the role of $\gamma$ protein in T lymphocyte activation

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Abstract

An analysis of the role of guanine nucleotide-binding proteins in T lymphocyte activation.

Proteins which bind and hydrolyse GTP are involved in the regulation of many aspects of cellular growth control and metabolism. For example, the heterotrimeric G proteins are involved in transducing signals from receptors to effect changes in cellular second messenger systems. Another class of guanine nucleotide binding proteins (p21\textsuperscript{ras}) are encoded by the \textit{ras} protooncogenes and have been implicated in the regulation of normal cell growth and oncogenic transformation. Using a permeabilised cell system which allowed cellular access for otherwise membrane-impermeant nucleotide and peptide reagents, while maintaining many intracellular signalling pathways, the role of guanine nucleotide binding proteins in T cell antigen receptor (TCR/CD3) complex coupling to phospholipase C (PLC) and in the regulation of protein kinase C (PKC) activity were investigated. In addition, the regulation of p21\textsuperscript{ras} by T cell surface receptors was analysed in both intact and permeabilised cells.

The data show that the TCR/CD3 complex and a population of G proteins can regulate PI-PLC in T cells but that the effects of guanine nucleotides on TCR/CD3 coupling are not compatible with a simple receptor→G protein→PI-PLC model. These results most likely reflect that the TCR/CD3 complex is not coupled to PLC via a G protein but that a guanine nucleotide binding protein can indirectly modulate TCR/CD3 coupling. An alternative TCR/CD3 coupling mechanism is suggested.

Receptor and G protein agonists were also observed to induce PKC-mediated phosphorylation of the CD3 γ subunit in permeabilised T lymphoblasts. These results are consistent with stimulation of PKC activity being explained by agonist effects on phosphatidylinositol metabolism.
However, the ability to dissociate G protein agonist effects on PI-PLC activity and phosphorylation of CD3 suggest that guanine nucleotide binding proteins also exert a regulatory effect on PKC activity by distinct mechanisms.

This study also demonstrates that the activation state of endogenous p21\textsuperscript{ras} can be regulated by triggering the TCR/CD3 complex, CD2 antigen or the high affinity receptor for the growth factor IL2. These results infer that normal p21\textsuperscript{ras} functions in the signalling pathways by which these receptors regulate T lymphocyte activation and proliferation. Evidence is provided to suggest that the TCR/CD3 complex regulates the GTPase activity of p21\textsuperscript{ras} and that both PKC-dependent and independent pathways exist for the regulation of p21\textsuperscript{ras} in T lymphocytes.
# Contents.
## Section 1: Introduction.

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Antigen recognition by T lymphocytes</td>
<td>1</td>
</tr>
<tr>
<td>1.2</td>
<td>Surface receptors that control T cell activation</td>
<td>1</td>
</tr>
<tr>
<td>1.3</td>
<td>The antigen-specific MHC-restricted T cell receptor complex (TCR/CD3)</td>
<td>3</td>
</tr>
<tr>
<td>1.3.1</td>
<td>The Ti components of the TCR/CD3 complex</td>
<td>3</td>
</tr>
<tr>
<td>1.3.2</td>
<td>The CD3 components of the TCR/CD3 complex</td>
<td>4</td>
</tr>
<tr>
<td>1.3.3</td>
<td>The TCR ( \zeta ) and ( \eta ) chains</td>
<td>6</td>
</tr>
<tr>
<td>1.4</td>
<td>The CD4 and CD8 antigens</td>
<td>8</td>
</tr>
<tr>
<td>1.5</td>
<td>The CD2 antigen</td>
<td>9</td>
</tr>
<tr>
<td>1.6</td>
<td>The CD45 antigen</td>
<td>11</td>
</tr>
<tr>
<td>1.7</td>
<td>The CD28 antigen</td>
<td>13</td>
</tr>
<tr>
<td>1.8</td>
<td>What are the consequences of T cell activation?</td>
<td>13</td>
</tr>
<tr>
<td>1.9</td>
<td>The IL2 system and T cell proliferation</td>
<td>15</td>
</tr>
<tr>
<td>1.9.1</td>
<td>Regulation of IL2 and IL2 receptor genes</td>
<td>17</td>
</tr>
<tr>
<td>1.9.2</td>
<td>The enhancer region of the IL2 receptor ( \alpha ) and ( \beta ) chain genes</td>
<td>18</td>
</tr>
<tr>
<td>1.9.3</td>
<td>The enhancer region of the IL2 gene</td>
<td>19</td>
</tr>
<tr>
<td>1.10</td>
<td>Intracellular biochemical changes that accompany T cell activation</td>
<td>21</td>
</tr>
<tr>
<td>1.11</td>
<td>The calcium signal and T cell activation</td>
<td>22</td>
</tr>
<tr>
<td>1.12</td>
<td>Protein kinase C and T cell activation</td>
<td>24</td>
</tr>
<tr>
<td>1.12.1</td>
<td>Structure and function of PKC isozymes</td>
<td>25</td>
</tr>
<tr>
<td>1.12.2</td>
<td>The role of PKC in T cells</td>
<td>26</td>
</tr>
<tr>
<td>1.13</td>
<td>Tyrosine phosphorylation</td>
<td>29</td>
</tr>
<tr>
<td>1.13.1</td>
<td>Tyrosine kinases and phosphatases in T lymphocytes</td>
<td>31</td>
</tr>
<tr>
<td>1.13.2</td>
<td>Regulation of src family protein tyrosine kinases</td>
<td>32</td>
</tr>
<tr>
<td>1.13.3</td>
<td>Regulation of Lck and Fyn in T lymphocytes</td>
<td>34</td>
</tr>
<tr>
<td>1.14</td>
<td>Signals that regulate expression of IL2R and IL2 secretion</td>
<td>39</td>
</tr>
<tr>
<td>1.14.1</td>
<td>The ( \Ca^{2+}/\text{PKC} ) model for IL2/IL2R expression</td>
<td>39</td>
</tr>
<tr>
<td>1.14.2</td>
<td>Evidence for the involvement of tyrosine phosphorylation in induction of IL2/IL2R expression</td>
<td>42</td>
</tr>
<tr>
<td>1.15</td>
<td>Signal transduction by the IL2 receptor</td>
<td>45</td>
</tr>
<tr>
<td>1.16</td>
<td>TCR/CD3 coupling to intracellular signaling pathways - role of the TCR ( \zeta ) and ( \eta ) subunits</td>
<td>47</td>
</tr>
<tr>
<td>1.17</td>
<td>Phosphatidylinositol specific phospholipase C</td>
<td>51</td>
</tr>
</tbody>
</table>
1.18 Inositol phospholipid lipid metabolism and cellular signalling.  
1.19 Regulation of PI-specific PLC.  
1.19.1 Regulation of PI-PLC by growth factor receptor tyrosine kinases.  
1.19.2 Guanine nucleotide binding proteins and receptor-effector coupling.  
1.19.3 Regulation of PI-PLC by G proteins.  
1.20 Evidence for PI-PLC regulation by tyrosine phosphorylation in T lymphocytes.  
1.21 Evidence for PI-PLC regulation by G proteins in T lymphocytes.  
1.22 The p21\textsuperscript{ras} guanine nucleotide binding proteins and growth regulatory pathways.  
1.22.1 Oncogenic mutations and the biological activity of p21\textsuperscript{ras}.  
1.22.2 Cellular factors which regulate p21\textsuperscript{ras}.  
1.22.3 The cellular effector of p21\textsuperscript{ras} action.  
1.22.4 The role of p21\textsuperscript{ras} in signal transduction.  
1.22.5 Possible links between growth regulatory pathways and p21\textsuperscript{ras}.  

**Diagrams.**

1.1 T cell activation in vivo.  
1.2 Structure of the TCR/CD3 complex.  
1.3 Structure of the high affinity IL2 receptor.  
1.4 The enhancer region of the IL2 receptor α chain gene.  
1.5 The enhancer region of the IL2 gene.  
1.6 Structure of PKC isozymes.  
1.7 The role of PKC and Ca\textsuperscript{2+} in T cell activation.  
1.8 Structure of src family protein tyrosine kinases.  
1.9 Regulation of tyrosine phosphorylation in T lymphocytes.  
1.10 T cell activation in vitro.  
1.11 Structure of PI-PLC isozymes.  
1.12 Inositol phospholipid hydrolysis by PI-PLC.  
1.13 Regulation of cellular DAG levels.  
1.13 Regulation of the G protein cycle of GTP binding and hydrolysis.  
1.15 Structure of (ras)GAP.  
1.16 The p21ras cycle of GTP binding and hydrolysis.  

**Aims of this study.**
Section 2: Materials and Methods.

2.1 Materials. 89
2.2 Antibodies. 90
2.3 Cell culture. 90
2.4 Cell permeabilisation. 91
2.5 Phosphorylation and immunoprecipitation of the γ subunit of the CD3 antigen. 92
2.6 [3H]inositol labelling and inositol phosphate determination. 92
2.7 Peptide assay for PKC activity in permeabilised cells. 93
2.8 Phospholipid labelling and analysis in permeabilised T lymphoblasts. 94
2.9 Determination of p21ras activity in T lymphocytes. 95
2.10 Cellular activity of p21ras GTPase activating protein. 96
2.11 Proliferation assay (3H-thymidine incorporation). 96

Section 3.
The role of G proteins in coupling the TCR/CD3 complex to phospholipase C.

Introduction. 97

Results.
3.1 Optimisation of conditions for permeabilisation of T lymphoblasts. 102
3.2 Inositol phospholipid metabolism in SLO-permeabilised T lymphoblasts. 104
3.3 Phosphorylation of an exogenous peptide substrate for PKC in SLO-permeabilised T lymphoblasts. 107
3.4 Time course of production of individual inositol phosphates in response to triggering of the TCR/CD3 complex with the anti-CD3 mAb UCHT1 and the G protein agonist GTP[S]. 110
3.5 Dose-response of UCHT1, hybrid anti-CD2 F(Ab’γ)3 and polyclonal mitogen PHA for inositol phosphate production. 112
3.6 Dose-response and guanine nucleotide specificity for inositol phosphate production. 114
3.7 Effect of varying [Ca2+] on UCHT1 and GTP[S]-induced inositol phosphate production. 116
3.8 Effect of varying [Mg^{2+}] on UCHT1 and GTP[S]-induced inositol phosphate production.

3.9 Effect of varying [Mg^{2+}] on the dose-response of UCHT1 and GTP[S] for inositol phosphate production.

3.10 Effect of varying [Mg^{2+}] on the time course of UCHT1 and GTP[S]-induced inositol phosphate production.

3.11 Effect of GDP and analogues of GDP on GTP[S]-induced inositol phosphate production.

3.12 Effect of GDP and analogues of GDP on UCHT1-induced inositol phosphate production.

3.13 Effect of GDP on the dose-response of GTP[S] and UCHT1 for induction of inositol phosphate production.

3.14 Guanine nucleotide dependency of UCHT1-induced inositol phosphate production.

3.15 Effect of UCHT1 on the dose-response of GTP[S]-induced inositol phosphate production.

3.16 Effect of UCHT1 on the timecourse of GTP[S]-induced inositol phosphate production.


3.18 Effect of analogues of cyclic AMP on TCR/CD3- and G protein-induced phosphatidylinositol hydrolysis.

3.19-3.20 Effect of TCR/CD3 down-regulation on GTP[S]-induced inositol phosphate accumulation in SLO-permeabilised T lymphoblasts.

**Figures.**

3.1 Permeabilisation of T lymphoblasts with the bacterial cytotoxin Streptolysin O.

3.2 Lipid phosphorylation and metabolism in T lymphoblasts permeabilised with Streptolysin O.

3.3 Time course of phosphorylation of a PKC substrate peptide induced by PKC activation, the mitogen PHA and triggering of TCR/CD3 or CD2 in SLO-permeabilised T lymphoblasts.

