containing endosomes that had not recruited AP–1.

**CCR5 traffics through Rab11-positive recycling endosomes
and the TGN**

The above data suggest that in the perinuclear region, where CCR5
accumulates in the constant presence of agonist, the receptor traffics
through both recycling endosomes, at least some of which are Rab11–
positive, and the TGN. These data also suggest that down-modulated CCR5 should be observable in both Rab11-positive and p230-positive compartments at steady-state. To confirm this, I performed similar immunofluorescence-based trafficking assays, staining for CCR5, Rab11 and p230 (Figure 3-X).

In unstimulated cells, Rab11 and p230 staining were largely separate, although a small amount of overlap was observed. This is consistent with the findings of Ullrich et al. (1996), who observed some Rab11 colocalisation with α-2,6-SialylT, a marker of the trans-Golgi cisterna and the TGN (Roth, Taatjes et al. 1985) in CHO cells. In cells stimulated with RANTES for 60 min, overlap of both CCR5 and Rab11, and CCR5 and p230 was observed in the perinuclear region. Some overlap between all three proteins was also observed. This may represent CCR5 in Rab11-containing regions of the TGN, or may result from close apposition of the TGN and Rab11-positive recycling endosomes, one of which containing CCR5. CCR5-positive puncta that were negative for both Rab11 and p230 were also found in the perinuclear region, which presumably represents CCR5 in Rab11-negative recycling endosomes.

Treatment with nocodazole led to the dispersal of Rab11 and p230 puncta, which, interestingly, were often observed closely apposed but not overlapping. This suggests that the compartments are distinct but that there may be some sort of physical link between them, which maintains their association despite disruption of the microtubule network. This pattern of Rab11 and p230 staining was maintained after 60 min RANTES treatment. Intriguingly, CCR5 colocalised well with p230 under these conditions but showed a much-reduced overlap with Rab11 compared with the overlap in RANTES-treated cells in the absence of nocodazole. This suggests that microtubules may be involved in the trafficking of CCR5 and that their disruption may lead to a slightly altered steady-state distribution in the presence of agonist.

Finally, as is best illustrated in the dual channel merged images in Figure 3-X, RANTES treatment did not alter the extent of p230 and Rab11 overlap.
Chapter 3: Morphological Outline of CCR5 Trafficking

Rab11  CCR5  p230  merge

unstimulated

DMSO

nocodazole

DMSO

nocodazole

60 min RANTES

DMSO  nocodazole

DMSO  nocodazole

CCR5 + Rab11  CCR5 + p230

Rab11 + p230

unstimulated  60 min RANTES

DMSO  nocodazole  DMSO  nocodazole

DMSO  nocodazole  DMSO  nocodazole
Figure 3-X In the constant presence of agonist, CCR5 accumulates in both Rab11- and p230-positive structures

CHO CCR5 cells were either treated for 3 h with nocodazole or DMSO in normal growth medium. For the last two hours of this incubation, CHX was added to clear newly synthesised CCR5 from the biosynthetic pathway. Cells were then either fixed (unstimulated) or treated with RANTES in BM containing CHX and either nocodazole or DMSO for 60 min (60 min RANTES), before being fixed. Cells were then permeabilised in blocking buffer and incubated with MC-5, anti-Rab11a, or anti-p230 primary antibodies to detect CCR5, Rab11 or p230, respectively. MC-5, anti-Rab11a and anti-p230 were detected with Alexa 488 GAM IgG2a, Alexa 594 GAR and 647 GAM IgG1, respectively. Single confocal sections are shown. Dual channel, green-red merges between CCR5 and Rab11 or p230 for RANTES-treated cells are shown, as are dual channel, green-red merges between Rab11 and p230. Bar, 5 μm.

**CCR5 traffics through the TGN in HOS cells**

The above data suggests that, unexpectedly, in CHO cells, CCR5 traffics through the TGN. To determine if this is a general feature of CCR5 trafficking, or whether the result is specific to CHO cells, I followed the agonist-induced trafficking of CCR5 in human osteosarcoma (HOS) cells stably expressing CCR5 as well as CD4 (Figure 3—XI).

I firstly investigated where CCR5 was located in unstimulated HOS CD4 CCR5 cells and whether the agonist–activated receptor trafficked in a similar manner to when expressed in CHO cells (Figure 3—XI A).

In unstimulated cells, CCR5 staining of permeabilised cells was almost exclusively restricted to the cell surface. After 60 min RANTES treatment, the receptor redistributed from the cell surface mainly into perinuclear puncta, although some of the receptor was seen in puncta throughout the rest of the cytoplasm. When cells that had been treated with RANTES were incubated for 60 min in TAK-779, conditions under which the receptor recovers to the cell surface in CHO cells, practically all of the CCR5 was observed on the cell surface, demonstrating that CCR5 recycled in this cell-line. Overall, in HOS cells, agonist–activated CCR5 traffics in a similar way to when it is expressed in CHO cells.
Chapter 3: Morphological Outline of CCR5 Trafficking

Figure 3-XI In the constant presence of agonist, some CCR5 accumulates in the TGN in HOS cells

A. HOS CD4 CCR5 cells were either fixed (unstimulated), treated with RANTES in BM for 60 min and fixed (60 min RANTES), or treated with RANTES in BM for 60 min followed by a 60 min incubation in BM containing TAK-779 before being fixed (60 min RANTES + 60 min TAK-779). Cells were then permeabilised and incubated with MC-5 primary antibody to detect CCR5, which was subsequently visualised with Alexa 488 GAM. Epifluorescence images are shown. Bar, 5 μm.

B. HOS CD4 CCR5 cells were treated with RANTES in BM for 60 min, fixed, permeabilised and stained for CCR5 and p230 using MC-5 and anti-p230 primary antibodies and Alexa 488 GAM IgG2a and Alexa 594 GAM IgG1 secondary antibodies, respectively. A single confocal section is shown. Bar, 5 μm.
To investigate whether CCR5 traffics through the TGN in HOS cells, I stained cells that had been treated with RANTES for 60 min for p230 and CCR5 (Figure 3–XI B). CCR5 showed a very strong overlap with p230 in the perinuclear region, suggesting that, like in the CHO cells, CCR5 traffics through the TGN in HOS cells. As observed in the CHO cells, nearly all of the p230-associated fluorescence was co-incident with CCR5 fluorescence, suggesting that at steady-state, agonist-activated CCR5 occupies most of the TGN elements in CHO cells. Some CCR5-positive only puncta were also observed around the perinuclear region and more peripherally in the cytoplasm, but these were in far lower abundance compared with the CCR5- and p230-positive puncta. The CCR5-positive only puncta may represent CCR5 present in recycling endosomes (perinuclearly-located) or CCR5 in early endosomes (peripherally-located).

**Discussion**

In this chapter I have described a series of experiments designed to chart the post-endocytic course taken by agonist-activated CCR5. I have shown that shortly after internalisation, CCR5 passes through early endosomes and that in the constant presence of agonist, the receptor accumulates in a perinuclear compartment. There is no agonist-induced receptor degradation: CCR5 recycles efficiently back to the cell surface, a process that is revealed by removal of agonist and incubation with the CCR5 antagonist, TAK-779. I have subsequently shown that Rab11 is involved in regulating CCR5 intracellular trafficking, but I have also been able to show that as well as trafficking through Rab11-positive recycling endosomes, CCR5 also traffics through the TGN. To my knowledge, this is the first evidence for the trafficking of a G protein-coupled receptor (GPCR) through the TGN after agonist-induced internalisation.

**Rab11 as a regulator of CCR5 trafficking**

Rab11 is a protein that is principally localised to perinuclear recycling endosomes and has been shown to play a role in regulating the trafficking of several receptors through this compartment, including the TfR (Ullrich, Reinsch et al. 1996; Ren, Xu et al. 1998) and the chemokine receptor,
CXCR2 (Fan, Lapierre et al. 2003; Fan, Lapierre et al. 2004). The results of the experiment where mutant forms of Rab11 were expressed in CHO CCR5 cells suggest that Rab11:GTP is required for CCR5 to access perinuclear recycling endosomes, because down-modulated CCR5 showed a far greater colocalisation with constitutively-active Rab11 Q70L than wild-type Rab11, but failed to colocalise with Rab11 S25N or cluster around the nucleus in the presence of this dominant-negative Rab11 mutant. This is similar to the functioning of Rab11 described for the TfR (Ren, Xu et al. 1998). Presumably, the more dispersed, punctate CCR5 staining observed in RANTES-treated cells expressing Rab11 S25N represents CCR5 held up in early endosomes, since this CCR5 staining did not colocalise with Rab11 S25N and Ullrich et al. (1996) found that despite Rab11 S25N expression leading to a dispersal of recycling endosomes throughout the cytoplasm, Rab11 S25N still labelled the compartment. It would be interesting to co-stain Rab11 S25N-expressing cells for EEA1, to confirm whether CCR5 exit from early endosomes is slowed in the presence of Rab11 S25N. Despite seemingly not being able access recycling endosomes, CCR5 was, nevertheless, able to recycle in Rab11 S25N-expressing cells with only a partial inhibition of recycling observed after 60 min incubation in TAK-779. If CCR5 truly did not reach recycling endosomes in Rab11 S25N-expressing cells, this suggests that CCR5 may be able to traffic directly from early endosomes to the plasma membrane. Alternatively, CCR5 trafficking to recycling endosomes at a reduced rate in the Rab11 S25N-expressing cells (mediated by endogenous Rab11 that can bind GTP) may be fast enough to result in a large amount of CCR5 having recycled after 60 min. Also in keeping with the functioning of Rab11 in TfR trafficking described by Ren et al., I found that the recycling of CCR5 was inhibited in the presence of Rab11:GTP but not by over-expression of wild-type Rab11, with a significant proportion of receptors remaining in Rab11 Q70L-positive clusters after 60 min incubation in TAK-779. This suggests that the recycling of CCR5 may require the hydrolysis of Rab11-bound GTP to GDP. The chemokine receptor, CXCR2, also undergoes agonist-induced internalisation and recycles back to the cell surface via a perinuclear, Rab11-positive compartment. Interestingly, recycling of this receptor was strongly inhibited by the expression of Rab11 S25N, whereas the expression
of a constitutively active Rab11 mutant (Rab11 S20V) had little effect on recycling (Fan, Lapierre et al. 2003). The reason why CXCR2 recycling is not inhibited by the constitutively-active Rab11 mutant, whereas CCR5 recycling is inhibited, is unclear but one possible explanation may lie in a differential ability to recycle from early endosomes: under conditions where CXCR2 recycling through the Rab11-positive recycling endosome was blocked by constitutively active RhoB expression, internalised CXCR2 could apparently recycle from early endosomes via a Rab4-controlled pathway (Neel, Lapierre et al. 2007). Perhaps Rab11 S25N expression blocks CXCR2 exit from early endosomes, whether en route to the recycling endosome or directly back to the cell surface, whereas CXCR2 can still recycle from early endosomes to the plasma membrane in the presence of Rab11:GTP, where the recycling block is at the recycling endosome. Indeed, Ren et al. (1998) concluded that Rab11:GTP is required for TfR exit from early endosomes whether en route to the plasma membrane or recycling endosome. That Rab11 S25N apparently had a more severe effect on CXCR2 recycling than it did on CCR5 recycling is most simply explained by considering that S25N expression did have a small inhibitory effect on CCR5 recycling and perhaps only assaying CCR5 recycling at one time-point missed a larger inhibitory effect of the mutant. A fuller kinetic analysis of recycling in the presence of Rab11 wild-type and mutants may resolve this issue. Finally, it must be borne in mind that the role of Rab11 in regulating CCR5 trafficking through recycling endosomes is complicated by the finding that the receptor also traffics through the TGN (see below), which may account for differences between CCR5 and CXCR2 trafficking. Moreover, from experiments following the retrograde trafficking of TGN38 and Shiga toxin in HeLa cells, it has been suggested that Rab11 may function in the sorting of proteins from recycling endosomes to the TGN (Wilcke, Johannes et al. 2000).

**Rab11 and recycling endosomes**

Yamashiro *et al.* (1984) demonstrated that the majority of the transferrin receptor population in CHO cells is present in recycling endosomes, making perinuclear TfR a good marker for the compartment. Rab11 is an accepted marker for recycling endosomes and has become synonymous with the
Chapter 3: Morphological Outline of CCR5 Trafficking

compartment (van Ijzendoorn 2006). Here, though, I have shown that only a small fraction of perinuclear TfR in CHO cells colocalises with Rab11, and, moreover, that this partial overlap in the perinuclear region is not restricted to CHO cells, demonstrating the same staining pattern in HeLa and HOS cells. Some of the lack of colocalisation (Rab11–positive, TfR–negative puncta) can be explained by the presence of a small amount of Rab11 on the TGN (Ullrich, Reinsch et al. 1996). However, I found significant amounts of perinuclear, TfR–containing endosomes that were Rab11 negative. This is in keeping with the demonstration in A431 cells that Rab11 is restricted to certain endosomal domains (Sönntichsen, De Renzis et al. 2000). It is important to be clear about terminology when referring to the recycling endosome and thus, I would suggest that the original description of a recycling endosomes as a perinuclear endosomal compartment with more tubular morphology than early endosomes, a higher pH (~ 6.5) and enrichment for recycling receptors but far lower levels of fluid phase material destined for the lysosome (Yamashiro, Tycko et al. 1984; Hopkins, Gibson et al. 1994; Marsh, Leopold et al. 1995), is a more complete definition than a compartment defined solely by the presence of Rab11. By this definition, Rab11 is only associated with a subset of the recycling endosome membranes. Moreover, the large amount of Rab11 staining in CHO, HeLa and HOS cells that does not colocalise with TfR, if not all labelling the TGN (which seems unlikely given the small overlap between Rab11 and p230 in CHO CCR5 cells), suggests some sort of functional compartmentalisation within the recycling endosome – Rab11–containing domains through which the TfR does not pass or passes very rapidly.

Model for the trafficking itinerary of agonist–activated CCR5

A model for CCR5 trafficking integrating the results of previous studies and all the data presented in this chapter is shown below in Figure 3–XII. After agonist–activation, CCR5 molecules are internalised through clathrin–coated pits and delivered to early endosomes. From there, receptors are sorted to a perinuclear recycling compartment and escape sorting to the lysosome. It was previously believed that the recycling compartment – the perinuclear compartment from which CCR5 recycles to the plasma membrane and in which down-modulated receptors accumulate at steady–
state - was the recycling endosome. However, from the immunofluorescence data presented in this chapter, the recycling compartment, by the above definition, appears to comprise membrane elements from both the recycling endosome and TGN. Moreover, as discussed above, the recycling endosome appears to contain elements that are both are positive and negative for Rab11. Agonist-activated CCR5 seems to traffic through both Rab11-positive and negative recycling endosomes, since it can be found in both populations of recycling endosomes as well as the TGN in cells stained for Rab11 and p230. To which component of the recycling compartment receptors sorted from early endosomes are initially delivered cannot be determined from the results of the experiments presented in this chapter. Nor can it be determined whether there is a sequential transfer of CCR5 between the recycling endosome and TGN components of this compartment, or from which element of the compartment CCR5 finally exits en route to the plasma membrane. However, it is tempting to speculate that as down-modulated CCR5 colocalises with p230 but not Rab11 when cells are treated with nocodazole, that CCR5 exits the recycling compartment from the TGN in a microtubule-dependent fashion - microtubules are believed to be involved in the extrusion of post-Golgi carriers from the TGN and their subsequent cytosolic transport (De Matteis and Luini 2008). It would be interesting to see if CCR5 is capable of recycling in nocodazole-treated cells.

The description of the role of Rab11 in CCR5 trafficking detailed above is compatible with a recycling compartment comprising recycling endosome and TGN components, where Rab11-GTP is required for trafficking to the perinuclear recycling compartment (little perinuclear clustering of CCR5 was observed in cells expressing Rab11 S25N) and hydrolysis of GTP on Rab11 is required for exit from the compartment. However, further morphological analysis is required here: of particular interest is the effect of the Rab11 mutants on TGN localisation of agonist-activated CCR5 at steady-state. Again, the results of the experiment with the Rab11 mutants shed no light on the specific sequence of trafficking events to, within and from the recycling compartment.
Chapter 3: Morphological Outline of CCR5 Trafficking

Figure 3-XII Model for CCR5 intracellular trafficking in CHO CCR5 cells

The figure shows a cartoon representing the most parsimonious model for the intracellular trafficking route taken by agonist-activated CCR5 based on previously published data and the results presented in this chapter. See main text for details. The site(s) of chemokine dissociation and receptor dephosphorylation are unclear. The MTOC is shown in the bottom right corner; dotted grey lines represent microtubules emanating from the MTOC. The Tfr cartoon representation is positioned in the compartments in which the Tfr can be readily found in CHO CCR5 cells.

Interestingly, the Rab11-positive recycling endosomes and the TGN are maintained in close proximity by a mechanism that appears to be, at least in part, independent of microtubules, since Rab11 and p230 puncta dispersed together in closely apposed, paired puncta in nocodazole-treated cells. This close proximity, which has also been observed by electron microscopy, may facilitate the transfer of CCR5 between the two compartments. The recycling endosome and TGN are morphologically hard to distinguish, and also have a similar pH of ~6.5 (Yamashiro, Tycko et al. 1984; van Ijzendoorn 2006; De Matteis and Luini 2008). The compartments are only really distinguished by
the markers they contain, although even many of these, e.g. AP-1 and Rab11, are common to the two compartments. This may be the reason that down-modulated CCR5 was not reported to localise to the TGN by Signoret et al. (2000): although CCR5 was observed in tubules and vesicles close to Golgi stacks on immunolabelled cryosections, no markers for the TGN were included in this study.

To my knowledge, this is the first evidence for a GPCR trafficking through the TGN after agonist-stimulation. Although the data presented here is very suggestive, it needs to be verified by ultrastructural analysis, e.g. electron microscopic observations of cryosections immunolabelled for CCR5 and p230/Rab11, to see if the observations at the light level hold true. The hypothesis that CCR5 traffics through the TGN could also be assessed by taking advantage of the fact that CCR5 is post-translationally modified by O-linked glycosylation of its N-terminus, preferentially on Ser6 (Farzan, Mirzabekov et al. 1999; Bannert, Craig et al. 2001). The O-linked oligosaccharides covalently attached to CCR5 contain terminal sialic acid residues that could be enzymatically removed before inducing CCR5 internalisation and then receptors recycling back to the plasma membrane could be assayed for the re-addition of sialic acid residues. This reaction would be predicted to occur in the TGN and would act as a tag demonstrating that the receptor had passed through the compartment. Indeed, the importance of the maintenance of N-terminal glycosylation for agonist binding may be the reason for CCR5 trafficking through the TGN (Bannert, Craig et al. 2001).

The clathrin adaptor protein, AP-1, as well as being present on the TGN elements to which CCR5 trafficked, also colocalised with down-modulated CCR5 on endosomal structures. Interestingly, AP-1 appeared to show an enhanced association with TfR-positive endosomes after RANTES treatment, which suggests that AP-1 is recruited to endosomes containing CCR5. A role for AP-1 in mediating CCR5 trafficking is further explored in Chapter 5.

Influence of antibody binding on CCR5 trafficking

As discussed in the results section, pre-binding the anti-CCR5 antibody, MC-5, to receptors before treating with agonist appears to subtly alter the steady-state distribution of agonist-activated receptors. Pre-binding MC-5
before RANTES treatment led to a strong overlap of down-modulated CCR5 with TfR in the perinuclear region (as seen in Figure 3-II) and very little overlap with \(\gamma\)-adaptin was observed (data not shown). In contrast, down-modulated CCR5 colocalised strongly with perinuclear \(\gamma\)-adaptin in cells where CCR5 was labelled post-fixation, some of which did not label for TfR (Figure 3-VIII), which led to the series of experiments showing that CCR5 traffics to the TGN (Figure 3-IX and Figure 3-X). Unfortunately, this difference between the two labelling methods and the ability of CCR5 to traffic through the TGN were only appreciated towards the end of the experimentation phase of this PhD project. This has had three consequences: (1) many immunofluorescence experiments described in the thesis were performed with pre-labelling of CCR5; (2) many experiments described in subsequent chapters were performed with the view that CCR5 trafficked to recycling endosomes and not the TGN; and (3) I have not had enough time to repeat trafficking experiments where CCR5 was pre-labelled with post-fixation staining of CCR5, to fully assess the effects of antibody pre-labelling.

However, in cells pre-labelled for CCR5 and stained with TfR and \(\gamma\)-adaptin, although the overlap of down-modulated CCR5 with \(\gamma\)-adaptin is very limited, a very small amount of CCR5 in some cells does appear to colocalise with \(\gamma\)-adaptin in TfR-negative structures, suggesting that down-modulated CCR5 can access the TGN when MC-5 is pre-bound (data not shown). In addition, given that CCR5 pre-bound with MC-5 is not targeted for degradation and recycles with similar kinetics to unbound CCR5, it is likely that antibody-bound CCR5 traffics through the same pathway as ‘naked’ CCR5, but that the trafficking rates within the recycling compartment are subtly altered.

**CCR5 trafficking in other cell-lines**

As well as supporting the notion that agonist-activated CCR5 traffics through the TGN, the demonstration that CCR5 internalises and recycles in HOS CD4 CCR5 cells in a manner similar to that observed in CHO cells, suggests that this cell-line could form a useful system for RNAi screens of proteins involved in CCR5 recycling. CHO cells suffer in this respect from
the fact that the hamster genome has not been sequenced. I have created a HOS cell-line in which only CCR5 (not CD4) is stably expressed, as there have been reports of CCR5–CD4 interactions, and the cell-line used for the experiments described above also expressed CD4 (Xiao, Wu et al. 1999). The observation that down-modulated CCR5 accumulates in the TGN in HOS CD4 CCR5 cells at steady-state should be repeated with this cell-line to rule out CD4 having an effect on CCR5 trafficking.
Chapter 4: ASSOCIATION OF CCR5 WITH β-ARRESTINS

"The aim of every artist is to arrest motion, which is life, by artificial means and hold it fixed so that a hundred years later, when a stranger looks at it, it moves again since it is life."

William Faulkner, Novelist, awarded the Nobel Prize for Literature in 1949

The data presented in Chapter 3 showed that soon after agonist activation, CCR5 molecules are internalised and traffic through early endosomes en route to a perinuclear recycling compartment. From this compartment, receptors recycle efficiently to the cell surface. As for many activated GPCRs, β-arrestins effect CCR5 internalisation by coupling receptors to the clathrin-mediated endocytic machinery (Huttenrauch, Nitzki et al. 2002; Fraile-Ramos, Kohout et al. 2003; Huttenrauch, Pollok-Kopp et al. 2005). There is also a growing body of evidence that suggests that the nature of the interaction of an activated GPCR with β-arrestins - transient or sustained - correlates with, and may in part dictate, the subsequent trafficking itinerary of a receptor (Oakley, Laporte et al. 2000; Oakley, Laporte et al. 2001). Here, I describe morphological and biochemical experiments investigating the nature of the interaction of CCR5 with β-arrestins 1 and 2. I then explore the role of C-tail phosphorylation in supporting β-arrestin association and the role that this phosphorylation plays in the trafficking of the receptor.
β-arrestins remain bound to CCR5 as it internalises and traffics to early endosomes

Mueller et al. previously showed by immunofluorescence of CHO CCR5 cells transiently transfected with GFP-β-arrestin1, that treatment for 60 min with the CCR5 agonist, MIP-1α, led to the redistribution of CCR5 from the plasma membrane into vesicular compartments and that GFP-βarr1 was found to colocalise with the internalised receptor (Mueller, Kelly et al. 2002). However, the authors did not determine in which intracellular compartment the down-modulated CCR5 was located, only observed a single time-point of agonist stimulation, and did not assess whether β-arrestin2 showed a similar agonist-induced redistribution.

To further study the nature of the interaction between agonist-activated CCR5 and β-arrestins, I performed a series of morphological trafficking experiments using CHO CCR5 cells stably expressing bovine β-arrestin1 or β-arrestin2, C-terminally tagged with yellow fluorescent protein (YFP) and green fluorescent protein (GFP), respectively. In addition to the Mueller et al. (2002) study, both N- and C-terminally GFP-tagged β-arrestins have been used extensively for fluorescence studies of β-arrestin recruitment to GPCRs: indeed, fluorescently-tagged β-arrestins were used for live-cell imaging performed by Oakley et al. in the experiments that led to the initial definition of two classes of GPCR that differed in the stability of their interaction with β-arrestins (Oakley, Laporte et al. 2000). Fluorescently-tagged β-arrestins are also routinely used for fixed-cell fluorescence imaging since endogenous β-arrestins are difficult to detect - principally due to a paucity of available antibodies that recognise the native forms of β-arrestins 1 and 2 in vivo.

I first looked by immunofluorescence to see whether agonist-activated CCR5 maintained an interaction with β-arrestins in early endosomes (Figure 4-1). Here, and in all the experiments described in this chapter, cells were pre-labelled for cell surface CCR5 with MC-5 before treating with agonist. In unstimulated cells, receptors were diffusely distributed at the cell surface and EEA1, a marker of early endosomes, had a punctate, cytoplasmic distribution. β-arrestin1-YFP was present in both the cytoplasm and the nucleus; in contrast, β-arrestin2-GFP was largely excluded from the
nucleus. This is consistent with the report that β-arrestin2 has a hydrophobic-rich region at its C-terminus that serves as a nuclear export signal (NES), resulting in an extra-nuclear β-arrestin2 localisation; in β-arrestin1, there is a single amino acid difference in sequence in this region that renders it incapable of supporting nuclear export, hence the difference in subcellular localisation (Wang, Wu et al. 2003). Pairs of juxtanuclear, fluorescent spots were also frequently observed in β-arrestin2-GFP-expressing cells, which likely represent β-arrestin2-GFP located at the microtubule-organising centre (MTOC) (A. Benmerah, unpublished results).

After 5 min RANTES treatment, some CCR5 remained diffusely distributed at the plasma membrane, but most was observed in punctate structures at or below the cell surface. Both β-arrestins 1 and 2 were recruited to the cell surface and clearly colocalised with CCR5 puncta, which presumably represent receptors recruited into clathrin-coated pits or flat clathrin lattices at the plasma membrane. Moreover, β-arrestins 1 and 2 colocalised with all of the cytoplasmic CCR5 puncta, many of which also colocalised with EEA1, demonstrating that β-arrestins 1 and 2 were bound to CCR5 in early endosomes. Peripheral cytoplasmic puncta that were positive for both CCR5 and β-arrestins but negative for EEA1, presumably represent internalised vesicles en route to early endosomes. Altogether, these data suggest that β-arrestins remain bound to CCR5 as it internalises and passes into early endosomes.

It is interesting to note that after 5 min RANTES treatment, much less diffuse cell-surface CCR5 staining was observed in CHO cells also expressing β-arrestin1-YFP or β-arrestin2-GFP than in those with just endogenous levels of β-arrestins (Figure 3–1). Over-expression of β-arrestins apparently led to an increase in the rate of CCR5 internalisation, which suggests that endogenous levels of β-arrestins are limiting for CCR5 internalisation in CHO CCR5 cells, which is not that surprising given the high levels of CCR5 expression.
Figure 4.1 β-arrestins remain bound to CCR5 as it internalizes and traffics to early endosomes

CHO CCR5 βarr1-YFP or CHO CCR5 βarr2-GFP cells were pre-labelled for cell surface CCR5 with MC-5 and then either fixed (unstimulated) or incubated with RANTES in BM for 5 min before being fixed (5 min RANTES). Cells were then incubated in permeabilising blocking buffer with anti-EEA1 primary antibody. MC-5 and EEA1 primary antibodies were detected with Alexa 488 GAM IgG2a and 647 GAM IgG1, respectively. Single confocal sections are shown. Bars, 5 μm
β-arrestins remain bound to internalised CCR5 as it traffics to a perinuclear, Tfr-positive compartment

To determine whether β-arrestins remain bound to CCR5 as it traffics to a perinuclear recycling compartment, I treated CHO CCR5 βarr1-YFP (Figure 4-I) or CHO CCR5 βarr2-GFP (Figure 4-III) cells with RANTES for different time-periods and co-stained for steady-state transferrin receptor (Tfr), the majority of which is located in perinuclear recycling endosomes in CHO cells (Yamashiro, Tycko et al. 1984).

In unstimulated cells, CCR5 was diffusely distributed over the cell surface and the Tfr population was often found clustered in the perinuclear region, although there was significant punctate staining observed throughout the rest of the cytoplasm. As before, β-arrestin1-YFP was found both in the cytoplasm and the nucleus, whereas β-arrestin2-GFP was largely excluded from the nucleus.

After 5 min RANTES stimulation, both β-arrestins were recruited to the cell surface and found in puncta together with CCR5. CCR5 was also found in punctate structures in the cytoplasm, all of which colocalised with βarr1-YFP or βarr2-GFP. Some cytoplasmic puncta also colocalised with Tfr, most likely present in early endosomes, given the results presented in Figure 4-I. In support of this conclusion, the confocal section through the middle of the of CHO CCR5 βarr2-GFP cells treated with RANTES for 5 min, shows how most of the cytoplasmic Tfr-containing, CCR5-positive puncta did not colocalise with Tfr located in the perinuclear region, so were likely early endosomal in nature.

After 30 min RANTES-treatment, in both CHO CCR5 βarr1-YFP and CHO CCR5 βarr2-GFP cells, CCR5 had accumulated in the perinuclear region and showed a very strong overlap with Tfr, whose own distribution appeared more concentrated around the nucleus compared with unstimulated cells. The distribution of CCR5 after 60 min RANTES-treatment was indistinguishable from that seen after 30 min, suggesting that a steady-state distribution for CCR5 under constant agonist stimulation was established by 30 min RANTES-treatment. The distribution of internal CCR5 after 60 min RANTES treatment was also the same as in CHO CCR5 cells treated in the same way (Figure 3-II). Both β-arrestin1-YFP and β-
arrestin2-GFP showed a strong colocalisation with CCR5 in the perinuclear region, suggesting that both β-arrestins maintained an interaction with CCR5 as it passed into perinuclear recycling endosomes. However, from these results it cannot be determined whether the β-arrestins were permanently bound to CCR5 throughout trafficking to the recycling endosome or underwent cycles of dissociation and re-association with the receptor - a situation that would provide a window for binding of other CCR5 C-tail-interacting proteins.

CCR5 recycling to the cell surface was also followed by treating cells for 60 min with RANTES to down-modulate CCR5 and then allowing receptors to recycle back to the plasma membrane in the presence of the CCR5 antagonist, TAK-779. Full recovery of CCR5 cell-surface fluorescence was seen in both CHO CCR5 βarr1-YFP and CHO CCR5 βarr2-GFP cells, although a little perinuclear CCR5 was observed in a minority of CHO CCR5 βarr2-GFP cells, which colocalised with β-arrestin2-GFP (data not shown). This suggests that over-expression of β-arrestins did not dramatically alter the trafficking itinerary of the receptor. After the incubation with TAK-779, β-arrestin1-YFP and β-arrestin2-GFP adopted similar distributions to that seen in unstimulated cells and no longer colocalised with CCR5, consistent with recycled, cell-surface-located receptors being stabilised in an inactive conformation in the presence of TAK-779. The TfR population also adopted a distribution similar to that observed in unstimulated cells, with a relaxation of the concentration of TfR staining around the nucleus seen in 30, 60 and 120 min RANTES-treated cells.

Recycling of CCR5 in cells treated with RANTES for 60 min followed by a 60 min incubation in TAK-779 contrasted with cells treated with RANTES for 120 min, where the CCR5 distribution and colocalisation with both β-arrestins and TfR was similar to that seen after 60 min RANTES treatment. This supports the conclusion drawn from the data presented in Chapter 3, that after long periods of RANTES treatment, CCR5 is not routed to a degradative pathway.
Figure 4-11 β-arrestin1-YFP remains bound to agonist-activated CCR5 as it traffics to a perinuclear, Tfr-containing compartment

CHO CCR5 βar1-YFP cells were pre-labelled for cell surface CCR5 with MC-5 and then treated with RANTES in BM for the indicated time-periods or treated with RANTES for 60 min followed by an incubation in BM containing TAK-779 for 60 min.
Cells were then fixed and incubated in permeabilising blocking buffer with H68.4 (anti-TfR) primary antibody. MC-5 and H68.4 primary antibodies were detected with Alexa 488 GAM IgG2a and 647 GAM IgG1, respectively. Single confocal sections are shown. Bars, 5 μm
Figure 4-III β-arrestin2-GFP remains bound to agonist-activated CCR5 as it traffics to a perinuclear, TfR-containing compartment

CHO CCR5 βarr2-GFP cells were pre-labelled for cell surface CCR5 with MC-5 and then treated with RANTES in BM for the indicated time-periods or treated with RANTES for 60 min followed by an incubation in BM containing TAK-779 for 60 min.
Cells were then fixed and incubated in permeabilising blocking buffer with H68.4 (anti-TfR) primary antibody. MC-5 and H68.4 primary antibodies were detected with Alexa 488 GAM IgG2a and 647 GAM IgG1, respectively. Single confocal sections are shown. Bars, 5 μm

**Endogenous β-arrestins are stably recruited to CCR5 in RBL cells**

Above, I have described experiments where I followed the association of over-expressed, tagged β-arrestins with agonist-activated CCR5. In order to determine whether endogenous β-arrestins exhibited the same pattern of sustained association with CCR5, immunofluorescence trafficking experiments were performed with rat basophilic leukaemia cells stably expressing CCR5 (RBL CCR5 cells), stained for endogenous β-arrestins. RBL CCR5 cells were chosen for this experiment for two reasons. Firstly, as CCR5 is endogenously expressed in a subset of leukocytes (Blanpain, Libert et al. 2002), RBL cells, being of a haematopoietic lineage, are a relevant system for studying physiological CCR5 trafficking. Secondly, RBLs have been utilised in studies of the trafficking of other GPCRs as they express high levels of β-arrestins (Santini, Penn et al. 2000).

CCR5 is expressed on the surface of unstimulated RBL CCR5 cells but, in contrast to CHO cells, RBL CCR5 cells have a significant intracellular pool of CCR5. This pool is located in compartments that label for the rat lysosomal membrane glycoproteins, Igp100/Igp80 (Silène Wavre, Endocytic Regulation of Chemokine Receptor Expression, PhD thesis, 2006), and correspond to secretory granules. Therefore, here, as with the CHO CCR5 cells, cell surface CCR5 was pre-labelled with MC-5 before inducing internalisation with RANTES.

As can be seen in Figure 4-IV, after 60 min RANTES treatment, CCR5 that was initially on the cell surface had accumulated in intracellular structures. Both β-arrestin1 and β-arrestin2, which did not colocalise with CCR5 in unstimulated cells, strongly colocalised with intracellular CCR5 after 60 min RANTES treatment, suggesting stable recruitment of the two proteins to agonist-activated CCR5.
Figure 4-IV Endogenous β-arrestins are stably recruited to agonist-activated CCR5 in RBL CCR5 cells

RBL CCR5 cells were pre-labelled for cell surface CCR5 with MC-5 and then either fixed (unstimulated) or treated with RANTES in BM for 60 min before being fixed (60 min RANTES). Cells were then incubated in permeabilising blocking buffer with primary antibodies, 178 (anti-β-arrestin1) and 182-4 (anti-β-arrestin2). MC-5 was detected with Alexa 488 GAM and 178 and 182-4 were detected with Alexa 594 GAR secondary antibody. Single confocal sections are shown. Bar, 5 μm.

Similar results were obtained in CHO CCR5 cells, but the β-arrestin staining was much weaker, especially for β-arrestin2 (data not shown). This is probably a reflection of lower endogenous β-arrestin levels in CHO cells than in RBL cells, and also possibly a lower affinity of the antibodies for hamster β-arrestins, the protein sequences of which are undetermined. Overall, these data support the view that the stable recruitment of fluorescently-tagged β-arrestins to CCR5 was not an over-expression artefact.

Stable recruitment of β-arrestins to CCR5 is not agonist-specific

CCR5 is activated upon binding of the CC chemokines, CCL3 (macrophage inflammatory protein [MIP]-1α), CCL4 (MIP-1β), CCL5 (regulated on activation normal T-cell expressed and secreted [RANTES]) and CCL8 (monocyte chemoattractant protein [MCP]-2) (Murphy, Baggioni et al. 2000). The results presented above show that β-arrestins are stably
recruited to RANTES (CCL5)-activated CCR5. To determine if this is an effect specific to RANTES activation or is a general property of agonist-activated CCR5, I performed immunofluorescence trafficking experiments with CHO CCR5 βarr1-YFP/βarr2-GFP cells treated with MIP-1α (CCL3) or MIP-1β (CCL4). As shown in Figure 4-V, both MIP-1α and MIP-1β induced down-modulation of cell surface-labelled CCR5 into a perinuclear, TfR-positive compartment after 60 min treatment. In addition, both β-arrestin1-YFP and β-arrestin2-GFP colocalised with CCR5 in this compartment. Hence, the stable recruitment of β-arrestins to CCR5 is a property common to CCR5 activated by 3 out of its 4 natural agonists, that is, it is a general property of agonist-induced CCR5 trafficking. Moreover, the staining pattern for CCR5 treated with MIP-1α and MIP-1β for 60 min strongly resembled that of RANTES-treated CCR5, suggesting that trafficking to a perinuclear recycling compartment is also a general feature of agonist-activated CCR5 trafficking.

Figure 4-V β-arrestins are stably recruited to MIP-1α- and MIP-1β-activated CCR5
CHO CCR5 βarr1-YFP or CHO CCR5 βarr2-GFP cells were pre-labelled for cell surface CCR5 with MC-5 and then treated with either MIP-1α or MIP-1β in BM for 60 min. Cells were then fixed and incubated in permeabilising blocking buffer with anti-TfR primary antibody (H68.4). MC-5 and H68.4 primary antibodies were detected with Alexa 488 GAM IgG2a and 647 GAM IgG1, respectively. Single confocal sections are shown. Bars, 5 μm

Biochemical evidence for a stable interaction between CCR5 and β-arrestins

The morphological data presented above suggest that β-arrestins remain bound to CCR5 as it internalises, passes through early endosomes and traffics to perinuclear recycling endosomes. However, colocalisation data only indicate that two proteins are present on the same intracellular structure and do not formally prove an interaction between two proteins. To confirm that β-arrestins maintain a sustained interaction with agonist-activated CCR5 as it traffics within the cell, I performed a series of co-immunoprecipitation experiments with CHO CCR5, CHO CCR5 βarr1–YFP and CHO CCR5 βarr2–GFP cells that had been treated with RANTES for various time-periods in a similar fashion to the immunofluorescence assays described above.

I was unable to co-immunoprecipitate β-arrestins (endogenous or over-expressed) with agonist-activated CCR5 using a variety of lysis buffers. This was probably because the membrane environment of CCR5 is crucial for maintaining a receptor conformation that supports high affinity β-arrestin binding and detergent solubilisation of membranes may influence receptor conformation. However, I was able to successfully co-immunoprecipitate β-arrestins with CCR5 using the membrane-permeant cross-linking agent, dithiobissuccinimidylpropionate (DSP). DSP comprises two N-hydroxysuccinimide (NHS) esters separated by a spacer of 12 Å length. The NHS esters react with primary amine groups (e.g. the primary amine group on lysine side-chains) to form covalent amide bonds with the release of N-hydroxysuccinimide. The spacer contains a central disulphide bond, which can be cleaved under reducing conditions so that cross-linked proteins can be separated on a gel.
Figure 4-VI A, shows a blot of an immunoprecipitation of CCR5 from CHO CCR5 β-arrestin1-YFP cells treated with RANTES for various time-periods. In unstimulated cells, a very small amount of β-arrestin1-YFP co-immunoprecipitated with CCR5. The bands for CCR5 and β-arrestin1-YFP were specific since they were both absent from a CCR5 immunoprecipitation from CHO-K1 cells, which express neither of the proteins, and the β-arrestin1-YFP band was absent from the lane containing the product of an immunoprecipitation of CCR5 from CHO CCR5 cells, which do not express β-arrestin1-YFP. RANTES-treated cells showed a reduction in CCR5 mobility, almost certainly due to phosphorylation (Oppermann, Mack et al. 1999), which was maintained throughout RANTES-treatment up to 120 min. β-arrestin1-YFP strongly associated with CCR5 after 5 min RANTES treatment, showing an approximately 7-fold increase in association over unstimulated cells. This degree of association was maintained throughout RANTES treatment, confirming a sustained interaction between β-arrestin1-YFP and CCR5 in RANTES-treated cells. In cells that had been treated with RANTES for 60 min followed by an incubation with TAK-779 for 60 min, the interaction between β-arrestin1-YFP and CCR5 was largely lost, with the amount of β-arrestin1-YFP co-immunoprecipitating with CCR5 reduced to a level below that seen in unstimulated cells. TAK-779 binding stabilises the receptor in an inactive conformation (Baba, Nishimura et al. 1999) and, hence, a small amount of basal activation of CCR5 was probably responsible for some of the β-arrestin1-YFP association with CCR5 in unstimulated cells.

As shown in Figure 4-VI B, β-arrestin2-GFP also co-immunoprecipitated with CCR5 in an agonist-dependent manner, the degree of association remaining approximately constant over time from 5 to 120 min. As with β-arrestin1-YFP, the interaction of β-arrestin2-GFP with CCR5 was reduced to below that detected in unstimulated cells after incubation of RANTES-treated cells in TAK-779 for 60 min.
Figure 4-VI The stable interaction between CCR5 and β-arrestins can be followed by co-immunoprecipitation

A, B, C + D. CHO K1, CHO CCR5, CHO CCR5 βarr1-YFP or CHO CCR5 βarr2-GFP cells were incubated for various time periods with RANTES in BM at 37°C (numbers above lanes indicate length of incubation in minutes; cells in C were treated with RANTES for 10 min) or treated with RANTES for 60 min followed by a 60 min incubation in BM containing TAK-779 (lanes marked TAK). Cells were then treated with the cross-linking agent, DSP before lysis and immunoprecipitation of either CCR5 or GFP, as indicated. Immunoprecipitates and lysates were run on gels under reducing conditions and separated proteins transferred to nitrocellulose membranes. Blots were probed with either MC-5, anti-GFP or 178 primary antibodies to detect CCR5,
parrl-YFP/parr2-GFP or parrl, respectively. Blots in A + D were subsequently incubated with IRDye® 800 GAM (recognises MC-5 and anti-GFP) or IRDye® 680 GAR (recognises 178) and proteins visualised using the Odyssey infra-red detection system. A shows a grayscale image; D shows a dual colour image, where IRDye® 800 GAM signal (CCR5) is shown in green and IRDye® 680 GAR signal (parrl) is shown in red. The blots shown in B + C were incubated with GAM-HRP secondary antibody and developed using a chemiluminescent substrate. E. Equal amounts of proteins from whole cell lysates of wild-type or β-arrestin knock-out MEFs were loaded on a gel. Separated proteins were transferred to nitrocellulose and probed with 178 (anti-parr 1) primary antibody. The blot was subsequently incubated with GAM-HRP and developed with chemiluminescent substrate.

IP, immunoprecipitate; IB, immunoblot, no Ab indicates a mock immunoprecipitation carried out without inclusion of the immunoprecipitating antibody.

To confirm the specificity of the CCR5-β-arrestin interaction, β-arrestin1-YFP and β-arrestin2-GFP were immunoprecipitated from CHO CCR5 β-arrestin1-YFP and CHO CCR5 β-arrestin2-GFP cells, respectively, using an anti-GFP antibody that recognises both GFP and YFP. Immunoprecipitates were then probed for GFP/YFP and CCR5. As can be seen in Figure 4–VI C, CCR5 immunoprecipitated with both β-arrestin1-YFP and β-arrestin2-GFP after 10 min RANTES stimulation. More β-arrestin1-YFP was immunoprecipitated than β-arrestin2-GFP from a volume of cell lysate containing an equal amount of protein mass, which is most likely due to the higher expression level of β-arrestin1-YFP than β-arrestin2-GFP in the CHO CCR5 cells used in this experiment (assessed from cell lysates of CHO CCR βarrl-YFP and CHO CCR5 βarr2-GFP cells probed with anti-GFP antibody; data not shown). Despite the larger amount of β-arrestin1-YFP immunoprecipitated compared with β-arrestin2-GFP, a greater amount of CCR5 co-immunoprecipitated with β-arrestin2-GFP, suggesting that β-arrestin2-GFP has a higher affinity for agonist-activated CCR5 than β-arrestin1-YFP. In this blot, a second, minor band of higher mobility was also recognised by the anti-GFP antibody in the lanes containing the products of the GFP immunoprecipitations. This band was not seen in the blots in A and B and was only observed in experiments where high levels of β-arrestins were present on the blots, hence its detection in an anti-GFP
immunoprecipitation. A recent study by Lee et al. found that β-arrestins undergo proteolytic cleavage after angiotensin II type 1 receptor (AT₁R) activation (Lee, Bhatt et al. 2008). The cleavage sites were determined to reside near the C-termini of β-arrestins 1 and 2. If the higher mobility band seen here resulted from a proteolytic cleavage, however, it must have occurred near the β-arrestin N-termini or in the GFP-protein, since the higher mobility protein was detected on the blot by an anti-GFP antibody. In cell lysates from cells where a large amount of protein had been loaded on a gel so that the lower band was visible, there was no change in the ratio between the intensity of the upper and lower bands between unstimulated and RANTES-treated cells, suggesting that CCR5 activation does not lead to a proteolytic cleavage event as demonstrated for the AT₁R (data not shown). Alternatively, the lower band may result from a post-translational modification of the β-arrestin molecule.

It was difficult to observe the co-immunoprecipitation of endogenous β-arrestins with CCR5, because the β-arrestin proteins, being around 50 kDa in mass, have a similar electrophoretic mobility to, and are masked by, the antibody heavy chain used in the immunoprecipitation (which is recognised by cross-reaction of the secondary antibody used for recognition of the anti-β-arrestin antibody). However, the Odyssey infra-red detection system offered an advantage here, in that secondary antibodies that recognise antibodies from different species can have different fluorophores attached, so that closely apposed bands can be more easily resolved. Figure 4-VI D shows a blot of an immunoprecipitation of CCR5 from CHO CCR5 cells treated for various time-periods with RANTES, in which mouse primary antibody (MC-5, anti-CCR5) was detected with IR Dye® 800 (pseudocoloured green) and a rabbit primary antibody against β-arrestin1 (178) was detected with IR Dye® 680 (pseudocoloured red) (178 was specific for mouse β-arrestin1, as shown from the blotting pattern it gave for lysates of mouse embryonic fibroblasts knock-out for β-arrestin1, 2 or 1 and 2; Figure 4-VI E). As seen before, agonist stimulation led to a decrease in CCR5 mobility, likely resulting from C-tail phosphorylation. Although the IR Dye® 680 anti-rabbit antibody cross-reacted with the heavy-chain of the anti-CCR5 mouse immunoprecipitating antibody, MC-5 (yellow band at 50 kDa),
part of the β-arrestin1 band was visible above this band (red band in area indicated by dashed white box). The blot clearly shows that endogenous β-arrestin1 was recruited to agonist-activated CCR5 after 5 min RANTES treatment and that the interaction was maintained over 120 min RANTES stimulation, in keeping with the data obtained with over-expressed, tagged β-arrestin1-YFP.

Despite being able to detect endogenous β-arrestin1 by this method, β-arrestin2 could not be detected with a rabbit immunoserum, since it migrates slightly faster than β-arrestin1 and is completely masked by the antibody heavy chain.

Altogether, the results from these biochemical assays are consistent with the morphological data and support the notion that both β-arrestins 1 and 2 are stably recruited to agonist-activated CCR5 as it traffics to a perinuclear recycling compartment.

**CCR5 C-tail phosphorylation correlates with β-arrestin association**

As is the case for numerous GPCRs (Ferguson 2001) C-tail phosphorylation has been shown to be required for β-arrestin recruitment to agonist-activated CCR5 (Kraft, Olbrich et al. 2001; Huttenrauch, Nitzki et al. 2002). Alanine scanning mutagenesis of CCR5 identified four C-terminal serine residues at positions 336, 337, 342 and 349, which are phosphorylated in a non-hierarchical manner upon receptor activation (Oppermann, Mack et al. 1999). Additionally, using phospho-site specific antibodies, Ser337 has been identified as a protein kinase C (PKC) substrate and Ser349 as a G protein-coupled receptor kinase (GRK) substrate (Pollok-Kopp, Schwarze et al. 2003).

The results presented above demonstrate that β-arrestins remain associated with agonist-activated CCR5 as it traffics to a perinuclear recycling compartment. To determine whether agonist-activated CCR5 remains phosphorylated throughout this trafficking, I performed immunofluorescence trafficking assays with RANTES-treated CHO CCR5 βarr1-YFP/βarr2-GFP cells, staining for phospho-serine337 (P-S337) and
phospho-serine349 (P-S349) residues with the site-specific antibodies described above.

Figure 4–VII shows the results of the trafficking assays using CHO CCR5 βarr1–YFP cells. In unstimulated cells, some P-S337 signal was detected at the cell surface (Figure 4–VII A). Although some of this fluorescence signal could be explained by low affinity binding of the anti-P-S337 antibody to its unphosphorylated epitope, in our hands, we find significant background activation of PKC in CHO CCR5 cells (N. Signoret, unpublished observation), so there may have been a small level of phosphorylation of cell surface CCR5. After 5 min RANTES treatment, a typical, punctate surface and peripheral cytoplasmic CCR5 staining pattern was observed, with β-arrestin1–YFP colocalising well with the receptor. The P-S337 signal was almost exclusively associated with CCR5 staining and substantially increased compared with unstimulated cells, demonstrating that significant phosphorylation of S337 had occurred. This phosphorylation was maintained after 60 min RANTES treatment, as receptors accumulated in the perinuclear region with β-arrestin1–YFP still bound.

Phosphorylation of S349 (Figure 4–VII B) proceeded in a similar fashion to that of S337. In unstimulated cells, a small amount of signal resulting from anti-P-S349 antibody binding was observed at the cell surface, probably reflecting low affinity binding of the antibody to its unphosphorylated epitope on CCR5. After 5 min, significant levels of phosphorylation of P-S349 were observed and this phosphorylation was maintained after 60 min RANTES treatment as receptors accumulated in the perinuclear region, all the time bound to β-arrestin1–YFP.

Similar results were obtained in CHO CCR5 βarr2–GFP cells (Figure 4–VIII). These results are in accordance with those of Pollok-Kopp et al. (2003) who showed that phosphorylation of S337 and S349 was maintained after 30 min RANTES treatment in RBL CCR5 cells, with the phospho-signal seen to accumulate in the perinuclear region (Pollok-Kopp, Schwarze et al. 2003).
Figure 4-VII CCR5 phosphorylation correlates with β-arrestin1-YFP association

CHO CCR5 βarr1-YFP cells were pre-labelled for cell surface CCR5 with MC-5 and then incubated for the indicated time-periods in BM containing RANTES at 37°C. Cells were then fixed and incubated in permeabilising blocking buffer with either V14/2 (anti-P-S337; A) or E11/19 (anti-P-S349; B) primary antibodies. MC-5, V14/2 and E11/19 were detected with Alexa 647 GAM IgG2a, Alexa 594 GAM IgG2b and Alexa 594 GAM IgG1, respectively. Single confocal sections are shown. Bars, 5 μm
Figure 4-VIII CCR5 phosphorylation correlates with β-arrestin2-GFP association

CHO CCR5 βarr2-YFP cells were pre-labelled for cell surface CCR5 with MC-5 and then incubated for the indicated time-periods in BM containing RANTES at 37°C. Cells were then fixed and incubated in permeabilising blocking buffer with either V14/2 (anti-P-S337; A) or E11/19 (anti-P-S349; B) primary antibodies. MC-5, V14/2 and E11/19 were detected with Alexa 647 GAM IgG2a, Alexa 594 GAM IgG2b and Alexa 594 GAM IgG1, respectively. Single confocal sections are shown. Bars, 5 μm.
C-tail phosphorylation is not required for CCR5 internalisation or recycling

To further investigate the importance of C-tail phosphorylation for the trafficking of CCR5, I compared the agonist-induced trafficking of a CCR5 mutant with all 4 C-tail serine residues shown to be phosphorylated upon receptor activation mutated to alanines (CCR5 4S→A), with that of the wild-type receptor (CCR5 wt) when transiently expressed in CHO-K1 cells (Figure 4–IX). As expected, 60 min RANTES treatment led to the down-modulation of cell surface CCR5 wt into a perinuclear compartment. CCR5 4S→A, like CCR5 wt, was principally expressed on the surface of unstimulated cells (data not shown). Cell surface-labelled CCR5 4S→A also underwent agonist-induced down-modulation to a similar extent as CCR5 wt and the pattern of intracellular CCR5 4S→A staining was indistinguishable from CCR5 wt. This suggests that the removal of the phosphorylation sites does not significantly alter the trafficking itinerary of the receptor. These results are in keeping with the report from Huttenrauch et al. (2002), who showed that CCR5 mutants with 3 or 4 phosphorylatable C-tail serine residues mutated to alanine, despite not being able to recruit β-arrestins to membranes, were able to internalise, albeit at a slower rate than the wild-type receptor (Huttenrauch, Nitzki et al. 2002).

In cells incubated with RANTES for 60 min to down-modulate CCR5, followed by incubation in TAK-779 to assay recycling, CCR5 wt, as expected, recycled fully to the cell surface in all cells. CCR5 4S→A also showed significant recovery to the cell surface, fully recycling in the majority of cells after a 60 min incubation in TAK-779, although a few cells showed incomplete recycling. These results demonstrate that receptor phosphorylation is not required for recycling.
The internalisation of CCR5 4S→A is β-arrestin-dependent

The phosphorylation of CCR5 C-tail serine residues has been shown to be necessary for β-arrestin binding (Kraft, Olbrich et al. 2001), with at least 2 intact phosphorylation sites determined to be necessary for β-arrestin recruitment to agonist-activated CCR5 (Huttenrauch, Nitzki et al. 2002). Moreover, CCR5 has been shown to internalise in a β-arrestin-dependent
fashion (Fraile-Ramos, Kohout et al. 2003). However, consistent with other reports, the data shown above indicate that C-tail phosphorylation (at least of the 4 previously reported phosphorylation sites) is not required for internalisation. To further clarify the dependency of CCR5 internalisation on β-arrestins and assess whether the phosphorylation-deficient mutant, CCR5 4S→A, internalises in a β-arrestin-dependent fashion, I transiently expressed either CCR5 wt or CCR5 4S→A in mouse embryonic fibroblasts (MEFs) isolated from mice where β-arrestin1, β-arrestin2 or β-arrestins 1 and 2 had been knocked out, and assessed the ability of the receptors to internalise upon agonist stimulation.

Figure 4-X A shows a blot of whole cell lysates from the different MEFs, probed with an antibody that recognises both β-arrestins 1 and 2, confirming that the cell-lines showed the correct pattern of β-arrestin expression.

Figure 4-X B shows the results of the trafficking assay. In all the cell-lines, CCR5 wt could be detected on the surface of unstimulated cells. After 60 min RANTES treatment, CCR5 wt accumulated in a perinuclear compartment in wild-type MEFs and in the single β-arrestin knock-out MEFs. Down-modulation proceeded to a significant extent in these cells, with practically no CCR5 found on the cell surface after 60 min RANTES treatment. There were also no gross differences in the distribution of down-modulated CCR5 in the wild-type and single β-arrestin knock-out MEFs. In contrast, CCR5 did not internalise in the β-arrestin 1 and 2 knock-out MEFs, confirming the result of Fraile-Ramos et al. (2003) that CCR5 internalisation, at least in MEFs, is β-arrestin dependent. However, I cannot rule out the unlikely possibility that CCR5 did internalise in the β-arrestin 1 and 2 double knock-out MEFs but that the recycling rate was very much enhanced compared with MEFs expressing β-arrestins, which could also result in an exclusively cell surface CCR5 distribution after 60 min RANTES treatment.
Chapter 4: Association of CCR5 with β-arrestins

Figure 4-X Agonist-induced internalisation of CCR5 wt and CCR5 4S→A is β-arrestin-dependent

A. Equal amounts of proteins from whole cell lysates of wild-type or knock-out MEFs were loaded on a gel. Separated proteins were transferred to nitrocellulose and probed with pan-arrestin (recognises β-arrestin1 and β-arrestin2) and 23 (anti-
clathrin heavy chain) primary antibodies. Blots were subsequently incubated with GAM-HRP and developed with chemiluminescent substrate. Bands representing β-arrestin1 and β-arrestin2 are indicated with arrows. IB, immunoblot B. MEFs transiently transfected with either CCR5 wt or CCR5 4S→A were pre-labelled for cell surface CCR5 with MC-5 and then either fixed (unstimulated) or treated with RANTES in BM for 60 min and then fixed (60 min RANTES). Cells were then incubated in permeabilising blocking buffer with Alexa 488 GAM secondary antibody to detect MC-5. Epifluorescence images are shown. Bar, 5 μm.

CCR5 4S→A was also highly expressed on the cell surface of all of the unstimulated MEFs and the pattern of CCR5 4S→A down-modulation directly matched that of the wild-type receptor. CCR5 4S→A accumulated in the perinuclear region after 60 min RANTES treatment in the wild-type and single β-arrestin knock-out MEFs, but failed to undergo down-modulation in the double β-arrestin knock-out cells. Again, the intracellular distribution of down-modulated CCR5 4S→A was indistinguishable in the wt and single β-arrestin knock-out MEFs and was also indistinguishable from CCR5 wt in the same cells, suggesting similar trafficking itineraries for the mutant and wild-type receptors.

In conclusion, these results suggest that CCR5 4S→A internalises in a β-arrestin-dependent fashion.

**β-arrestin recruitment to agonist-activated CCR5 4S→A**

Given that CCR5 4S→A internalised in a β-arrestin-dependent fashion in MEFs, I assessed whether the recruitment of β-arrestins to the mutant receptor could be observed by immunofluorescence and whether the interaction with β-arrestins was sustained during redistribution of the receptor into the recycling compartment, as seen for the wild-type receptor. CCR5 wt or CCR5 4S→A were transiently co-expressed in combination with either β-arrestin1-YFP or β-arrestin2-GFP in CHO-K1 cells and treated with RANTES for either 5 or 60 min. As expected, both β-arrestin1-YFP and β-arrestin2-GFP were strongly recruited to CCR5 wt after 5 min RANTES treatment in all cells and this interaction was maintained after 60 min.
RANTES treatment as wild-type receptors accumulated in a perinuclear compartment (Figure 4-XI A).
Figure 4-XI β-arrestin recruitment to CCR5 wt and CCR5 4S→A

CHO CCR5 cells were transiently transfected with either CCR5 wt (A) or CCR5 4S→A (B) in combination with either β-arrestin1-YFP or β-arrestin2-GFP. Cells were pre-labelled for cell surface CCR5 with MC-5 and then incubated for the indicated time-periods in BM containing RANTES at 37°C. Cells were then fixed and incubated in
permeabilising blocking buffer with Alexa 594 GAM secondary antibody to detect MC-5. Single confocal sections are shown. Bars, 5 μm

Interestingly, as shown in Figure 4-XI B, β-arrestin2-GFP was also recruited to CCR5 4S→A after 5 min RANTES treatment, and, moreover, this association was observed after 60 min RANTES treatment. However, the strength of the recruitment of β-arrestin2-GFP to CCR5 4S→A at both time-points was variable in different cells, suggesting that the interaction was not as strong as that between β-arrestin2-GFP and CCR5 wt. β-arrestin1-YFP was also recruited to CCR5 4S→A after 5 min RANTES treatment, although far more weakly, with only a small amount of colocalisation seen, and after 60 min RANTES treatment, β-arrestin1-YFP failed to show an association with CCR5 4S→A, which had accumulated in the perinuclear region. Moreover, after 5 min RANTES treatment, CCR5 4S→A did not cluster at the plasma membrane or internalise to the same extent in cells co-expressing β-arrestin1-YFP as in cells co-expressing β-arrestin2-GFP. This suggests that β-arrestin2-GFP expression can accelerate the rate of CCR5 4S→A internalisation above that supported by endogenous levels of β-arrestins, whereas β-arrestin1-YFP, which is only poorly recruited to agonist-activated CCR5 4S→A, is less capable of doing so. CCR5 also co-immunoprecipitated to a greater extent with β-arrestin2-GFP than β-arrestin1-YFP in RANTES-treated cells (Figure 4-VI C). Together, these observations are consistent with the notion that β-arrestin2 has a higher affinity for the activated conformation of CCR5 than β-arrestin1.

The key point from this experiment, though, is that both β-arrestin1-YFP and β-arrestin2-GFP were recruited to agonist-activated CCR5 4S→A, strengthening the notion that phosphorylation is not strictly required for β-arrestin binding to CCR5.

Discussion

In this chapter I have presented morphological and biochemical data that demonstrate that β-arrestins, after facilitating the internalisation of agonist-activated CCR5, remain bound to receptors as they traffic to a perinuclear
recycling compartment. This association appears to be a general effect of CCR5 agonist-activation, since the stable interaction was observed for RANTES-, MIP-1α- and MIP-1β-stimulated CCR5. As expected by the prevailing view that like most GPCRs, CCR5 C-tail phosphorylation is required for high-affinity β-arrestin binding, phosphorylation of the C-tail serine residues, S337 and S349, was detected while CCR5 remained bound to β-arrestins. However, contrary to the dogma, a CCR5 mutant with all 4 serine residues shown to be phosphorylated upon receptor activation mutated to alanines, internalised in a β-arrestin-dependent manner and was observed to form a stable association with β-arrestin2-GFP co-expressed in the same cells.

**Association of CCR5 with β-arrestins**

**β-arrestin association with CCR5 in the recycling compartment**

In Chapter 3, I provided evidence suggesting that the perinuclear compartment to which agonist-activated CCR5 traffics may comprise elements of the TGN as well as the recycling endosome. Moreover, I described how pre-binding of MC-5 to cell surface receptors before inducing internalisation, may subtly perturb the steady-state down-modulated distribution of CCR5, with fewer receptors residing in the TGN. All the immunofluorescence experiments in this chapter were performed with pre-labelling of cell surface receptors, as the effect of antibody pre-binding was not known at the time. Here, I have shown that β-arrestin-bound CCR5 traffics to a perinuclear compartment that shows a good overlap with TfR at the level of light microscopy. The distribution of internal, down-modulated CCR5 in CHO CCR5 cells stably expressing either β-arrestin1-YFP or β-arrestin2-GFP after 60 min RANTES treatment was indistinguishable from that in CHO CCR5 cells expressing only endogenous levels of β-arrestins (Figure 3-11). This suggests that the receptor traffics to the same compartment in the presence of high levels of β-arrestins.