3.4 Time course of inositol phosphate production in response to UCHT1 and GTP[S] in permeabilised T lymphoblasts.

3.5 Dose-response of UCHT1, PHA and hybrid anti-CD2 F(Ab'γ)3 for inositol phosphate production.
3.6 Nucleotide specificity of inositol phosphate production in SLO-permeabilised T lymphoblasts.
3.7 Effect of variations in the concentration of free Ca$^{2+}$ on UCHT1- and GTP[S]-induced inositol phosphate production in SLO-permeabilised T lymphoblasts.
3.8 Effect of variations in the concentration of free Mg$^{2+}$ on UCHT1- and GTP[S]-induced inositol phosphate production in permeabilised T lymphoblasts.
3.9 Effect of varying [Mg$^{2+}$] on the dose-response of UCHT1 and GTP[S] for inositol phosphate production.
3.10 Effect of varying [Mg$^{2+}$] on the time course of UCHT1 and GTP[S]-induced inositol phosphate production.
3.11 Dose-response and nucleotide specificity for inhibition of GTP[S]-induced inositol phosphate production by exogenous nucleotides in permeabilised T lymphoblasts.
3.12 Dose-response and nucleotide specificity for inhibition of UCHT1-induced inositol phosphate production by exogenous nucleotides in permeabilised T lymphoblasts.
3.13 Effect of GDP on the dose-response of GTP[S] and UCHT1 for induction of inositol phosphate production in permeabilised T lymphoblasts.
3.14 Effect of GTP[S] on UCHT1-induced inositol phosphate production in permeabilised T lymphoblasts.
3.15 Effect of UCHT1 on the dose-response for GTP[S]-induced inositol phosphate production in permeabilised T lymphoblasts.
3.16 Effect of UCHT1 on the time course of GTP[S]-induced inositol phosphate production in permeabilised T lymphoblasts.
3.18 Effect of pre-treatment with forskolin or dibutryl-cyclic AMP on the dose response for UCHT1 and GTP[S]-induced inositol phosphate production in permeabilised T lymphoblasts.
3.19 Effect of downregulation of the TCR/CD3 complex on the dose-response for UCHT1 and GTP[S]-induced inositol phosphate production in permeabilised T lymphoblasts.
3.20 Effect of downregulation of the TCR/CD3 complex on the time course of UCHT1 and GTP[S]-induced inositol phosphate production in permeabilised T lymphoblasts.
Section 4.

Phosphorylation of the γ-subunit of the CD3 antigen: Regulation of protein kinase C activity in SLO-permeabilised T lymphoblasts.

Introduction.

Results.

4.1-4.2 Effect of the phorbol ester PDBu, G protein agonist GTP[S] and polyclonal mitogen PHA on phosphorylation of the γ-subunit of the CD3 antigen in permeabilised T lymphoblasts.

4.3 Dose response and specificity of guanine nucleotide-induced CD3 γ-subunit phosphorylation.

4.4 Inhibition of CD3 γ-subunit phosphorylation by a pseudo-substrate inhibitor of PKC.

4.5-4.6 Effect of variations in the concentration of free Ca²⁺ and Mg²⁺ on PDBu, PHA and G protein-mediated CD3γ-subunit phosphorylation.

4.7 Comparison of the dose-response for CD3γ-subunit phosphorylation and inositol phosphate production by GTP[S] and PHA.

4.8 Comparison of the Ca²⁺-dependence of CD3 γ-subunit phosphorylation and inositol phosphate production in permeabilised T lymphoblasts and Jurkat.

Figures.

4.1 Time course of CD3γ-subunit phosphorylation in permeabilised T lymphoblasts.
4.2 Dose-response of PHA for induction of CD3γ-subunit phosphorylation in permeabilised T lymphoblasts. 158
4.3 Nucleotide specificity of CD3γ-subunit phosphorylation in permeabilised T lymphoblasts. 160
4.5 Effect of variations in the concentration of free Ca²⁺ on CD3 γ-subunit phosphorylation in permeabilised T lymphoblasts. 164
4.6 Effect of variations in the concentration of free Mg²⁺ on CD3 γ-subunit phosphorylation in permeabilised T lymphoblasts. 165
4.7 Comparison of the dose-response of GTP[S] and PHA for induction of CD3 γ-subunit phosphorylation and inositol phosphate production in permeabilised T lymphoblasts. 167
4.8 Comparison of the effects of variations in [Ca²⁺] on CD3 γ-subunit phosphorylation and inositol phosphate production in permeabilised T lymphoblasts and Jurkat. 170

Discussion. 172

Diagrams.
4.1 Proposed models for the regulation of protein kinase C activity in permeabilised T lymphoblasts. 175

Section 5.
The regulation of p21ras during T cell activation: Analysis in SLO-permeabilised T lymphoblasts.

Introduction. 178

Results.
5.1 Effect of triggering the TCR/CD3 complex with the anti-CD3 mAb UCHT1 or CD2 triggering with hybrid anti-CD2 F(Ab′γ)3 on guanine nucleotide bound to p21ras in T lymphoblasts. 181
5.2-5.3 Effect of phorbol dibutyrate and ionomycin on guanine nucleotide bound to p21ras in T lymphoblasts. 183
5.4 Time course of activation of p21ras on stimulation of PKC and triggering the TCR/CD3 complex or CD2 antigen. 186
5.5 Effect of PKC activation or TCR/CD3 triggering on p21ras
in the T leukaemic cell line HPBALL.

5.6-5.7 Stimulation of p21\textsuperscript{ras} in SLO-permeabilised T lymphoblasts.

5.8-5.10 Effect of PKC activation and triggering of the TCR/CD3 complex or CD2 antigen on the guanine nucleotides bound to p21\textsuperscript{ras} in permeabilised T lymphoblasts.

5.11 Specificity of nucleotide exchange onto p21\textsuperscript{ras} in permeabilised T lymphoblasts.

5.12 Inhibition of (p21\textsuperscript{ras} )GAP activity by agents that stimulate p21\textsuperscript{ras} in T lymphoblasts.

5.13-5.15 Effect of a PKC pseudosubstrate peptide inhibitor on PDBu, CD2 and TCR/CD3-induced stimulation of p21\textsuperscript{ras} in permeabilised T lymphoblasts.

5.16 Stimulation of p21\textsuperscript{ras} by the T cell growth factor IL2.

5.17 Kinetics and persistence of p21\textsuperscript{ras} stimulation by IL2 in T lymphoblasts.

5.18-5.19 Dose-response of IL2 for stimulation of p21\textsuperscript{ras} and DNA synthesis in T lymphoblasts.

5.20 Effect of IL2 on guanine nucleotide bound to p21\textsuperscript{ras} in permeabilised T lymphoblasts.

5.21 Effect of IL2 on (p21\textsuperscript{ras} )GAP activity in lysates from T lymphoblasts.

5.22 Relationship between IL2 stimulation and other agents that activate p21\textsuperscript{ras} in T lymphoblasts.

5.23-5.24 Exchange rate of guanine nucleotide onto p21\textsuperscript{ras} in permeabilised Rat-1 and NIH-3T3 fibroblasts.

5.25 Effect of PKC activation on the activation state of p21\textsuperscript{ras} in permeabilised Rat-1 fibroblasts.

**Figures.**

5.1 Effect of TCR/CD3 and CD2 triggering on guanine nucleotide bound to p21\textsuperscript{ras} in T lymphoblasts.

5.2 Effect of the calcium ionophore ionomycin or phorbol ester PDBu on guanine nucleotide bound to p21\textsuperscript{ras} in T lymphoblasts and the T-leukaemic cell line Jurkat.

5.3 Effect of TCR/CD3 or CD2 triggering, the mitogen PHA or activation of PKC on guanine nucleotide bound to p21\textsuperscript{ras} in T lymphoblasts.
5.4 Time course of activation of p21\textsubscript{ras} on stimulation of PKC or triggering the TCR/CD3 complex and CD2 antigen in T lymphoblasts. 187
5.5 Effect of PKC activation or TCR/CD3 triggering on guanine nucleotide bound to p21\textsubscript{ras} in the T cell line HPB-ALL. 188
5.6 Effect of PKC activation and TCR/CD3 or CD2 triggering on guanine nucleotide bound to p21\textsubscript{ras} in SLO-permeabilised T lymphoblasts. 190
5.7 Time course of hydrolysis of GTP in SLO-permeabilised T lymphoblasts. 191
5.8 Time course of p21\textsubscript{ras} activation on stimulation of PKC and triggering the TCR/CD3 complex or CD2 antigen in SLO-permeabilised T lymphoblasts. 194
5.9 Analysis of the effect of PKC activation or triggering of the TCR/CD3 complex and CD2 antigen on the guanine nucleotide bound to p21\textsubscript{ras} in permeabilised T lymphoblasts. 195
5.10 Analysis of the effect of PKC activation on p21\textsubscript{ras} in permeabilised T lymphoblasts at 27°C. 196
5.11 Rate and specificity of nucleotide exchange onto p21\textsubscript{ras} in permeabilised T lymphoblasts. 198
5.12 Comparison of (p21\textsubscript{ras} )GAP activity in lysates from quiescent and activated T lymphoblasts. 200
5.13 Effect of a pseudosubstrate peptide inhibitor of PKC on PDBu and TCR/CD3-induced stimulation of p21\textsubscript{ras} in permeabilised T lymphoblasts. 203
5.14 Effect of a pseudo-substrate peptide inhibitor of PKC on stimulation of p21\textsubscript{ras} in permeabilised T lymphoblasts. 204
5.15 Effect of a pseudo-substrate peptide inhibitor of PKC on stimulation of PKC substrate peptide phosphorylation in permeabilised T lymphoblasts. 205
5.16 Effect of IL2 treatment on the guanine nucleotide bound to p21\textsubscript{ras} in T lymphocytes. 207
5.17 Time course and persistence of IL2-induced activation of p21\textsubscript{ras} in T lymphoblasts. 209
5.18 Dose-response of IL2 for stimulation of p21\textsubscript{ras} in permeabilised T lymphoblasts. 211
5.19 Dose-response of IL2 for stimulation of p21\textsubscript{ras} and DNA synthesis in T lymphoblasts. 212
5.20 Effect of IL2 treatment on p21\textsuperscript{ras} in permeabilised T lymphoblasts. 214

5.21 Comparison of (p21\textsuperscript{ras} )GAP activity in lysates from quiescent and IL2-stimulated T lymphoblasts. 216

3.22 Effect of IL2 stimulation in combination with PKC activation or receptor stimulation on guanine nucleotide bound to p21\textsuperscript{ras} in T lymphoblasts. 217

5.23 Rate of guanine nucleotide exchange onto p21\textsuperscript{ras} in permeabilised Rat-1 and Swiss 3T3 fibroblasts. 219

5.24 Comparison of the rate of guanine nucleotide exchange onto p21\textsuperscript{ras} in permeabilised Rat-1, Swiss 3T3 fibroblasts and T lymphoblasts. 220

5.25 Effect of PKC activation on guanine nucleotide bound to p21\textsuperscript{ras} in SLO-permeabilised Rat-1 fibroblasts. 222

Discussion. 223

Diagrams.
5.1 Regulation of p21\textsuperscript{ras} in T lymphocytes. 231
5.2 Possible involvement of p21\textsuperscript{ras} in signalling pathways from the TCR/CD3 complex and IL2R. 233

Section 6.
An analysis of the role of guanine nucleotide-binding proteins in T cell activation.

Conclusions and perspectives. 239

Diagrams.
6.1 A speculative model for the involvement of guanine nucleotide-binding proteins in T cell activation. 246

References. 249
Publications.

Some of the work presented in this thesis has been published in the following:


Abbreviations.

Section 1: Introduction.

1.1 Antigen recognition by T lymphocytes.

T cells recognise proteolytically-derived fragments of protein antigens at the surface of an antigen presenting cell in association with molecules of the major histocompatibility complex (MHC) (Sprent and Webb, 1987; Hedrick, 1988). MHC molecules can be divided into two groups by virtue of their structure and tissue distribution; MHC class I, which are expressed on most nucleated cells and MHC class II, whose expression is restricted to B lymphocytes, macrophages, dendritic cells, thymic epithelium and activated T cells. The requirement for presentation of processed antigen at the cell surface in conjunction with MHC molecules, termed MHC restriction, constitutes one of the fundamental concepts of T cell biology and is essential to an understanding of T cell function. The phenomenon of MHC restriction is derived from the original observations of Zingernagel and Doherty who demonstrated that virus-specific murine cytotoxic T lymphocytes would only kill virally infected cells of the same MHC haplotype (Zingernagel and Doherty, 1974).