However, from these experiments I can only conclude that CCR5 remains bound to β-arrestins in recycling endosomes and, without the inclusion of TGN markers in similar trafficking experiments, I cannot determine whether β-arrestins maintain an association with CCR5 in the TGN. However, given
that antibody pre-binding only subtly affects the steady-state down-modulated receptor distribution and that the recycling endosome and TGN are often difficult to distinguish, it would not be surprising if the interaction of CCR5 with β-arrestins were maintained in the TGN. 

Re-enforcing a point made in Chapter 3, in the CHO CCR5 βarr1–YFP/βarr2–GFP cells, the TfR population showed a concentration around the nucleus as CCR5 accumulated in the compartment, consistent with that observed in CHO CCR5 cells. This further illustrates the plasticity of the compartment and highlights a general property of endosomes, that, to paraphrase Ira Mellman in his discussion of early to late endosome maturation in *Endosomes Come of Age*, ‘given the prolific amounts of membrane known to move through the [endosomal] system each hour (Steinman, Mellman et al. 1983), it is almost a semantic impossibility to consider the endosomal apparatus as being anything other than subject to dynamic remodelling’ (Mellman 2006).

**CCR5 as a Class C receptor**

β-arrestins couple numerous agonist-activated GPCRs to the clathrin-mediated endocytic machinery and there is various evidence to suggest that the nature of the interaction of a GPCR with β-arrestin may, in part, regulate the subsequent trafficking itinerary of the receptor. Initial work by Laporte and co-workers following fluorescently-tagged β-arrestins, led to the identification of 2 major classes of GPCRs, which differed in the nature of their interaction with β-arrestins (Oakley, Laporte et al. 2000). Class A receptors, including the β2-adrenergic receptor (β2AR), μ-opioid receptor (MOR), and endothelin type A receptor, have a higher affinity for β-arrestin2 compared with β-arrestin1 and they dissociate from the β-arrestin during, or immediately after, endocytosis. These receptors then recycle rapidly to the plasma membrane. Class B receptors, including the angiotensin II type 1 receptor, vasopressin type 2 receptor (V2R) and neurotensin receptor, bind both β-arrestin1 and β-arrestin2 with equally high affinity and maintain an interaction with β-arrestins into endosomes. These receptors recycle very slowly, if at all. Receptor mutagenesis performed on the C-tails of a variety of Class B receptors showed that specific clusters of phosphorylated
serine/threonine residues (Ser/Thr residues occupying 3/3, 3/4, or 3/5 consecutive positions), which are conserved in their position relative to the NPXXY motif, are responsible for the sustained β-arrestin association (Oakley, Laporte et al. 1999; Oakley, Laporte et al. 2001). These clusters are absent from Class A receptors, although potential phosphate acceptor sites are present. Interestingly, swapping the C-tails of the Class A receptor, β2AR, and the Class B receptor, V2R, not only reversed the stability of the receptor–β-arrestin complex but also the corresponding rates of receptor dephosphorylation, recycling and resensitisation, which are much faster for the β2AR than the V2R (Oakley, Laporte et al. 1999). Hence, it was suggested that the stability of the receptor–β-arrestin interaction regulates the rate of receptor dephosphorylation, recycling and resensitisation, presumably via bound β-arrestins restricting the access to the receptor of phosphatases. However, unmentioned by the authors is that the results of these studies do not rule out the possibility that the interaction with β-arrestins may be coincidental and that the phosphorylated serine residues dictate these properties by some other mechanism.

This classification of GPCRs into two classes and the simple role of β-arrestins in impeding receptor recycling and resensitisation has proved too simplistic (see below), and, moreover, does not accurately describe the trafficking behaviour of CCR5 as presented in this chapter. Although CCR5 forms a stable interaction with β-arrestins as it traffics to a perinuclear recycling compartment, typical of a Class B receptor, CCR5 recycles rapidly to the plasma membrane, a process that can be seen upon agonist washout and incubation with the CCR5 antagonist, TAK-779. This is in stark contrast with other Class B GPCRs, which recycle very slowly, if at all (Oakley, Laporte et al. 1999; Anborgh, Seachrist et al. 2000). Moreover, CCR5 does not contain (a) cluster(s) of serine/threonine residues as defined by Oakley et al. (2001), supposed to mediate the stable β-arrestin association. Indeed, based on the results of the trafficking experiments with CCR5 4S→A, CCR5 appears to be able to stably associate with β-arrestin2-GFP in a non-phosphorylated state (see below for further discussion). Finally, CCR5 appears to have a higher affinity for β-arrestin2 than β-arrestin1 – a hallmark of Class A receptors – although, unlike the β2AR, a typical Class A
receptor, it does not show a significantly impaired internalisation rate in β-arrestin2 knock-out MEFs (Kohout, Lin et al. 2001). Whether this differential affinity for the two β-arrestins has a regulatory bearing on CCR5 trafficking is open to investigation.

Other reports have recently emerged of receptors that show similar behaviour to CCR5 in terms of β-arrestin association, including members of the kinin family. The bradykinin type 2 receptor (B2R) also internalises into endosomes with β-arrestins but upon agonist removal, the receptor is efficiently recycled to the plasma membrane. Interestingly, expression of a β-arrestin2 mutant that has an increased affinity for agonist-activated receptors, prevented recycling of the B2R, which remained colocalised with the mutant β-arrestin2 on endosomes after agonist wash-out. From this, it was concluded that the dissociation of β-arrestins from the B2R is necessary for its recycling (Simaan, Bédard-Goulet et al. 2005). The neurokinin 1 receptor also behaves like the B2R in terms of its β-arrestin association and recycling behaviour (Garland, Grady et al. 1996; McConalogue, Dery et al. 1999). The activity of these receptors has led Laporte and colleagues to suggest an extension of their initial classification system to include a third group, the so-called Class C receptors (Simaan, Bédard-Goulet et al. 2005). These GPCRs internalise with β-arrestins into endosomes but the β-arrestin can dissociate from the receptor in endosomes and the receptor can recycle rapidly to the cell surface. The SST2A somatostatin receptor also exhibits behaviour consistent with this definition (Tulipano, Stumm et al. 2004).

By this updated classification system, CCR5 would have to be included as a Class C receptor, although in contrast to CCR5, the aforementioned Class C receptors have serine/threonine clusters in their C-tails like those described for Class B receptors.

**β-arrestins as regulators of GPCR intracellular trafficking**

The original description of Class B receptors suggested that β-arrestin association with a receptor on endosomes impedes receptor recycling and more recent reports have linked sustained β-arrestin interaction with receptor ubiquination and lysosomal targeting (Shenoy, McDonald et al. 2001; Martin, Lefkowitz et al. 2003). Moreover, the experiments described
above with the B2R, a Class C receptor like CCR5, suggest that dissociation of β-arrestins from the B2R is required for recycling. There is evidence, however, for β-arrestins acting as facilitators of GPCR recycling for the A_{2B} adenosine receptor (Mundell, Matharu et al. 2000) and N-formyl peptide receptor (FPR) (Vines, Revankar et al. 2003). In the case of the FPR, although β-arrestins are not required for its internalisation, it nevertheless co-internalises with β-arrestins and maintains this interaction into a Rab11-positive compartment. From there, the FPR recycles efficiently to the plasma membrane. Interestingly, there is a complete inhibition of recycling in β-arrestin 1 and 2 knock-out MEFs, which can be relieved by co-expression of either β-arrestin1 or β-arrestin2, suggesting that β-arrestins are required for the recycling of the FPR. In support of this notion, the authors also found a greater concentration of the FPR in the perinuclear recycling compartment in β-arrestin 1 and 2 knock-out cells compared with wild-type MEFs. However, a subsequent study from the same group also concluded that dissociation of β-arrestin from the FPR is required for its recycling (Key, Vines et al. 2005).

Although a clear description of the role of β-arrestins in the intracellular trafficking of GPCRs is lacking, these studies do suggest that β-arrestins may well be playing a regulatory role and that this function is likely to be different for particular receptors. For CCR5, I have clearly shown a sustained interaction with β-arrestins into the perinuclear recycling compartment. Unfortunately, these observations do not indicate where the receptor and β-arrestin dissociate, and as such, I have tried to follow CCR5 trafficking and β-arrestin2–GFP association in live cells. However, these experiments were hampered by the photo-sensitivity of the CHO cells used and no conclusive results were obtained, so this remains an open question.

In Chapter 6, I expand on the observations reported in this chapter and describe experiments where I have attempted to determine if β-arrestins regulate CCR5 intracellular trafficking.

β-arrestin ubiquitination and association with GPCRs

Besides the pattern of serine/threonine clusters in the C-tails of GPCRs, another potential regulatory mechanism governing transient versus stable
association of β-arrestins with GPCRs has emerged, in that there appears to be a correlation between the stability of β-arrestin ubiquitination and the stability of the β-arrestin association (Shenoy and Lefkowitz 2003; Shenoy and Lefkowitz 2005). Indeed, expression of β-arrestin2 with ubiquitin fused to its C-tail imparts Class B characteristics on the β2AR, a Class A receptor (Shenoy and Lefkowitz 2003). It may be that post-translational modifications are superimposed on the intrinsic binding affinities of β-arrestin-receptor complexes to regulate their association. It would be interesting, therefore, to follow β-arrestin ubiquitination throughout CCR5 trafficking.

**Studying a regulatory role for β-arrestins in CCR5 trafficking in β-arrestin knock-out MEFs**

Unfortunately, although the β-arrestin knock-out MEFs are an ideal system to study a differential role for β-arrestin1 and β-arrestin2 in CCR5 recycling, little recycling was observed in the transiently-transfected MEFs (data not shown). This may partially account for the high extent of CCR5 down-modulation observed in these cells. Also, this may or may not reflect an inability of the receptor to recycle in MEFs, *per se*: in my hands, CCR5 recycled poorly in a variety of transiently-transfected cell-lines so this may be more a reflection of the inability of transiently-transfected cells to support robust CCR5 recycling.

**CCRS phosphorylation and recycling**

**Phosphorylation-independent β-arrestin binding**

Consistent with sustained β-arrestin binding to agonist-activated CCR5, I have shown by immunofluorescence that CCR5 molecules remain phosphorylated on serines 337 and 349 as they traffic to the perinuclear recycling compartment. That the intensity of the phospho-antibody signals was maintained from 5 to 60 min RANTES treatment and a sustained change in electrophoretic mobility of CCR5 on polyacrylamide gels over long periods of RANTES treatment was observed (Figure 4-VI), suggests that the majority of receptors maintain their phosphorylated status as they pass into
the perinuclear recycling compartment. These results are consistent with those of Pollok-Kopp et al. (2003), who showed a sustained phosphorylation of serines 337 and 349 in RBL CCR5 cells in the constant presence of RANTES for 30 min. Moreover, they are harmonious with data obtained in CHO cells, showing that a 10 min incubation with RANTES induced a rapid and sustained phosphorylation of CCR5 for at least up to 4 h (Signoret, Christophe et al. 2004).

I have also shown that a CCR5 mutant with all 4 serines shown to undergo agonist-induced phosphorylation mutated to alanines (CCR5 4S→A), can undergo internalisation, again, in keeping with previous reports (Kraft, Olbrich et al. 2001). However, my finding that fluorescently-tagged β-arrestins were recruited to agonist-activated CCR5 4S→A is in contrast to previous reports demonstrating the requirement of intact phosphorylation sites for high affinity β-arrestin binding. Firstly, following the interaction between CCR5-ECFP and β-arrestin-EYFP in HEK293 cells using fluorescence resonance energy transfer (FRET), Kraft et al. (2001) found that although an interaction between wild-type CCR5 and β-arrestin could be detected after agonist treatment, there was no significant change in the FRET ratio upon RANTES stimulation of cells expressing CCR5 4S→A-ECFP and β-arrestin-EYFP. However, the authors remarked on the relative inefficiency of this particular system using transiently transfected cells, with only a modest increase in FRET ratio seen in RANTES-treated cells expressing wild-type CCR5-ECFP and β-arrestin-EYFP. Thus, perhaps the assay was not sensitive enough to detect an interaction between CCR5 4S→A and β-arrestin, which would be predicted to be weaker than binding to wild-type CCR5. Moreover, the proteins were C-terminally tagged: the addition of CFP to the C-tail of CCR5 might have weakened the strength of β-arrestin binding, resulting in undetectable binding of β-arrestin to CCR5 4S→A.

Secondly, through immunoblot detection of the presence of endogenous β-arrestins in membranes isolated from whole cell lysates of RBL cells expressing various serine to alanine mutants of CCR5, Huttenrauch et al. (2002) found that at least two intact phosphorylation sites were required to support β-arrestin binding. Again, the difference between these authors' results and mine is most simply explained by the difference in assay, where
it may be argued that the assay used by Huttenrauch et al. was not sensitive enough to detect binding of endogenous β-arrestins to CCR5 mutants lacking 3 or 4 phosphorylation sites, which would be assumed to have a lower affinity for β-arrestins than wild-type CCR5. Conversely, it could be argued that the association of β-arrestins with CCR5 4S→A in my assay was an over-expression artefact. However, I favour the first explanation, since CCR5 4S→A did not internalise in MEFs lacking endogenous β-arrestins, whereas it did internalise in wild-type MEFs or MEFs lacking either β-arrestin 1 or β-arrestin2, suggesting that β-arrestins do mediate its internalisation.

A surprising number of GPCRs have been shown to bind β-arrestins (and in most cases also internalise) in the absence of phosphorylation, including the D6 non-signalling chemokine receptor (Galliera, Jala et al. 2004), protease activated receptor 2 (Stalheim, Ding et al. 2005), lutropin receptor (Mukherjee, Palczewski et al. 1999; Min and Ascoli 2000; Min, Galet et al. 2002; Mukherjee, Gurevich et al. 2002), substance P receptor (Richardson, Balius et al. 2003), orexin 1 receptor (Milasta, Evans et al. 2005), leukotriene B4 receptor 1 (Jala, Shao et al. 2005) and several splice variants of the serotonin 5-HT4 receptor (Barthet, Gaven et al. 2005). However, for the substance P, orexin-1 and protease activated 2 receptors, phosphorylation has been shown to enhance the stability of the β-arrestin-receptor complex (Richardson, Balius et al. 2003; Milasta, Evans et al. 2005; Stalheim, Ding et al. 2005). Likewise, it does seem that phosphorylation of CCR5 enhances the affinity and stability of its association with β-arrestins, especially with β-arrestin1: β-arrestin1-YFP was only weakly recruited to CCR5 4S→A and the receptor appeared to have undergone less endocytosis after 5 min RANTES treatment than the wild-type receptor (CCR5 wt). Moreover, no association between β-arrestin1 and CCR5 4S→A was observed after 60 min RANTES treatment, when the receptor had accumulated in the perinuclear recycling compartment. A stronger interaction was observed between β-arrestin2-GFP and CCR5 4S→A, which was maintained into the recycling compartment, but the strength of this interaction was variable between cells and was not as consistently strong as the interaction between CCR5 wt and either of the β-arrestins. So although agonist binding may push CCR5 into a conformation where it can bind β-arrestins in a phosphorylation-independent manner,
phosphorylation may further stabilise this state and/or increase the affinity or stability of the interaction.

What should be made clear, though, is that just because agonist-activated receptors are capable of binding β-arrestins and internalising in a phosphorylation-independent manner, this mechanism may not operate in vivo, where phosphorylation occurs rapidly after agonist-activation. This is also goes for most of the receptors shown to undergo β-arrestin-dependent internalisation in the absence of receptor phosphorylation, where this ability has mainly been demonstrated using phosphorylation-deficient receptor mutants, although the D6 chemokine receptor, which undergoes constitutive β-arrestin-dependent internalisation, does not appear to be phosphorylated in vivo (Galliera, Jala et al. 2004).

One final consideration with respect to phosphorylation-independent β-arrestin binding is that, although unlikely, there is the possibility that CCR5 is phosphorylated on residues other than serines 336, 337, 342 and 349 in CHO cells and MEFs. The C-terminus of CCR5 contains 5 serine and 2 threonine residues that could be phosphorylated upon receptor activation. The phospho-amino acid analysis that revealed that agonist-induced phosphorylation of CCR5 occurs exclusively on serine residues and alanine scanning mutagenesis that showed that only serines 336, 337, 342 and 349 undergo agonist-induced phosphorylation, were carried out in COS-7 cells (Oppermann, Mack et al. 1999). Moreover, the subsequent experiments that showed that S337 is a PKC substrate and S349 is a GRK substrate were conducted in RBL cells (Pollok-Kopp, Schwarze et al. 2003). Although S325 would be an unlikely candidate for phosphorylation due to its proximity to cysteines 321, 323 and 324, which have been shown to be palmitoylated (Blanpain, Wittamer et al. 2001; Kraft, Olbrich et al. 2001; Percherancier, Planchenault et al. 2001), it remains to be determined if the threonine residues are phosphorylated upon agonist activation in CHO cells or MEFs. $^{32}$P-labelleing of RANTES-treated cells expressing CCR5 4S→A could confirm whether this is the case.
Phosphorylation, resensitisation and recycling

As alluded to above, internalisation and recycling is generally considered to be necessary for GPCR resensitisation, which involves agonist dissociation, dephosphorylation of C-tail Ser/Thr residues and recycling back to the plasma membrane (Hanyaloglu and von Zastrow 2007). For instance, β2AR, the prototypical GPCR, undergoes agonist dissociation in the low pH of the early endosome, and dephosphorylation and recycling of the receptor are inhibited by raising endosomal pH (Krueger, Daaka et al. 1997; Signoret, Christophe et al. 2004). Recycled receptors are competent to respond to further rounds of agonist stimulation. However, unlike the β2AR, a pH-dependent agonist-dissociation mechanism does not operate for CCR5 agonists and the recycling of CCR5 is not affected by raising endosomal pH (Signoret, Christophe et al. 2004). Moreover, receptors can recycle to the plasma membrane in an agonist-bound form, which results in their recycling (Signoret, Pelchen-Matthews et al. 2000). Dephosphorylation of CCR5 does not appear to be required for recycling either (nor is it inhibited by raising endosomal pH (Signoret, Christophe et al. 2004)), since an N-terminally modified form of RANTES, AOP–RANTES, which shows an enhanced association with GRKs and increased phosphorylation of C-tail serine residues (Oppermann, Mack et al. 1999; Vila-Coro, Mellado et al. 1999), recycles with similar kinetics to RANTES-treated CCR5 (Signoret, Pelchen-Matthews et al. 2000).

The results presented in this chapter are consistent with these previous observations, in that most receptors appear to remain phosphorylated as they traffic to the perinuclear recycling compartment, but both a phosphorylation-deficient receptor and a wild-type receptor, which has the potential to remain phosphorylated, are able to recycle. The results also demonstrate that a dephosphorylated receptor could traffic to the recycling compartment and also engage the recycling machinery, since agonist-activated CCR5 4S→A was seen to accumulate in the recycling compartment under constant agonist stimulation and was also able recycle back to the plasma membrane. This raises the possibility that dephosphorylation could occur as early in post-endocytic trafficking as in the early endosome, without perturbing the trafficking itinerary of the receptor. Whether phosphorylation actually plays a role in regulating post-endocytic CCR5
trafficking, though, is not certain. The simplest role for phosphorylation in controlling trafficking of a GPCR is one exercised through \( \beta \)-arrestin binding (as described above), although dephosphorylation may be more controlled by \( \beta \)-arrestin dissociation, allowing phosphatases to access phosphorylated residues, rather than the other way round (although perhaps not for CCR5, given the accessibility of phospho-sites to antibodies - see below). This simple view of phosphorylation exerting its effect through \( \beta \)-arrestin binding has been challenged, though, by mutational analysis of the V2R, which has shown that phosphorylated residues in the C-tail required for a sustained association with \( \beta \)-arrestins are distinct from those slowing receptor recycling (Innamorati, Le Gouill et al. 2001; Le Gouill, Innamorati et al. 2002). Moreover, a recent study with the MOR (a Class A receptor), suggests that phosphorylated and non-phosphorylated MORs recycle through distinct pathways mediated by Rab4 and Rab11, respectively (Wang, Chen et al. 2008). Despite this, for most GPCRs, dephosphorylation may simply be a requisite step in their resensitisation process and specific sequences present in their C-tails may be far more important in mediating recycling (Hanyaloglu and von Zastrow 2007). However, there is some data suggesting that phosphorylation may control the interaction of regulatory proteins with these sequences (see below).

Unfortunately, the phosphorylation assays described in this chapter provide little kinetic data for either down-modulation or recycling. The down-modulation of CCR5 wt and CCR5 4S\( \rightarrow \)A were only assessed after 60 min RANTES treatment in both CHO-K1 cells and MEFs, and recycling only after 60 min in CHO-K1 cells. These time-points were chosen as they have been shown to be sufficient to allow a steady-state down-modulated distribution of CCR5 to be established and allow enough time for the recovery of the vast majority of CCR5 molecules to the cell surface in CHO CCR5 cells, respectively. The immunofluorescence results may belie significant differences in trafficking rates for the two receptors and for the receptors in the absence of either \( \beta \)-arrestin1 or \( \beta \)-arrestin2. These differences may be revealed by a kinetic analysis of cell surface CCR5 levels at numerous time-points by flow cytometry, as has been employed in Chapters 5 and 6 for other investigations.
Certainly, though, CCR5 does seem to be unique among GPCRs, in that a significant proportion of agonist-activated, internalised receptors recycle to the plasma membrane in an agonist-bound, activated form, escaping resensitisation. Why this should be the case is unclear, but it may have something to do with its role in chemotaxis. Perhaps a migrating cell exposed to a chemokine gradient regulates the rate of resensitisation of its chemotactic receptors as part of its gradient-sensing mechanism. Alternatively, both vesicles emanating from the recycling endosome and the TGN have been shown to be delivered to the front of the lamellipodium in migrating cells (Hopkins, Gibson et al. 1994; Schmoranzer, Kreitzer et al. 2003; Prigozhina and Waterman-Storer 2004), and there is evidence for the accumulation of CCR5 at the leading edge of migrating T cells (Nieto, Frade et al. 1997). Moreover, there is growing recognition that β-arrestins may scaffold signalling molecules involved in cytoskeletal reorganisation to promote localised actin assembly events leading to the formation of a leading edge (Defea 2006), so perhaps the lack of CCR5 resensitisation (and continued β-arrestin association) is a mechanism to deliver bound β-arrestins to the lamellipodium via targeted exit from the recycling compartment.

**CCRS recycling and sequence-directed recycling**

A recent study conducted by Delhaye et al. identified the last four amino acids of the CCR5 C-terminus as crucial for receptor recycling (Delhaye, Gravot et al. 2007). Removal of these residues resulted in a re-routing of internalised receptors to lysosomes for degradation. These last four amino acids, SVGL, which contain Ser349, conform to a type II PDZ ligand (X−φ−X−φ, where X is any amino acid and φ is a hydrophobic amino acid). The authors propose that this putative PDZ-ligand may engage PDZ domain-containing proteins, such as the Na+/H+ exchanger regulatory factor / ezrin/radixin/moesin-binding phosphoprotein of 50 kDa (NHERF/EBP50), which regulates recycling of the β2AR by binding to a type I PDZ ligand (DSLL) at its C-terminus (Cao, Deacon et al. 1999). Interestingly, recycling of the β2AR is inhibited by the phosphorylation of the serine residue after GRK5 over-expression, which may prevent NHERF/EBP50 binding. Moreover,
replacement of Ser411 with a phospho-mimetic aspartic acid residue blocked the interaction of the receptor with NHERF and also inhibited recycling, leading to the degradation of internalised receptors. In contrast, Delhaye et al. found that CCR5 recycling seemed to be independent of the phosphorylation state of S349: in Jurkat cells, a CCR5 phospho-mimetic mutant in which Ser349 had been mutated to Glu, recycled just as well as the wild-type receptor and the recycling of a Ser349→Ala mutant was only partially inhibited in its ability to recycle. The results of this study are compatible with the data presented herein, as the CCR5 4S→A mutant would effectively contain an intact PDZ ligand with the serine residue mutated to alanine. PDZ ligand-based recycling signals have been suggested to regulate the recycling of numerous other GPCRs, such as the MOR (Tanowitz and von Zastrow 2003) and the endothelin type A receptor (Paasche, Attramadal et al. 2005), and a sequence conforming to a type II PDZ ligand is present in the chemokine receptor, CCR2b (Hung and Sheng 2002). Such a sequence-dependent recycling mechanism may operate for CCR5, perhaps superimposed on a β-arrestin-dependent regulatory mechanism (see below).

Accessibility of phospho-sites and implications for control of CCR5 trafficking

Despite the observation that β-arrestin2-GFP is stably recruited to CCR5 4S→A, C-tail serine phosphorylation probably participates in high affinity β-arrestin binding, as is the case for numerous other GPCRs (Gurevich and Gurevich 2006). As such, it may be expected that β-arrestin binding would preclude the binding of phosphosite-specific antibodies. That antibody binding was observed in the immunofluorescence experiments described above can be explained in at least two ways. The first explanation is related to the ability of CCR5 to dimerise, which has been reported by several different groups (Blanpain, Vanderwinden et al. 2002; Chelli and Alizon 2002; Issafras, Angers et al. 2002; Huttenrauch, Pollok-Kopp et al. 2005). In a CCR5 dimer, perhaps only one activated receptor need bind β-arrestin1/2 to effect internalisation, leaving a β-arrestin-free C-tail that is accessible to phospho-site specific antibodies.
The second explanation is that the phosphorylated residues, although required for high-affinity β-arrestin binding, may become accessible (solvent exposed) after transition of the β-arrestin molecule from its basal, cytosolic conformation into its active, high-affinity receptor binding conformation upon engagement with an activated, phosphorylated receptor. Phospho-residues on the receptor are believed to disrupt the polar core of the β-arrestin molecule, a process necessary for β-arrestin activation. Interestingly, mutation of the phosphate-sensitive residue, Arg169, in the polar core of β-arrestin1 results in an arrestin molecule that binds agonist-activated but non-phosphorylated β2AR with similar affinity to which wild-type β-arrestin1 binds the agonist-activated, phosphorylated receptor (Kovoor, Celver et al. 1999). This suggests that after the conformational change in the β-arrestin molecule promoted by phosphate binding, ionic interactions between the phospho-residues and the β-arrestin molecule in the finally rearranged receptor-β-arrestin complex are not required for high-affinity binding. Moreover, this also appears to be true for the sustained interaction of β-arrestins with Class B receptors (Oakley, Laporte et al. 2001). Oakley et al. showed that β-arrestin1 formed stable complexes with agonist-activated neurotensin receptor (NTR) and V2R as they trafficked into endosomes (Class B receptors), whereas mutation of one of the serine clusters in their C-tails, which are phosphorylated upon agonist-activation, prevented this stable interaction (β-arrestin1 dissociated at or near the plasma membrane). However, a truncated β-arrestin mutant, in which the structural constraint of the β-arrestin C-tail involved in maintaining β-arrestin in its basal conformation was removed, stably associated with the mutant receptors as they redistributed into endosomes, suggesting that ionic interactions between the phospho-residues and the β-arrestin molecule in the finally rearranged receptor-β-arrestin complex are not required for the sustained, high-affinity β-arrestin interaction but that the phospho-residues may, instead, function to promote conformational changes in the β-arrestin molecule that promote this sustained interaction.

If, after a stable CCR5-β-arrestin complex has been formed, the phosphorylation status of the CCR5 C-tail serine residues becomes redundant for high-affinity β-arrestin binding - and phospho-residues
become solvent-exposed - CCR5 dephosphorylation and β-arrestin
dissociation may not be tightly coupled, as is suggested for other GPCRs
(Oakley, Laporte et al. 1999). A second intriguing implication is that the
putative PDZ ligand at the extreme C-terminus, which includes S349, would
also be accessible on a β-arrestin-engaged receptor. This would also be the
case in a receptor dimer with only one CCR5 C-tail bound to β-arrestin.
Either way, the fact that at least some of the P-S349 epitopes are accessible
to antibody binding demonstrates that a PDZ sequence-directed regulatory
mechanism could act alongside a β-arrestin-dependent regulatory
mechanism for CCR5 trafficking.

**Summary of CCR5 C-tail phosphorylation and trafficking with β-
arrestins**

Although many aspects of the phosphorylation of CCR5 and its association
with β-arrestins are unclear, Figure 4—XII shows a cartoon model of CCR5
trafficking and association with β-arrestins that attempts to integrate the
data presented in this chapter with previous reports.

In this model, agonist binding leads to receptor activation but CCR5
molecules are rapidly desensitised, a process initiated by phosphorylation of
C-tail serine residues by PKC and CRKs 2 and 3. This increases the affinity
of receptors for β-arrestins 1 and 2, which bind and couple CCR5 molecules
to the clathrin-mediated endocytic machinery. Receptors internalise with β-
arrestins and traffic through early endosomes to a perinuclear recycling
compartment, maintaining an interaction with β-arrestins throughout this
trafficking. A significant proportion of the receptors reaching this
compartment appear to be phosphorylated. From here, receptors recycle
back to the plasma membrane. Some receptors returning to the cell surface
are agonist-free, dephosphorylated and, presumably β-arrestin-free (i.e.
resensitised), although the site(s) of these resensitisation processes are
unclear - an agonist-free, dephosphorylated receptor is tentatively drawn in
the perinuclear recycling compartment in the model. Some CCR5 molecules,
however, recycle back to the plasma membrane in an agonist-bound and
presumably phosphorylated state. In this case, they can re-engage the
clathrin-mediated endocytic machinery and undergo a further round of
internalisation and recycling. Whether β-arrestins remain engaged with agonist-bound receptors as they recycle back to the cell surface is unknown. It may take several cycles of internalisation and recycling for the average receptor to be resensitised.

Figure 4-XII Cartoon illustrating the association of β-arrestins with CCR5 throughout intracellular trafficking

See main text for details. Receptor activation is indicated by a colour change from black to red. The recycling compartment, which may comprise elements of the TGN and recycling endosome (see Chapter 3) is shown in a simplified form. The MTOC is shown in the bottom right corner; dotted grey lines represent microtubules emanating from the MTOC.
Chapter 5: THE CONTROL OF CCR5 RECYCLING

“You can contain drug trafficking by the immobilisation of the few cartels who truly control it.”

James Mills, The Underground Empire - Where Crime and Governments Embrace

The data presented in the previous two chapters demonstrate that after agonist activation, CCR5 is internalised and traffics to a perinuclear recycling compartment comprising recycling endosome and TGN elements. β-arrestins, which are recruited to activated receptors at the cell surface, remain bound to CCR5 molecules as they traffic to this compartment. In this chapter, I focus on the trafficking machinery that may operate to bring about CCR5 recycling from the recycling compartment, providing evidence that the passage of CCR5 through this compartment is both clathrin- and dynamin-dependent.

Down-modulated CCR5 accumulates in compartments decorated with clathrin

Very little is currently known about the mechanisms operating at the recycling compartment for sorting internalised CCR5 for recycling. Indeed, the experiments showing that CCR5 likely traffics through the TGN in addition to the recycling endosome (see Chapter 3) do not indicate whether the recycling endosome or TGN is the site of this sorting mechanism. A clue to a possible sorting mechanism, though, comes from the observations of immunolabelled cryosections by Signoret et al. (2000), where after 60 min
RANTES treatment in CHO CCR5 cells, CCR5 was observed in tubules and vesicles in the perinuclear region, with the vesicles often displaying a prominent coat, highly reminiscent of clathrin. However, no immunolabelling was carried out to confirm the nature of the coat.