1.2 Surface receptors that control T cell activation.

The physiological ligand for T cell activation is antigen presented on the surface of antigen presenting cells. An important consequence of this interaction is that recognition of antigen by T lymphocytes brings the T cell and antigen presenting cell into close proximity. The key cognitive event involves the T cell receptor for antigen. However, analysis of receptor signaling function has revealed that triggering of the T cell antigen receptor alone is insufficient to induce full T cell activation and that additional amplifying signals, derived from antigen non-specific receptors, are required to stimulate the proliferative response (see diagram 1.1).
The conclusion that multiple receptors play a co-stimulatory role in T cell activation is based largely on the agonistic or antagonistic properties of mAb directed against such receptors. T cell surface molecules that, by this criterion, have been identified to be involved in T cell activation include the CD2, CD4, CD5, CD8, CD28 and CD45 antigens. Many accessory receptors have been identified to interact with a physiological ligand to mediate the stimulatory association between T cells and antigen presenting cells. For example, CD4 and CD8 interact with MHC class I and II respectively, the CD2 antigen interacts with it's ligand LFA-3 and the CD28 molecule binds to the B7/BB1 B cell surface antigen (Linsley et al., 1990). In addition, the intercellular adhesion molecules I-CAM and LFA-1 promote adhesive interaction between T cell and antigen presenting target (reviewed by Springer et al., 1987). Many of these accessory molecular interactions are thought to contribute to the T cell response by both passively enhancing cellular adhesion, thereby increasing
the avidity of interaction between the T cell receptor and antigen/MHC, and by generating intercellular signals that actively contribute to the biological response of T cells to antigen. Cytokines such as IL1 and IL6 also provide amplifying signals for T cell activation. Produced by a wide variety of different cell types, including macrophages and B cells, these pleiotropic soluble mediators do not appear to be obligatory for T cell activation but can synergise with other stimuli for the activation of T cells (Weaver and Unanue, 1990; Holsti and Raulet 1989).

A major question in T cell biology concerns the nature of the amplifying signals generated by accessory receptors. Despite intensive investigation, it remains unclear whether these accessory molecules utilize intracellular signaling pathways in common with the T cell receptor for antigen or whether they generate distinct biochemical signals.

1.3 The antigen-specific MHC-restricted T cell receptor complex (TCR/CD3).

The T cell antigen receptor (TCR) is a multisubunit complex whose polypeptide components can be divided into three groups on the basis of structure and presumed function. The antigen/MHC recognition components of the receptor (Ti) consist of αβ or γδ disulphide-linked heterodimers. The second group of receptor components are the invariant δ, ε and γ chains, collectively referred to as the CD3 complex. The third group of TCR components are the invariant ζ and η chains. Although the TCR/CD3 complex comprises at least seven subunits, multiple receptor isoforms exist and the exact stoichiometry of the complex is not well understood (reviewed by Clevers et al., 1988) (see diagram 1.2).

1.3.1 The Ti components of the TCR/CD3 complex.

The majority of T lymphocytes express an antigen-specific MHC-restricted T cell receptor, comprising disulphide-linked α and β subunits which
are the products of rearranged genes and are expressed in a clonally restricted manner. However, a small proportion of T cells (~5-10%) express a distinct class of Ti, consisting of subunits termed γ and δ. Significant homology to immunoglobulins, as well as similarities in terms of germ line organisation and mechanism of rearrangement, have been revealed by the molecular cloning of the genes that encode Ti α, β, γ and δ (reviewed by Kronenberg et al., 1986 and Raulet et al., 1989).

Diagram 1.2 Structure of the TCR/CD3 complex.

1.3.2 The CD3 components of the TCR/CD3 complex.

Ti αβ and γδ heterodimers are expressed at the cell surface in conjunction with non-covalently associated invariant polypeptides that are collectively referred to as the CD3 complex. The human CD3 antigen comprises three subunits; the 25-28kDa γ polypeptide, 20kDa δ glycoprotein and 20kDa ε polypeptide. Evidence for the association of the Ti heterodimer
with the CD3 complex is partly derived from co-modulation, co-immunoprecipitation and chemical crosslinking studies. In addition, analysis of variants of the Jurkat cell line which had been selected for surface expression of Ti or the CD3 complex revealed that these molecules were interdependent with respect to surface expression (Weiss and Stobo, 1984).

Molecular cloning of the components of the human CD3 complex and their murine homologues has revealed that the three CD3 components have a small extracellular domain and, unlike the TCR components, a large intracellular domain. Although these structural features are suggestive of a role in signal transduction, little information concerning their function can be derived from sequence analysis since CD3γ, δ and ε exhibit no homology with known signal transduction molecules. The γ and δ subunits of the CD3 complex are markedly homologous, especially in their transmembrane and intracellular domains (Krissansen et al., 1986). The intracellular domain of the CD3 ε chain is quite distinct from that of γ and δ but the molecules share some limited homology in their extracellular domain proximal region (Gold et al., 1987). In particular, the conservation of certain cystein residues between CD3γ, δ and ε has led to their inclusion in the immunoglobulin supergene family (reviewed in Williams and Barclay, 1988). The transmembrane domains of the CD3 subunits contain a centrally located acidic residue (Glutamic acid in γ and Aspartic acid in δ and ε). These are considered to form stabilizing non-covalent associations with the basic residues found in the transmembrane regions of the Ti components. Mutation of these amino acids to non-charged residues did not affect αβ heterodimer formation but prevented interaction with the CD3 complex and subsequent transport to the cell surface (Morley et al., 1988; John et al., 1989; Alcover et al., 1990).
1.3.3 The TCR $\zeta$ and $\eta$ chains.

The third and most recently described and cloned group of TCR components are the invariant 16kDa $\zeta$ and 22kDa $\eta$ polypeptides which are derived from the same gene locus by alternative splicing events (Weissman et al., 1988a; Orloff et al., 1989; Jin et al., 1990). Although the invariant components of the TCR share a presumed role as signal transduction elements, $\zeta$ and $\eta$ share no homology with immunoglobulin or the CD3 components and have some interesting characteristics that require their consideration as a distinct group (Weissman et al., 1988b; Jin et al., 1990). TCR$\zeta$ and $\eta$ share a 9 amino acid extracellular segment and a 21 residue transmembrane segment that includes a negatively charged aspartic acid residue that is characteristic of the CD3 subunits (Clevers et al., 1988). The 113 amino acid $\zeta$ cytoplasmic domain contains six potential tyrosine phosphorylation sites and a region which bears a weak homology to a consensus ATP binding site. The first 92 amino acids of the $\eta$ chain cytoplasmic domain are identical to the corresponding region of the $\zeta$ chain but the C terminal regions of the two proteins diverge significantly. The cytoplasmic domain of $\eta$, 42 amino acids larger than that of $\zeta$, contains 5 tyrosine residues and lacks the putative nucleotide binding site that has been tentatively identified in the cytoplasmic domain of $\zeta$ (Jin et al., 1990; Weissman et al., 1988b).

In the murine system, $\zeta$ exists primarily as a disulphide linked homodimer (80-90%) or as a $\zeta$-$\eta$ heterodimer (10-20%) within the TCR/CD3 complex (Baniyash et al., 1988a). Expression studies have demonstrated that $\zeta$ and $\eta$ can form $\zeta$-$\zeta$, $\zeta$-$\eta$ and $\eta$-$\eta$ disulphide linked dimers which associate with the Ti/CD3 pentamer to generate 3 possible receptor isoforms (Clayton et al., 1990). In murine T cell hybridomas that lack expression of the $\zeta$ chain, most incomplete TCR/CD3 complexes are retained and degraded in a lysosomal compartment (Lippincott-Schwartz et al., 1988). Transfection of these mutants
with a ζ cDNA restores TCR/CD3 expression (Weissman et al., 1989). Thus, ζ has been suggested to be the limiting subunit for receptor assembly and could regulate overall receptor levels. Recent studies have suggested that transfection of these ζ-negative hybridomas with an η cDNA, resulting in the formation of η-η homodimers, can also rescue TCR/CD3 complexes from lysosomal degradation and restore surface expression (Clayton et al., 1990). However, transfectants expressing only η-η always demonstrate lower surface expression of the TCR/CD3 than those expressing ζ-ζ. The preference for subunit assembly into stable TCR/CD3 complexes appears to be ζ-ζ > ζ-η > η-η. It is unclear whether this arises as a consequence of different affinities of these dimers for the Ti/CD3 pentamer or differential abilities to salvage incomplete TCR/CD3 complexes from degradation.

The ζ chain is also expressed in natural killer cells as a homodimer in association with CD16, the ligand binding subunit of the FcγRIII receptor (Lanier et al., 1989; Anderson et al., 1989). Furthermore, the γ subunit of the FcεRI receptor, which is expressed on mast cells and basophils, contains regions which are highly homologous to ζ. The γ chain of the FcεRI receptor has also been reported to be expressed in T cells as a heterodimer with either ζ or η (Orloff et al., 1990). Thus, it is possible that a family of ζ-related molecules exist and function within different receptor complexes in a variety of different cells. While it is clear that ζ and η play an important role in signal transduction from the TCR/CD3 complex and may also regulate surface expression of the complex, the architectural complexity of the TCR/CD3 complex has hampered attempts to elucidate the interrelationship and functions of these molecules. It has also proved difficult to clearly differentiate between the effects of these components on expression and signaling function.
1.4 The CD4 and CD8 antigens.

The T cell-specific CD4 and CD8 glycoproteins have been cloned and well characterized. CD4 is a 55-67kDa single-chain polypeptide whereas CD8 can be expressed as a heterodimer of 34-38kDa α plus 28-30kDa β subunits or as an α-α homodimer (reviewed by Parnes, 1989). Originally considered to be phenotypic markers of T lymphocyte functional subsets, CD4 and CD8 are now known to determine T cell MHC specificity and have been shown to bind monomorphic determinants on MHC class II and class I molecules respectively (Rosenstein et al., 1989; Doyle and Strominger, 1987).

The observation that certain CD4 or CD8 mAbs blocked antigen specific T lymphocyte responses provided the first indication that CD4 and CD8 were involved in T cell activation (reviewed in Bierer et al., 1989). Whether the role of CD4 and CD8 in T cell activation is purely adhesive or whether they actively participate in signal transduction has been the subject of much research. In support of an adhesive function, transfection of CD8 together with the appropriate Tiα and β chains into a donor T cell line was sufficient for expression of antigen-specific cytolytic function (Gabert et al., 1988). Furthermore, expression of CD4 or CD8 cDNA in antigen-specific T cell hybridomas augments the class II MHC- or class I MHC-restricted responses of these cells to antigen (Gay et al., 1987; Sleckman et al., 1987, Ballhausen et al., 1988; Dembic et al., 1987). However, the powerfully synergistic mitogenic signal delivered by crosslinking of either CD4 or CD8 to the TCR/CD3 complex suggests that aggregation of CD4 and CD8 with TCR/CD3 might be an important step during T cell activation in vivo (Emmrich 1988). In support of this hypothesis, it has been demonstrated that CD4 and CD8 physically interact with TCR/CD3 during activation (Saizawa et al., 1987, Kupfer and Singer, 1988, Mittler et al., 1989) and transfection studies have suggested that CD8 and the TCR/CD3 complex must interact with the same MHC class I molecule.
for an optimal activation signal to be delivered (Connoly et al., 1990, Salter et al., 1990).

Thus, it appears that CD4 and CD8 contribute two functions in T cell activation. Firstly, increasing the effective affinity of TCR for antigen by adhesive interaction with MHC and secondly, by the delivery of accessory activation signals.

1.5 The CD2 antigen.

CD2, originally identified as a T cell-specific sheep red blood cell receptor on human T cells, was later defined as a 50kDa glycoprotein by a specific antibody (Kamoun et al., 1981; Howard et al., 1984). Molecular cloning of the human (Sewell et al., 1986; Sayre et al., 1987), rat (Williams et al., 1987) and murine (Sewell et al., 1987; Clayton et al., 1987) CD2 antigen has revealed that the protein consists of a 185-186 extracellular domain, a 25 amino acid transmembrane segment and a cytoplasmic domain of 116-126 residues. The protein contains limited regions that show homology with some members of the immunoglobulin supergene family (Sewell et al., 1986; Sayre et al., 1987; Williams et al., 1987). The physiological ligand for CD2 is lymphocyte function-associated antigen-3 (LFA-3) (Dustin et al., 1987; Plunkett et al., 1987; Hunig et al., 1987; Bierer et al., 1988b), a widely expressed 55-70kDa cell surface glycoprotein that is also a member of the immunoglobulin supergene family (reviewed in Springer et al., 1987; Springer, 1990).

Accumulated evidence suggests that CD2/LFA-3 binding plays an important role in T cell activation. Thus, mAbs to CD2 or its ligand LFA-3 can inhibit antigen-independent association of T cells with their targets, antigen-specific cytotoxic T cell killing (Krensky et al., 1983; Shaw et al., 1986) or mitogen-induced T cell proliferation (Palacios and Martinez-Maza, 1982). These studies suggested that the interaction between CD2 and LFA-3 functions to increase the avidity of the interaction between T cell and potential
targets. Transfection studies have supported this conclusion by demonstrating that expression of complete human CD2, but not of a mutant CD2 molecule unable to bind LFA-3, greatly enhanced the responsiveness of an antigen-specific murine T cell hybridoma towards target cells expressing LFA-3 (Bierer et al., 1988c). Another study has demonstrated that expression of LFA-3, in transfected L cells already expressing MHC class II molecules, dramatically reduces the concentration of nominal antigen required for the L cells to present antigen to antigen-specific T cell hybridomas (Koyasu et al., 1990).