Endosomal clathrin-coated buds (which label positively for TfR) have been observed in A431 and HeLa cells (Stoorvogel, Oorschot et al. 1996; van Dam and Stoorvogel 2002), and TfR recycling from recycling endosomes (but not early endosomes) appears to involve the formation of clathrin-coated vesicles in a dynamin-dependent manner (van Dam and Stoorvogel 2002). In addition, endosomal clathrin-coated buds have been observed in polarised Madin–Darby canine kidney (MDCK) cells, where they have been proposed to play a role in basolateral TfR trafficking (Futter, Gibson et al. 1998). More recently, there has also been a report that ACAP1 (ARFGAP with coiled coil, ANK repeat, and pleckstrin homology domains), which has been shown to be involved in the recycling of the TfR from the recycling endosome (Dai, Li et al. 2004), acts as a clathrin adaptor in stimulation-dependent integrin recycling and insulin-stimulated recycling of glucose transporter type 4 (GLUT4) from the recycling endosome (Li, Peters et al. 2007). Clathrin has long been known to be present at the TGN, although its role has been best described in terms of mediating sorting from the TGN to endosomes (Robinson 2004).

Based on the above evidence, a reasonable hypothesis is clathrin-coated vesicle formation at the recycling compartment is involved in CCR5 recycling, perhaps with CCR5-containing vesicles being directly targeted to the plasma membrane. I have also shown that β-arrestins, which internalise with CCR5, remain bound to receptors in the recycling compartment, so a more tantalising hypothesis is that β-arrestins function as clathrin adaptors for CCR5 at the recycling compartment.

To test the plausibility of the hypothesis that CCR5 transit through the recycling compartment requires clathrin, I initially assessed by immunofluorescence the extent to which clathrin could be found on steady-state down-modulated CCR5-containing compartments (Figure 5–1). Figure 5–1 A shows the results of an experiment using CHO CCR5 cells stably expressing clathrin light chain a fused to dsRed (CHO CCR5 CLCa–dsRed cells), which has previously been shown to label clathrin-coated pits without
interfering with clathrin-mediated endocytosis (Gaidarov, Santini et al. 1999; Merrifield, Feldman et al. 2002; Perrais and Merrifield 2005). This experiment was performed in a similar manner to experiments following the association of β-arrestins with CCR5, in that cell surface CCR5 was pre-labelled with MC-5 and cells were co-stained for TfR. The figure shows representative single confocal sections through the middle of cells. In unstimulated cells, CCR5 was present diffusely over the cell surface. Clathrin signal was localised to punctate structures that were predominantly located in the perinuclear region. Clathrin puncta were also observed at the plasma membrane, but these were only really visible in basal sections, where a greater proportion of the plasma membrane was covered in the field of view (data not shown). TfR staining was punctate and frequently concentrated in the perinuclear region, although, as previously noted in CHO cells used in the experiments described in this thesis, significant amounts of punctate staining was seen throughout the rest of the cytoplasm. TfR and clathrin exhibited a partial overlap in both the perinuclear region and in the rest of the cytoplasm; separate TfR-positive and clathrin-positive puncta were observed in both regions. Perinuclear clathrin- and TfR-positive puncta presumably represented clathrin-decorated recycling endosomes; more peripheral puncta positive for both proteins were more likely early endosomes. The perinuclear clathrin signal that did not colocalise with TfR was likely localised to the TGN.

After 60 min RANTES treatment, CCR5 redistributed from the cell surface mainly into the perinuclear region, although some more peripherally-located CCR5 puncta were observed and some cell surface CCR5 was also seen. As observed in CHO CCR5 and CHO CCR5 βarr1-YFP/βarr2-GFP cells, CCR5 showed an almost complete overlap with TfR, which appeared more concentrated around the nucleus than in unstimulated cells. Significantly, clathrin colocalised well with CCR5 that had accumulated in the perinuclear region, suggesting the presence of clathrin on CCR5-containing recycling endosomes. Clathrin was also present on many of the more peripheral CCR5- and TfR-positive puncta. As in unstimulated cells, some clathrin puncta that did not colocalise with TfR and CCR5 were also observed in both the perinuclear region and more peripheral cytoplasm.
Chapter 5: The Control of CCR5 Recycling

A

unstimulated

60 min RANTES
Figure 5.1 In the constant presence of agonist, down-modulated CCR5 accumulates in intracellular compartments decorated with clathrin

A. CHO CCR5 clathrin LCa-dsRed cells were pre-labelled for cell surface CCR5 with MC-5 and then either fixed (unstimulated) or incubated with RANTES in BM for 60 min before being fixed (60 min RANTES). Cells were then incubated in permeabilising blocking buffer with H68.4 (anti-TfR) primary antibody. MC-5 and H68.4 were detected with Alexa 488 GAM IgG2a and Alexa 647 GAM IgG1 secondary antibodies, respectively. B. CHO CCR5 cells were pre-labelled for cell surface CCR5 with MC-5 and then either fixed (unstimulated) or treated with RANTES in BM for 60 min and then fixed (60 min RANTES). Cells were then incubated in permeabilising blocking buffer with MC-5 (anti-CCR5) and X22 (anti-clathrin heavy chain) primary antibodies. MC-5 and X22 were detected with Alexa 488 GAM IgG2a and Alexa 594 GAM IgG1, respectively. C. CHO CCR5 cells were...
treated with CHX to clear newly synthesised CCR5 from the biosynthetic pathway. Cells were then treated with RANTES in BM containing CHX for 60 min, before being fixed. Cells were then permeabilised in blocking buffer and incubated with MC-5 (anti-CCR5) and X22 (anti-clathrin heavy chain) primary antibodies. MC-5 and X22 were detected with Alexa 488 GAM IgG2a and Alexa 594 GAM IgG1, respectively. Single confocal sections are shown in A, B and C. Bars, 5 μm.

To ensure that the overlap between down-modulated CCR5 and clathrin was not an overexpression artefact, I repeated the trafficking assay in CHO CCR5 cells, staining for endogenous clathrin heavy chain (Figure 5-1 B; TfR staining was omitted from this experiment). In unstimulated cells, clathrin staining was very similar to that of CLCa-dsRed in the CHO CCR5 CLCa-dsRed cells: punctate and strongest in the perinuclear region, with some puncta found in the rest of the cytoplasm and at the plasma membrane. CCR5 was present over the whole of the cell surface. After 60 min RANTES treatment, CCR5 accumulated in the perinuclear region and showed a significant overlap with clathrin staining, in agreement with the overlap observed between CCR5 and CLCa-dsRed in the CHO CCR5 CLCa-dsRed cells.

I also performed the experiment without pre-binding MC-5 (Figure 5-1 C). Here, CHO CCR5 cells were pre-treated with CHX to clear CCR5 from the biosynthetic pathway, treated with RANTES, fixed and labelled for CCR5 with MC-5 post-fixation. Under these conditions, a significant amount of down-modulated CCR5 staining was observed to overlap with a TGN marker, p230 (Figure 3–IX). As expected, CCR5 and clathrin distributions in unstimulated cells were indistinguishable from cells pre-labelled for CCR5 as in Figure 5–I B (data not shown). After 60 min RANTES treatment, CCR5 accumulated in the perinuclear region and, again, showed a strong overlap with clathrin. This suggests that regions of the TGN containing CCR5 are probably also coated with clathrin.

Altogether, these data show that the recycling compartment through which CCR5 passes is extensively coated with clathrin at the point CCR5 is traversing it.
Down-modulated CCR5 accumulates in intracellular compartments positive for AP-1

In Chapter 3, I showed that after 60 min RANTES treatment, down-modulated CCR5 accumulated in compartments that were positive for AP-1. This colocalisation is again illustrated in Figure 5-II A, where CHO CCR5 cells were either untreated or treated with RANTES for 60 min, fixed and subsequently stained for CCR5 and γ-adaptin, a component of AP-1. Not only did CCR5 colocalise with γ-adaptin in the perinuclear region, but some more peripherally located CCR5 puncta also colocalised with γ-adaptin, which perhaps represents AP-1 on CCR5-containing early endosomes. In Chapter 3, I also showed that some of the perinuclear CCR5- and γ-adaptin-positive puncta were largely negative for TfR (this is illustrated with another example in Figure 5-II B; most easily observed in the dual channel, green-red merges) and that significant overlap of down-modulated CCR5 with p230, a TGN marker, could be observed. However, some CCR5 could also be detected in γ-adaptin-positive but p230-negative perinuclear puncta, so altogether, after 60 min RANTES treatment, CCR5 could be found in the TGN and recycling endosomes, which are both positive for AP-1. Overall, the clear message is that agonist-activated CCR5 certainly passes through multiple AP-1-positive compartments.

In keeping with the data presented in this chapter, S. Wavre, by electron microscopy (EM) observations of immunolabelled cryosections, has more recently observed down-modulated CCR5 in AP-1-positive coated vesicles adjacent to other CCR5-containing coated structures in the perinuclear region, whose coat also labels for AP-1 (Silène Wavre, Endocytic Regulation of Chemokine Receptor Expression, PhD thesis, 2006). The presence of AP-1 in these coats provides strong evidence that the coat is clathrin.
Chapter 5: The Control of CCR5 Recycling

A

unstimulated

60 min RANTES
Figure 5-11 In the constant presence of agonist, down-modulated CCR5 accumulates in intracellular compartments positive for AP-1

A + B. CHO CCR5 cells were treated with CHX to clear newly synthesised CCR5 from the biosynthetic pathway. Cells were then either fixed (unstimulated) or treated with RANTES in BM containing CHX for 60 min before being fixed (60 min RANTES). Cells in A were then permeabilised in blocking buffer and incubated with MC-5 (anti-CCR5) and Mary (anti-γ-adaptin) primary antibodies. MC-5 and Mary were detected with Alexa 488 GAM and Alexa 594 GAR, respectively. Cells in B were permeabilised in blocking buffer and incubated with MC-5 (anti-CCR5), Mary (anti-γ-adaptin) and H68.4 (anti-TfR) primary antibodies. MC-5, Mary and H68.4 were detected with Alexa 488 GAM IgG2a, Alexa 594 GAR and Alexa 647 GAM IgG1, respectively. Single confocal sections are shown. Dual channel, green-red merges between CCR5 and γ-adaptin, and CCR5 and TfR are also shown. Bars, 5 μm.

Significantly, there is evidence from other systems for AP-1 acting as a clathrin adaptor protein at both the recycling endosome and TGN. For recycling endosomes, the strongest evidence comes from polarised cells,
where a variant of AP-1 containing the μ1B subunit, AP-1B (Fölsch, Ohno et al. 1999), has been shown to mediate TfR recycling to the basolateral membrane (Rodriguez-Boulan, Müssch et al. 2004) and has been suggested to function in the basolateral delivery of newly synthesised proteins in epithelial cells (Fölsch 2005). In non-polarised cells, AP-1 has been shown to be involved in TGN to endosome sorting (Robinson 2004), although no role has been demonstrated for AP-1 in TGN to plasma membrane trafficking in non-polarised cells. Interestingly, The Alliance for Cellular Signaling (www.signaling-gateway.org) recently reported a yeast two-hybrid interaction between β-arr1 and the β1 subunit of AP-1. Moreover, in COS cells over-expressing GFP-tagged β-arr1 or 2, AP-1 co-immunoprecipitates with the tagged β-arrs (Julie Pitcher, personal communication), although AP-1 shows a stronger interaction with β-arr1 than β-arr2. I also have preliminary data suggesting an interaction between β-arr1–YFP and AP-1 in CHO CCR5 βarr1–YFP cells using the same immunoprecipitation protocol as for the CCR5–β-arr co-immunoprecipitations described in Chapter 4 (data not shown). However, the extent of this interaction did not appear to be agonist dependent. Given that β-arrs accompany CCR5 as it traffics to the perinuclear compartment, there is the possibility for a situation highly analogous to that which exists at the plasma membrane, where β-arrs couple CCR5 to both AP-2 and clathrin, in that β-arrs could couple CCR5 to AP-1 and clathrin in the recycling compartment, resulting in the formation of transport vesicles involved in the recycling of the receptor to the plasma membrane.

**Brefeldin A partially inhibits CCR5 recycling**

To investigate a role for AP-1 in CCR5 recycling, I initially assessed CCR5 recycling in the presence of the fungal metabolite, brefeldin A (BFA). BFA interferes with the GTP exchange function of GTP exchange factors (GEFs) for ADP-ribosylation factor (ARF) family proteins (Donaldson, Finazzi et al. 1992; Helms and Rothman 1992). This prevents the binding of ARF proteins to membranes and the proteins they, in turn, recruit (Klausner, Donaldson et al. 1992). AP-1 is recruited to membranes in part by the action of ARF
proteins and, indeed, BFA treatment causes the loss of γ-adaptin from the TGN (Robinson and Kreis 1992; Wong and Brodsky 1992; Wagner, Rajasekaran et al. 1994) and from endosomes (Futter, Gibson et al. 1998). Moreover, in MDCK cells, where Tf was found in γ-adaptin-containing, endosomal clathrin-coated buds, BFA treatment led to the loss of γ-adaptin from endosomes and an inhibition of the basolateral sorting of Tf, with a corresponding apical Tf release (Futter, Gibson et al. 1998).

To determine whether BFA treatment led to the loss of AP-1 from membranes in CHO CCR5 cells and to determine the lowest concentration that gave a rapid redistribution (to avoid concentration-dependent non-specific effects), I assessed the effect of various concentrations of BFA on γ-adaptin distribution by immunofluorescence (Figure 5—III A). After 1 min treatment with 10 μg/ml BFA, a significant redistribution of γ-adaptin from a principally punctate, perinuclear distribution to a disperse, cytoplasmic staining pattern had occurred; however, some punctate, perinuclear staining remained. Treatment with 100 ng/ml BFA for 1 min did not result in any additional redistribution, but the loss of punctate, perinuclear γ-adaptin staining was far less in cells treated with 1 μg/ml BFA, where the γ-adaptin distribution was largely unchanged. After 5 min incubation with 1 μg/ml BFA, γ-adaptin staining had largely become diffuse and cytoplasmic, but some perinuclear puncta still remained. In cells treated with 10 or 100 μg/ml BFA, nearly all of the γ-adaptin staining had become diffuse, suggesting that the majority of AP-1 had been lost from TGN and endosomal membranes after 5 min.

From these results, 10 μg/ml BFA was chosen for use in subsequent CCR5 recycling assays given the faster loss of γ-adaptin membrane staining than with 1 μg/ml BFA but no apparent increase in rate of loss of γ-adaptin membrane-association with 100 μg/ml BFA.

To assay recycling, I firstly used a FACS-based recycling assay. In this assay, CHO CCR5 cells were treated in suspension with RANTES for 60 min in order for CCR5 to reach a down-modulated steady-state. Cells were then washed and incubated with TAK-779 for 60 min. Aliquots of cells were removed from suspension at various time-points during the RANTES and TAK-779 incubation phases and their cell surface CCR5 levels measured by FACS.
As previously shown in this thesis by immunofluorescence, after 60 min RANTES treatment, the majority of the internal receptor pool is located in the perinuclear region (recycling compartment). In addition, Signoret et al. (2000) estimated that at least 75% of the internal CCR5 under these conditions was present in perinuclear tubules and vesicles, by morphometric analysis of immunolabelled cryosections. Hence, a subsequent incubation of 60 min RANTES-treated cells with the CCR5 antagonist, TAK-779, which prevents re-internalisation of receptors (and internalisation of receptors transiently present at the cell surface at the end of this 60 min RANTES treatment), allows receptor recycling from the recycling compartment to the cell surface to be followed.

Figure 5—III B shows the results of a FACS-based trafficking experiment, where BFA has been included in, or omitted from, the TAK-779 incubation. Here, cell surface CCR5-associated fluorescence has been expressed as a percentage of the initial cell surface fluorescence. Unfortunately, individual experiments showed a variable extent of recycling after 60 min incubation with TAK-779 (120 min time-point on graph). In all cases, the recycling curve reached a plateau by 120 min or began to plateau by this time-point. Why all of the initial cell surface fluorescence was not recovered in all experiments is unknown, but this variation with respect to the extent of recycling was a problem experienced in FACS-based recycling experiments reported in the remainder of this chapter and in Chapter 6.

Figure 5—III B is representative of multiple experiments, though, in the proportional difference between recycling in the presence and absence of BFA. As can be seen in the graph, BFA had a partial inhibitory effect on CCR5 recycling, with the curves diverging to an increasing extent over time. Over multiple experiments, BFA treatment led to an average reduction in recycling after 60 min incubation in TAK-779 of 33.2 ± 9.6% (where the difference in cell surface CCR5 level at 120 min and 60 min in the absence of BFA = 100%; n=6).

The shapes of the recycling curves suggest that CCR5 recycles via a single pathway in both the presence and absence of BFA. In order to test this argument, I used the following equation to describe the recycling process:

\[ f_t = f_{\text{max}}(1 - \frac{f_{\text{max}} - f_0}{f_{\text{max}}} e^{-\frac{t}{t_{\text{max}}}}) \]
The equation assumes that the rate of recycling is proportional to a single, first-order rate constant \( k_{\text{rec}} \) and the difference between the maximum cell surface fluorescence reached after recycling \( (f_{\text{max}}) \) and the cell surface fluorescence at any time-point \( (f) \) (see Chapter 2 for derivation of equation). This equation was linearised to give:

\[
\ln \left| \frac{f_{\text{max}} - f}{f_{\text{max}}} \right| = -k_{\text{rec}} t + \ln \left| \frac{f_{\text{max}} - f_0}{f_{\text{max}}} \right|
\]

The data from the recycling phases in the presence and absence of BFA were used in a plot of \( \ln((f_{\text{max}}-f)/f_{\text{max}}) \) against \( t \), as shown in Figure 5-III C. Curves were fitted to the data by linear regression and an \( R^2 \) value, the coefficient of determination (the square of the correlation coefficient; takes values between 0 and 1) obtained. This gives an indication of how well the obtained data describe the recycling equation and thus, whether CCR5 recycling from the recycling compartment likely occurs through a single pathway. In the absence of BFA, \( R^2 \) was 0.98, indicating that the data strongly describe a single-pathway recycling process. In the presence of BFA, \( R^2 \) was 0.79: a good, but nonetheless weaker fit. This analysis requires the arbitrary definition of the \( f_{\text{max}} \) value. In this first analysis, it was set to 100, as in the absence of BFA, all of the initial cell surface fluorescence was recovered. However, in the presence of BFA, extrapolation of the recycling curve would predict a plateau at around 75%. If a \( f_{\text{max}} \) of 75 is used for the data of recycling in the presence of BFA, an \( R^2 \) value of 0.95 is obtained, suggesting that recycling in the presence of BFA occurs via a single pathway but that a certain amount of CCR5 is retained within cells/recycles extremely slowly (Figure 5-III D).

As can be inferred from the linearised equation, the slope of the line from such a plot corresponds to the rate constant for recycling, \( k_{\text{rec}} \). As would be predicted from the recycling curves in Figure 5-III B, \( k_{\text{rec}} \) is reduced in the presence of BFA.

Next, I performed a morphological recycling assay by immunofluorescence to determine the effect of BFA on recycling (Figure 5-III E). Cell surface CCR5 was pre-labelled with MC-5 and then cells were treated for 60 min with
RANTES to down-modulate CCR5 before incubation in BM containing TAK-779 with or without BFA for 60 min to assess recycling. In keeping with the FACS results, CCR5 showed an almost complete recovery to the cell surface after 60 min incubation in TAK-779 in the absence of BFA; however, in the presence of BFA, although some CCR5 recycled to the plasma membrane, internal CCR5 signal was also observed, frequently concentrated in perinuclear clusters. The pattern of staining is slightly unexpected, since other studies have shown that BFA causes tubulation of endosomes (Stoorvogel, Oorschot et al. 1996; Futter, Gibson et al. 1998; Sönnichsen, De Renzis et al. 2000) and the TGN (Lippincott-Schwartz, Yuan et al. 1991).

In summary, BFA partially inhibited CCR5 recycling, suggesting that AP-1 may be involved in this process.

---

**A**

untreated

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Chapter 5: The Control of CCR5 Recycling

B

**B**

**RANTES**
(down-modulation)

**TAK-779**
(recycling)

![Graph showing cell surface fluorescence (% of untreated cells) over time (min)]

- **BFA**
- + BFA

Cell surface fluorescence (% of untreated cells)

0 20 40 60 80 100 120

0 20 40 60 80 100 120

0 10 20 30 40 50 60

0.0 -0.5 -1.0 -1.5 -2.0 -2.5 -3.0 -3.5 -4.0

ln((IU - Ayf)**(1)**/(IU**(1)**)) = -0.0134t - 0.5389

R^2 = 0.7893

ln((IU - Ayf)**(1)**/(IU**(1)**)) = -0.0475t - 0.4802

R^2 = 0.9813

C

**C**

![Graph showing ln((IU - Ayf)**(1)**/(IU**(1)**)) over time [t] (min)]

- **BFA**
- + BFA

In((IU - Ayf)**(1)**/(IU**(1)**)) = -0.0134t - 0.5389

R^2 = 0.7893

In((IU - Ayf)**(1)**/(IU**(1)**)) = -0.0475t - 0.4802

R^2 = 0.9813

195
Chapter 5: The Control of CCR5 Recycling

Figure 5-III BFA treatment inhibits CCR5 recycling
A. CHO CCR5 cells were either untreated or treated with 1, 10 or 100 μg/ml BFA in BM at 37°C for 1 or 5 min, before being fixed and stained for γ-adaptin with Adaptin γ primary antibody and Alexa 488 GAM secondary antibody in permeabilising blocking buffer. Epifluorescence images are shown. Images were captured with the same acquisition settings and processed identically. Two fields of untreated cells are shown; 1 field of cells is shown for each BFA treatment. Bar, 5 μm. B. FACS-based trafficking assay. CHO CCR5 cells were treated with RANTES in BM for 60 min before being washed and further incubated in BM containing TAK-779 and either 10 μg/ml BFA (+BFA) or ethanol (-BFA) for 60 min. Aliquots of cells were removed at various time-points and assayed for cell surface CCR5-associated fluorescence by FACS, using 44'MC-5 to detect CCR5. Cell surface CCR5 fluorescence is expressed as a percentage of the initial cell surface CCR5 fluorescence and plotted against time. A representative experiment is shown, with individual data points representing the mean of triplicate samples; error bars represent 1 standard deviation of the means. C. Graph showing a plot of ln((f_max - f)/f max) against time [t] for the data in B covering the recycling phase (TAK-779 incubation, 60 to 120 min). f_max, the maximum extent of recycling, was set to 100. Curves were fitted by linear regression and straight-line equations are shown next to the fitted curves. R², the coefficient of determination, obtained by regression analysis, is also shown. The coefficient of t is the recycling rate constant, k_rec, with units of min⁻¹. D. Graph showing a plot of ln((f_max - f)/f max) against time [t] for recycling in the presence of BFA, with f_max set to either 100 or 75. E. Immunofluorescence-based trafficking assay. CHO CCR5 cells were either fixed (unstimulated), treated with RANTES for 60 min and then fixed (60 min RANTES) or treated with RANTES for 60 min followed by an incubation in BM containing TAK-779 and either 10 μg/ml BFA (+BFA) or ethanol (-BFA). Single confocal sections are shown for unstimulated and 60 min RANTES treated cells; series of confocal sections are shown for cells treated for 60 min with RANTES followed by a 60 min incubation with TAK-779. Bar, 5 μm.

**AP-1 knock-down does not inhibit CCR5 recycling**

The above data suggest that AP-1 may play a role in CCR5 recycling. However, although it has been shown that BFA prevents the formation of clathrin-coated buds at the recycling endosome and inhibits the recycling of TfR from this compartment (Stoorvogel, Oorschot et al. 1996; van Dam and Stoorvogel 2002; van Dam, Ten Broeke et al. 2002), the same group reporting these findings also reported that endosomal clathrin-coated buds
were largely devoid of γ-adaptin (Stoorvogel, Oorschot et al. 1996). Hence, the BFA result could be interpreted as an inhibitory effect on clathrin-coated pit formation, rather than on the function of AP-1. Moreover, BFA has multiple other effects, such as the release of COPI components from the Golgi (Donaldson, Lippincott-Schwartz et al. 1990; Wong and Brodsky 1992) and redistribution of Golgi elements into the endoplasmic reticulum (Lippincott-Schwartz, Donaldson et al. 1990), and may also have an effect on other ARF effectors.

I therefore performed a knock-down of an AP-1 subunit as an alternative approach to assess the role of AP-1 in CCR5 recycling. AP-1 is a heterotetrameric protein complex, whose clathrin adaptor activity can be effectively removed by knock-down of its μ1A subunit (Hirst, Motley et al. 2003). However, the most commonly used siRNA oligonucleotide against the μ1A subunit, described in Hirst et al. (2003), failed to knock down the protein in CHO CCR5 cells (data not shown). As the hamster genome has not yet been sequenced, I designed three siRNA oligos against the mouse μ1A DNA sequence. I also checked that the target sequences were conserved in rat, as mice, rats and hamsters belong to the Momorpha sub-order of rodents. As can be see in Figure 5-IV A, the three oligonucleotides, Dharma1, Dharma2 and Inv, all showed a partial knock-down of μ1A-adaptin, with the Dharma2 oligonucleotide giving the highest efficiency of knock-down.

![A](image-url)
Chapter 5: The Control of CCR5 Recycling

B

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AllStars negative control siRNA

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<td>60 min RANTES</td>
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<td><img src="image13.png" alt="Image" /></td>
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<tr>
<td>60 min RANTES + 60 min TAK-779</td>
<td><img src="image14.png" alt="Image" /></td>
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μ1A siRNA

199
Figure 5-IV Knock-down of AP-1 does not affect CCR5 internalisation or recycling

A. CHO CCR5 cells were transfected with the indicated siRNA oligonucleotides using the Oligofectamine protocol described in Materials and Methods. Cell lysates were prepared in RIPA buffer and volumes of cell lysates containing equal amounts of proteins loaded on a gel. Separated proteins were transferred to nitrocellulose and probed with 23 (anti-clathrin heavy chain, C-HC), Adaptin γ (anti-γ-adaptin) and μ1A (anti-μ1A) primary antibodies. The blot was subsequently incubated with IRDye® 800 GAM to detect 23 and Adaptin γ, and IRDye® 680 GAR to detect μ1A, and proteins visualised using the Odyssey infra-red detection system. B. CHO CCR5 cells were transfected with either AllStars negative control or Dharma2 μ1A siRNA oligonucleotides using the Oligofectamine protocol described in Materials and Methods. Cells were either fixed (unstimulated), treated with RANTES in BM for 60 min and then fixed (60 min RANTES), or treated with RANTES for 60 min and then further incubated in BM containing TAK-779 for 60 min, before being fixed (60 min RANTES + 60 min TAK-779). Cells were then incubated in permeabilising blocking buffer containing MC-5 (anti-CCR5) or Mary (anti-γ-adaptin) primary antibodies. MC-5 and Mary were detected with Alexa 488 GAM and Alexa 594 GAR, respectively. Single confocal sections are shown. Knock-down cells are indicated with white asterisks. Bar, 5 μm. C. CHO CCR5 cells were transfected with either AllStars negative control or Dharma2 μ1A siRNA oligonucleotides using the Oligofectamine protocol described in Chapter 2 and assayed for CCR5 trafficking by FACS. A blot showing the extent of μ1A-adaptin knockdown, prepared as in A, except with a
tubulin loading control (blot probed with anti-tubulin primary antibody followed by incubation with IRDye® 800 GAM) is shown. For the FACS-based trafficking assay, CHO CCR5 cells were treated with RANTES in BM for 60 min before being washed and further incubated in BM containing TAK-779 for 60 min. Aliquots of cells were removed at various time-points and assayed for cell surface CCR5-associated fluorescence by FACS, using **MC-5 to detect CCR5. Cell surface CCR5 fluorescence is expressed as a percentage of the initial cell surface CCR5 fluorescence and plotted against time. Data points represent the mean of triplicate samples from a single experiment; error bars represent 1 standard deviation of the means. Also shown is a bar graph showing the initial cell surface CCR5 fluorescence in the AllStars negative control siRNA- and μ1A siRNA-transfected cells, as a percentage of the CCR5 fluorescence in the AllStars negative control siRNA-transfected cells. The data represent the means of triplicate samples from the same experiment; error bars represent 1 standard deviation of the means.

Using the Dharma2 siRNA oligonucleotide, I performed trafficking assays on control and knock-down cells, initially by immunofluorescence (Figure 5—IV B). Although I could assess the knock-down efficiency by immunoblot by detection of the μ1A subunit, I was unable to detect any specific staining in CHO CCR5 cells with the anti-μ1A antibody by immunofluorescence. I therefore used γ-adaptin staining to identify knock-down cells. Hirst et al. (2003) reported that a μ1A knock-down in COS cells resulted in the formation of partial AP-1 complexes that lacked the β1 and μ1A subunits and that knock-down cells had diffuse rather than membrane bound γ-adaptin. To assess whether γ-adaptin levels were changed by knocking down μ1A-adaptin in CHO CCR5 cells, I additionally probed cell lysates of knock-down and control cells with an anti-γ-adaptin antibody (Figure 5—IV A). In accordance with Hirst et al., γ-adaptin levels were unchanged in μ1A knock-down cells.

In unstimulated control siRNA treated cells, γ-adaptin staining was punctate and largely concentrated in the perinuclear region, although smaller, more peripheral puncta were also observed. After 60 min RANTES treatment, CCR5, which was initially present on the cell surface, accumulated in the perinuclear region, where it showed a significant overlap with γ-adaptin. After a subsequent incubation with TAK-779, the vast majority of CCR5 recycled to the cell surface.
In cells transfected with μ1A siRNA, although around 50% of cells showed a similar punctate, perinuclear γ-adaptin staining pattern to that of control siRNA-transfected cells, the other 50% showed no perinuclear punctate γ-adaptin staining and instead, a faint, diffuse cytoplasmic staining pattern was observed (cells marked with white asterisks). This is consistent with these cells having μ1A-adaptin knocked down: at least some γ-adaptin remained but it was unable to localise to membranes in the absence of μ1A adaptin. The immunofluorescence also suggested that the partial knock-down effect observed at the population level by immunoblot was a result of near complete knock-down in a fraction of the cells. Transfection efficiency was, thus, the limiting factor to knock-down efficiency. It was also likely that only around 50% of the control siRNA-transfected cells contained the control siRNA. In knock-down cells, there was no obvious effect on cell surface CCR5 levels in unstimulated cells. In cells treated with RANTES for 60 min, CCR5 internalised in knock-down cells, although perhaps did not cluster so tightly around the nucleus as in untransfected cells or in control siRNA-transfected cells. After 60 min TAK-779 incubation, CCR5 recycled fully to the cell surface in μ1A knock-down cells, suggesting that AP-1 may not be involved in CCR5 recycling.

I next performed a FACS-based trafficking assay to compare the trafficking of CCR5 in control and μ1A knock-down cells. Figure 5—IV C shows the result of this experiment, which remains unrepeated. Here, as shown in the immunoblot, an approximately 55% μ1A knock-down was achieved, which, given the results of the immunofluorescence in Figure 5—IV B, probably meant that a near complete knock-down occurred in around 50% of cells, but that 50% of cells probably were not transfected. This was probably also the case for the control siRNA-transfected cells. Cell surface CCR5 levels were unaffected by μ1A knock-down, as shown in the bar graph, which is in accordance with the immunofluorescence results. Also in agreement with the immunofluorescence results, CCR5 down-modulated in the μ1A knock-down cells to a similar extent as control cells, although very slightly less down-modulation was seen in the μ1A knock-down cells. Recycling was quite similar in both knock-down and control cells, although the knock-down cells showed a slightly greater recovery of cell surface CCR5 fluorescence after 60 min incubation in TAK-779 and a greater initial
recycling rate, measured over the first 10 min of recycling (2.61 ± 0.02% min⁻¹ versus 2.21 ± 0.02% min⁻¹).

Overall, these results show that AP-1 knock-down had little effect on CCR5 recycling; indeed, if anything, recycling occurred slightly faster in knock-down cells.

**Dynasore blocks CCR5 recycling**

Clathrin-coated vesicle formation at the recycling compartment would be predicted to require the GTPase, dynamin, in the 'pinching off' of nascent clathrin-coated pits to form clathrin-coated vesicles. There are 3 isoforms of dynamin, of which dynamin-2 is ubiquitously expressed (Cook, Urrutia et al. 1994; Sontag, Fykse et al. 1994). Dynamins -1 and -3 have more restricted distributions, with dynamin-1 being a neuron-specific isoform (Nakata, Iwamoto et al. 1991) and dynamin-3 found primarily in the brain, lung and testis (Nakata, Takemura et al. 1993; Cook, Mesa et al. 1996). Hence, only dynamin-2 would be expected to be expressed in CHO cells. Although best characterised for its role in endocytosis (Conner and Schmid 2003), dynamin has been shown to act at the recycling endosome in TfR recycling, in the formation of TfR-containing clathrin-coated vesicles (van Dam and Stoorvogel 2002), and is also present at the TGN (McNiven, Cao et al. 2000), where it functions, for instance, in the export of the mannose 6-phosphate receptor (Cao, Weller et al. 2005).

CCR5 internalisation in CHO CCR5 cells is clathrin-dependent (Signoret, Hewlett et al. 2005) and, as such, would most probably require dynamin. Hence, methods of inhibiting dynamin activity, such as expression of dominant-negative dynamin mutants, would inhibit the internalisation of CCR5 and prevent an assessment of dynamin's involvement in receptor recycling. So, to determine whether dynamin plays a role in CCR5 recycling, I initially used the membrane-permeant drug, dynasore, which has been reported to be a non-competitive inhibitor of the GTPase function of dynamin (Macia, Ehrlich et al. 2006). This reagent has also been shown to inhibit the internalisation of transferrin and low density lipoprotein, ligands whose uptake is completely dependent on the action of dynamin (Macia, Ehrlich et al. 2006).
Figure 5-V Dynasore inhibits CCR5 internalisation and recycling

A. CHO CCR5 cells were incubated in BM containing MC-5 and either dynasore
(+dynasore) or DMSO (-dynasore) for 20 min at 37°C. Cells were then washed and either fixed (unstimulated) or further incubated in BM containing either dynasore (+dynasore) or DMSO (-dynasore) with 125 nM RANTES for 40 min at 37°C, before being fixed. Cells were then incubated in permeabilising blocking buffer with Alexa 594 GAM to detect MC-5. Epifluorescence images are shown. Bar, 5 μm. B. CHO CCR5 cells were either fixed (unstimulated), treated with RANTES in BM for 60 min and fixed (60 min RANTES) or treated with RANTES for 60 min followed by an incubation in BM containing TAK-779 and either dynasore (+dynasore) or DMSO (-dynasore) for 60 min, before being fixed (60 min RANTES + 60 min TAK-779). Cells were then permeabilised in blocking buffer and incubated with MC-5 to detect CCR5, followed by incubation with Alexa 594 GAM to detect MC-5. Single confocal sections are shown. Bar, 5 μm. C. CHO CCR5 cells were treated with RANTES for 60 min followed by an incubation in BM containing TAK-779 and dynasore for 60 min. Cells were then fixed and incubated with MC-5 and Mary in permeabilising blocking buffer to detect CCR5 and γ-adaptin, respectively. MC-5 and Mary were detected with Alexa 594 GAM and Alexa 647 GAR, respectively. A single confocal section is shown. Bar, 5 μm. D. CHO CCR5 cells were treated with RANTES in BM for 60 min before being washed and further incubated in BM containing TAK-779 for 60 min. Aliquots of cells were removed at various time-points and assayed for cell surface CCR5-associated fluorescence by FACS, using MC-5 primary antibody and Alexa 647 GAM secondary antibody. Cell surface CCR5 fluorescence is expressed as a percentage of the initial cell surface CCR5 fluorescence and plotted against time. Data points represent the mean of triplicate samples from a single experiment; error bars represent 1 standard deviation of the means.

Firstly, I tested the effect of dynasore on CCR5 internalisation by immunofluorescence (Figure 5–V A). Cells were pre-treated with MC-5 for 20 min at 37°C to label cell surface CCR5 with or without dynasore and then treated with RANTES for 40 min in the presence or absence of dynasore. Dynasore pre-treatment did not affect the initial cell surface distribution of CCR5 but continued treatment with dynasore led to a significant inhibition of CCR5 internalisation (compare RANTES-treated cells +/- dynasore). Interestingly, bright fluorescent puncta were observed at the plasma membrane after 40 min RANTES in the presence of dynasore. This suggests that some CCR5 might have entered clathrin-coated pits or flat clathrin
lattices, but that the pits failed to pinch off into vesicles, which is consistent with an inhibition of dynamin.

I next assessed the effect of dynasore on the recycling of CCR5 (Figure 5-V B). CHO CCR5 cells were treated with RANTES for 60 min and then incubated in BM containing TAK-779 with or without dynasore. The inclusion of dynasore in the recycling step led to an almost complete inhibition of CCR5 recycling, with the distribution of CCR5 being very similar to cells just treated with RANTES for 60 min, suggesting that CCR5 had been prevented from exiting the recycling compartment. In agreement with this, when cells were co-stained for γ-adaptin, CCR5 showed a strong overlap with γ-adaptin in the perinuclear region after 60 min TAK-779 incubation in the presence of dynasore (Figure 5-V C). However, some relatively large intracellular accumulations of CCR5 that did not colocalise with γ-adaptin were also observed in cells treated in this manner.

Finally, I performed a FACS-based trafficking assay to assess the effect of dynasore on CCR5 recycling (Figure 5-V D). In keeping with the immunofluorescence results, inclusion of dynasore in the recycling phase led to an almost complete inhibition of CCR5 recycling, with the recycling curve for CCR5 in the presence of dynasore failing to show any increase in cell surface CCR5-associated fluorescence after 10 min of recycling, suggesting that most of the CCR5 was trapped inside the cells.

In summary, dynasore strongly inhibited CCR5 recycling, which suggests that dynamin is involved in CCR5 exit from the recycling compartment.

**Dyngo-4a, but not Bis-T-23, inhibits the recycling of CCR5**

Although the results described above suggest a role for dynamin in CCR5 recycling, the specificity of dynasore has recently been brought into question by P. Robinson and co-workers (personal communication, unpublished results). They found that although dynasore inhibited clathrin-mediated endocytosis, it had little inhibitory effect on dynamin GTPase activity, as initially reported by Macia et al. (2006), suggesting that it targets a different component of the clathrin-mediated endocytic machinery. Based on this result, the inhibition of CCR5 recycling by dynasore could indicate
that there is a shared component between the machinery effecting clathrin-mediated CCR5 internalisation and that mediating its recycling.

Robinson and colleagues have recently generated a series of dynamin inhibitors, the two most potent being Dyngo-4a (*Dev. Cell*, in review), which is structurally similar to dynasore, and Bis-T-23, which is an unrelated bistyrphostin molecule (Hill, Odell et al. 2005). Initial results suggest that both target the dynamin ring assembly domain (P. Robinson, personal communication). Significantly, Bis-T-22, another bistyrphostin, highly related to Bis-T-23, has been shown to inhibit the epidermal growth factor receptor (EGFR) tyrosine kinase (*IC*₅₀ = 0.4 μM) and block EGF-induced cell proliferation (*IC*₅₀ = 3 μM) (Gazit, Osherov et al. 1996). Bis-T-23 may also exhibit activity against tyrosine kinases.

I used these inhibitors in trafficking assays to further investigate whether dynamin plays a role in CCR5 trafficking. I firstly assessed the effect of the inhibitors on agonist-induced CCR5 internalisation in CHO CCR5 cells, including whether the drugs could inhibit dynamin activity rapidly after addition to the extracellular medium. I used a concentration of Dyngo-4 and Bis-T-23 that produced maximal inhibition of transferrin uptake in U2OS cells (P. Robinson, personal communication): the drugs had not previously been tested on CHO cells. CHO CCR5 cells were incubated with MC-5 to label cell surface receptors for 15 min at 37°C, followed by a 5 min incubation at 37°C in BM containing either the dynamin inhibitors or DMSO (the solvent for the two drugs) as a control. Cells were then treated with RANTES for 30 min in the presence or absence of the drugs. As shown in Figure 5–VI A, both Dyngo-4a and Bis-T-23 completely inhibited CCR5 internalisation. However, unlike the results seen with the inhibition of internalisation in the presence of dynasore, clustering of CCR5 was not observed at the cell surface. Moreover, as shown in Figure 5–VI B for unstimulated cells, when the dynamin inhibitors were included throughout a 20 min pre-incubation with MC-5, a significant reduction in CCR5 staining intensity was seen, suggesting that, remarkably, given their different chemical structures, both drugs interfered with MC-5 binding. At the concentrations used, Bis-T-23 appeared to interfere more strongly with MC-5 binding than Dyngo-4a, with very little specific cell surface CCR5 staining visible above background staining. MC-5 binds to the extreme N-
terminus of CCR5 (Blanpain, Vanderwinden et al. 2002) and although MC-5 binding does not interfere with RANTES binding (Blanpain, Vanderwinden et al. 2002), the N-terminal domain of CCR5 has been shown to participate in RANTES binding and the interaction of RANTES with this domain is required for RANTES-induced CCR5 signalling (Blanpain, Doranz et al. 1999). Thus, although it appeared that the uptake of the drugs into cells occurred very quickly and that the drugs rapidly inhibited the action of hamster dynamin, thus preventing CCR5 internalisation, there is the possibility that the dynamin inhibitors caused an inhibition of internalisation by interfering with RANTES binding and therefore activation of CCR5.

Figure 5-VI The effect of the dynamin inhibitors, Dyngo-4a and Bis-T-23, on CCR5 internalisation
A. CHO CCR5 cells were incubated in BM containing MC-5 for 15 min at 37°C followed by 5 min incubation in BM containing either DMSO, Dyno-4a or Bis-T-23 at 37°C. Cells were then washed and either fixed (unstimulated) or further incubated in BM containing 125 nM RANTES with either DMSO, Dyno-4a or Bis-T-23 for 30 min at 37°C, before being fixed. Cells were then incubated in permeabilising blocking buffer with Alexa 594 GAM to detect MC-5. Epifluorescence images are shown. Bar, 5 μm. B. CHO CCR5 cells were incubated in BM containing MC-5 and either DMSO, Dyno-4a or Bis-T-23 for 20 min at 37°C before being fixed. Cells were then incubated in permeabilising blocking buffer with Alexa 594 GAM to detect MC-5. Epifluorescence images are shown. Images were acquired with the same microscope settings. ‘Enhanced’ images show the same fields of cells but with increased exposure time (longer exposure time for Bis-T-23-treated cells than Dyno-4a-treated cells, as signal was weaker). Bar, 10 μm.

Although the effects of the dynamin inhibitors on CCR5 internalisation were inconclusive, I nevertheless tested their effect on CCR5 recycling. In this assay, I also included dynasore for comparison. Here, CHO CCR5 cells were treated with RANTES for 60 min and then incubated in BM containing TAK-779 with the various drugs or DMSO as a control, for either 30 or 60 min to assess recycling. CCR5 was labelled post-fixation. As can be seen in Figure 5-VII, after 60 min incubation in BM containing TAK-779 and DMSO, almost full recycling was achieved with most of the CCR5 population present on the cell surface; a slightly lower extent of recycling seen after 30 min. Inclusion of dynasore in the recycling phase severely inhibited CCR5 recycling, with the distribution of CCR5 after 30 and 60 min TAK-779 incubation being very similar to cells just treated with RANTES for 60 min, again, suggestive of a block in exit from the recycling compartment. Inclusion of Dyno-4a in the recycling phase also led to a strong inhibition of CCR5 recycling. However, the intracellular distribution of CCR5 was altered compared with cells treated only with RANTES for 60 min, in that a tubulation of the CCR5-containing compartment was observed. This suggests that the mechanism of inhibition of CCR5 recycling is different for Dyno-4a and dynasore. In contrast to the inhibitory effects of dynasore and Dyno-4a on CCR5 recycling, Bis-T-23 did not inhibit CCR5 recycling. Since the internalisation assay did not prove the ability of Bis-T-23 to rapidly and potently inhibit
dynamin at the concentration used in CHO cells, the inability of Bis-T-23 to inhibit recycling could be due to either slow uptake of the drug into CHO cells (slow enough for most of the internal CCR5 population to have returned to the cell surface) or lack of efficacy on hamster dynamin.

![Unstimulated and 60 min RANTES](image)

**Figure 5-VII The effect of various dynamin inhibitor drugs on CCR5 recycling**

CHO CCR5 cells were either fixed (unstimulated), treated with RANTES in BM for 60 min and then fixed (60 min RANTES) or treated with RANTES in BM for 60 min followed by an incubation in BM containing TAK-779 and either DMSO, Dyngo-4a, Bis-T-23 or dynasore for 30 or 60 min, before being fixed (30 min TAK-779 and 60 min TAK-779, respectively). Cells were then incubated with MC-5 in permeabilising blocking buffer. MC-5 was detected with Alexa 594 GAM. Epifluorescence images are shown. Bar, 5 μm.

I also performed the recycling assay with pre-labelling of cell surface CCR5 molecules with MC-5: the results were essentially identical (data not shown).
In summary, the inhibition of CCR5 recycling by Dyngo-4a strongly suggests the involvement of dynamin in CCR5 recycling. The difference in the internal CCR5 distribution in cells where recycling was blocked by either dynasore or Dyngo-4a suggests that dynasore is probably hitting a different target from dynamin, likely another component of the machinery involved in clathrin–coated vesicle formation.

**Discussion**

In this chapter I have shown that the recycling compartment through which agonist-activated CCR5 recycles to the plasma membrane is extensively coated with clathrin at the point that CCR5 traverses it. AP-1 also shows a strong colocalisation with down-modulated CCR5, but despite BFA partially inhibiting CCR5 recycling, an AP-1 knock-down had no effect on the ability of the receptor to recycle. The dynamin inhibitor, Dyngo-4a, did, however, have a strong inhibitory effect on the ability of down-modulated CCR5 to recycle, suggesting that dynamin functions in CCR5 trafficking at the recycling compartment. Dynasore, a drug previously shown to inhibit the dynamin GTPase, also completely blocked CCR5 recycling, although new data from the lab of Phil Robinson suggests that dynasore may target a different component of the clathrin–coated vesicle-forming machinery. These results, together with the electron microscopy observations of down-modulated CCR5 in coated vesicles in the perinuclear region of the cell, with the coat likely being clathrin (Silène Wavre, Endocytic Regulation of Chemokine Receptor Expression, PhD thesis, 2006), suggest that the formation of clathrin–coated vesicles in a dynamin-dependent fashion at the recycling compartment is involved in CCR5 recycling. To my knowledge, this is the first evidence for such a mechanism functioning in the post-endocytic trafficking of a GPCR.

**A role for clathrin and dynamin in CCR5 intracellular trafficking**

Several lines of evidence suggest that CCR5 recycling from the recycling compartment involves clathrin and dynamin. The first indication for clathrin involvement came from observations of immunolabelled cryosections by Signoret et al. (2000), where after 60 min RANTES treatment in CHO CCR5
cells, CCR5 was observed in tubules and vesicles in the perinuclear region, with the vesicles often displaying a prominent coat, highly reminiscent of clathrin. Here, I have shown that the recycling compartment is extensively coated with clathrin at the time CCR5 is passing through it. Recently, using a similar EM technique to that used by Signoret et al., S. Wavre, has observed down-modulated CCR5 in AP-1-positive coated vesicles adjacent to other CCR5-containing coated structures in the perinuclear region, whose coats also label for AP-1 (Sîlène Wavre, Endocytic Regulation of Chemokine Receptor Expression, PhD thesis, 2006). Given that AP-1 is a clathrin adaptor, this suggests that the coat is, indeed, clathrin and the presence of coated vesicles containing CCR5 adjacent to larger coated structures also containing CCR5 suggests that these vesicles have arisen from these structures. Clathrin-coated vesicle formation at the plasma membrane requires the action of dynamin functioning as a mechano-enzyme to constrict the neck of a nascent clathrin-coated pit, resulting the release of a clathrin-coated vesicle from the plasma membrane (McNiven, Cao et al. 2000; Mears, Ray et al. 2007). Dynamin has also been shown to act at the recycling endosome in TfR recycling, in the formation of TfR-containing clathrin-coated vesicles (van Dam and Stoorvogel 2002), and is present at the TGN (McNiven, Cao et al. 2000), where it functions, for instance, in the export of the mannose 6-phosphate receptor (Cao, Weller et al. 2005). In this chapter I have shown that the dynamin inhibitor, Dyngo-4a, potently inhibits CCR5 recycling. Moreover, Dyngo-4a caused a tubulation of the intracellular compartment from which CCR5 exit is blocked. This is reminiscent of the tubulation of recycling endosomes that van Dam et al. (2002) observed by immunofluorescence upon shifting cells expressing a temperature sensitive dynamin mutant to the non-permissive temperature. Whole-mount EM confirmed that in temperature sensitive dynamin mutant-expressing cells at the non-permissive temperature, endosomal tubules of the recycling endosome increased in length, and also showed that they had greater numbers of clathrin-coated buds and labelled more strongly for dynamin than cells over-expressing wild-type dynamin. Dynasore also inhibited CCR5 recycling from the recycling compartment. In contrast to Dyngo-4a, though, no tubulation effect was seen in dynasore-treated cells: cells in which CCR5 had been down-modulated for 60 min
with RANTES and then subsequently incubated in BM containing TAK-779 and dynasore, looked remarkably similar to cells treated only with RANTES for 60 min. My preliminary EM data also suggest that there are no gross morphological differences between cells in which dynasore or DMSO has been included in the recycling phase (data not shown). This suggests that although dynasore inhibits recycling, it does so in a different manner from Dyngo-4a. Indeed, P. Robinson and colleagues found that although dynasore inhibited clathrin-mediated endocytosis, it had little inhibitory effect on dynamin GTPase activity, as initially reported by Macia et al. (2006), suggesting that it targets a different component of the clathrin-mediated endocytic machinery. Thus, the inhibitory effect of dynasore on CCR5 recycling can be interpreted as an inhibition of a component of the machinery responsible for the formation of CCR5-containing clathrin-coated vesicles involved in receptor recycling. If Dyngo-4a and dynasore inhibit CCR5 exit from the same compartment, the tubulation effect caused by Dyngo-4a is probably more a function of dynamin inhibition, rather than the inhibition of clathrin-coated vesicle formation, and may reflect a wider role for dynamin in the formation of other (CCR5-negative) carriers, independently of clathrin. The inhibitory effect of BFA on CCR5 recycling may also be indicative of the involvement of clathrin in CCR5 recycling (see below).

Bis-T-23, another dynamin inhibitor from the laboratory of Phil Robinson, failed to show an inhibitory effect on CCR5 recycling. However, nothing is known about the speed at which this drug is taken up into CHO cells or its effect on hamster dynamin at the concentration used. Experiments designed to assess the effects of the dynamin inhibitors from Dr. Robinson on CCR5 internalisation, which should have acted as a positive control for the rapid action of the drugs, were inconclusive. Remarkably, both Dyngo-4a and Bis-T-23 were found to interfere with MC-5 binding to CCR5. Although the MC-5 epitope is located at the extreme N-terminus of CCR5 and MC-5 binding does not interfere with RANTES binding (Blanpain, Vanderwinden et al. 2002), the N-terminal domain of CCR5 participates in RANTES binding and the interaction of RANTES with this domain is required for RANTES-induced CCR5 signalling (Blanpain, Doranz et al. 1999). Thus, the inhibitory effect on CCR5 internalisation that both drugs exhibited could be explained by an
inhibition of RANTES binding rather than an inhibition of dynamin. Indeed, if dynamin were being targeted, it may be expected that activated CCR5 molecules would still redistribute within the plane of the membrane into flat clathrin lattices, as observed shortly after agonist addition by Signoret et al. (2005), or clathrin-coated pits, which are prevented from pinching off from the membrane due to the inhibition of dynamin. In the presence of dynasore, CCR5 that was prevented from internalising was observed to cluster at the membrane. To determine if RANTES can bind to receptors in the presence of Dyngo-4a and Bis-T-23, cells treated with RANTES in the presence of the dynamin inhibitors could be fixed and stained with the antibodies that recognise the phosphorylated forms of serines 337 and 349, which should be phosphorylated if receptors have been activated. Interference with RANTES binding is not an issue with the effect of the drugs on recycling, as in this situation, receptors have already internalised before the drugs are added. The simplest explanation, therefore, for the differential effects of Dyngo-4a and Bis-T-23 on CCR5 recycling is that Dyngo-4a is able to enter CHO cells rapidly and inhibit hamster dynamin, whereas Bis-T-23 is either taken up slowly into cells or is a poor inhibitor of hamster dynamin. Presumably, as CCR5 was detectable with no apparent loss of staining intensity in cells in which recycling had been assayed in the presence of Dyngo-4a or Bis-T-23, free drug molecules were removed in the wash steps before antibody labelling with MC-5.

The kinetic data of CCR5 recycling fit very well to an equation describing a single pathway recycling process, suggesting that CCR5 molecules recycling to the cell surface only exit through one element of the recycling compartment, either from the TGN or the recycling endosome. The most straightforward conclusion from all of the data as a whole, is that dynamin-dependent clathrin-coated vesicle formation at the recycling compartment participates in CCR5 recycling. However, the site at which CCR5-containing clathrin-coated vesicles are formed (TGN or recycling endosome) and what exactly their role is in CCR5 recycling is unclear. The almost complete inhibition of CCR5 recycling in the presence of Dyngo-4a and dynasore is consistent with such vesicle formation being involved in the direct trafficking of CCR5 to the plasma membrane. Additionally, an inhibitory effect on CCR5 recycling could be produced if the vesicles were involved in
transport within the recycling compartment - from the recycling endosome to the TGN, or vice versa - because inhibiting their formation could block transfer to the particular component of the recycling compartment from which recycling carriers are born. However, inhibition of intra-recycling compartment trafficking in isolation would probably only produce a partial inhibition of CCR5 recycling, since down-modulated CCR5 is present in both recycling endosomes and the TGN at steady-state. Thus, it is likely that dynamin-dependent clathrin-coated vesicle formation is involved at least in exit from the recycling compartment, but may also play a role in intra-recycling compartment trafficking. Also, although highly likely that clathrin and dynamin function in the same clathrin-coated vesicle formation step, dynamin could, in addition, function in a clathrin-independent step occurring at the recycling compartment, which Dyno-4a may inhibit. Figure 5-VIII shows a cartoon illustrating the possible sites of dynamin-dependent clathrin-coated vesicle formation (clathrin-independent, dynamin-dependent events are not shown).

Figure 5-VIII Possible sites of dynamin-dependent clathrin-coated vesicle formation at the recycling compartment
CCR5 trafficking through the perinuclear recycling compartment is likely to involve dynamin-dependent clathrin-coated vesicle formation. Transport vesicles may be formed at the recycling endosome or at the TGN and directly targeted towards the plasma membrane. In addition, transport vesicles so formed may mediate CCR5 trafficking in either direction between recycling endosomes and the TGN. CCR5-containing clathrin-coated pits in a late stage of development with dynamin assembled at their necks are shown to illustrate the possible sites of clathrin-coated vesicle formation. β-arrestins, which remain bound to CCR5 molecules as they traffic to the perinuclear recycling compartment, may function as clathrin adaptors in the compartment. CCR5 molecules are shown in a phosphorylated state because most receptors appear to remain phosphorylated in the recycling compartment, but the site(s) of receptor dephosphorylation is unclear. The MTOC is shown in the bottom-right corner; dashed grey lines indicate microtubules emanating from the MTOC.

Immunostaining of cells in which CCR5 is down-modulated and then allowed to recycle in the presence of Dyngo-4a or dynasore with antibodies against Rab11, TfR and p230, may help to determine the site at which these drugs inhibit CCR5 trafficking and thus, where CCR5-containing transport vesicles are formed. Moreover, further ultrastructural analysis is desirable to confirm the involvement of clathrin and dynamin in CCR5 recycling and perhaps provide further details of the site of clathrin-coated vesicle formation and the potential role of dynamin in this process. EM analysis of the effect of Dyngo-4a on CCR5 recycling, should, if the drug is truly specific for dynamin, and the hypothesis that a dynamin-dependent clathrin-coated vesicle formation step is correct, show CCR5 trapped in coated pits that have failed to undergo scission. Labelling with anti-clathrin antibodies could then confirm if the coat is clathrin and labelling with compartment markers could determine whether the vesicles are formed at the TGN or recycling endosome. This sort of analysis would also assess the possibility that CCR5 recycling is clathrin- and dynamin-independent but that dynamin inhibitors block CCR5 recycling by tying up accessory proteins in arrested clathrin-coated pits elsewhere in the cell, which may also be required for clathrin- and dynamin-independent CCR5 recycling.

Lastly, CCR5 recycling in a clathrin-dependent fashion suggests a possible role for CCR5-bound β-arrestins, which I have shown accompany CCR5 to
the recycling compartment, as clathrin adaptors at the recycling compartment. This is also illustrated in Figure 5-VIII. A potential role for β-arrestins in CCR5 recycling is further investigated in Chapter 6.

**A role for AP-1 in CCR5 intracellular trafficking**

There is evidence from other systems for AP-1 acting as a clathrin adaptor protein at both the recycling endosome and TGN (Fölisch, Ohno et al. 1999; Robinson 2004; Rodriguez-Boulan, Müsch et al. 2004; Fölisch 2005). Interestingly, The Alliance for Cellular Signaling (www.signalinggateway.org) recently reported a yeast two-hybrid interaction between β-arrestin1 and the β1 subunit of AP-1, and in COS cells over-expressing GFP-tagged β-arrestin 1 or 2, AP-1 co-immunoprecipitates with the tagged β-arrestins (Julie Pitcher, personal communication). Given that β-arrestins accompany CCR5 as it traffics to the perinuclear compartment, there is the possibility for a situation where β-arrestins could couple CCR5 to AP-1 and clathrin in the recycling compartment. My immunofluorescence data shows that agonist-activated CCR5 passes through multiple AP-1-positive compartments, including both components of the perinuclear recycling compartment – recycling endosomes and the TGN. I therefore investigated the potential involvement of AP-1 in CCR5 recycling.

Although BFA partially inhibited the recycling of CCR5, a knock-down of the μ1A subunit of AP-1 did not show any inhibition of CCR5 recycling after 60 min TAK-779 incubation. The FACS-based trafficking assay also suggests that, if anything, recycling proceeded at a slightly faster rate and to a slightly greater extent in knock-down cells. In addition, the μ1A knock-down efficiency in the FACS-based recycling assay was only about 55% and given that this assay does not, within a population of μ1A siRNA-transfected cells, discriminate between knock-down cells and cells that have normal levels of μ1A, this experiment may have underestimated any effect on CCR5 recycling. However, I have only conducted this FACS-based trafficking assay once and, especially given the very small difference between CCR5 trafficking in control and knock-down cells, repeats are necessary before firm conclusions are made. I can only conclude at this stage that a μ1A knock-down has no inhibitory effect on CCR5 recycling.
A small effect of the \( \mu 1A \) knock down was that down-modulated CCR5 was not quite as concentrated around the nucleus as in control siRNA-treated cells or cells treated with \( \mu 1A \) siRNA that failed to show a knock-down (i.e. cells that probably did not take up the \( \mu 1A \) siRNA). Moreover, CCR5 and AP-1 showed some colocalisation on structures in the periphery of the cytoplasm after 60 min RANTES treatment (Figure 5-II and Figure 3-VIII), which could be early endosomes. It is possible that AP-1 is involved in trafficking to the recycling compartment and that this is why \( \mu 1A \) knock-down leads to a slightly more dispersed steady-state down-modulated CCR5 distribution. Further immunofluorescence experiments with early endosomal markers and markers of the recycling compartment may be able to confirm this proposal.

Although these results suggest that AP-1 is not required for recycling because CCR5 recycles in its absence, I cannot rule out the possibility that either there is enough residual \( \mu 1A \) protein in knock-down cells to be able to form sufficient functional AP-1 complexes to support an AP-1-dependent CCR5 recycling pathway or that partially-formed AP-1 complexes could function adequately in this regard. If CCR5 is considered to recycle in a clathrin-dependent fashion, though, the second proposal is unlikely, since immunoprecipitated AP-1 complexes (using an anti-\( \gamma \)-adaptin antibody) lacked the \( \beta 1 \) and \( \mu 1A \) subunits in \( \mu 1A \) knock-down cells (Hirst, Motley et al. 2003) and the \( \beta 1 \) subunit is responsible for clathrin binding (Kirchhausen 2000). Despite its implausibility, this possibility could be addressed by simultaneous knock-down of multiple AP-1 subunits. However, this would not rule out the further possibility that AP-1 is ordinarily involved in CCR5 trafficking, but that in its absence, another recycling route can be taken. Indeed, the knock-down protocol used might have allowed cells to adapt to the absence of this adaptor protein complex.