However, in addition to mediating intracellular adhesion, CD2 actively contributes to the activation of T cells. Combinations of antibodies directed against different epitopes of the CD2 molecule have been demonstrated to induce T cell proliferation (Meuer et al., 1984b; Huet et al., 1986; Yang et al., 1986) and antigen-specific or -nonspecific cytolytic activity by cytotoxic T cells or NK cells respectively (Siliciano et al., 1985; Schmidt et al., 1985). At least six distinct CD2 epitopes, some of which are induced upon activation of resting T cells, have been defined by the use of mAbs. Only some anti-CD2 antibodies can activate T cells and a combination of two antibodies with distinct epitope specificities is required. More recently, it has been shown that purified LFA-3 can synergise with certain anti-CD2 mAbs to induce T cell activation (Hunig et al., 1987; Denning et al., 1988; Bierer et al., 1988b). That the synergistic effect of anti-CD2 and anti-CD3 ligands for T cell activation (Bierer et al., 1988a) requires an active signaling role for CD2 is suggested by studies in which deletion of the cytoplasmic tail of the CD2 molecule, without altering LFA-3 binding, abolished this cooperative effect (Bierer et al., 1988a). Perhaps most significantly, crosslinking of the CD2 molecule overcomes the requirement for accessory cell interaction for highly purified resting T lymphocytes to proliferate in response to immobilised CD3 mAbs (Anderson et al., 1988).

The apparent independence of the CD2 pathway from the presence of nominal antigen led to its description as an "alternative pathway" of T cell
activation (Meuer et al., 1984b). However, compelling evidence exists to indicate that the expression of a functional TCR/CD3 complex is required for CD2-mediated signal transduction and activation (Pantaleo et al., 1987; Breitmeyer et al., 1987; Alcover et al., 1988; Blockenstedt et al., 1988). Thus, the CD2 molecule cannot function in TCRβ chain-negative (and consequently CD3-negative) mutant of the Jurkat T leukaemic line and transfection of this mutant with a TCRβ chain cDNA restored TCR/CD3 expression and the signaling capacity of the CD2 molecule (Alcover et al., 1988; Blockenstedt et al., 1988). The ability of CD2 agonists to activate CD3-negative thymocytes (Fox et al., 1985), natural killer cells (Siliciano et al., 1985) and some CD3-negative Jurkat mutants (Moretta et al., 1987) raises the possibility that, under some cellular circumstances, CD2 can function in the absence of TCR/CD3. Interestingly, CD2-mediated NK cell activation requires crosslinking of CD2 with the low affinity receptor for immunoglobulin G, FcγRIII (CD16) (Anasetti et al., 1987) which has been shown to interact with the ζ chain originally identified as a component of the TCR/CD3 complex in T cells (Lanier et al., 1989; Anderson et al., 1989). Thus, in this cell system, the CD2 pathway also appears to be dependent on the presence of another receptor. Whether CD2 function requires expression of ζ in this TCR/CD3-negative environment remains to be determined.

Although stabilisation of binding between T cells and other cell types by interaction of CD2 and LFA-3 is an important element of CD2 function, the CD2 antigen is also clearly involved in providing co-stimulatory signals which synergise with signals generated by the TCR/CD3 complex.

1.6 The CD45 antigen.

CD45, previously referred to as T200 or leukocyte common antigen, is a family of cell surface glycoproteins expressed on all haematopoietic cells except erythrocytes and platelets (Thomas and Lefrancois, 1988). Members of
this family, which range in molecular weight from 180 to 220kDa, vary in their degree of glycosylation and can be differentiated by their antigenicity. This heterogeneity results from differential splicing of a single gene which generates differences in the extracellular domain (Barclay et al., 1987; Ralph et al., 1987; reviewed in Thomas 1989). The molecule is composed of a large invariant intracellular domain of 705-707 amino acids, a membrane spanning region and an extracellular domain of 400-500 amino acids depending on the extent of RNA splicing. The fact that CD45 isoforms are expressed in an activation-state and lineage-dependent manner suggests that the heterogeneity may have functional relevance. In particular, it is possible that the isoforms interact differentially with putative natural ligand for CD45.

Evidence for a signalling role for CD45 is provided by the fact that antibody mediated crosslinking of CD45 with the TCR/CD3 complex and CD2 antigen inhibits transmembrane signaling events and the proliferative response initiated by these antigens (Ledbetter et al., 1988; 1991; Keiner and Mittler, 1989). In contrast, soluble CD45 mAbs have been reported to selectively enhance CD2-induced proliferation and can replace the accessory cell requirement for proliferation in response to both CD3 mAb and PHA (Martorell et al., 1987, Marvel et al., 1989). Further evidence is provided by studies employing murine T cell clones that have been selected for lack of expression of CD45. These cells do not proliferate in response to antigen or CD3 mAbs. Spontaneous revertants, re-expressing CD45, regain their ability to respond to antigen (Pingel and Thomas, 1989). The accumulated evidence suggests that the CD45 molecule can modulate T cell signaling in response to mitogenic stimulation and is required for the T cell response to antigen. The observation that the cytoplasmic domain of CD45, common to all isoforms, encodes a protein-tyrosine phosphatase activity provides a potential mechanism for these effects (Charbonneau et al., 1988).
1.7 The CD28 antigen.

CD28 (previously T44, Tp44 or the 9.3 antigen), a homodimeric glycoprotein of 44kDa subunits, is a member of the immunoglobulin superfamily (Hara et al., 1985, Aruffo and Seed, 1987). It is expressed on ~95% of CD4+ cells and ~50% of CD8+ T lymphocytes (Hansen et al., 1980). Although mAbs against CD28 are not mitogenic for T cells, they appear to be able to replace the requirement for accessory cells in CD3 mAb-induced T cell proliferation, suggesting that CD28 might deliver an accessory signal (Ledbetter et al., 1985, Martin et al., 1986, Weiss et al., 1986; Pierres et al., 1988). In this regard, the natural ligand for CD28 has been identified as the B7/BB-1 molecule, expressed on activated B cells (Linsley et al., 1990). Unlike CD2-mediated activation, CD28 does not appear to require cell surface expression of the TCR/CD3 complex to mediate its effects (Hara et al., 1985, Weiss et al., 1986, Pierres et al., 1990). Thus, CD28 appears to represent an accessory activation pathway that is biochemically-distinct from that employed by the TCR/CD3 complex or CD2 antigen.

1.8 What are the consequences of T cell activation?

T cell activation in response to antigen in the presence of accessory cells involves the integration of intracellular signals, generated by multiple surface receptors, to initiate a program of genetic events that lead to cell cycle progression, proliferation and functional differentiation. Recent studies have identified more than 60 distinct cDNA clones that constitute part of the transcriptional response of resting human peripheral blood-derived T cells to mitogenic stimulation (Zipfel et al., 1989; Irving et al., 1989). Eighty percent of these genes were also induced in human lung fibroblasts, suggesting that the majority might be ubiquitous whereas the others might be T cell-specific. Although the pathway along which a particular cell differentiates may be influenced by both the nature of the stimulus and the ontogenic state of the
cell, the genes that are induced during this process can be considered to belong to several functional categories (reviewed by Ullman et al., 1990).

Amongst the earliest genes to be induced following T cell activation are those whose protein products function to modulate the activity of other genes. These include the ubiquitous nuclear protooncogenes c-fos and c-myc which are induced during the movement of resting T cells from G0→G1 phase of the cell cycle. The c-fos gene product participates in DNA binding protein complexes containing the product of the proto-oncogene c-jun/AP-1 which have been implicated in both the positive and negative trans-activation of other genes (Curran and Franza, 1988; Angel et al., 1988; reviewed by Karin, 1991). c-fos mRNA can be detected within 15 minutes of T cell stimulation, peaks at approximately 30 minutes and is undetectable after 1-2 hours (Reed et al., 1986; Moore et al., 1986). The product of the c-myc protooncogene is also thought to function as a nuclear transcriptional trans-activating factor with an important and ubiquitous role in cell cycle regulation. In T cells, c-myc mRNA is detectable after 30 minutes of stimulation, peaks after 3 hours and declines to undetectable levels on entry into S phase. The importance of c-myc expression for T cell responses is demonstrated by a study in which DNA synthesis, but not progression from G0→G1, was observed to be dependent on c-myc expression (Heikkila et al., 1987). In addition, the nuclear DNA binding factor NF-AT, a transcriptional trans-activating factor that appears to be unique to T cells, appears after 20 minutes of activation (Shaw et al., 1988; Durand et al., 1988; reviewed by Ullman et al., 1990).

Another group of genes, induced between 30 minutes and 6 hours after activation, encode soluble factors which mediate the effector functions of some T cell subsets. These include interleukin 4 (IL4), IL5 and IL6, which stimulate activated B cells to proliferate, differentiate and produce immunoglobulin. In addition, some T cells produce granulocyte macrophage colony stimulating factor (GM-CSF) which stimulates the proliferation of bone marrow precursor
cells or gamma interferon (γ-IFN) which induces granulocytes and macrophages to express Fc receptors and phagocytose (reviewed by Mosmann and Coffman, 1989). Genes encoding proteins that mediate T cell cytotoxic effector function, such as lymphotoxins and enzymes found in the secretory granules of cytotoxic T cells are also induced.

1.9 The IL2 system and T cell proliferation.

Another category of genes induced upon T cell activation from $G_0 \rightarrow G_1$ phase of the cell cycle are those which regulate further progression to DNA synthesis and proliferation. The key event for T cell cycle progression is induction of secretion of the growth factor interleukin-2 (IL2) and acquisition of IL2 receptors (reviewed by Smith, 1988). The transcriptional activation of the IL2 and IL2 receptor genes takes place about 45 minutes and 2 hours after T cell activation respectively. Upon a critical threshold of interaction between IL2 and it's receptor, T cells progress to DNA synthesis (Cantrell and Smith, 1984). Critical features of the autocrine IL2 system are that quiescent T cells neither produce IL2 nor express IL2 receptors and that both IL2 production and receptor expression in response to cellular stimulation is transient (Cantrell and Smith, 1983). This is particularly important in the light of the observation that IL2 can induce expression of it's own receptor (Smith and Cantrell, 1985 and reviewed by Smith, 1988).

Two IL2 binding proteins have been cloned, IL2Rα (also called TAC or p55) and IL2Rβ (also known as p70 or p75) (Leonard et al., 1984; Nikaido et al., 1984; Cosman et al., 1984; Hatakeyama et al., 1989). When expressed together on the cell surface, the IL2Rα and IL2Rβ chains associate non-covalently to form the high affinity IL2R heterodimer ($K_d=10^{-11}M$) (see diagram 1.3). In addition, IL2 binds with low ($K_d=10^{-8}M$) and intermediate ($K_d=10^{-9}M$) affinity to IL2Rα and IL2Rβ chains expressed singly (Hatakeyama et al., 1989). Antigenic activation of resting T cells results in the expression of high affinity
IL2Rs which then signal the proliferation of antigen-selected cells (reviewed by Smith, 1989). Previous studies have suggested that the IL2Rβ chain is constitutively expressed and functionally active on resting populations of T cells (Leonard et al., 1984; Hatakeyama et al., 1989). However, employing radiolabelled IL2 binding assays, northern blot analyses, flow cytometric analyses and IL2 dose-response proliferation assays, a recent report has demonstrated that the vast majority (>98%) of resting T cells lack constitutive expression of either IL2Rα or β subunits before antigen activation (Caliguri et al., 1990). Thus, resting T cells neither produce IL2 nor are they capable of responding to it.

Diagram 1.3 Structure of the high affinity IL2 receptor.

While the TCR complex can be considered to determine the specificity, the magnitude of the clonal response is determined by the concentration of
IL2, the density of high affinity IL2R expression and the duration of IL2 interaction with IL2R (Cantrell and Smith, 1984). In physiological terms, the magnitude of the T cell response will be regulated by the concentration of specific antigen since expression of IL2 and IL2R genes are controlled by the TCR/CD3 complex. The phenotypic differentiation process, initiated by appropriate contact with antigen, is concluded by acquisition of specialized effector functions.

1.9.1 Regulation of IL2 and IL2 receptor genes.

Since commitment of T cells to the proliferative pathway requires induction of the IL2 and IL2R genes, understanding the events that regulate their expression is crucial to elucidating the molecular mechanisms of commitment in T cells. Although many of the cell surface receptors involved in T cell activation have been identified, together with some genes which undergo modulation of expression in response to signaling through these receptors, the precise mechanism by which receptors control the transcriptional activity of target genes remains unclear.