Despite the caveats associated with the \( \mu 1A \) knock-down, given the multiple effects of BFA (see Brefeldin A partially inhibits CCR5 recycling) the \( \mu 1A \) knock-down is a more reliable indicator of the role of AP-1. Actually, that an AP-1 knock-down did not inhibit CCR5 recycling but BFA did, chimes well with the description of TfR recycling from recycling endosomes by Stoorvogel and co-workers, where AP-1 does not appear to play a role (Stoorvogel, Oorschot et al. 1996). Visualising endosomes by whole-mount
EM using a technique for the selective fixation of the endosomal apparatus, they found that BFA treatment led to a loss of clathrin-coated buds from endosomal tubules and, moreover, they found that BFA treatment inhibited TfR recycling from recycling endosomes to the plasma membrane (Stoorvogel, Oorschot et al. 1996; van Dam and Stoorvogel 2002). In addition, using their whole-mount EM technique, they also reported that clathrin-coated buds containing Tf on tubular endosomes are largely devoid of γ-adaptin, although it could be argued that this study was biased towards the observation of peripheral endosomal tubules rather than perinuclear ones, which are more likely to represent bone fide recycling endosomes (Stoorvogel, Oorschot et al. 1996). In summary, the authors essentially describe a BFA-sensitive, but probably AP-1-independent, recycling event for the TfR, which is what the above results suggest for CCR5. Given the effect Stoorvogel and colleagues see of BFA treatment on the loss of clathrin from endosomal tubules, the inhibition of CCR5 recycling in the presence of BFA may argue simply for the involvement of clathrin in this process. However, the authors also found that BFA led to a tubulation of the TfR-containing endosomal network (Stoorvogel, Oorschot et al. 1996; van Dam and Stoorvogel 2002). In contrast, CCR5 that had been prevented from recycling back to the plasma membrane in the presence of BFA did not appear to occupy extended tubular structures, suggesting a difference in the way BFA affects CCR5 trafficking. Others have also witnessed a tubulation of the endosomal network in the presence of BFA (Stoorvogel, Oorschot et al. 1996; Futter, Gibson et al. 1998; Sönichsen, De Renzis et al. 2000) as well as a tubulation of the TGN (Lippincott-Schwartz, Yuan et al. 1991; Futter, Gibson et al. 1998). However, Futter et al. (1998) saw a far less marked tubulation of the TGN compared with the endosomal network in the presence of BFA in MDCK cells, so perhaps BFA treatment prevents CCR5 exit from the TGN. Repetition of this experiment with markers of the TGN and recycling endosome, e.g. p230 and Rab11, respectively, may shed some light on the point of inhibition of CCR5 trafficking by BFA. Also, it is interesting to note that Sönichsen et al. (2000) reported that although Rab11 and Rab4 redistributed into a tubular network in the presence of BFA, a fraction of Rab4-containing endosomes did not form tubules but remained in distinct globular structures. In conclusion, although BFA
inhibition of CCR5 recycling suggests the involvement of clathrin in this process, the exact mechanism of BFA inhibition remains unclear.
There is evidence that β-arrestins play a role in controlling the intracellular trafficking of several GPCRs, although a clear description of their function is currently lacking. In Chapter 4, I showed that CCR5 is best described as a Class C GPCR in terms of its association with β-arrestins: β-arrestins maintain an interaction with agonist-activated CCR5 molecules as they traffic to a perinuclear recycling compartment, from where they recycle rapidly to the cell surface. Moreover, the data in the previous chapter suggest that CCR5 recycling from the recycling compartment is clathrin- and dynamin-dependent. Thus, there is the possibility that β-arrestins couple CCR5 to clathrin at the recycling compartment in a process required for recycling. Here, I describe experiments that begin to address the hypothesis that β-arrestins regulate CCR5 intracellular trafficking.
Chapter 6: The Role of β-arrestins in the Intracellular Trafficking of CCR5

The effect of over-expression of wild-type β-arrestin2 and expression of constitutively active β-arrestin2 mutants on CCR5 recycling

One of the strongest pieces of evidence that β-arrestins are involved in GPCR recycling is the demonstration that the N-formyl peptide receptor (FPR) does not recycle in β-arrestin 1 and 2 knock-out MEFs (Vines, Revankar et al. 2003). This demonstration was only possible, though, because the FPR can internalise in a β-arrestin-independent manner. CCR5 internalisation, however, is β-arrestin-dependent (Fraile-Ramos, Kohout et al. 2003), so a β-arrestin knock-out/knock-down approach to the role of β-arrestins in CCR5 intracellular trafficking is not feasible. An alternative approach is to assay the effect of β-arrestin over-expression or expression of characterised β-arrestin mutants on CCR5 intracellular trafficking. Indeed, two studies have used constitutively active β-arrestin mutants to investigate a role for β-arrestins in the trafficking of the FPR and the bradykinin 2 receptor (B2R), two Class C GPCRs (Key, Vines et al. 2005; Simaan, Bédard-Goulet et al. 2005).

Simaan et al. (2005) used a constitutively active β-arrestin2 mutant truncated at residue 381 (β-arrestin2(1–381)). In this mutant, the structural constraint imposed by the β-arrestin C-tail, which is involved in stabilising the β-arrestin molecule in the 'closed', inactive conformation through intramolecular interactions with the globular domains, is removed. An equivalent truncation mutant of β-arrestin1, showed a greatly enhanced affinity for phosphorylated, agonist-activated β2-adrenergic receptor (β2AR) (Kovoor, Celver et al. 1999) and translocated to the β2AR faster and to a greater extent than wild-type β-arrestin1 (Oakley, Laporte et al. 2001). In addition, whereas wild-type β-arrestin1 dissociated from the agonist-activated β2AR during, or shortly after endocytosis, the C-terminally truncated β-arrestin1 remained bound to the β2AR in endosomes, suggesting a higher affinity for the receptor (Oakley, Laporte et al. 2001). Expression of β-arrestin2(1–381) prevented recycling of the B2R, which remained associated with β-arrestin2(1–381) in endosomes after agonist wash-out. Thus, making the assumption that the truncated β-arrestin2...
mutant also had a higher affinity for the activated B2R, the dissociation of β-arrestins from the B2R was proposed to be required for its recycling. However, it must be noted that as well as showing a higher affinity for agonist-activated, phosphorylated β2AR, the β-arrestin1 truncation mutant also bound activated, non-phosphorylated β2AR with equal affinity to that which wild-type β-arrestin1 bound activated, phosphorylated CCR5 (Kovoor, Celver et al. 1999), as one might predict from the mechanism of β-arrestin activation (see Chapter 1). Thus, the effects of this β-arrestin mutant may be more complicated.

Key et al. (2005) used both a truncated form of β-arrestin1 and a mutant where an IVF motif in the C-tail was mutated to 3 alanines (β-arrestin1(IVF/AAA)). Through its interaction with a hydrophobic pocket on the N-terminal globular domain, this motif participates in locking the C-tail back onto the globular domains in the inactive, 'closed' conformation (Granzin, Wilden et al. 1998; Hirsch, Schubert et al. 1999; Han, Gurevich et al. 2001; Milano, Pace et al. 2002), and has been demonstrated to contribute significantly towards the basal inactivity of the protein (Vishnivetskiy, Schubert et al. 2000). Both of these mutants displayed a slightly enhanced affinity in vitro for agonist-activated, phosphorylated FPR, compared with wild-type β-arrestin1, and displayed significant affinity for an agonist-activated, partially phosphorylated mutant, in contrast to wild-type β-arrestin1, which did not bind this mutant. In this study, expression of the β-arrestin1(IVF/AAA) mutant inhibited the recycling of both wild-type FPR and the partially phosphorylated mutant; however, expression of the truncation mutant had no effect on recycling of wild-type or the partially phosphorylated mutant. Based on the results with the β-arrestin1(IVF/AAA) mutant, the authors argued that, as with the B2R, the dissociation of β-arrestins from the FPR is a critical determinant in receptor recycling. The

[6 In a separate study, β-arrestin1(IVF/AAA) also showed stronger binding to activated, phosphorylated β2AR than wild-type β-arrestin1, and bound the activated, non-phosphorylated β2AR with as high affinity as wild-type β-arrestin1 binds activated, phosphorylated β2AR (Pan, L., E. Gurevich, et al. (2003). "The Nature of the Arrestin-Receptor Complex Determines the Ultimate Fate of the Internalized Receptor." J. Biol. Chem. 278(13): 11623-11632.)

In the FPR study, the constitutively active β-arrestin1 mutants showed no enhanced binding over wild-type β-arrestin1 to agonist-activated, non-phosphorylated FPR.

223
observation that the truncation mutant did not affect FPR recycling may be explained by the possible presence of regulatory sites in the \( \beta \)-arrestin1 C-tail.

To investigate whether \( \beta \)-arrestins play a regulatory role in CCR5 recycling, I initially assayed CCR5 trafficking in CHO CCR5 cells over-expressing GFP-tagged versions of the constitutively active \( \beta \)-arrestin2 mutants, \( \beta \)-arrestin2(IVF/AAA)-GFP and GFP-\( \beta \)-arrestin2(1-380), a C-terminal truncation mutant. I also assayed trafficking in cells expressing the equivalent wild-type proteins, \( \beta \)-arrestin2-GFP and GFP-\( \beta \)-arrestin2. In Chapter 4, I showed that both \( \beta \)-arrestin1 and \( \beta \)-arrestin2 are capable of individually supporting CCR5 internalisation and that both \( \beta \)-arrestins traffic with the receptor to the recycling compartment. Thus, the choice of \( \beta \)-arrestin2 over \( \beta \)-arrestin1 for this analysis was quite arbitrary, although the fact that \( \beta \)-arrestin2 binds down-modulated CCR5 4S→A in the recycling compartment, whereas \( \beta \)-arrestin1 does not, gives it the potential to interact with a wider population of CCR5 molecules in terms of phosphorylation status.

![Figure 6-1 Diagram of the C-termini of wild-type \( \beta \)-arrestin2 and constitutively active \( \beta \)-arrestin2 mutants](image)

Amino acid sequences from residue 370 to the C-termini of \( \beta \)-arrestin2 wild-type and mutant proteins are shown; GFP is not shown. Point mutations are indicated in red. Residues demonstrated to participate in clathrin (Krupnick, Goodman et al. 1997) and AP-2 binding (Laporte, Oakley et al. 2000; Kim and Benovic 2002; Burtey, Schmid et al. 2007) are shown, as are residues required for NES activity (Scott, Le Rouzic et al. 2002; Wang, Wu et al. 2003).

Diagrams of the C-terminal residues of wild-type \( \beta \)-arrestin2 and the two constitutively active mutants can be seen in Figure 6-1. It is noteworthy that
the IVF motif has, since the publication of the reports by Simaan et al. (2005) and Key et al. (2005), been shown to participate in AP-2 binding, with the phenylalanine directly binding the β2-adaptin ear domain and the isoleucine and valine residues appearing to negatively regulate this interaction (Burtey, Schmid et al. 2007). Mutation of all 3 residues, as in the mutant used in this study, resulted in a β-arrestin2 mutant with significantly attenuated AP-2 binding capacity (Burtey, Schmid et al. 2007). The β-arrestin2 (1-380) mutant would also be expected to show impaired or completely abolished binding to AP-2, since all of the residues shown to participate in binding to β2-adaptin are removed from this mutant. Both mutants, however, retain their ability to couple activated GPCRs to clathrin, since the clathrin binding box, responsible for this interaction is intact in both mutants.

Initially, I transiently transfected the β-arrestin2 proteins and CFP alone into CHO CCR5 cells and assayed CCR5 trafficking by FACS, as described in Chapter 5. Cells were treated in suspension with RANTES for 60 min to down-modulate CCR5, before being washed and resuspended in medium containing TAK-779 for 120 min, to follow CCR5 recycling. Aliquots of cells were removed at various time-points and cell surface CCR5-associated fluorescence determined. Since the transfection efficiency was quite low, I gated for cells expressing the exogenous proteins based on their GFP fluorescence. Using this method, the trafficking of CCR5 in cells not expressing detectable levels of the exogenous proteins was almost identical in the differentially transfected samples (data not shown).

Figure 6-11 shows the pooled results of a series of trafficking experiments. As can be seen in Figure 6-11 A, expression of β-arrestin2 wild-type or mutant proteins did not greatly alter the cell surface expression of CCR5 relative to expression of GFP alone, although cell surface CCR5 levels were slightly lower in both β-arrestin2 wild-type and mutant expressing cells. This suggests that β-arrestin2 wild-type or constitutively active mutant protein expression does not grossly affect basal CCR5 trafficking or CCR5 biosynthetic trafficking.

Figure 6-11 B and C show the results of CCR5 trafficking assays with the transfected cells. For clarity, the data for the two mutant β-arrestin2 proteins and their wild-type counterparts are displayed over two graphs,
with the same data for the GFP-expressing cells shown on both graphs. The trafficking data and rates/rate constants derived from the data are shown in Table 6-1.

From Figure 6-II B, it can be seen that in cells expressing GFP, a 69.13 ± 0.89% loss of initial cell surface CCR5 was seen after 60 min RANTES treatment. Both cells expressing wild-type β-arrestin2-GFP and β-arrestin2(IVF/AAA)-GFP down-modulated CCR5 to a greater extent (84.37 ± 1.55% and 80.30 ± 2.74%, respectively), and there was a smaller percentage change in cell surface CCR5 fluorescence between 30 and 60 min RANTES treatment compared with GFP-expressing cells, suggesting that the approach to a steady-state down-modulated CCR5 distribution was quicker in these cells. Cells expressing GFP showed a recovery of cell surface CCR5 after 120 min in the presence of TAK-779 to 82.14 ± 3.48% of the initial level, with a plateau reached after 60 min recycling in TAK-779. Why CCR5 did not recover to 100% of its initial cell surface level is not known. It was assumed for the purposes of this analysis that the maximum cell surface CCR5 fluorescence value reached by the GFP-transfected cells in the recycling phase represented the maximum level of CCR5 recycling in all the differentially transfected cells. In fitting data to the equation describing a single pathway recycling process, detailed in Chapter 5, $f_{\text{max}}$ was based on this value. Using this value, the data describing recycling of CCR5 in the GFP-transfected cells fitted the equation well ($R^2 = 0.97$, linear regression of a plot of $\ln((f_{\text{max}}-f)/f_{\text{max}})$ against time [t]). $k_{\text{rec}}$, the recycling rate constant, in GFP-transfected cells was 0.047 ± 0.014 min$^{-1}$. In contrast, both wild-type β-arrestin2-GFP- and β-arrestin2(IVF/AAA)-GFP-expressing cells showed more sluggish CCR5 recycling, with recycling of CCR5 after 120 min in TAK-779 to only 67.84 ± 6.25% and 73.77 ± 5.89% of the initial cell surface CCR5 levels. The recycling curves for CCR5 in these cells had not reached a plateau after 120 min in TAK-779, suggesting that CCR5 was still recycling at this time-point. These curves also showed good fits to the single pathway recycling equation ($R^2 = 0.95$ and 0.92, respectively) and their slower recycling of CCR5 was reflected in reduced recycling rate constants compared with GFP-transfected cells ($k_{\text{rec}}$(wt βarr2-GFP) = 0.12 ± 0.0028 min$^{-1}$, $k_{\text{rec}}$(βarr2(IVF/AAA)-GFP) = 0.015 ± 0.0039 min$^{-1}$, $k_{\text{rec}}$(GFP) = 0.047 ± 0.014 min$^{-1}$; see bar graph in Figure 6-II D). It could, perhaps, be argued.
that the cells expressing β-arrestin2(IVF/AAA)-GFP recycled CCR5 slightly faster than cells expressing wild-type β-arrestin2-GFP, which might account for the slightly lower extent of CCR5 down-modulation in cells expressing β-arrestin2(IVF/AAA)-GFP compared with those expressing wild-type β-arrestin2-GFP, since down-modulation is a balance of internalisation and recycling rates. However, the difference between the two rate constants is within the error associated with the data, so cannot be concluded to be significant.

One point to note, is that given that differentially transfected cells down-modulated CCR5 to different extents, comparing rate constants for recycling \( (k_{\text{rec}}) \) is more relevant than a comparison of initial recycling rates calculated for the first 10 min of recycling \( (r_{\text{rec}}) \), as these are, in their simplest form, a product of a rate constant and the amount of internal CCR5, which varied between the differentially transfected cells. Moreover, the recycling rate constant is calculated from a fit to all of the recycling data, which minimises error compared with the initial recycling rate, which is calculated from only two values. Nevertheless, this value is listed in Table 6-1 for comparison.

From Figure 6-11 C, it can be seen that the trafficking curve for cells expressing GFP-β-arrestin2 wild-type closely matched that of cells expressing wild-type β-arrestin2-GFP: cells down-modulated CCR5 to a similar extent (84.37 ± 1.78% and 84.37 ± 1.55%, respectively), recovered cell surface CCR5 to a similar extent after 120 min incubation in TAK-779 (71.51 ± 8.97% and 67.84 ± 6.25% of initial cell surface CCR5, respectively) and had similar recycling rate constants (0.012 ± 0.0028 min⁻¹). Expression of GFP-βarrestin2(1-380) also led to an enhanced CCR5 down-modulation relative to GFP-expressing cells (76.96 ± 3.58% versus 69.13 ± 0.89%) although not as great as cells expressing GFP-β-arrestin2 wild-type (84.37 ± 1.78%). However, in contrast to the inhibitory effect on recycling seen in cells expressing wild-type β-arrestin2, there was only a mild inhibitory effect on recycling in the presence of GFP-β-arrestin2(1-380): cell surface CCR5 reached 81.69 ± 2.27% of the initial cell surface level after 120 min incubation in TAK-779 (in GFP-expressing cells, CCR5 recovered to 82.14 ± 3.48%) and \( k_{\text{rec}} \) was 0.037 ± 0.013 min⁻¹ \( (k_{\text{rec}} \text{(GFP)} = 0.047 ± 0.014 \text{ min}^{-1}) \). A graphical comparison of the recycling rate
constants for all of the differentially transfected cells is shown in Figure 6-II D. The recycling curves for CCR5 in cells expressing GFP-β-arrestin2 wild-type and GFP-β-arrestin2(1-380) also fitted well to the single pathway recycling model ($R^2$ (GFP-βarr2 wt) = 0.96, $R^2$ (GFP-βarr2(1-380)) = 0.99).

A

![Graph A](image)

B

![Graph B](image)
Figure 6-11 The effect of over-expression of wild-type and constitutively active mutants of β-arrestin2 in CHO CCR5 cells

CCR5 trafficking in CHO CCR5 cells transiently expressing wt βarr2-GFP, GFP-βarr2 wt, βarr2(IVF/AAA)-GFP or GFP-βarr2(1-380) was followed by FACS, as described in Materials and Methods. A. Bar graph showing mean cell surface CCR5-associated fluorescence in unstimulated cells expressing the various exogenous proteins. Mean cell surface CCR5 fluorescence is expressed as a percentage of the mean.
fluorescence from CHO CCR5 cells expressing βarr2-GFP. B and C. Trafficking assays. Cells were treated with RANTES in BM for 60 min before being washed and further incubated in BM containing TAK-779 for 120 min. Aliquots of cells were removed at various time-points and assayed for cell surface CCR5-associated fluorescence by FACS, using MC-5 and Alexa 647 GAM to detect CCR5. Cell surface CCR5 fluorescence is expressed as a percentage of the initial cell surface CCR5 fluorescence and plotted against time. The same curve for cells expressing GFP is shown in both B and C. The data for CCR5 trafficking in cells over-expressing β-arrestin2 wild-type and mutant proteins are displayed over two graphs for clarity. D. Bar graph showing the recycling rate constant, \( k_r \), for CCR5 in CHO cells expressing the various exogenous proteins, derived from fitting the data points encompassing the recycling phases (60 to 180 min) shown in B and C to a first-order rate equation.

All data shown represent means of 3 or 4 independent experiments. Error bars represent 1 standard deviation of the means.

In summary, over-expression of wild-type β-arrestin2 led to an enhanced down-modulation of CCR5 relative to GFP-expressing cells. Moreover, over-expression of wild-type β-arrestin2 significantly inhibited the recycling of CCR5, suggesting that β-arrestin2 is a negative regulator of CCR5 recycling. Interestingly, in cells expressing GFP–β-arrestin2(1–380), CCR5 was only slightly inhibited in its ability to recycle, suggesting that the ability to inhibit CCR5 recycling may lie in the C-tail of β-arrestin2. That neither constitutively active β-arrestin2 mutant led to a complete block in CCR5 recycling is in contrast to the behaviour of the FPR and B2R.

It should be noted that wild-type β-arrestin2 proteins expressed at slightly higher levels than the mutant proteins. A similar analysis gating for cells expressing low or medium levels of the exogenous proteins (i.e. excluding high-expressing cells that were more frequent in β-arrestin2 wild-type-transfected cells than mutant-transfected cells) returned almost identical results to those where all cells expressing the exogenous proteins were considered (data not shown). Hence, the difference in CCR5 recycling kinetics in the presence of the different β-arrestin2 wild-type and mutant proteins was not due to differences in expression levels.
Table 6-1: The effect of over-expression of wild-type and constitutively active mutants of β-arrestin2 on CCR5 trafficking

<table>
<thead>
<tr>
<th>Protein over-expressed</th>
<th>% down-modulation [100 - f_{l60} min]</th>
<th>f_{l180 min} (%) initial</th>
<th>r_{rec} (% min⁻¹)</th>
<th>k_{rec} (min⁻¹)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt βarr2-GFP</td>
<td>84.37 ± 1.55</td>
<td>67.84 ± 6.25</td>
<td>1.39 ± 0.50</td>
<td>0.012 ± 0.0028</td>
<td>0.95</td>
</tr>
<tr>
<td>βarr2(IVF/AAA)-GFP</td>
<td>80.30 ± 2.74</td>
<td>73.77 ± 5.89</td>
<td>2.27 ± 0.34</td>
<td>0.015 ± 0.0039</td>
<td>0.92</td>
</tr>
<tr>
<td>GFP-βarr2 wt</td>
<td>84.37 ± 1.78</td>
<td>71.51 ± 8.97</td>
<td>1.85 ± 0.16</td>
<td>0.014 ± 0.0050</td>
<td>0.96</td>
</tr>
<tr>
<td>GFP-βarr2(1-380)</td>
<td>76.96 ± 3.58</td>
<td>81.69 ± 2.27</td>
<td>2.77 ± 0.25</td>
<td>0.037 ± 0.013</td>
<td>0.99</td>
</tr>
<tr>
<td>GFP</td>
<td>69.13 ± 0.89</td>
<td>82.14 ± 3.48</td>
<td>2.96 ± 0.01</td>
<td>0.047 ± 0.014</td>
<td>0.97</td>
</tr>
</tbody>
</table>

The table summarises the data obtained from the quantitative analysis of CCR5 trafficking in CHO CCR5 cells over-expressing wt βarr2-GFP, βarr2(IVF/AAA)-GFP, GFP-βarr2 wt, or GFP-βarr2(1-380), as shown in Figure 6-II. f_{l}, cell surface fluorescence at time, t; r_{rec}, initial rate of recycling (from 60 to 70 min); k_{rec}, rate constant for recycling obtained by fitting recycling data to a first-order rate equation; R², coefficient of determination from regression analysis of a plot of ln((f_{lmax}-f)/f_{lmax}) against time [t] (i.e. degree of fit of data to a first-order recycling rate equation, R² max = 1). See Materials and Methods for further details. All data represent the mean of 3 or 4 independent experiments; errors represent 1 standard deviation of the mean.

I also checked to see if the mutant β-arrestin2 proteins could be seen to associate with CCR5 in RANTES-treated cells and whether there was any difference in distribution of CCR5 in the β-arrestin2 mutant-expressing cells. Figure 6-III shows CHO CCR5 cells transiently-transfected with the GFP-tagged β-arrestin2 wild-type and mutant proteins, which were pre-labelled for cell surface CCR5 and treated with RANTES for 60 min. CCR5 down-modulated in both cells expressing the wild-type β-arrestin2 proteins and those expressing the constitutively active β-arrestin2 mutants. Moreover, both wild-type and mutant β-arrestin2 proteins colocalised with nearly all of the internal CCR5 after 60 min RANTES treatment, demonstrating that the like wild-type β-arrestin2, the constitutively active mutants are stably recruited to agonist-activated CCR5. In cells expressing wild-type β-arrestin2, CCR5 was concentrated in the perinuclear region, although some punctate CCR5 fluorescence was also observed in the more peripheral cytoplasm. In cells expressing β-arrestin2(IVF/AAA)-GFP, CCR5 also largely accumulated in the perinuclear region, but was sometimes observed in slightly larger structures than in wild-type β-arrestin2-GFP-
expressing cells. In cells expressing GFP-β-arrestin2(1–380), the mutant β-arrestin2 was concentrated in the nucleus, most likely as a result of the absence of the nuclear export signal (NES) that is present after residue 380 in the C-tail of wild-type β-arrestin2 (see Figure 6-I). Interestingly, in cells expressing the truncation mutant, CCR5 showed a slightly more scattered cytoplasmic distribution, with less concentration around the nucleus, suggesting a possible defect in intracellular trafficking of GFP-β-arrestin2(1–380)-bound CCR5.

Figure 6-III The effect of constitutively active β-arrestin2 mutants on the
distribution of down-modulated CCR5

CHO CCR5 cells were transiently transfected with wt βarr2-GFP, GFP-βarr2 wt, βarr2[(IVF/AAA)-GFP or GFP-βarr2(1-380). Cells were pre-labelled for cell surface CCR5 with MC-5 and then treated with RANTES in BM at 37°C for 60 min. Cells were then fixed and incubated in permeabilising blocking buffer with Alexa 594 GAM secondary antibody to detect MC-5. Single confocal sections are shown. Bars, 5 μm

The effect of β-arrestin2 mutants defective in clathrin or AP-2 binding on CCR5 trafficking

The above results show that unlike the B2R and FPR, constitutively active β-arrestin mutants do not block CCR5 recycling. However, over-expression of β-arrestin2 does inhibit CCR5 recycling and this inhibition of recycling was partially relieved by truncating the C-tail of β-arrestin2 to residue 380. Despite the inhibitory effect of β-arrestin2 over-expression on CCR5 recycling, β-arrestins may still be required for recycling - consider the FPR, where β-arrestin1[(IVF/AAA) inhibits recycling (Key, Vines et al. 2005) but recycling does not occur in the absence of β-arrestins (Vines, Revankar et al. 2003). As previously discussed, one way in which β-arrestins could facilitate CCR5 recycling is by coupling receptors to a clathrin-mediated trafficking step required for exit from the recycling compartment. In Chapter 5, I also addressed the possibility that CCR5 may, via β-arrestins, couple to the clathrin adaptor, AP-1, in the recycling compartment, to effect such a clathrin-mediated transport step. The binding site for β1-adaptin on the β-arrestin molecule is not known. However, the β2-adaptin site has been mapped and involves Arg396 in β-arrestin2 (see Figure 6–IV, residues in blue). Mutation of this residue to Glu results in a mutant incapable of binding β2-adaptin and thus AP-2. The yeast two-hybrid interaction between the β1 subunit of AP-1 and β-arrestin reported by the Alliance for Cellular Signaling used residues 190–410 of β-arrestin1 as a bait, suggesting a site of interaction between residue 190 and the C-terminus. Unpublished data from Julie Pitcher (MRC LMCB) indicates that an AP-2 binding mutant of β-arrestin1, with Arg395 mutated to Glu (equivalent to
Arg396 in β-arrestin2), also shows reduced binding to β1-adaptin, suggesting that β1 and β2-adaptin share closely-related binding sites.

<table>
<thead>
<tr>
<th></th>
<th>370</th>
<th>VDTNLIEFDTNYATDDDIVEDPARLRLKGMKDDDCCDQLC</th>
<th>410</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>370</td>
<td>VDTNLIEFDTNYATDDDIVEDPARLRLKGMKDDDCCDQLC</td>
<td>410</td>
</tr>
<tr>
<td>LIEF/AAEA</td>
<td>370</td>
<td>VDTNLIEFDTNYATDDDIVEDPARLRLKGMKDDDCCDQLC</td>
<td>410</td>
</tr>
<tr>
<td>R396A</td>
<td>370</td>
<td>VDTNLIEFDTNYATDDDIVEDPARLRLKGMKDDDCCDQLC</td>
<td>410</td>
</tr>
</tbody>
</table>

*Figure 6-IV Diagram of the C-termini of wild-type β-arrestin2 and mutants defective for clathrin and AP-1/A-2 binding*

Amino acid sequences from residue 370 to the C-termini of β-arrestin2 wild-type and mutant proteins are shown; GFP is not shown. Point mutations are indicated in red. Residues demonstrated to participate in clathrin and AP-2 binding are shown, as are residues required for NES activity. See Figure 6-1 for references.

To investigate the possibility that β-arrestins couple CCR5 to clathrin in the recycling compartment to facilitate recycling, and the possibility that AP-1 is involved, I over-expressed in CHO CCR5 cells a GFP-tagged mutant of β-arrestin2 defective for clathrin binding, β-arrestin2(LIEF/AAEA) (Goodman, Krupnick et al. 1997), and a GFP-tagged Arg396→Ala mutant, β-arrestin2(R396A), which is defective for AP-2 binding (Laporte, Oakley et al. 2000) and likely AP-1 binding.

I initially assessed the effects of the β-arrestin mutants using the FACS-based trafficking assay. Again, I gated for cells expressing the exogenous proteins based on their GFP-fluorescence. Figure 6-V shows the pooled results of a series of trafficking experiments. In this set of experiments, although consistent between individual experiments, CCR5 did not recycle to as great an extent as in the experiments performed with the constitutively active β-arrestin2 mutants, making possible only relative comparisons with that data set. Expression levels of wild-type β-arrestin2-GFP, β-arrestin2(LIEF/AAEA)-GFP and β-arrestin2(R396A)-GFP were all similar (data not shown).

As can be seen in Figure 6-V, although the cells expressing wild-type β-arrestin2 and the β-arrestin2 mutants had slightly lower cell surface CCR5
levels than GFP-expressing cells, over-expression of the β-arrestin proteins did not grossly alter cell surface CCR5 expression levels.

Binding of β-arrestin1/2 to both AP-2 and clathrin are believed facilitate clathrin-mediated endocytosis. In this series of experiments, to assess whether the over-expression of β-arrestin mutants defective in clathrin or AP-2 binding affected the internalisation rate of CCR5, I also assayed cell surface CCR5 levels after 10 min RANTES. Little recycling of CCR5 should have occurred in this small time-window and, hence, initial internalisation rates could be calculated. Perfect comparison of initial internalisation rates, though, would require the differentially transfected cells to have the same absolute initial levels of cell surface CCR5, so this analysis is slightly complicated by the fact that the GFP-expressing cells had slightly higher levels of cell surface CCR5 expression. Figure 6-V B shows the results of the trafficking assays in cells expressing the exogenous proteins; initial internalisation rates are compared in the bar graph in Figure 6-V C. Over-expression of wild-type β-arrestin2-GFP enhanced the initial internalisation rate compared with GFP-expressing cells (7.36 ± 0.21% min⁻¹ versus 4.46 ± 0.34% min⁻¹, ~ 1.65-fold increase). Expression of β-arrestin2(LIEF/AAEA)-GFP or β-arrestin2(R396A)-GFP also increased the initial rate of internalisation compared with cells expressing GFP (*r* \(_{\text{int}}\) (βarr2(LIEF/AAEA)-GFP) = 6.58 ± 0.30% min⁻¹, *r* \(_{\text{int}}\) (βarr2(R396A)-GFP) = 6.85 ± 0.17% min⁻¹), although not quite as much as over-expression of wild-type β-arrestin2-GFP. Thus, the amount of β-arrestins in CHO CCR5 cells was rate-limiting for CCR5 internalisation.

That expression of the β-arrestin2 mutants defective in either clathrin or AP-2 binding also increased the rate of CCR5 internalisation, almost to the same extent as over-expression of wild-type β-arrestin2, suggests that either the clathrin or the AP-2 binding site on the β-arrestin molecule is sufficient to effect the clathrin-mediated internalisation of CCR5 and either site can function alone almost as efficiently as both sites together. In keeping with the pattern of initial internalisation rates, CCR5 down-modulated to the greatest extent in cells over-expressing wild-type β-arrestin2-GFP (84.06 ± 2.34% down-modulation), with β-arrestin2(R396A)-GFP- and β-arrestin2(LIEF/AAEA)-GFP- expressing cells down-modulating...
81.70 ± 1.03% and 79.40 ± 3.24% of cell surface CCR5 after 60 min RANTES treatment. The extent of down-modulation in the cells expressing the β-arrestin2 wild-type and mutant proteins was in excess of that achieved in GFP-expressing cells, where only 73.48 ± 2.79% of the initial cell surface CCR5 was down-modulated after 60 min.
Figure 6-V Trafficking of CCR5 in cells expressing β-arrestin2 mutants defective in clathrin or AP-2 binding

CCR5 trafficking in CHO CCR5 cells transiently expressing wt βarr2-GFP, βarr2(LIEF/AEAA)-GFP or βarr2(R396A)-GFP was followed by FACS, as described in Materials and Methods. A. Bar graph showing mean cell surface CCR5-associated fluorescence in unstimulated cells expressing the various exogenous proteins. Mean cell surface CCR5 fluorescence is expressed as a percentage of the mean fluorescence from CHO CCR5 cells expressing βarr2-GFP. B. Trafficking assay. Cells were treated with RANTES in BM for 60 min before being washed and further incubated in BM containing TAK-779 for 120 min. Aliquots of cells were removed at various time-points and assayed for cell surface CCR5-associated fluorescence by FACS, using MC-5 and Alexa 647 GAM to detect CCR5. Cell surface CCR5 fluorescence is expressed as a percentage of the initial cell surface CCR5 fluorescence and plotted against time. C. Bar graph showing initial rates of CCR5 internalisation (over first 10 min of RANTES treatment) in CHO CCR5 cells expressing the various exogenous proteins, derived from the data shown in B. D. Bar graph showing the recycling rate constant, $k_{rec}$, for CCR5 in CHO cells expressing the various exogenous proteins, derived from fitting the data points encompassing the recycling phases (60 to 180 min) shown in B to a first-order rate equation.