Experimental analyses of this crucial question have focussed on the characterization of intracellular signals generated by receptors, the cis-acting promoter-enhancer regions regions found upstream of target genes and the trans-acting regulatory protein complexes that interact with specific sequences within these regions and regulate the transcriptional activity of the gene (reviewed by Karin, 1991). The approaches used to identify these regulatory sequences include DNA mobility shift assays, mapping of DNaseI-sensitive regions and the ability of upstream sequences to direct transcription and translation of an indicator gene, such as chloramphenicol acetyltransferase, linked to a ubiquitously-expressed promoter (reviewed by Crabtree, 1989).

Characterization of the transcriptional enhancer regions of the IL2 and IL2R genes has revealed that regulation of their transcription is complex,
mediated by multiple positive and negative regulatory elements that respond to a variety of trans-acting regulatory factors.

1.9.2 The enhancer region of the IL2 receptor α and β chain genes.

The IL2Rα promoter is encompassed by nucleotides -476 to -225 relative to the site of transcriptional initiation and includes both positive and negative response elements. An 11 nucleotide sequence (-265 to -255) is very similar to enhancers found in the promoter region of the Ig κ light chain gene, HIV-1 and several other cell surface molecules (Sen and Baltimore, 1986; Nabel and Baltimore, 1987; Leung and Nabel, 1988). These enhancer sequences bind and cross-compete for binding to at least 2 inducible proteins, NF-κB (Cross et al., 1987; Leung and Nabel, 1988) and HIVEN86A (Bohnlein et al., 1988) which activate the HIV-1 long terminal repeat. NF-κB, constitutively expressed in mature Ig-producing B cells, can be induced by a variety of stimuli in pre-B cells, T cells and non-lymphoid cells but acts as a lymphoid-specific enhancer (Pierce et al., 1988). This factor is induced by a posttranslational mechanism that involves its dissociation from a specific cytoplasmic inhibitor, IkB, and subsequent translocation to the nucleus (Baeuerle and Baltimore, 1988).

Diagram 1.4 The enhancer region of the IL2 receptor α chain gene.

Another enhancer sequence, found close to the κB enhancer upstream of the IL2Rα coding region (-253 to -244), is represented by the "CArG" box
(where Ar is a six-nucleotide A,T-rich sequence)(Phan-Dinh-Tuy et al., 1988; Toledano et al., 1990). This sequence, a site for the transcription factor AP-1, is also found in the promoter region of the c-fos gene where it mediates the response of c-fos to serum by binding a factor know as SRF (Norman et al., 1988).

Little is known about the regulation of the gene for IL2Rβ and analysis of the promoter-enhancer region of the IL2Rβ gene is in its early stages. However, mapping of the 5'-flanking region has revealed the existence of at least 5 sites which may be targets for the binding of regulatory factors (Gnarra et al., 1990; Shibuya et al., 1990). Sequence analysis of this region suggests the presence of potential sites for Octamer binding transcription factors and for the AP-1, AP-2 transcription factors. The occurrence, within the IL2Rβ promoter, of novel repetitive "GC-clusters" and the absence of the NF-κB binding motif suggests that the mechanism for gene regulation of the IL2Rβ gene may be different from that of the IL2Rα gene.

1.9.3 The enhancer region of the IL2 gene.

The IL2 transcriptional enhancer is located between -319 and -52 nucleotides upstream of the transcriptional initiation site. Five major regulatory sites have been identified within this region and shown to interact with specific nuclear DNA-binding proteins (Fujita et al., 1986; Durand et al., 1988; Shaw et al., 1988 and reviewed by Crabtree, 1989) (see diagram 1.6). Deletion analysis suggests that these sites collectively determine IL2 gene activity (Hoyos et al., 1989 and reviewed by Ullman et al., 1990). Sequences include a site for the ubiquitous NF-κB (-208 to -188) and AP-1 (-185 to -177) transcription factors. Two regulatory sites bind a nuclear factor termed NF-IL2A, which is identical to the ubiquitous octamer binding protein oct-1 and is found in the nuclei of both stimulated and unstimulated T cells (Kamps et al., 1990). One of these sequences (-93 to -66) has been demonstrated to be
transcriptionally responsive to TCR/CD3 signaling and has been termed antigen receptor response element 1 (ARRE-1). The ARRE-1 site also corresponds to an element that has been reported to negatively regulate IL2 transcription (Nabel et al., 1988). Thus, it appears possible that the constitutively expressed NF-IL2A factor participates, with an inducible component, in a DNA-binding complex that has dual regulatory activity; suppressing expression of the IL2 promoter in resting cells and activating it upon TCR/CD3 stimulation. In addition, a TCR/CD3-responsive site (-286 to -257) termed ARRE-2, binds the nuclear factor NFAT-1 which appears to be unique to T cells and only appears on activation (Shaw et al., 1988; reviewed by Ullman et al., 1990).

Analysis of deletion mutants suggests that all the protein binding sites must be occupied to allow full activity of the IL2 enhancer and that removal of any single site leads to a substantial reduction in activity (Hoyos et al., 1989; Durand et al., 1988). This observation contrasts with the characteristics of the IL2Rα enhancer and typical viral enhancers where redundancy of binding sites enables multiple signals to substitute and induce transcriptional activation.

The relative cytoplasmic level of mRNA encoding a specific gene can generally be considered to be an index of transcriptional activation. However, changes in specific mRNA levels can also be effected by alterations in the frequency of initiation and/or the overall rate of gene transcription and by
modulation of the stability of the resulting mRNA (reviewed by Crabtree, 1989). Although activation of T cells results in the transient transcriptional activation of the genes that encode both IL2 and IL2Rα chain, there is also evidence for regulation of IL2 production at the level of mRNA stability.

Studies have demonstrated that mAbs directed against the CD28 antigen can enhance the production of IL2 under conditions of optimal TCR/CD3 signaling (June et al., 1989, Thompson et al., 1989). These findings are consistent with the observation that CD28 appears, at least in part, to mediate its stimulatory effects on lymphokine production by specifically enhancing mRNA stability (Lindsten et al., 1989). The recent identification of a CD28-responsive transcriptional enhancer element in the promoter region of the IL2 gene suggests that CD28 can also stimulate transcriptional activation of the IL2 gene by a pathway that is independent of TCR/CD3 (Fraser et al., 1991).

1.10 Intracellular biochemical changes that accompany T cell activation.

The use of agonistic monoclonal antibodies against T cell surface structures, such as TCR/CD3, CD2, CD4, CD8 and CD45, has allowed the contribution of many of these receptor pathways to T cell activation to be investigated. Amongst the immediate intracellular consequences that have been established to occur on stimulation of T lymphocytes by antigen, antibodies directed against the TCR/CD3 complex or combinations of antibodies specific for the CD2 antigen are the induction of tyrosine kinase activity, elevation of intracellular free calcium concentration and activation of the serine/threonine protein kinase C, as determined by phosphorylation of cellular proteins considered to be PKC substrates.
1.11 The calcium signal and T cell activation.

It has been apparent for many years that Ca\(^{2+}\) plays an important and almost universal role in signaling and the initiation of cell growth. Activation of T lymphocytes with appropriately-presented antigen (Nisbet-Brown et al., 1985) or with mAbs directed against either the TCR/CD3 complex (Weiss, M.J. et al., 1984, Weiss, A. et al., 1984) or CD2 antigen (Alcover et al., 1986, Pantaleo et al., 1987) results in a rapid increase in the concentration of intracellular free calcium ([Ca\(^{2+}\)]\(_i\)). Much of this information has been derived from the use of fluorescent dyes, such as Fura2, which on introduction into cells bind Ca\(^{2+}\) and act as indicators of the [Ca\(^{2+}\)]\(_i\) (Gryniewicz et al., 1985).

The increase in [Ca\(^{2+}\)]\(_i\) observed on T cell activation can be considered to consist of two components. The initial [Ca\(^{2+}\)]\(_i\) peak, lasting for about 1 minute, is independent of the concentration of extracellular calcium and consequently results from the release of Ca\(^{2+}\) from intracellular sources. The second component, a lower sustained plateau phase, demonstrates a strict requirement for extracellular calcium and most probably results from the activation of a membrane Ca\(^{2+}\) transporter or ion channel. Patch-clamp studies have demonstrated that T cells, unlike many other excitable cells, do not express classical plasma membrane voltage-gated Ca\(^{2+}\) channels. Several studies have revealed the existance of Ca\(^{2+}\) channels in T cells that are regulated by the mitogen PHA or mAbs directed against either the TCR/CD3 complex or CD2 antigen (Alcover et al., 1986; Pecht et al., 1987, Gardner et al., 1989). However, since the observed properties of these channels differ, both in terms of their electrophysiology and mechanism of coupling to surface receptors, the relationship between TCR/CD3 or CD2 stimulation and Ca\(^{2+}\) influx remains unclear (reviewed by Gardner 1989).

The mechanism by which the initial [Ca\(^{2+}\)]\(_i\) peak occurs is, by comparison, relatively well understood. Triggering of T cell surface receptors known to induce the mobilisation of intracellular calcium, such as the
TCR/CD3 complex and the CD2 antigen, results in the production of inositol 1,4,5 triphosphate (Ins(1,4,5)P₃) as a consequence of phospholipase C-mediated hydrolysis of membrane phosphatidylinositol 4,5 bisphosphate (Imboden and Stobo, 1985; Cockcroft et al., 1987; Pantaleo et al., 1987). Evidence has accumulated over a long period of time to suggest a causal relationship between Ins(1,4,5)P₃ production and Ca²⁺ release from intracellular stores (reviewed by Berridge and Irvine). However, it is only relatively recently that the interaction of Ins(1,4,5)P₃ with it's receptor, localized to the rough and smooth endoplasmic reticulum, has been demonstrated to stimulate the release of Ca²⁺ from these intracellular stores (Ferris et al., 1989; reviewed by Rink and Merritt). Since addition of Ins(1,4,5)P₃ to saponin-permeabilised Jurkat cells induces the release of Ca²⁺, it appears likely that this mechanism extends to T lymphocytes (Imboden and Stobo, 1985). In addition, Ins(1,4,5)P₃ has been reported to regulate a plasma membrane Ca²⁺ channel in patch clamp studies. It is possible that, in addition to the initial Ca²⁺ transient, Ins(1,4,5)P₃ may stimulate Ca²⁺ influx and therefore contribute to the sustained elevation of [Ca²⁺]ᵢ observed on TCR/CD3 or CD2 triggering (Kuo and Gardner, 1987).

Further complication is provided by the existence of a distinct Ins(1,4,5)P₃-insensitive intracellular calcium pool. The relationship of this pool to extracellular calcium entry and the Ins(1,4,5)P₃-sensitive pool is not clear. However, a role for GTP and Ins(1,3,4,5)P₃ in regulating the movement of calcium between these pools has been suggested to explain some of the observations that have been made as a consequence of investigations into intracellular calcium regulation (reviewed by Berridge and Irvine, 1989).
1.12 Protein kinase C and T cell activation.

The PKC family of protein serine/threonine kinases demonstrate a requirement for Ca$^{2+}$, an acidic phospholipid such as phosphatidylserine and a neutral lipid such as the phospholipid metabolite diacylglycerol. Diacylglycerol, considered to be the endogenous activator of PKC, serves to increase the enzyme's affinity for phospholipid and Ca$^{2+}$. (reviewed by Nishizuka 1988; Bell, 1986). In addition, PKC is the cellular receptor for tumour-promoting phorbol esters, which bind and directly activate the enzyme (Kikkawa et al., 1983). The ability of phorbol esters to mimic some aspects of T cell activation, including the phosphorylation of a variety of cellular substrates, suggests that PKC is activated on triggering of receptors such as the TCR/CD3 complex or CD2 antigen.

T cell activation with phorbol esters, antigen, mitogenic lectins or mAbs specific for the TCR/CD3 complex or CD2 antigen can induce phosphorylation on serine/threonine residues of the $\gamma$ subunit (and $\epsilon$ in mice) of the CD3 complex (Cantrell et al., 1987, Samelson et al., 1985a, 1987), CD4, CD8 (Acres et al., 1986; 1987; Minami et al., 1987; Blue et al., 1987), CD45 and MHC class I molecules (Autero and Gahmberg, 1987; Schackleford and Trowbridge, 1986). Two cytoplasmic proteins, of 19 and 80kDa, have also been documented as being rapidly phosphorylated (Friedrich et al., 1988). Further evidence to suggest that PKC is activated under these circumstances is derived from the fact that stimulation of T lymphocytes with antigen or antibodies against the TCR/CD3 complex or CD2 antigen is known to induce inositol phospholipid hydrolysis by PLC with consequent production of diacylglycerol (Imboden and Stobo, 1985; Cockcroft et al., 1987; Pantaleo et al., 1987).
1.12.1 Structure and function of PKC isozymes.

Molecular cloning techniques have revealed the existence of several isozymes of PKC termed $\alpha$, $\beta_1$, $\beta_2$, $\gamma$, $\delta$, $\zeta$, and $\epsilon$ (Parker et al., 1986; Coussens et al., 1986; Knopf et al., 1986 and reviewed by Nishizuka, 1988). Ranging in molecular weight from 67-83kDa, all contain a regulatory and catalytic domain but can be divided into two groups according to certain conserved structural features. PKC $\alpha$, $\beta_1$, $\beta_2$ and $\gamma$ share conserved sequences in four regions; C1 and C2 constituting the regulatory domains and C3 and C4 forming the catalytic domains. The $\delta$, $\zeta$ and $\epsilon$ isozymes of PKC possess C1, C3 and C4 sequences but lack the C2 region (see diagram 1.8). In addition, all PKC isozymes contain a sequence in the regulatory domain which acts as a pseudosubstrate inhibitor of the enzymes catalytic activity and has been demonstrated to inhibit PKC in vitro (House and Kemp, 1987). Areas of the molecules that are not conserved, termed V1-V5, have been suggested to play a role in determining the possible functional and localizational heterogeneity of PKC isozymes (reviewed in Nishizuka 1988).

Diagram 1.6 Structure of PKC isozymes.

- Isolation of $\alpha$, $\beta$ and $\gamma$ PKC by column chromatography, Northern blot analysis with specific oligonucleotide probes and Western blotting or
immunocytochemical staining with isozyme-specific antisera has allowed a comparison of the biochemical characteristics and tissue distribution of these isozymes. In contrast, characterisation of the δ,ζ and ε subspecies is at an early stage and awaits the development of discriminatory reagents. PKC has been found in all tissues examined and although most cell types appear to express more than one isozyme, α PKC appears to be the most widely-distributed subspecies. Studies indicate that peripheral blood-derived T lymphocytes or leukaemic T cells express at least two PKC isozymes, α and β (Beyers et al., 1988; Shearman et al., 1988). Despite some evidence to the contrary, it is likely that T cells do not express γ PKC since this isozyme is thought to be expressed specifically in central nervous tissue (Ohno et al., 1987; reviewed by Nishizuka, 1988).

The implications of PKC heterogeneity for cellular signaling are unclear. However, evidence is emerging to suggest that cellular circumstances might exist in which the isozymes may be differentially activated. In particular, the β isozyme but not the α isozyme of PKC can be significantly activated in the nominal absence of Ca²⁺ (Shearman et al., 1988). In addition, the γ isozyme, in the presence of phospholipid and Ca²⁺ can be activated by arachidonic acid (Nishizuka, 1988). These results, obtained from in vitro assays using mixed lipid micelles or vesicles in conjunction with exogenous protein substrates, must be interpreted with caution since reported enzymic properties differ widely according to experimental conditions.

1.12.2 The role of PKC in T cells.

Much of the evidence that PKC is stimulated during T cell activation stems from the observation that phorbol esters and TCR/CD3 or CD2 agonists induce the phosphorylation of common cellular substrates. However, unequivocal identification of the kinase responsible for receptor-stimulated phosphorylation of a particular cellular substrate or substrates is difficult to
establish since many substrates can be phosphorylated by multiple protein kinases. For example, the serine/threonine Ca\(^{2+}\)/calmodulin-dependent kinase could also be involved in TCR/CD3- or CD2-induced phosphorylation. In this respect, there is evidence that Ca\(^{2+}\)-regulated kinases distinct from PKC can regulate the phosphorylation of the CD3\(\gamma\) subunit, CD4 and CD8 antigens (Breitmeyer et al., 1987; Cantrell et al., 1989).

Diagram 1.7 The role of PKC and Ca\(^{2+}\) in T cell activation.

Furthermore, if TCR/CD3 or CD2 stimulation is observed to induce phosphorylation of the same cellular substrates as phorbol ester treatment, this does not necessarily mean that ligand-stimulated phosphorylation is directly effected by PKC. For example, the ribosomal S6 protein is phosphorylated in response to phorbol ester stimulation of T lymphocytes.
This phosphorylation event, which takes place in many cell types in response to a variety of stimuli, probably reflects the activation of the 42kDa MAP kinase by PKC. The MAP kinase, an intermediate in a cascade of kinases, is regulated by phosphorylation on both tyrosine and threonine residues and in turn activates the S6 kinase (Anderson et al., 1990; Ely et al., 1990). Interestingly, activation of PKC with phorbol esters has been demonstrated to stimulate the phosphorylation of MAP kinase on tyrosine in fibroblasts (Kazlauskas and Cooper, 1988). Thus, it is possible that further components of this kinase pathway remain to be discovered and that PKC may activate a tyrosine kinase under some circumstances. Although the tyrosine kinase p56\textsuperscript{ck} is phosphorylated in response to phorbol ester treatment of T lymphocytes, the functional consequences of this event are unclear (Veillette et al., 1988a). Another important target for PKC-mediated phosphorylation may be the serine/threonine protein kinase encoded by the c-raf protooncogene. Phorbol ester treatment and T cell activation via the TCR/CD3 complex have both been described to result in phosphorylation and activation of the Raf kinase (Morrison et al., 1988; Siegel et al., 1989).

One approach that has been used to implicate PKC in receptor-stimulated phosphorylation events has been to down-regulate PKC expression and function by prolonged treatment with phorbol esters (Rodriguez-Pena and Rozengurt, 1986). Using this technique, antigen-induced phosphorylation of CD3\textsubscript{y} has been demonstrated to require the presence of functional PKC in murine T cells (Patel et al., 1987). Unfortunately, in human T lymphocytes it is not possible to effectively down-regulate PKC in this manner.

PKC has been implicated in the activation-associated phosphorylation of several T cell surface molecules known to be involved in the activation process. The best characterised of these are the γ subunit of the CD3 complex (Cantrell et al., 1987), the CD4 and CD8 antigens (Acres et al., 1986, Blue et
al., 1987) and the CD45 antigen (Autero and Ghamberg 1987).
Phosphorylation of CD3 and CD4 is accompanied by the downregulation of
surface expression which, in the case of CD3, is due to an increased rate of
internalisation (Cantrell et al., 1985, Blue et al., 1987, Minami et al., 1987). It is
possible that these events are associated with reduced responsiveness to the
appropriate ligand and that they represent a negative feedback pathway
(Cantrell et al., 1985).

1.13 Tyrosine phosphorylation.

The importance of tyrosine phosphorylation as a primary signal
transduction event is suggested by the fact that the surface receptors for many
peptide growth factors possess cytoplasmic domains with intrinsic protein
tyrosine kinase activity. Examples include the receptors for epidermal growth
factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor
(FGF), Insulin-like growth factor (IGF)-1 and colony stimulating factor (CSF)-1.
In each of these cases, receptors with impaired tyrosine kinase activity
demonstrate attenuated ability to induce mitogenesis (Reviewed by Hunter,
1989 and Ullrich and Schlessinger, 1990). Another major family of protein
tyrosine kinases are structurally and functionally similar to the protooncogene
p60c-src. These non receptor tyrosine kinases are localized to the inner face of
the plasma membrane via amino-terminal myristylation and are also thought to
play a role in signal transduction (reviewed by Cooper, 1990).

Ligation of the TCR/CD3 complex, CD2, CD4 or CD8 antigens
stimulates the rapid phosphorylation of a number of proteins on tyrosine
residues (Samelson et al., 1986; Patel et al., 1987; Hsi et al., 1989; June et al.,
1990; Veillette et al., 1989b; Luo and Sefton, 1990). The pattern of intracellular
substrate phosphorylation observed on CD4/CD8 and TCR/CD3 stimulation
have been observed to be different. This suggests that these pathways may
activate distinct tyrosine kinases with different substrate specificity (Veillette et
Identification of the kinases responsible for these modifications, the identity of the substrate proteins and the consequences of their phosphorylation during T cell activation is in its early stages. The sensitive and specific detection of phosphotyrosine-containing proteins has been a major problem in studies designed to identify tyrosine kinase substrates. One approach commonly employed for the detection of proteins containing phosphotyrosine utilizes phosphoamino acid analysis to identify phosphotyrosine in $^{32}$P-labelled protein samples (Cooper et al., 1983). The major disadvantage of this method is the low phosphotyrosine content of cellular proteins in comparison with phosphoserine and phosphothreonine. Immunological detection of tyrosine phosphoproteins by Western blotting with antibodies specific for this phosphoamino acid is also frequently employed (Frackleton et al., 1983). The use of this technique requires rigorous characterisation of the specificity of the antibody employed and is limited in its sensitivity.

A protein that has been identified to be phosphorylated on TCR/CD3-, CD2- and CD4/CD8-triggering is the 16kDa $\zeta$ subunit of the TCR/CD3 complex (Baniyash et al., 1988b; Monostori et al., 1990; Veillette et al., 1989a). In contrast to some of the other substrates, which are phosphorylated within seconds of stimulation (June et al., 1990), half maximal phosphorylation of $\zeta$ occurs after about 15 minutes. The accumulated evidence that the $\zeta$-chain functions to transduce signals from the T cell receptor suggests that this phosphorylation event has regulatory potential. In particular, it has been suggested that $\zeta$ phosphorylation represents a negative feedback pathway for the uncoupling of TCR/CD3 from intracellular effectors (Samelson et al., 1986; Scholz et al., 1988).
1.13.1 Tyrosine kinases and phosphatases in T lymphocytes.

Significantly, none of the T cell surface molecules that have been implicated in T cell activation contain consensus sequences suggestive of intrinsic tyrosine kinase activity. Nevertheless, T cells have been demonstrated to express at least three p60c-src family tyrosine kinases; p59\(^{fyn}\), p60\(^{yes}\) and p56\(^{lck}\) (Cooke and Perlmutter, 1989; Samelson \textit{et al}., 1990). All these molecules possess a highly-conserved C-terminal kinase domain but are distinguished by unique N-terminal sequences thought to be responsible for their association with other proteins (reviewed by Cooper, 1990) (see diagram 1.8). While p60\(^{yes}\) is found in a wide variety of tissues and cell types, p56\(^{lck}\) and one isoform of p59\(^{fyn}\), \textit{fyn}(T) are found primarily in lymphocytes (Cooke and Perlmutter, 1989).

p56\(^{lck}\) has been demonstrated to interact with a sequence in the cytoplasmic domains of the CD4 antigen and \(\alpha\) chain of the CD8 antigen. (Rudd \textit{et al}., 1988, Shaw \textit{et al}., 1989; Veillette \textit{et al}., 1988). The potential significance of these interactions has been demonstrated by the finding that truncation of most of the cytoplasmic domain of either CD4 or CD8 prevents their association with \textit{lck} and impairs their ability to enhance TCR/CD3-mediated responses (Sleckman \textit{et al}., 1988; Zamoyska \textit{et al}., 1989; Glaichenhaus \textit{et al}., 1990). Recently, p59\(^{fyn}\) has been observed to co-immunoprecipitate with the TCR/CD3 complex under mild detergent solubilization conditions (Samelson \textit{et al}., 1990). Whereas up to 90\% of cellular p56\(^{lck}\) can exist in a CD4-associated form, around 0.1\% of cellular p59\(^{fyn}\) is immunoprecipitated with the TCR/CD3 complex (although this figure is apparently rising!). The significance of this stoichiometric difference is unclear but the fact of association between these molecules provides a potential mechanism for the induction of tyrosine phosphorylation on T cell activation.
Another potential regulatory component of T lymphocyte tyrosine kinase signal transduction pathways was identified when the cytoplasmic domain of CD45 was found to contain two tandem repeats that were homologous to a tyrosine phosphatase isolated from placenta (Charbonneau et al., 1988). Subsequently, CD45 immunoprecipitates were demonstrated to possess intrinsic tyrosine phosphatase activity (Tonks et al., 1988). These observations suggest possible mechanisms for the modulatory effects of CD45 mAbs on T cell activation (Ledbetter et al., 1988; 1991; Keiner and Mittler, 1989; Martorell et al., 1987, Marvel et al., 1989). Although CD45 has been suggested to be a receptor protein-tyrosine phosphatase, no physiological ligand has been identified and nothing is known about the regulation of its activity in vivo. CD45 is known to be a substrate for PKC but the consequences of this event for CD45 activity are unknown (Schackelford and Trowbridge, 1986; Autero and Gahmberg, 1987).