All data shown represent means of 4 independent experiments. Error bars represent 1 standard deviation of the means.

Consistent with the effects of wild-type β-arrestin2-GFP and GFP-β-arrestin2 wild-type shown in Figure 6-II, over-expression of β-arrestin2-GFP in this series of experiments led to a decreased rate of recycling compared with GFP-expressing cells ($k_{rec} (βarr2-GFP) = 0.015 \pm 0.0081 \text{ min}^{-1}$, $k_{rec} (GFP) = 0.032 \pm 0.018 \text{ min}^{-1}$). For this analysis, I also based $f_{\text{max}}$ for recycling on the maximum cell surface fluorescence value reached by the
GFP-expressing cells. All of the curves describing the recycling of CCR5 in the differentially transfected cells fitted well to the single pathway recycling equation. Cells expressing the β-arrestin2 clathrin or AP-2 binding mutants also had a reduced recycling rate compared with GFP-expressing cells ($k_{rec}$ ($\beta$arr2(LIEF/AAEA)-GFP) = $0.018 \pm 0.0067$ min$^{-1}$, $k_{rec}$ ($\beta$arr2(R396A)-GFP) = $0.018 \pm 0.0075$ min$^{-1}$, $k_{rec}$ (GFP) = $0.032 \pm 0.018$ min$^{-1}$). However, the β-arrestin2 clathrin and AP-2 binding mutant-expressing cells had a slightly faster rate of CCR5 recycling than cells expressing wild-type β-arrestin2-GFP ($k_{rec}$ ($\beta$arr2-GFP) = $0.015 \pm 0.0081$ min$^{-1}$), although this difference was well within the errors associated with the data, so cannot be considered significant. Unfortunately, given the poor extent of CCR5 recycling in this series of experiments, only large effects, like the inhibitory effect of β-arrestin2-GFP expression on CCR5 recycling, would likely fall out of the range of the error associated with the data. A graphical comparison of the recycling rate constants is shown in Figure 6-V D.

Table 6-II The effect of β-arrestin2 mutants defective for clathrin or AP-2 binding on CCR5 trafficking

<table>
<thead>
<tr>
<th>Protein overexpressed</th>
<th>% down-modulation $f_{180 \min}$ (100 - $f_{60 \min}$)</th>
<th>$f_{180 \min}$ ($%$ initial)</th>
<th>$r_{int}$ ($%$ min$^{-1}$)</th>
<th>$r_{rec}$ ($%$ min$^{-1}$)</th>
<th>$k_{rec}$ (min$^{-1}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt $\beta$arr2-GFP</td>
<td>84.06 ± 2.34</td>
<td>52.33 ± 11.58</td>
<td>7.36 ± 0.21</td>
<td>1.45 ± 0.24</td>
<td>0.015 ± 0.0081</td>
<td>0.97</td>
</tr>
<tr>
<td>$\beta$arr2(LIEF/AAEA)-GFP</td>
<td>79.40 ± 3.24</td>
<td>55.24 ± 10.17</td>
<td>6.58 ± 0.30</td>
<td>1.71 ± 0.27</td>
<td>0.018 ± 0.0067</td>
<td>0.95</td>
</tr>
<tr>
<td>$\beta$arr2(R396A)-GFP</td>
<td>81.70 ± 1.03</td>
<td>55.15 ± 5.41</td>
<td>6.85 ± 0.17</td>
<td>1.50 ± 0.20</td>
<td>0.018 ± 0.0075</td>
<td>0.95</td>
</tr>
<tr>
<td>GFP</td>
<td>73.48 ± 2.79</td>
<td>58.49 ± 4.63</td>
<td>4.46 ± 0.34</td>
<td>2.03 ± 0.24</td>
<td>0.032 ± 0.018</td>
<td>0.94</td>
</tr>
</tbody>
</table>

The table summarises the data obtained from the quantitative analysis of CCR5 trafficking in CHO CCR5 cells over-expressing wt $\beta$arr2-GFP, $\beta$arr2(LIEF/AAEA)-GFP or $\beta$arr2(R396A)-GFP, as shown in Figure 6-V. $f_{60 \min}$ cell surface fluorescence at time $t$; $r_{int}$, initial rate of internalisation (from 0 to 10 min); $r_{rec}$, initial rate of recycling (from 60 to 70 min); $k_{rec}$, rate constant for recycling obtained by fitting recycling data to a first-order rate equation; $R^2$, coefficient of determination from regression analysis of a plot of ln($f_{\max}$-$f_{t}$/$f_{\max}$) against time $t$ (i.e. degree of fit data to a first-order recycling rate equation, $R^2$ max = 1). See Materials and Methods for further details. All data represent the mean of 4 independent experiments; errors represent 1 standard deviation of the mean.

I also checked to see whether the β-arrestin2 clathrin and AP-2 binding mutants formed stable associations with down-modulated CCR5 by
immunofluorescence (Figure 6–VI). In CHO CCR5 cells transiently-transfected with either wild-type β-arrestin2-GFP, β-arrestin2(LIEF/AAEA)-GFP or β-arrestin2(R396A)-GFP and treated with RANTES for 60 min, CCR5 accumulated in the perinuclear region, where it colocalised with the wild-type and mutant β-arrestin2 proteins, suggesting that CCR5 trafficked normally in complex with the clathrin or AP-2 binding defective β-arrestin2 mutants. The only difference between the down-modulated CCR5 distribution in mutant- and wild-type-expressing cells, was that in cells expressing β-arrestin2(LIEF/AAEA)-GFP, much of the CCR5 was often found concentrated in a single, large perinuclear cluster (as shown in Figure 6–VI), which was rarely observed in cells expressing wild-type β-arrestin2-GFP or β-arrestin2(R396A)-GFP.

Figure 6–VI The effect of β-arrestin2 mutants defective for clathrin and AP-2 binding on the distribution of down-modulated CCR5

CHO CCR5 cells were transiently transfected with wt βarr2-GFP, βarr2(R396A) or
βarr2(LIEF/AAEA)-GFP. Cells were pre-labelled for cell surface CCR5 with MC-5 and then treated with RANTES in BM at 37°C for 60 min. Cells were then fixed and incubated in permeabilising blocking buffer with Alexa 594 GAM secondary antibody to detect MC-5. Single confocal sections are shown. Bars, 5 μm

Overall, this series of experiments strengthens the argument that β-arrestin2 is a negative regulator of CCR5 recycling. Although not excluding the possibility that β-arrestins act as facilitators of CCR5 recycling by coupling to clathrin and/or AP-1, they provide no support for this hypothesis.

**Discussion**

In this chapter I have presented biochemical data showing that over-expression of β-arrestin2 impedes CCR5 recycling in CHO CCR5 cells. In contrast to their previously demonstrated inhibitory effect on the recycling of the Class C receptors, B2R and FPR, expression of two constitutively active β-arrestin2 mutants did not prevent CCR5 recycling. Indeed, truncation of the β-arrestin2 C-tail partially relieved the inhibitory effect of β-arrestin2 over-expression and morphological analysis showed that the pattern of down-modulated CCR5 complexed with the truncated β-arrestin2 mutant was slightly different from CCR5 bound to wild-type β-arrestin2. β-arrestin2 over-expression also increased the rate of CCR5 internalisation and, unexpectedly, given the accepted role of β-arrestin interaction with AP-2 and clathrin in endocytosis, expression of β-arrestin2 mutants defective in AP-2 or clathrin binding also increased the rate of CCR5 internalisation. These mutants, however, did not significantly affect the rate of CCR5 recycling compared with over-expression of wild-type β-arrestin2. Altogether, although not excluding the possibility that β-arrestins facilitate CCR5 recycling through a clathrin-dependent mechanism, these experiments provide no evidence in support of this theory.

**β-arrestin2 is a negative regulator of CCR5 recycling**

Over-expression of β-arrestin2-GFP/GFP-β-arrestin2 significantly slowed the recycling of CCR5, with around a 3.5-fold decrease in the rate constant
for recycling compared with cells expressing GFP. As previously shown by immunofluorescence, over-expression of GFP-tagged β-arrestin2 does not alter the distribution of CCR5 after 60 min RANTES treatment compared with cells expressing endogenous levels of β-arrestin and in this chapter I have shown that the recycling of CCR5 in cells over-expressing β-arrestin2 is just as well described by a single pathway recycling process as CCR5 recycling in cells expressing endogenous levels of CCR5. Together, these data argue that β-arrestin2 over-expression retards the rate of CCR5 recycling but does not alter its trafficking itinerary. This suggests that in the normal trafficking of CCR5, β-arrestins function as negative regulators of CCR5 recycling. This is in accordance with the original proposition by Oakley et al., that β-arrestins function to impede receptor recycling (Oakley, Laporte et al. 1999). However, in CHO CCR5 cells expressing endogenous levels of β-arrestins, CCR5 recycles with very similar kinetics to the prototypical Class A receptor, β2AR, which does not maintain an interaction with β-arrestins in endosomes (Oakley, Laporte et al. 1999; Anborgh, Seachrist et al. 2000; Signoret, Christophe et al. 2004). Of particular note is that Signoret et al. expressed CCR5 and β2AR in CHO cells, so if β-arrestins function as negative regulators of CCR5 recycling, then other factors must either negatively regulate the recycling of β2AR or positively regulate the recycling of CCR5 to account for the similarity in their recycling rates.

Another implication of CCR5 recycling being slowed in cells over-expressing β-arrestin2 is that β-arrestin expression levels in different cellular backgrounds may modulate the CCR5 recycling rate. Importantly, when considering the physiological relevance of CCR5 expression in CHO cells, the rate of CCR5 recycling in this cell-line is similar to that in lymphocytes and monocytes, where CCR5 is endogenously expressed (Mack, Luckow et al. 1998).

One significant limitation of this study is that only the effect β-arrestin2 over-expression has been assessed, and not that of β-arrestin1. Most studies addressing the role of β-arrestins in intracellular GPCR trafficking have not addressed whether there may be differential roles for β-arrestin1 and β-arrestin2. However, the expression of β-arrestin anti-sense constructs in HEK293 cells led to significant defects in A2B adenosine.
receptor recycling, which although restored by either β-arrestin1 or β-arrestin2 reconstitution, was faster when rescued with β-arrestin2 compared with β-arrestin1 (Mundell, Matharu et al. 2000). Hence, redundancy of the functions of the two β-arrestin proteins should not be assumed and the effect of β-arrestin1 over-expression should be separately assessed. As yet, I have no data concerning the mechanism through which β-arrestin2 negatively regulates the recycling of CCR5. One interesting observation made through electron microscopy observations of immunolabelled cryosections, is that after 60 min RANTES treatment, a small amount of CCR5 was found in large, flat-coated patches on endosomal structures. Interestingly, these coated areas also labelled for AP-1, suggesting that the coat might be clathrin (Silène Wavre, Endocytic Regulation of Chemokine Receptor Expression, PhD thesis, 2006). Flat clathrin coats have also been observed on early endosomes, which contain hepatocyte growth factor regulated tyrosine kinase substrate (HRS) and mediate sorting of proteins for lysosomal degradation (Raiborg, Wesche et al. 2006). Perhaps β-arrestin2 couples CCR5 to flat clathrin patches to retain it in endosomes and inhibit its recycling. However, the rate of CCR5 recycling was very similar in cells expressing the clathrin binding mutant of β-arrestin2 and those over-expressing wild-type β-arrestin2, suggesting that either this mechanism does not operate or that β-arrestins also couple CCR5 to clathrin to facilitate recycling (through the formation of CCR5-containing clathrin-coated vesicles), so that an inability of β-arrestin2 to couple to clathrin has both positive and negative effects on the ability of CCR5 to recycle, which cancel out.

The function of a β-arrestin-dependent retention mechanism is uncertain, although it may have something to do with the maintenance of signalling from the β-arrestin molecule; for instance, β-arrestins have been shown to scaffold mitogen-activated protein kinases (MAPKs) (Shenoy and Lefkowitz 2005).
Chapter 6: The Role of β-arrestins in the Intracellular Trafficking of CCR5

The β-arrestin C-tail may contain regulatory site(s) for CCR5 trafficking

The expression of constitutively active β-arrestin2 mutants did not inhibit the recycling of CCR5 to a greater extent than over-expression of wild-type β-arrestin, in apparent contrast with results obtained with B2R and FPR (Key, Vines et al. 2005; Simaan, Bédard-Goulet et al. 2005). There are several caveats with the expression of these mutants and the interpretation of their effects is not straightforward. Firstly, although I have demonstrated that the constitutively active mutants bind agonist-activated CCR5 and remain stably associated with receptors as they traffic to endosomes, I have not determined their CCR5 binding affinities in relation to wild-type β-arrestin2. Moreover, rather than increasing the affinity for agonist-activated, phosphorylated GPCRs, probably the most significant effect of truncating the β-arrestin C-tail or mutating the IVF motif is to render the β-arrestin molecule’s binding to activated GPCRs phosphorylation insensitive: both of the β-arrestin mutants have been shown to bind agonist-activated, non-phosphorylated β2AR with similar affinity to that which wild-type β-arrestin1 shows towards activated, phosphorylated β2AR (Kovoor, Celver et al. 1999; Pan, Gurevich et al. 2003). Both mutants also bound activated, partially phosphorylated mutant FPR with high affinity (Key, Vines et al. 2005).

Furthermore, it is difficult to compare the data presented in this chapter with that of the B2R and FPR studies because these studies did not assess the effect of wild-type β-arrestin over-expression compared with endogenous β-arrestin expression on the rate of receptor recycling.

For the B2R, the expression of a β-arrestin1 truncation mutant significantly reduced the rate of recycling relative to wild-type β-arrestin1 over-expression, suggesting that dissociation of the β-arrestin is required for recycling (Simaan, Bédard-Goulet et al. 2005). In contrast, the expression of the constitutively active mutants did not further inhibit CCR5 recycling over the effect produced by wild-type β-arrestin2 expression. This difference, and the apparent need for β-arrestin dissociation from the B2R before recycling, may well be a reflection of the requirement for B2R to be dephosphorylated before it is recycled. It has been shown that agonist-induced phosphorylation of B2R is followed by its rapid dephosphorylation.
Chapter 6: The Role of β-arrestins in the Intracellular Trafficking of CCR5

(Blaukat, Alla et al. 1996). Perhaps, in keeping with the classical model of β-arrestins impeding GPCR recycling, the presence of a β-arrestin1 mutant with higher affinity for the B2R prevents its dephosphorylation and, as a consequence, its recycling. CCR5, on the other hand, seems to be able to recycle in a phosphorylated state, so such a mechanism is unlikely to exist (Signoret, Pelchen-Matthews et al. 2000).

The effect of the constitutively active mutants are perhaps more easily reconcilable with those of the FPR, considering the effects of the constitutively active mutants in their own right, rather than relative to wild-type β-arrestin2, since the FPR study did not assess the effect of wild-type β-arrestin over-expression, only the expression of the constitutively active mutants. The affinities of the constitutively active β-arrestin1 mutants for activated, phosphorylated FPR were only slightly higher than wild-type β-arrestin1 anyway (K_d for constitutively active mutants = 0.22 µM and 0.18 µM, K_d for wild-type β-arrestin1 = 0.6 µM) (Key, Vines et al. 2005). By this method of comparison, the results of this study and that with the FPR are strikingly similar, since β-arrestin1(IVF/AAA) inhibited the recycling of the FPR and β-arrestin2(IVF/AAA) inhibited the recycling of CCR5, whereas the expression of β-arrestin1(1-382) did not affect the recycling of the FPR and β-arrestin2(1-380) expression led to a much reduced inhibitory effect on CCR5 recycling (perhaps this small inhibitory effect on CCR5 recycling reflects a greater sensitivity of CCR5 to β-arrestin over-expression than the FPR). Given that the constitutively active β-arrestin1 mutants had similar affinities for the activated, phosphorylated FPR (and for partially phosphorylated mutants), the reason for the difference between the effects of the mutants may lie in the residues deleted from the β-arrestin C-tail in the truncated mutants. Similarly, such an explanation can be invoked for CCR5 recycling: C-tail residues in β-arrestin2 may be required for the negative regulation of CCR5 recycling. Immunofluorescence analysis showed that after 60 min RANTES treatment, internalised CCR5, which colocalised strongly with β-arrestin2(1-380), had a slightly more dispersed cytoplasmic staining pattern than CCR5 down-modulated in cells over-expressing wild-type β-arrestin2, suggesting an alteration in trafficking. Although quite speculative, one interpretation of these results is that residues in the C-tail
of β-arrestin2 are responsible for directing the trafficking of CCR5 to the perinuclear recycling compartment where an inhibitory mechanism operates. However, co-staining of CHO CCR5 cells expressing β-arrestin2(1–380) treated with RANTES for 60 min with recycling compartment markers is needed to confirm this (a lack of colocalisation would be expected). Directing CCR5 to the recycling compartment is probably not the inhibitory mechanism in itself, since CCR5 recycling was faster in cells expressing GFP, where it passes through this compartment (data not shown), than in those expressing β-arrestin2(1–380). The recycling data for CCR5 in the presence of β-arrestin2(1–380) fitted well to the equation describing single pathway recycling mechanism, suggesting that CCR5 still recycled primarily through a single pathway in the presence of the β-arrestin2 truncation mutant. Whether this is the same pathway as it ordinarily takes cannot be determined from this analysis.

It is unlikely that the AP-2/AP-1 binding site, which is removed in the β-arrestin2 truncation mutant, is involved in regulating CCR5 intracellular trafficking, such as mediating trafficking to the perinuclear recycling compartment, since in cells expressing either β-arrestin2(IVF/AAA) or β-arrestin2(R396A), which have mutations in the AP-2/AP-1 binding site resulting in attenuated AP-2 binding (and presumed reduction in binding to AP-1), CCR5 recycling was inhibited almost to the same extent as cells over-expressing wild-type β-arrestin2, and immunofluorescence analysis revealed that down-modulated CCR5 accumulated in the perinuclear region. One potential regulatory site present in the β-arrestin2 C-tail is Thr383. Cytoplasmic β-arrestin2 is constitutively phosphorylated on Thr383 (rodent amino acid sequence numbering, as shown in Figure 6-1 and Figure 6-IV; bovine Thr382) by casein kinase II (CKII) (Lin, Krueger et al. 1997; Kim, Barak et al. 2002). Both β-arrestin2 and β-arrestin1, which is phosphorylated by extracellular-related kinase 1/2 (ERK1/2) on Ser412 become dephosphorylated upon recruitment to activated β2AR (Lin, Krueger et al. 1997; Lin, Miller et al. 1999; Kim, Barak et al. 2002). However, whereas β-arrestin1 dephosphorylation regulates interaction with clathrin (Lin, Krueger et al. 1997; Lin, Miller et al. 1999), the dephosphorylation of β-
arrestin2 has not been linked with any functional consequence other than interaction with an unidentified protein (Kim, Barak et al. 2002).

It is interesting that the trafficking behaviour of CCR5 in the presence of the β-arrestin mutants more closely resembled that of the FPR than the B2R, as it suggests a similarity in the regulatory role of β-arrestins for chemotactic GPCRs (CCR5 and FPR) versus non-chemotactic GPCRs (B2R). This is especially noteworthy as there is growing evidence for β-arrestins as master regulators of chemotaxis.

Crucially, although these data suggest that β-arrestin2 is a negative regulator of CCR5 recycling, they do not rule out the possibility that β-arrestins may be required for recycling. Without the potential to perform β-arrestin knock-downs to assess the requirement for CCR5 in recycling – because CCR5 does not internalise in their absence – determining whether β-arrestins are required for CCR5 recycling will be difficult. It may be necessary to engineer an alternative internalisation motif, such as the AP-2 binding motif of CXCR4, which can mediate CXCR4 internalisation in a phorbol myristate acetate- (PMA-) inducible manner (Signoret, Rosenkilde et al. 1998).

**β-arrestin binding to AP-2 and clathrin in CCR5 internalisation**

The rate of CCR5 internalisation was enhanced by β-arrestin2 over-expression, indicating that β-arrestins are limiting for CCR5 internalisation. This is in keeping with immunofluorescence data presented in Chapter 4, where a greater degree of internalisation and plasma membrane clustering of CCR5 was visible after 5 min RANTES-treatment in CHO CCR5 cells stably over-expressing GFP-tagged β-arrestins compared with CHO CCR5 cells.

This is unsurprising given the high levels of CCR5 expression in the CHO CCR5 cells used in this study. Given the accepted role of β-arrestins in coupling to AP-2 and clathrin to effect GPCR clathrin-mediated endocytosis, it was, however, slightly surprising that expression of both β-arrestin2(LIEF/AAEA) and β-arrestin2(R396A), mutants defective in clathrin and AP-2 binding, respectively, led to an increased rate of CCR5 internalisation over GFP-expressing cells, almost to the same extent as over-expression of wild-type β-arrestin2. This suggests that for CCR5
internalisation, either AP–2 or clathrin–binding by CCR5–bound β–arrestins is sufficient to mediate internalisation. Nevertheless, the possibility that both AP–2 and clathrin binding sites are required for β–arrestin–sponsored CCR5 internalisation but that in β–arrestin2 mutant–expressing cells, there are sufficient numbers of wild–type β–arrestin molecules that can bind activated CCR5 molecules and provide either the AP–2 or clathrin binding site in nascent clathrin–coated pits, cannot be excluded.

β–arrestin–mediated coupling of CCR5 to clathrin and AP–1 in CCR5 recycling

Previous data presented in this thesis has pointed to the formation of CCR5–containing clathrin–coated vesicles at the recycling compartment as a step involved in CCR5 recycling. As β–arrestins are bound to CCR5 in the recycling compartment, they may facilitate this step by direct binding to clathrin heavy chain. Although the data hitherto presented have provided no evidence for the additional involvement of AP–1 in this process, the recent finding that AP–1 binds β–arrestins 1 and 2 warranted investigation of this possibility. Expression of β–arrestin2 mutants defective in clathrin or AP–2 binding (assumed from various lines of evidence to be also defective in AP–1 binding, as discussed above), did not inhibit CCR5 recycling any more than over–expression of wild–type β–arrestin2, suggesting that neither clathrin nor AP–1 binding are essential for CCR5 recycling. However, although clathrin and AP–2 binding have been shown to be involved in β–arrestin–mediated GPCR internalisation, expression of β–arrestin2 mutants defective in either clathrin or AP–2 binding led to an enhancement of the rate of CCR5 internalisation over cells expressing GFP, albeit a slightly smaller enhancement than that produced by wild–type β–arrestin2 over–expression. By analogy, if β–arrestins couple CCR5 to AP–1 and clathrin in the recycling compartment to facilitate a clathrin–dependent trafficking step, ablation of β–arrestin interaction with just one of these proteins may not significantly affect recycling.

If, however, it is assumed that AP–1 plays no role in CCR5 recycling (the simplest interpretation of the data presented in Chapter 5) it could be
argued that β-arrestin-mediated coupling to clathrin is not required for CCR5 recycling.

**Potential roles for β-arrestins in CCR5 trafficking**

Figure 6-VII shows a cartoon illustrating the potential involvement of β-arrestins in regulating the trafficking of CCR5.

![Figure 6-VII Possible roles for β-arrestins in regulating CCR5 trafficking](image)

β-arrestins may control several steps in CCR5 trafficking. (1) β-arrestins couple agonist-activated CCR5 to the clathrin-mediated endocytic machinery. (2) The C-terminal region of β-arrestins may regulate trafficking from early endosomes to the perinuclear recycling compartment. (3) β-arrestins appear to act as negative regulators of CCR5 recycling, perhaps by coupling receptors to flat clathrin coats in the perinuclear recycling compartment (4) β-arrestins may couple the receptor to clathrin, as part of mechanism for progress through the recycling compartment,
e.g. final exit from the compartment, as shown in the cartoon. However, the data presented in this chapter provide no evidence for the occurrence of such a mechanism. See main text for further details.

The perinuclear recycling compartment, which may comprise elements of the TGN and recycling endosome, is shown in simplified form. The MTOC is shown in the bottom right corner; dotted grey lines represent microtubules emanating from the MTOC.

The data presented in this thesis clearly support the notion that β-arrestins facilitate the agonist-induced internalisation of CCR5 (Step 1 in Figure 6-VII). In addition, β-arrestins maintain an interaction with CCR5 as it traffics to a perinuclear recycling compartment, raising the possibility that they may play a further regulatory role in controlling CCR5 intracellular trafficking. The exact role that β-arrestins play is hard to discern from the data presented in this chapter, but the clearest conclusion is that β-arrestin2 acts as negative regulator of CCR5 trafficking and that residues in the C-tail of β-arrestin2 are required for this inhibitory function. β-arrestin2 may be responsible for directing trafficking of CCR5 to the recycling compartment, a function requiring the C-tail of β-arrestin2 (Step 2), where an inhibitory mechanism operates. As speculated above, this inhibitory mechanism may involve coupling the receptor to flat clathrin lattices (Step 3). Although providing no evidence for a role of β-arrestins in facilitating CCR5 recycling at the recycling compartment (Step 4), the data presented above do not exclude such a role.
Chapter 7: FINAL DISCUSSION

"Alzheimer's usually comes later than AIDS, but I decline to call that progress."
Mason Cooley, US apologist (d. 2002)

The notion that down-regulating CCR5 cell surface expression could be an effective anti-HIV strategy has driven research that has mapped out a basic trafficking pathway for agonist-activated CCR5, teased out some of the molecular players involved in its internalisation, and determined several properties of its recycling behaviour (Mack, Luckow et al. 1998; Signoret, Rosenkilde et al. 1998; Signoret, Pelchen-Matthews et al. 2000; Signoret, Christophe et al. 2004; Signoret, Hewlett et al. 2005). Since its discovery as a co-receptor for R5-tropic strains of HIV, CCR5 has been the target of several small-molecule HIV entry-inhibitor drugs. One of the first of these was TAK-779, whose interaction with CCR5 blocks that of the viral envelope protein, gp120, but its poor pharmacological and toxicological profile has relegated it to a more humble role in laboratory experiments, such as those described in this thesis (Baba, Nishimura et al. 1999; Dragic, Trkola et al. 2000). A more promising CCR5 antagonist is Pfizer’s compound, Maraviroc (originally designated UK-427857), which has been approved for use by the US Food and Drug Administration (FDA) and the European Commission (EC) (Dorr, Westby et al. 2005). But the notion of down-regulating CCR5 cell surface expression by interfering with its intracellular trafficking is yet to be realised and is unlikely to be while the waters beneath the cell surface remain murky. Moreover, a more complete description of CCR5 intracellular trafficking is needed to understand how CCR5 performs its physiological role in recruiting subsets of leukocytes to sites of inflammation.
In this study I have added to the current understanding of CCR5 trafficking by providing a fuller description of the path taken by internalised CCR5 molecules, focusing on the nature of the perinuclear recycling compartment through which they pass en route to the cell surface, and I have identified some of the molecules involved in CCR5 progression through this compartment. Moreover, I have shown that like a growing number of GPCRs, β-arrestins, which participate in CCR5 internalisation, remain associated with the receptor during its intracellular trafficking with potential regulatory consequences.

**CCR5 recycling: endocytic meets biosynthetic**

Much of the data regarding CCR5's post-endocytic trafficking itinerary has been obtained using CHO cells stably expressing the receptor. In these cells, it has been shown that after clathrin-mediated internalisation, CCR5 molecules traffic through early endosomes en route to a perinuclear recycling compartment, whence they recycle back to the cell surface (Signoret, Pelchen-Matthews et al. 2000; Signoret, Christophe et al. 2004; Signoret, Hewlett et al. 2005). It was previously thought that the recycling compartment - the perinuclear compartment from which CCR5 recycles to the plasma membrane and in which down-modulated receptors accumulate at steady-state - was the recycling endosome. Indeed, CCR5 was the first GPCR demonstrated to traffic through the recycling endosome (Signoret, Pelchen-Matthews et al. 2000). However, my morphological analysis of CCR5 trafficking in CHO CCR5 cells suggests that in addition to trafficking through recycling endosomes, CCR5 also traffics through the TGN and the perinuclear recycling compartment defined as above may be considered to comprise membrane elements from both the recycling endosome and TGN. To my knowledge, this is the first evidence for an agonist-activated GPCR trafficking through the TGN.

Although the data presented herein are reasonably convincing, follow-up experiments should be conducted to confirm the passage of CCR5 through the TGN. These could either take the form of an ultrastructural analysis, for instance electron microscopy performed on cryosections immunolabelled for p230/Rab11, or a biochemical assay taking advantage of the fact that CCR5
has O-linked oligosaccharides added to its N-terminus (Farzan, Mirzabekov et al. 1999; Bannert, Craig et al. 2001). In the latter case, the terminal sialic acid residues associated with these oligosaccharides could be removed from cell surface receptors by enzymatic cleavage and receptors returning to the cell surface after agonist-induced internalisation assayed for re-addition of sialic acid.

It should be noted that although the demonstration of CCR5 trafficking through the TGN is presented in Chapter 3, this trafficking behaviour was only fully appreciated towards the end of the experimental stage of the thesis project in combination with a realisation of the effects of antibody pre-labelling of cell surface CCR5 on its subsequent agonist-induced trafficking (discussed in Influence of antibody binding on CCR5 trafficking in Chapter 3). Thus, many immunofluorescence experiments described in this thesis, particularly those investigating the role of β-arrestins, were performed with pre-labelling of CCR5 and with the view that CCR5 trafficked through recycling endosomes and not the TGN. Thus, I have had to re-interpret some data in the context of CCR5 trafficking to a recycling compartment composed of recycling endosome and TGN components, which both complicates interpretation and begs further experimentation.

The obvious question that sorting to the biosynthetic pathway raises is what is the order of the trafficking events taking place in the perinuclear region of the cell? Unfortunately, the experiments described in this thesis do not provide the answer to this question. It may be expected from previously described trafficking itineraries of other recycling membrane proteins that the pathway is likely early endosome to recycling endosome to TGN, since there are examples of proteins trafficking from recycling endosomes to the TGN, whereas recycling membrane proteins taking an early endosome to TGN route are not well documented (Maxfield and McGraw 2004). In addition, there may be bidirectional transport of CCR5 between the TGN and recycling endosomes. From which element of the recycling compartment CCR5 molecules destined for the plasma membrane exit, is also unclear. Kinetic data of CCR5 recycling from the recycling compartment fit very well to an equation describing a single-pathway recycling process. The simplest interpretation of this analysis is that exit of CCR5 molecules destined for the plasma membrane only occurs from one of the two components of the
recycling compartment - either from the TGN or the recycling endosome - but it is also consistent with CCR5 recycling from both the TGN and recycling endosome with the same kinetics, perhaps with the same machinery required for exit functioning on both compartments. It should be noted that CCR5 recycling from the recycling compartment had a half-time of around 10 min, which is very similar to previous reports of TfR recycling from recycling endosomes (Mayor, Presley et al. 1993).

The trafficking of TfR through recycling endosomes, and that of several other recycling membrane proteins, such as the chemokine receptor, CXCR2, is regulated by Rab11 (Ullrich, Reinsch et al. 1996; Ren, Xu et al. 1998; Fan, Lapierre et al. 2003). In this thesis, I have shown that Rab11 also regulates CCR5 trafficking. Rab11:GTP was found to be necessary for trafficking of CCR5 to recycling endosomes and hydrolysis of the bound GTP to GDP required for CCR5 recycling, which is in keeping with the proposed role of Rab11 in TfR recycling (Ren, Xu et al. 1998). My data are also completely compatible with a description of Rab11:GTP being required for trafficking to a perinuclear recycling compartment comprising TGN and recycling endosome elements and hydrolysis of Rab11-bound GTP to GDP being required for exit from this compartment. Thus, the problem of not knowing the exact trafficking route that CCR5 takes means that I cannot exclude the possibility that Rab11 plays an additional role in trafficking within the perinuclear recycling compartment. Interestingly, results of experiments following the retrograde trafficking of TGN38 and Shiga toxin in HeLa cells, suggest that Rab11 may function in the sorting of proteins from recycling endosomes to the TGN (Wilcke, Johannes et al. 2000); it is possible that this is also the case for CCR5.

This discussion demonstrates the need for further clarification of the trafficking pathway taken by CCR5. This could begin with a quantitative immunofluorescence assay, similar to those performed by Ghosh et al. (1998) and Ghosh et al. (2003) to elucidate the trafficking itineraries of TGN38 and Cl–MPR, respectively. By following the patterns of association of CCR5 with compartmental markers after agonist treatment for various periods of time, perhaps in combination with pulses of chemokine stimulation followed by chase periods in the absence of stimulation, it may be possible to ascertain the direction of the trafficking steps. Also, the
intriguing observation that in the presence of the microtubule depolymerising agent, nocodazole, down-modulated CCR5 was found only to colocalise with the TGN marker, p230, and not the recycling endosome marker, Rab11, warrants further investigation as this may suggest a microtubule dependent exit pathway for CCR5 from the TGN, as has been shown to function for many other proteins (De Matteis and Luini 2008).

Why CCR5 traffics through the TGN in addition to recycling endosomes is an open question. Perhaps CCR5 must cycle through this compartment to maintain carbohydrate modifications and sulphation of its N-terminus, which are critical for agonist binding (Farzan, Mirzabekov et al. 1999; Bannert, Craig et al. 2001; Farzan, Chung et al. 2002). Turnover of these post-translational modifications is not well understood. Interestingly, tyrosine sulphation contributes significantly to HIV envelope protein binding and HIV co-receptor activity (Farzan, Mirzabekov et al. 1999; Cormier, Persuh et al. 2000; Farzan, Chung et al. 2002; Huang, Lam et al. 2007). Thus, if CCR5 trafficking through the TGN is required to maintain this modification, this may represent a potential target for an anti-viral strategy.

Alternative hypotheses for the need to traffic through the TGN are the same as those for CCR5 recycling through recycling endosomes: why CCR5 recycles back to the cell surface through recycling endosomes and not simply via early endosomes is not clear. For CXCR2, at least, passage of recycling receptors through recycling endosomes and not just recycling per se, is crucial for chemotaxis. Perturbation of CXCR2 recycling with a constitutively active mutant of RhoB, which prevented trafficking through Rab11–positive recycling endosomes but did not affect the extent of receptor recycling (which, under these conditions probably occurred through a Rab4–controlled pathway from early endosomes), led to impaired chemotaxis in HEK293 cells (Neel, Lapierre et al. 2007).

At least 2 reasons can be postulated for why CCR5 traffics through the recycling endosome. Firstly, trafficking through this compartment may be a necessary step in CCR5 resensitisation, which appears to be very differently regulated compared with the prototypical GPCR, the β2AR. After agonist-induced internalisation of the β2AR, agonist dissociation and dephosphorylation are promoted by the low pH of early endosomes; raising endosomal pH inhibits dephosphorylation and recycling to the cell surface.
I. Na i D iscussion

(Pippig, Andexinger et al. 1995; Krueger, Daaka et al. 1997). From early
endosomes, the receptor can recycle to the plasma membrane in a process
controlled by PDZ domain-containing proteins (Cao, Deacon et al. 1999;
Cong, Perry et al. 2001; Gage, Kim et al. 2001; He, Bellini et al. 2005);
however, it must be noted that there is some evidence for the β2AR recycling
through recycling endosomes after prolonged agonist stimulation (Moore,
Millman et al. 2004). In contrast, the recycling of CCR5 is not regulated by a
pH-dependent agonist dissociation mechanism and may not require
dephosphorylation (Signoret, Pelchen-Matthews et al. 2000; Signoret,
Christophe et al. 2004). Also, CCR5 molecules, which can recycle back to the
plasma membrane in an agonist-occupied state (Signoret, Pelchen-
Matthews et al. 2000), probably undergo multiple rounds of internalisation
and recycling before they are returned to – and remain at – the cell surface
in a fully resensitised form. A condition of the previous statement, though,
is that resensitisation does take place, albeit inefficiently. Given that
resensitisation does not seem to require the low pH of the early endosome,
the events involved in this process may occur – inefficiently – in recycling
endosomes, perhaps involving proteolytic degradation of bound chemokines
by recycling endosome-located proteases. In a similar fashion, one could
argue that passage through the TGN may be involved in CCR5
resensitisation. Indeed, passage through the recycling endosome may just
be necessary for endocytosed receptors to reach the TGN, where such a
mechanism could operate.

A requirement for passage through the recycling endosome for
resensitisation may not be the only reason for passage through the
compartment. Indeed, the resensitisation of CXCR2, which also passes
through recycling endosomes, appears to be quite different from that of
CCR5, suggesting that a common mechanism involved in chemokine
receptor desensitisation in the recycling endosome does not exist. CXCR2
has been shown to associate with okadaic-acid sensitive protein
phosphatase 2A (PP2A) after endocytosis, an interaction responsible for its
dephosphorylation. Disruption of this interaction not only abrogated
receptor dephosphorylation but also impaired chemotaxis (Fan, Yang et al.
2001). Dephosphorylation of Ser337 (a PKC-substrate) on CCR5, on the
other hand, although being sensitive to okadaic acid, can occur without
endocytosis and dephosphorylation of Ser349 (a GRK substrate) is not sensitive to okadaic acid (Pollok-Kopp, Schwarze et al. 2003). An alternative hypothesis for the reason that CCR5 traffics through recycling endosomes is that this is responsible for targeted recycling of endocytosed CCR5 molecules to the leading edge of cells. CCR5 has been seen to accumulate at the leading edge of leukocytes undergoing RANTES–induced chemotaxis (Nieto, Frade et al. 1997; Gómez-Moutón, Lacalle et al. 2004) and in migrating fibroblasts, recycling TfR has been shown to be selectively delivered from recycling endosomes to the leading lamellae (Hopkins, Gibson et al. 1994). Moreover, passage through the TGN may fulfil a similar leading edge targeting role. In Swiss 3T3 fibroblasts it was found that protein kinase D (PKD)–mediated anterograde membrane traffic from the TGN to the plasma membrane is required for localised Rac1–dependent leading edge activity and fibroblast locomotion (Prigozhina and Waterman-Storer 2004) and by following newly-synthesised low density lipoprotein receptor (LDLR) tagged with GFP using total internal reflection fluorescence microscopy (TIR–FM), exocytic events were observed to be polarised towards the leading edge in migrating fibroblasts (Schmoranzer, Kreitzer et al. 2003). Thus, although speculative, CCR5 may engage a targeted recycling pathway from recycling endosomes and/or the TGN for delivery to the leading edge of cells undergoing chemotaxis. However, despite CCR5 having been observed to accumulate at the leading edge of migrating leukocytes, concentration of chemotactic receptors at the leading edge of migrating cells is a controversial issue, with several studies suggesting that other chemotactic receptors are evenly distributed across the surface of migrating cells (Xiao, Zhang et al. 1997; Servant, Weiner et al. 1999; Jin, Zhang et al. 2000; Janetopoulos, Jin et al. 2001). The simplest explanation for these observations is that different receptors are regulated in different ways. My results also go a fair way towards describing the machinery that may operate to bring about CCR5 recycling. The simplest conclusion from my morphological data and functional data using various drugs to inhibit CCR5 recycling, and the EM observations of others, is that dynamin–dependent clathrin–coated vesicle formation at the recycling compartment is involved in CCR5 recycling. A clathrin– and dynamin–dependent recycling pathway from recycling endosomes to the plasma membrane has been described for
TfR (van Dam and Stoorvogel 2002; van Dam, Ten Broeke et al. 2002), but this is the first evidence for the involvement of clathrin and dynamin in the recycling of a GPCR. One key experiment is necessary to test my hypothesis, though. Ultrastructural analysis of the effect of Dyngo-4a on CCR5 recycling, should, if the drug is truly specific for dynamin, and the above hypothesis correct, show CCR5 trapped in coated pits that have failed to undergo scission. Labelling with anti-clathrin antibodies could then confirm if the coat is clathrin and labelling with compartment markers could be used to determine the site of this inhibition - TGN or recycling endosome - which is currently unknown. Although transport vesicles formed in such a process could be targeted directly to the plasma membrane, my data do not discount the possibility that such a mechanism also operates in intra-recycling compartment transport, i.e. trafficking from the recycling endosome to TGN or vice versa. With the ultrastructural analysis and a better understanding of the precise trafficking pathway taken by CCR5, which could be provided from the quantitative immunofluorescence assay described above, it may be possible to discern on which step in CCR5 trafficking this clathrin- and dynamin-dependent process acts.

Lastly, if CCR5 does use a clathrin-dependent trafficking step, then there is likely to be an adaptor protein to link CCR5 to such a pathway. Given that ACAP1 has been shown to be involved in TfR recycling (Dai, Li et al. 2004) and that it has subsequently been shown to function as a clathrin adaptor in stimulation-dependent integrin recycling and insulin-stimulated recycling of glucose transporter type 4 (GLUT4) from recycling endosomes (Li, Peters et al. 2007), a potential role for its involvement in such a capacity for CCR5 should be investigated. That β-arrestins remain bound to CCR5 in the recycling compartment also raises the possibility that in a beautiful symmetry with their role as clathrin adaptors in CCR5 endocytosis at the plasma membrane, β-arrestins couple CCR5 to a clathrin-mediated trafficking step involved in CCR5 recycling.

**β-arrestins: regulators of CCR5 intracellular trafficking**

There is now much evidence suggesting that in addition to sponsoring clathrin-mediated endocytosis of many agonist-activated GPCRs, β-arrestins
also play a role in regulating their subsequent trafficking itineraries. Initial experiments defined two classes of GPCRs, Class A and Class B, which differ in the nature of their association with β-arrestins in that Class A receptors have a higher affinity for β-arrestin2 than β-arrestin1 and dissociate from the β-arrestin molecule during, or immediately after, endocytosis, whereas Class B receptors have an equally high affinity for both β-arrestin1 and β-arrestin2 and maintain an interaction with β-arrestins into endosomes (Oakley, Laporte et al. 2000). Also, whereas Class A receptors recycle rapidly to the plasma membrane after internalisation, Class B receptors recycle very slowly, if at all, presumably undergoing eventual degradation (Oakley, Laporte et al. 1999; Anborgh, Seachrist et al. 2000). Experiments swapping the C-tails of the Class A receptor, β2AR, and the Class B receptor, V2R, not only reversed the stability of the receptor–β-arrestin complex but also reversed the corresponding rates of receptor dephosphorylation, recycling and resensitisation, leading to the idea that β-arrestins impede receptor recycling, presumably by restricting the access of phosphatases to receptors (Oakley, Laporte et al. 1999). Along the same lines of β-arrestins inhibiting recycling, it has, more recently, been shown that β-arrestin binding can facilitate receptor ubiquitinination, targeting receptors for degradation (Shenoy, McDonald et al. 2001; Martin, Lefkowitz et al. 2003). However, despite the data linking sustained β-arrestin interaction with very slow receptor recycling/degradation (Oakley, Laporte et al. 1999; Anborgh, Seachrist et al. 2000), more recent studies have identified receptors that despite maintaining an interaction with β-arrestins in endosomes, nevertheless recycle rapidly to the cell surface - the so-called Class C receptors (Garland, Grady et al. 1996; McConalogue, Dery et al. 1999; Mundell, Matharu et al. 2000; Vines, Revankar et al. 2003; Tulipano, Stumm et al. 2004; Simaan, Bédard-Goulet et al. 2005). Although there is evidence that dissociation of β-arrestins from Class C receptors may be required for their recycling (Key, Vines et al. 2005; Simaan, Bédard-Goulet et al. 2005), which is in keeping with the early view that a sustained interaction with β-arrestins inhibits recycling, evidence from β-arrestin knock-out and knock-down studies have demonstrated the requirement for β-arrestins in the
recycling of the \(N\)-formyl peptide receptor (FPR) and the \(A_{2B}\) adenosine receptor (Mundell, Matharu et al. 2000; Vines, Revankar et al. 2003).

Following on from the observation by Mueller et al. (2002) that GFP-\(\beta\)-arrestin1 redistributed with agonist-activated CCR5 into endosomes, I have shown that both \(\beta\)-arrestins 1 and 2 stably associate with CCR5 as it traffics through early endosomes and on to the perinuclear recycling compartment. Given that CCR5 rapidly recycles from the recycling compartment to the cell surface, CCR5 can thus be classified as a Class C receptor. Whether this sustained interaction between \(\beta\)-arrestins and CCR5 plays a role in regulating CCR5 trafficking and what this role is has been harder to pin down. A tantalising hypothesis is that \(\beta\)-arrestins could act as facilitators of CCR5 recycling by coupling the receptor to a clathrin-mediated trafficking step that my data suggest operates at the recycling compartment. However, over-expression of \(\beta\)-arrestin2 resulted in an inhibition of CCR5 recycling, suggesting that \(\beta\)-arrestins act as negative regulators of CCR5 recycling. Although the significance of the results of CCR5 recycling in the presence of \(\beta\)-arrestin2 mutants is unclear, one of the simplest interpretations of the data as a whole is that residues in the C-tail of \(\beta\)-arrestin2 are responsible for mediating trafficking of CCR5 to the recycling compartment and, perhaps, the recycling compartment is the site where a \(\beta\)-arrestin-mediated inhibitory mechanism operates. The mechanism through which \(\beta\)-arrestins would exercise this role is unclear but it may involve the coupling of the receptor to flat clathrin patches, since on immunolabelled cryosections, down-modulated CCR5 has been observed in large, flat coated patches on endosomal structures, which contained the clathrin adaptor protein complex, AP-1 (Silène Wavre, Endocytic Regulation of Chemokine Receptor Expression, PhD thesis, 2006).

It is also interesting to note that \(\beta\)-arrestin1 has been implicated in early endosome to lysosome targeting of CXCR4, which is related to its ability to interact directly with the E3 ubiquitin ligase, AIP4 (Bhandari, Trejo et al. 2007). Thus, \(\beta\)-arrestins may play a crucial sorting role at the early endosome, dictating whether receptors are targeted for degradation or to a recycling pathway.
A major criticism of the experiments addressing the role of \(\beta\)-arrestins in CCR5 post-endocytic trafficking is that I have only assessed the effect on CCR5 recycling of \(\beta\)-arrestin2 over-expression, and not that of \(\beta\)-arrestin1. For the \(A_{2b}\) adenosine receptor, expression of \(\beta\)-arrestin anti-sense constructs in HEK293 cells led to significant defects in recycling, which although restored by either \(\beta\)-arrestin1 or \(\beta\)-arrestin2 reconstitution, was faster when rescued with \(\beta\)-arrestin2 compared with \(\beta\)-arrestin1 (Mundell, Matharu et al. 2000), so there may be differential roles for the two \(\beta\)-arrestin proteins in intracellular trafficking of GPCRs. Although CCR5 did not recycle in transiently-transfected MEFs, experience with transient versus stably-transfected cell lines suggests that this inability to support CCR5 recycling may be a function of transient transfection rather than MEFs in particular. Hence, a MEF cell-line stably expressing CCR5 should be made to see if the receptor can recycle in these cells. If it can, \(\beta\)-arrestin knock-out MEFs stably-expressing CCR5 should also be made, which may be useful in determining whether there are differential effects of the \(\beta\)-arrestin proteins on CCR5 recycling. Moreover, a \(\beta\)-arrestin knock-out background would be more desirable for assessing the effect of \(\beta\)-arrestin mutants on CCR5 trafficking. If CCR5 were unable to recycle in MEFs stably expressing the receptor, this would be an interesting result in itself, since it would suggest that MEFs lack (or express very low levels of) a component(s) of the recycling machinery. This would also suggest that CCR5 recycling is quite a specialised process as presumably other recycling membrane proteins, such as TfR and TGN38 can recycle in MEFs. Of course, an absolute requirement for \(\beta\)-arrestins in CCR5 recycling could still not be assessed in \(\beta\)-arrestin 1 and 2 knock-out MEFs stably-expressing CCR5, since CCR5 does not internalise in the absence of \(\beta\)-arrestins. More ambitious experiments may be necessary here, such introducing an alternative internalisation motif into CCR5, such as the AP-2 binding motif of CXCR4, which can mediate CXCR4 internalisation in a phorbol myristate acetate- (PMA-) inducible manner (Signoret, Rosenkilde et al. 1998).

The sustained association of CCR5 with \(\beta\)-arrestins must also be viewed in a physiological context, particularly with a consideration of the potential role of \(\beta\)-arrestins in chemotaxis by scaffolding signalling molecules involved in
cytoskeletal reorganisation to promote localised actin assembly events leading to the formation of a leading edge (Defea 2006). As discussed above, CCR5 has been seen to accumulate at the leading edge of migrating cells (Nieto, Frade et al. 1997; Gómez-Moutón, Lacalle et al. 2004) and there is evidence for targeted trafficking of other proteins from recycling endosomes and the TGN to the leading edge of cells during cell migration (Hopkins, Gibson et al. 1994; Schmoranzer, Kreitzer et al. 2003; Prigozhina and Waterman-Storer 2004). Moreover, increased rates of clathrin-mediated internalisation at the leading edge compared with the trailing edge have also been observed in migrating cells (Rappoport and Simon 2003), so in leukocytes undergoing chemotaxis, CCR5 may effectively be constrained to a recycling loop between the leading edge and the perinuclear recycling compartment. Such a model, including the additional proposal that there is slow lateral diffusion in the plasma membrane at the leading edge, has been proposed to contribute to the generation of polarity in migrating cells (Jones, Caswell et al. 2006). Thus, signalling from β-arrestins bound to CCR5 may, as a consequence, be restricted to within this loop. Moreover, a significant proportion of agonist-activated, internalised CCR5 molecules recycle to the plasma membrane in an agonist-bound, activated form, escaping resensitisation (Signoret, Pelchen-Matthews et al. 2000). It is possible, therefore, that β-arrestins accompany agonist-bound receptors back to the cell surface and may thus target signalling complexes to the leading edge that are responsible for actin reorganisation. Of course, this could also be achieved through retention of CCR5 complexed with β-arrestins at the cell surface but this would not allow for CCR5 resensitisation, which would be predicted to play a role in gradient sensing. Thus, a recycling mechanism with some CCR5 molecules recycling in a β-arrestin-bound state and some being returned in a resensitised state may fulfil both the requirement for localised β-arrestin signalling at the leading edge and enable the population of CCR5 molecules to collectively ‘sense’ a chemokine gradient. Interestingly, constant retargeting of internalised EGFR (epidermal growth factor receptor) and PVR (platelet-derived growth factor/vascular endothelial growth factor [PDGF/VEGF] receptor) to the leading edge of Drosophila border cells, which keeps downstream signalling localised, is crucial for their migration (Jékely, Sung et al. 2005).
Alternatively, but not mutually exclusive with the previous proposal, \( \beta \)\-arrestin\-mediated signalling in the recycling compartment may control some other aspect of cell migration or physiological response to chemokine challenge. This may explain why \( \beta \)\-arrestins act as negative regulators of recycling: retention of CCR5 in the recycling compartment may be a mechanism to prolong \( \beta \)\-arrestin\-mediated signalling at the recycling compartment.

I have tried to address the issue of whether \( \beta \)\-arrestins accompany CCR5 in CHO cells as it recycles back to the cell surface by live-cell fluorescence microscopy but have experienced problems with severe phototoxicity in CHO cells. This could perhaps be overcome by using a multi-photon microscope.

Despite over-expression of \( \beta \)\-arrestin2 impeding CCR5 recycling, the data presented in Chapter 6 do not rule out the possibility that \( \beta \)\-arrestins also function to facilitate CCR5 recycling, perhaps in a clathrin adaptor role. However, the sequential multi-site binding model of \( \beta \)\-arrestin interaction with an activated GPCR proposes that agonist-dissociation leading to a conformational change in the receptor is the catalyst for \( \beta \)\-arrestin dissociation (Gurevich and Benovic 1993). Although I have presented data that \( \beta \)\-arrestin binding may be able to occur independently of receptor phosphorylation, receptor activation is a pre-requisite of \( \beta \)\-arrestin binding, since agonist-free receptors (wild-type or phosphorylation-deficient) on the cell surface do not bind \( \beta \)\-arrestins. After agonist dissociation, wherever that may occur, the theory would suggest that CCR5 molecules should not retain an interaction with \( \beta \)\-arrestins, or at least the complex should be severely destabilised. Although CCR5 molecules can return to the surface in an agonist-bound state and may undergo several cycles of internalisation and recycling before returning to the surface in a resensitised state, agonist dissociation must occur at some stage in this iterative cycling, since resensitised receptors do re-appear on the cell surface after agonist wash-out (Signoret, Pelchen-Matthews et al. 2000). If agonist dissociation were to occur prior to the \( \beta \)\-arrestin\-mediated trafficking step, then this would require an alternative mechanism to effect CCR5 trafficking at this particular step. Thus, a \( \beta \)\-arrestin\-mediated recycling mechanism may be a
specialised form of recycling restricted to agonist-bound receptors. The significance of this is not clear.

In keeping with the previously-demonstrated importance of receptor phosphorylation in supporting high-affinity β-arrestin binding, most CCR5 molecules that traffic to the recycling compartment seem to remain phosphorylated on serines 337 and 349. However, I have also shown that a CCR5 mutant with all 4 serine residues previously demonstrated to undergo agonist-induced phosphorylation mutated to alanines, is also capable of recruiting β-arrestins and undergoing internalisation when stimulated with agonist. Moreover, its ability to recruit β-arrestins was not an over-expression artefact, since it failed to undergo agonist-induced internalisation in β-arrestin 1 and 2 knock-out MEFs, yet underwent internalisation in wild-type and single β-arrestin knock-out MEFs. Although it is necessary to confirm that this mutant is, indeed, not phosphorylated on other threonine residues when expressed in CHO cells or MEFs, these data suggest that CCR5 can bind β-arrestins independently of phosphorylation. My data do, however, suggest that phosphorylation increases the affinity and - particularly in the case of β-arrestin1 - the stability of the interaction. CCR5 is not alone in its ability to bind β-arrestins in the absence of phosphorylation: a surprising number of GPCRs have been shown to bind β-arrestins (and in most cases also internalise) in the absence of phosphorylation, including the D6 non-signalling chemokine receptor (Galliera, Jala et al. 2004), protease activated receptor 2 (Stalheim, Ding et al. 2005), lutropin receptor (Mukherjee, Palczewski et al. 1999; Min and Ascoli 2000; Min, Galet et al. 2002; Mukherjee, Gurevich et al. 2002), substance P receptor (Richardson, Balius et al. 2003), orexin 1 receptor (Milasta, Evans et al. 2005), leukotriene B4 receptor 1 (Jala, Shao et al. 2005) and several splice variants of the serotonin 5-HT4 receptor (Barthet, Gaven et al. 2005). Whether phosphorylation-independent β-arrestin binding to CCR5 actually occurs in vivo, though, is questionable. It is likely that because receptors are rapidly phosphorylated after agonist-activation, that most receptors binding β-arrestins are phosphorylated. Indeed, most of the experiments showing that the above receptors can undergo β-arrestin-
dependent internalisation in the absence of receptor phosphorylation have used phosphorylation–deficient receptor mutants, so it is not known whether any non–phosphorylated receptors are internalised in a β–arrestin–dependent fashion in vivo. However, there remains the possibility that some CCR5 molecules could engage β–arrestins without having undergone phosphorylation. What the significance of this would be is unclear.

Similar to CCR5, for the substance P, orexin–1 and protease activated 2 receptors, phosphorylation has been shown to enhance the stability of the β–arrestin–receptor complex; that is, mutation of key phospho–acceptor residues converts the receptors from a Class B to a Class A phenotype (Richardson, Balius et al. 2003; Milasta, Evans et al. 2005; Stalheim, Ding et al. 2005). It should be noted, though, that although the stability of the interaction between β–arrestin1 and phosphorylation–deficient CCR5 was clearly reduced compared with wild–type CCR5, phosphorylation–deficient CCR5 showed only a mildly reduced stability with β–arrestin2, maintaining an interaction with β–arrestin2 into the recycling compartment in most cells observed. Thus, CCR5 seems to be unique in its ability to maintain an interaction with β–arrestins into endosomes without receptor phosphorylation. Additionally, CCR5 differs from the previously characterised Class B and Class C receptors in that it does not contain (a) cluster(s) of serine/threonine residues (Ser/Thr residues occupying 3/3, 3/4, or 3/5 consecutive positions), so does appear to be very different from other previously characterised receptors with respect to its interaction with β–arrestins. Stable β–arrestin binding has been linked to ubiquitination of the β–arrestin molecule. Rapid internalisation of the β2AR, a Class A receptor, requires transient ubiquitination of β–arrestin2 and the time–course of ubiquitination is consistent with β–arrestin2 carrying a ubiquitin moiety only when bound to the receptor (Shenoy, McDonald et al. 2001). Activation of the V2 vasopressin receptor (V2R), a Class B receptor, results in more prolonged β–arrestin ubiquitination and switching the C–tails of the β2AR and V2R reverses the kinetics of β–arrestin ubiquitination (Shenoy and Lefkowitz 2003). Importantly, expression of a β–arrestin2–ubiquitin fusion protein, which cannot be de–ubiquinated in the cell and mimics the stably ubiquitinated form of arrestin, imparts Class B characteristics on the β2AR
(Shenoy and Lefkowitz 2003). Perhaps agonist-activated CCR5 can interact with β-arrestins in a way that induces a β-arrestin conformation that promotes its stable ubiquitination despite the receptor lacking serine/threonine clusters in its C-tail. At least for β-arrestin2, it would be predicted that this conformation can be achieved without the requirement for C-tail phosphorylation of CCR5.

β-arrestin binding to unphosphorylated receptors at all requires an explanation consistent with the activation mechanism of β-arrestin. The D6 chemokine receptor does not appear to be phosphorylated in vivo but nevertheless undergoes constitutive β-arrestin-dependent internalisation (Galliera, Jala et al. 2004). For this receptor, an acidic region in its C-terminus is involved in β-arrestin binding, presumably activating the β-arrestin phosphate sensor in the absence of phospho-residues on the receptor (Galliera, Jala et al. 2004). There are several glutamic acid residues in the CCR5 C-tail that may function in a similar way, although they are spread along the length of the tail and do not constitute a cluster.

One particularly interesting result, which was a by-product of the experiment demonstrating that CCR5 molecules remain phosphorylated on serines 337 and 349 as they traffic to the perinuclear recycling compartment, is that at least serines 337 and 349 appear to be accessible after β-arrestin binding. Whether this is due to these residues becoming solvent-exposed after the conformational change in the β-arrestin molecule associated with the transition to its active, high-affinity receptor-binding state or whether in a dimer only one receptor binds a β-arrestin molecule, this means that CCR5 dephosphorylation and β-arrestin dissociation may not be tightly coupled, as has been suggested for other GPCRs (Oakley, Laporte et al. 1999). Moreover, the putative PDZ ligand at the extreme C-terminus, which includes S349 and whose integrity has been shown to be necessary for CCR5 recycling (Delhaye, Gravot et al. 2007), would also be accessible despite sustained β-arrestin interaction with internalised receptors and thus a PDZ sequence-directed regulatory mechanism could act alongside a β-arrestin-dependent regulatory mechanism for CCR5 trafficking. This may also be the case for other GPCRs.
Chapter 7: Final Discussion

**The way forward**

This study and others have demonstrated various features of CCR5 trafficking in CHO cells. However, the major limitation of this cell-line is the difficulty with performing routine siRNA knock-down studies because the hamster genome has not been sequenced. I have established a human osteosarcoma (HOS) cell-line that appears to traffic the receptor similarly to CHO cells and should prove useful for investigating the role of candidate proteins in CCR5 recycling – given the likely involvement of clathrin in a CCR5 recycling pathway, investigating a role for ACAP1 should be prioritised. When a clearer molecular picture of CCR5 intracellular trafficking has emerged in non-motile cells, the relevance of this trafficking for physiological processes such as chemotaxis should then be assessed. However, more sophisticated assays than the simple Boyden chamber assay commonly used to assess chemotaxis should be explored, which can better reflect the 3-dimensional cellular matrix through which leukocytes must migrate within an inflamed tissue and also recapitulate the *in vivo* situation where chemokines are presented on the surface of cells through interaction with glycosaminoglycans (GAGs). Moreover, chemotaxis assays that assess the ability of cells to undergo chemotaxis in a gradient of chemokine, although perhaps being relevant for the chemotactic migration of leukocytes within an inflamed tissue, do not really reflect well the other steps involved in the recruitment of leukocytes to sites of inflammation including the steps prior to extravasation, where the recruitment of leukocytes from the blood stream begins with the selectin-mediated capture and rolling of leukocytes to the vessel wall, which allows immobilised chemokines to interact with their cognate receptors on the leukocyte, resulting in integrin activation and firm arrest. Moreover, chemokines also promote transendothelial migration in a process that may involve leukocyte movement down an immobilised chemokine gradient – so-called haptorepulsion – since the highest concentrations of chemokines are found on the luminal surface. Indeed, CXCR3 has been shown to mediate the migration of plasmacytoid dendritic cells, which required the encounter of its cognate ligands immobilised, optimally by heparan sulfate, in the form of a negative gradient (Kohrgruber, Groger et al. 2004).
Additionally, leukocytes express CCR5 along with many other GPCRs, including many $G_{\alpha_4}$-coupled chemoattractant receptors, which may be simultaneously activated during a response to inflammation. Interestingly, the activation of CXCR1 and FPR have been shown to not only lead to cross-phosphorylation and desensitisation of CCR5 but also cross-internalisation of CCR5 in cells co-expressing the receptors (Shen, Li et al. 2000; Li, Wetzel et al. 2001; Richardson, Tokunaga et al. 2003). Moreover, CCR5 has been shown to heterodimerise with the complement 5a receptor (C5aR) in RBL cells and agonist-induced internalisation of C5aR led to the co-internalisation of CCR5. Importantly, internalisation of CCR5 was also observed in C5a-stimulated macrophages, demonstrating that such a co-internalisation mechanism operates in cells where CCR5 is endogenously expressed. How such hetero-oligomerisation affects the intracellular trafficking of CCR5 is unknown, as is the extent of heterodimerisation and cross-regulation in leukocytes. Thus, once the fundamental properties of CCR5 trafficking have been determined in cell-lines, the task of understanding how the receptor traffics in cells where it is endogenously expressed can start and this is where the real fun will begin!
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