1.13.2 Regulation of src family tyrosine kinases.

The kinase activity of members of the pp60c-src family can be positively or negatively regulated by tyrosine phosphorylation at multiple sites. For example, phosphorylation of a tyrosine residue close to the carboxy terminus and catalytic domain of p60c-src correlates with reduced kinase activity (Cooper et al., 1986; Piwnica-Worms et al., 1987; reviewed by Cooper, 1990). In p60c-src this residue is Tyr527 whilst the corresponding residue in p56\(^{\text{Lck}}\) and p59\(^{\text{Fyn}}\) are Tyr505 and Tyr531 respectively. In common with Tyr527 of p60c-src, both residues are highly phosphorylated in quiescent cells and are thought to maintain p56\(^{\text{Lck}}\) and p59\(^{\text{Fyn}}\) in an inactive state (Veillette et al., 1988a; Kawakami et al., 1988). The importance of these residues for the regulation of p56\(^{\text{Lck}}\) and p59\(^{\text{Fyn}}\) activity has been demonstrated in studies where mutation of Tyr505 of p56\(^{\text{Lck}}\) or Tyr531 of p59\(^{\text{Fyn}}\) to phenylalanine, preventing phosphorylation at these positions, enhances the in vitro kinase activity.
activity of both kinases and confers oncogenic potential upon their transfection into fibroblasts (Amrein and Sefton, 1988; Marth et al., 1987; Kawakami et al., 1988).

Diagram 1.8 Structure of src family protein tyrosine kinases.

Phosphorylation of p60<sup>c-src</sup> at Tyr527 is apparently mediated by a distinct protein tyrosine kinase rather than by autophosphorylation. (Okada and Nakagawa, 1988). Thus, Tyr505 of p56<sup>lk</sup> and Tyr531 of p59<sup>fyn</sup> represent sites for inhibitory modifications by other, as yet unidentified, protein tyrosine kinases.

Phosphorylation of Tyr416 of pp60<sup>c-src</sup> or the corresponding residue, Tyr394, of p56<sup>lk</sup> correlates with increased activity of these kinases (Veillette and Fournel, 1990; reviewed by Cooper, 1990). The analogous residue in p59<sup>fyn</sup> is Tyr420 (Semba et al., 1986). Interestingly, this tyrosine residue is conserved within the catalytic domain of all tyrosine kinases and corresponds to one of the autophosphorylation sites of many growth factor receptor tyrosine kinases. For these kinases, the mechanism of ligand-induced stimulation is thought to involve the formation of a receptor dimer and mutual trans-phosphorylation of dimer partners. Ligand-induced autophosphorylation has been suggested to stimulate receptor phosphotransferase activity by removing internal constraints which hinder interaction with substrate (reviewed by Ullrich and Schlessinger, 1990). Further support for the regulatory significance of
Tyr394 is derived from the observation that this residue, a major site of p56\textit{lck} autophosphorylation \textit{in vitro}, is not significantly phosphorylated in unstimulated T lymphocytes (Veillette \textit{et al.}, 1988b; 1989b). However, \textit{in vitro} phosphorylation of Tyr394 is observed on p56\textit{lck} activation by mutation of Tyr505 to phenylalanine (Amrein and Sefton, 1988; Marth \textit{et al.}, 1987) or, to a lesser extent, on crosslinking of CD4 or CD8 on T lymphocytes (Veillette \textit{et al.}, 1989b; Luo and Sefton, 1990). The oncogenic potential of a Phe505 mutant \textit{lck} protein is greatly reduced by the subsequent mutation of Tyr394 (Veillette and Fournel, 1990). Thus, it appears likely that dephosphorylation of Tyr505 may serve a "permissive" role in the regulation of p56\textit{lck}, allowing subsequent phosphorylation of Tyr394 and correlating with enhanced biological activity. Although phosphorylation of Tyr394 is usually an intramolecular event, other p56\textit{lck} molecules and distinct tyrosine kinases may also be able to phosphorylate this residue and regulate kinase activity.

In addition to phosphorylation on tyrosine, p56\textit{lck} also appears to be a substrate for PKC. Thus, TCR/CD3-triggering or phorbol ester treatment stimulates phosphorylation of p56\textit{lck} on multiple serine residues in the amino-terminal of the molecule resulting in altered electrophoretic mobility to an apparent size of 59kDa (Villette \textit{et al.}, 1988a). The significance of these modifications is unclear.

1.13.3 Regulation of \textit{lck} and \textit{fyn} in T lymphocytes.

Antibody mediated cross-linking of CD4 results in a rapid increase in tyrosine phosphorylation of p56\textit{lck} and certain cellular substrates in several murine T cell lines. This correlates with a 3- to 5-fold increase in the tyrosine kinase activity of receptor-bound p56\textit{lck} molecules under certain \textit{in vitro} assay conditions (Veillette \textit{et al.}, 1989b; Luo and Sefton, 1990). Phosphorylation of \textit{lck} has also been observed to occur on crosslinking of CD4 or CD8 on murine thymocytes and splenic T cells. Cross-linking is apparently essential since Fab
fragments of anti-CD4 or CD8 antibodies do not induce phosphorylation of p56/ck or of other cellular substrates. The importance of the positive regulatory Tyr394 residue in this process is suggested by studies demonstrating that Try394 is absolutely required for CD4 cross-linking to activate p56/ck (Veillette and Fournel, 1990). Thus, a speculative model for the regulation of p56/ck kinase activity by CD4/CD8 is that ligation and aggregation of CD4 or CD8 molecules promotes phosphorylation of Tyr394 and activation of receptor-associated kinase activity (Mustelin and Altman, 1989; Rudd, 1990). Although it is unclear whether Tyr394 phosphorylation is an intramolecular or intermolecular event it appears possible that, by analogy to growth factor receptor tyrosine kinases, phosphorylation of this critical residue occurs between receptor-associated kinases within receptor aggregates (Ullrich and Schlessinger, 1990).

An important role for the dephosphorylation of Tyr505 in the activation of p56/ck has emerged from several studies which suggest that Tyr505 is a substrate for CD45. CD45 has been observed to dephosphorylate p59/ck at Tyr505 in vitro and co-crosslinking of CD4 and CD45 results in the dephosphorylation of p56/ck at Tyr505, correlating with enhanced in vitro kinase activity (Ostergaard et al., 1989; Mustelin et al., 1989). These findings suggest that dephosphorylation of Tyr505 may play a primary role in the activation of p56/ck. Paradoxically, increased phosphorylation of Tyr505, in addition to Tyr394, is observed on antibody-mediated aggregation of CD4 (Veillette et al., 1989a; 1989b; Luo and Sefton, 1990). A possible explanation for this increase is the prevention of phosphatase action against Tyr505 on CD4 aggregation. The role of Tyr505 in the regulation of p56/ck activity by CD4 or CD8 requires further experimental clarification.

Antibody-mediated cross-linking of the TCR/CD3 complex has not been observed to result in phosphorylation of p56/yn or in enhanced receptor-associated kinase activity (Samelson et al., 1990). This result could reflect the
low stoichiometry of TCR/CD3-fyn association. Nevertheless, high level expression of p59/72\textit{fyn} has been observed to correlate with constitutive phosphorylation of an uncharacterised src-family kinase and phosphorylation of the \(\zeta\) subunit of the TCR/CD3 complex in CD4\(^+\) CD8\(^-\) T cells from lpr/lpr mice which demonstrate lymphoproliferative disorders (Samelson \textit{et al.}, 1986; Katagiri \textit{et al.}, 1989). Although these results do not directly implicate p59/72\textit{fyn} in phosphorylation of \(\zeta\), they are strongly suggestive of an important role for this kinase. The most convincing evidence for \textit{fyn} involvement in TCR/CD3-induced tyrosine phosphorylation has been obtained in a recent study employing transgenic mice that over-express p59\textit{fyp} in developing T lineage cells (Cooke \textit{et al.}, 1991). In transgenic animals relative to control animals, induction of tyrosine phosphorylation on stimulation by anti-CD3 mAb was observed to be identical in terms of substrate specificity but dramatically increased in terms of both kinetics and abundance. These results suggest that ligation of TCR/CD3 resulted in activation of both endogenous p59\textit{fyn} and the transgene-encoded enzyme in lymphocytes derived from control and transgenic animals respectively.

The recent studies in which the ability of TCR/CD3 or CD2 triggering to induce tyrosine phosphorylation was found to be impaired in a CD45-deficient mutant derived from the the human T-leukaemic line Jurkat (Koretzky \textit{et al.}, 1991). Although these authors were unable to confirm the mutational defect by CD45 transfection and demonstration of reconstituted coupling, the simplest interpretation of these results is that CD45-mediated dephosphorylation, possibly of p59\textit{fyn} Tyr420, is the primary event required for activation of tyrosine kinase activity by the TCR/CD3 complex or CD2 antigen. Although it's activity against p59\textit{fyn} is unknown, another potential substrate for CD45 tyrosine phosphatase could be the \(\zeta\) chain of the TCR/CD3 complex. Phosphorylation of this subunit has been suggested to modulate TCR/CD3 coupling to signal transduction pathways. A model in which the proximity of
CD45 to the TCR/CD3 complex could influence signaling through TCR/CD3 might provide an explanation for the inhibitory or enhancing effects that have been observed for CD45 mAbs in conjunction with stimulation via CD3 or CD2 agonists (Ledbetter et al., 1988; 1991; Keiner and Mittler, 1989; Martorell et al., 1987, Marvel et al., 1989) (see diagram 1.9). In addition to a proximal role in the regulation of tyrosine phosphorylation, CD45 could antagonize distal tyrosine kinase-mediated biological events by dephosphorylating critical effector substrates.

Diagram 1.9 Regulation of tyrosine phosphorylation in T lymphocytes.

For explanation of model see supporting text.
Under physiological conditions of T cell activation, CD4/CD8 and the TCR/CD3 complex are brought into close proximity by the appropriate presentation of antigen in the context of self-MHC. In addition, a physical interaction between CD4 and TCR/CD3 has been observed to occur on TCR/CD3 ligation (Gallagher et al., 1989). This interaction could serve to bring p59\textsuperscript{lck} into a position where it could phosphorylate the ζ subunit of TCR/CD3 and modify it's coupling to intracellular pathways. Alternatively, or in addition, this juxtaposition might allow for a functional interaction between p59\textsuperscript{lck} and p56\textsuperscript{fyn}. A model in which CD4/CD8- and TCR/CD3-associated tyrosine kinase pathways interact could provide an explanation for the synergistic effect of crosslinking mAbs against CD4 and CD3 on the proliferative response of T cells (Emmrich, 1988) (see diagram above). In this respect, co-crosslinking of CD4 and the TCR/CD3 complex has been observed to synergise for the induction of tyrosine phosphorylation (June et al., 1990; Abraham et al., 1991). Although crosslinking of the TCR/CD3 complex does not result in the activation of p56\textsuperscript{lck} (Veillette et al., 1989a), the finding that CD4 association with p56\textsuperscript{lck} is a requirement for TCR/CD3-mediated responses in certain antigen-specific T cell hybridomas supports the concept that p56\textsuperscript{lck} could interact to regulate the TCR/CD3 pathway (Glaichenhaus et al., 1990). Further evidence is provided by a recent study in which a Phe505 p56\textsuperscript{lck} mutant was introduced into an antigen specific, CD4-negative, murine T cell line. Although no constitutive elevation in tyrosine phosphorylation was observed, TCR/CD3-induced tyrosine phosphorylation was observed to be enhanced in cells expressing Phe505 p56\textsuperscript{lck} (Abraham et al., 1991). Interestingly, the substrate pattern of tyrosine phosphorylation induced by TCR/CD3 ligands in these cells was identical to that observed on co-crosslinking of TCR/CD3 and CD4 in a CD4-positive variant, suggesting that the amplifying effect of Phe505 p56\textsuperscript{lck} may be relevant to the normal function of CD4-associated p56\textsuperscript{lck}.
1.14 Signals that regulate expression of IL2R and IL2 secretion.

Since the induction of expression of high affinity receptors for IL2 and secretion of IL2 are critical events for the regulation of T cell proliferation, analysis of the signalling requirements for the induction of this growth factor pathway are essential to an understanding of the molecular mechanisms of T cell activation. Although triggering of the TCR/CD3 complex is one critical signal and will stimulate highly purified resting T lymphocytes to initiate IL2R expression it is insufficient to induce secretion of IL2 under these circumstances (Meuer et al., 1984; Weiss et al., 1984a; 1984b). Additional signals, provided by the inclusion of antigen-presenting cells, the provision of lymphokines such as IL1 or co-stimulation of T cell surface molecules such as CD2, CD4/CD8 or CD28 are required to induce IL2R expression, IL2 production and hence proliferation of resting T cells (reviewed by Weiss and Imboden, 1987). The basal activation state of the cells used appears to influence the requirements for induction of the IL2/IL2R pathway. For example, crosslinking or immobilisation of anti-CD3 monoclonal antibodies is required to activate many T cell hybridomas and antigen-specific T cell clones to produce and respond to IL2 (Manger et al., 1988). Whether the differences arise from the fact that these cells have less stringent requirements for induction of IL2 production or whether they are already activated to produce an additional signal is not understood.

1.14.1 The Ca\(^{2+}\)/PKC model for IL2/IL2R expression.

The use of pharmacological agents, to mimic aspects of the intracellular consequences of TCR/CD3 occupancy, has suggested an important role for activation of PKC and elevation of [Ca\(^{2+}\)]\(_i\) in the induction of IL2R expression and secretion of IL2. Thus Ca\(^{2+}\) ionophores, such as ionomycin which which elevates [Ca\(^{2+}\)]\(_i\), in combination with phorbol esters, such as phorbol 12,13-dibutyrate that activate PKC can stimulate T cell proliferation by induction of
IL2R expression and secretion of IL2 (Truneh et al., 1985; Kaibuchi et al., 1985). Further studies with these agents revealed that stimulation of IL2R expression and IL2 secretion differed with respect to their signaling requirements. Although treatment with phorbol esters alone was sufficient to induce expression of IL2 receptors, initiation of IL2 secretion by pure resting T cells was found to require the simultaneous presence of both phorbol esters and ionomycin (Kumagai et al., 1987). Although ligation of the TCR/CD3 complex results in elevation of [Ca^{2+}]_{i} and activation of PKC, one of the paradoxes of signal transduction in T cells concerns the requirement for accessory signals for the induction of IL2R and IL2 secretion. The observation that phorbol ester treatment synergises with triggering of the TCR/CD3 complex for the induction of both IL2R expression and IL2 secretion (Weiss et al., 1984a; 1984b; reviewed by Weiss and Imboden, 1987) suggests that TCR/CD3 stimulation fails to initiate T cell proliferation because of a failure to optimally activate PKC. Thus, the requirement for accessory signals for commitment of T cells to proliferation could reflect the need for enhanced activation of PKC for the induction of IL2 production (see diagram 1.10).

These findings led to the suggestion of a "two signal" model in which the effects of TCR/CD3 on the expression of IL2R and secretion of IL2 could be explained by TCR/CD3-mediated elevation of the [Ca^{2+}]_{i}. Thus, one signal can be provided by activation of PKC with phorbol esters and the other can be provided either by TCR/CD3 triggering or ionomycin treatment (reviewed by Weiss et al., 1986). Further evidence for the importance of elevated [Ca^{2+}]_{i} in the regulation of T cell activation was provided by experiments in which depletion of extracellular Ca^{2+} or the use of Ca^{2+} channel blockers was observed to inhibit both IL2 production and DNA synthesis induced by TCR/CD3 stimulation (Gelfand et al. 1986). These conditions eliminate extracellular sources of Ca^{2+} but do not prevent the initial mobilisation that originates from intracellular stores. Although the elevation of [Ca^{2+}]_{i} is required
for the induction of IL2 production, the initial rise is not necessary (Gelfand et al. 1988), suggesting that the sustained elevation of [Ca^{2+}]_{i} may play a significant role in induction of IL2 gene expression and ultimately in growth regulation.

**Diagram 1.10 T cell activation *in vitro.***

The concept that prolonged stimulation is required for optimal proliferation of T cells was supported by work demonstrating that although a short incubation with phorbol ester and ionomycin was sufficient to induce expression of high affinity IL2R by resting human lymphocytes, endogenous IL2 production required a more prolonged exposure (2-6 hrs) to these agents (Kumagai et al., 1987). In agreement with this study, a period of 2-4 hours stimulation via the TCR/CD3 complex in the presence of phorbol ester was required to commit the human T leukaemic line Jurkat to IL2 production and proliferation (Weiss et al., 1987). Using somatic mutants of the Jurkat line, which exhibit impaired signaling function via the TCR/CD3 complex, Weiss and
colleagues further investigated the relationship between receptor-mediated elevation of \([\text{Ca}^{2+}]_i\) and IL2 production. Selected for it's inability to increase the \([\text{Ca}^{2+}]_i\) in response to stimulation by anti-TCR/CD3 mAbs, one mutant was found to transiently elevate \([\text{Ca}^{2+}]_i\) in response to certain combinations of mAbs. However, this attenuated response was insufficient to induce IL2 production in combination with phorbol esters (Goldsmith and Weiss, 1988).

Since an important mechanism by which the TCR/CD3 complex regulates \(\text{Ca}^{2+}\) and PKC is stimulation of PI-PLC activity, it has been suggested that PI-PLC activity is the receptor-linked biochemical pathway responsible for lymphokine gene expression (Weiss et al., 1984c, Truneh et al., 1985). Recent data suggests that stimulation of PI-PLC is sufficient to induce both IL2R\(\alpha\) expression and IL2 expression in the T leukaemic cell line Jurkat (Desai et al., 1990). Transfection of a heterologous muscarinic receptor, which regulates PI metabolism in neuronal cells, can functionally substitute for the TCR/CD3 complex in Jurkat. Stimulation of transfectants with the muscarinic agonist carbachol, inducing production of inositol phosphates accompanied by elevation of \([\text{Ca}^{2+}]_i\), resulted in IL2R\(\alpha\) expression and production of IL2 (Goldsmith et al. 1989; Desai et al., 1990). Furthermore, muscarinic receptor triggering strongly synergised with phorbol esters for the induction of these events. Although this study demonstrates that, for this cell type, activation of PI-PLC could explain the effects of TCR/CD3 stimulation on the proliferative response, a role for distinct signaling pathways that may be triggered by this receptor cannot be excluded.

1.14.2 Evidence for the involvement of tyrosine phosphorylation in induction of IL2/IL2R expression.

Evidence is accumulating to suggest the involvement of a receptor-regulated pathway distinct from the metabolism of phosphatidylinositol and elevation of \([\text{Ca}^{2+}]_i\) in the induction of late biological responses such as IL2.
production. For example, in the presence of phorbol esters, a nonmitogenic anti-CD2 mAb or mAbs directed against CD28 can stimulate proliferation and IL2 production in the absence of detectable changes in [Ca2+]i (Holter et al., 1986; June et al., 1989). Studies employing the murine T cell hybridoma 2B4.11 and a variant which is deficient for expression of the ζ and η subunits of the TCR/CD3 complex have also provided evidence for TCR/CD3-induced IL2 production in the absence of changes in [Ca2+]i (Sussman et al., 1988). By apparently dissociating the ability of the variant cells to metabolise PI from their ability to induce IL2 secretion in response to anti-CD3 mAbs, the authors conclude that this pathway is not essential for IL2 production. Taken together, these results seriously question the Ca2+/PKC paradigm and suggest that another signal, such as receptor-induced tyrosine phosphorylation, may function to regulate IL2 production.

Investigation of the possibility that receptor-mediated changes in tyrosine phosphorylation may be involved in regulation of the IL2 pathway has been hampered by the fact that no pharmacological means exist to directly activate tyrosine kinases. However, several gene transfer studies have provided strong evidence to support a role for tyrosine kinases in the regulation of these distal events. Firstly, expression of a constitutively active v-src gene in the murine antigen-specific hybridoma 2B4, resulting in elevated tyrosine phosphorylation of cellular substrates, stimulates spontaneous production of IL2 (O'Shea et al., 1991). Although this data suggests that tyrosine phosphorylation may be sufficient for induction of IL2 gene expression, the amount of IL2 made by p60v-src-expressing cells was small in comparison to that produced on antigen stimulation. Two possible interpretations are that p60v-src is inefficient with respect to the TCR/CD3-mediated tyrosine kinase pathway or, more plausibly, that additional signals are required for optimal induction of IL2 production. Secondly, introduction of a constitutively active mutant of p56lck into a murine CD4-negative antigen-
specific hybridoma results in enhanced tyrosine phosphorylation and IL2-induction upon TCR/CD3 triggering (Abraham et al., 1991). Thirdly, transgenic mice which over-express p59\(\text{fyn}\) in developing T lineage cells have been observed to be sensitized with respect to antigenic stimulation for tyrosine phosphorylation and IL2 production (Cooke et al., 1991).

Further supportive evidence can be derived from a number of recent studies which have employed a variety of tyrosine kinase inhibitors to probe the function of tyrosine phosphorylation in the initiation of IL2 secretion by TCR/CD3 triggering. Pretreatment with the isoflavone compound genistein, which inhibits TCR/CD3-induced tyrosine phosphorylation, has been observed to block stimulation of IL2 production by a combination of anti-CD3 mAbs and phorbol esters (Trevillyan et al., 1990). Since TCR/CD3-mediated inositol phosphate production and elevation of \([\text{Ca}^{2+}]_i\) were not significantly inhibited under these conditions, the results imply that \(\text{Ca}^{2+}\) mobilisation is insufficient for the induction of IL2 production and suggest an obligatory role for TCR/CD3-mediated tyrosine phosphorylation. However, this conclusion is weakened by another study in which profound inhibition of TCR/CD3-mediated inositol phosphate production and elevation of \([\text{Ca}^{2+}]_i\) in response to genistein treatment was observed (Mustelin et al., 1990). In addition, the biological effects of genistein cannot be exclusively attributed to it's inhibition of tyrosine kinases. Thus, a concentration of genistein that abolished IL2 production induced by TCR/CD3 triggering in the presence of phorbol ester inhibited the response to ionomycin and phorbol ester by 40% (Trevillyan et al., 1990). Although several other pharmacological compounds have been used to investigate the role of tyrosine phosphorylation in the induction of IL2 production, the effects of these agents on early TCR/CD3 signaling events could explain the effects observed (Stanley et al., 1990; June et al., 1990b).

In summary, it is clear that multiple intracellular signals are necessary for the initiation of IL2 production in T lymphocytes. One critical signal appears to
be activation of protein kinase C but a second signal can apparently be provided by agonists that elevate [Ca$^{2+}$]$\text{ij}$ or by those that do not. Since the TCR/CD3 complex can regulate PKC activity, [Ca$^{2+}$]$\text{ij}$ and tyrosine phosphorylation it has the capacity to contribute multiple signals to the cellular control of IL2 production. While it is clear that these pathways may explain the regulatory influence of the TCR/CD3 complex or CD2 and CD4 antigens on IL2 production, the mechanism by which receptors such as CD28 exert their modulatory effects is unknown.

**1.15 Signal transduction by the IL2 receptor.**

Because of the different cellular consequences of stimulation via the TCR/CD3 complex and IL2R, these two receptors might be expected to couple to different intracellular effectors. In this respect, there is considerable evidence to suggest that IL2 binding to the IL2R does not activate PI-PLC as measured by PI metabolism, production of InsPs or elevation of [Ca$^{2+}$]$\text{ij}$ (Mills et al., 1986; Kozumbo et al., 1987). Furthermore, unlike coupling of the TCR/CD3 complex to PI-PLC, surface expression of CD45 is not required for IL2-induced proliferation (Pingel and Thomas, 1989). However, several studies have detected changes on IL2 stimulation, such as increases in diacylglycerol and PKC translocation from the cytosol to the membrane, which are usually considered to accompany PKC activation. (Mills et al., 1985; 1986; Farrar and Anderson, 1985). In addition, IL2 treatment results in the phosphorylation on serine and threonine residues of several cellular proteins (Gaulton and Eardley, 1986; Evans et al., 1987). Despite the evidence for activation of PKC in response to IL2, studies using cells which lack PKC or cells in which PKC has been downregulated by phorbol ester treatment, indicate that PKC activation is not an obligatory step for IL2-induced proliferation (Mills et al., 1988; Valge et al., 1988). It remains possible that a kinase distinct from PKC is the mediator of serine/threonine phosphorylation observed on IL2 stimulation.
One recent suggestion concerns the involvement of membrane glycosylphosphatidylinositol (GPI) metabolism (Eardley and Koshland, 1990; Merida et al., 1990). These studies demonstrated IL2-stimulated hydrolysis of GPI by a GPI-specific PLC, with coordinate production of inositolphosphoglycan (IPG) and diacylglycerol in B lymphoma and T cell line. It is unclear whether DAG or IPG produced as a consequence of the metabolism of GPI lipids mediate the biological effects of the stimulating agonists. However, the ability of exogenous IPG to synergise with IL2 for the induction of T cell proliferation suggests that this metabolite may play a significant role in mediating the biological effects of this agonist (Merida et al., 1990).

The involvement of a tyrosine kinase in mediating the biological effects of IL2 is suggested by the observation that IL2 treatment increases tyrosine phosphorylation of a number of intracellular substrates (Saltzman et al., 1990; Mills et al., 1990; Merida and Gaulton, 1990). A recent study has demonstrated that the 72-74kDa serine/threonine kinase Raf-1 is phosphorylated on tyrosine residues, rapidly and with high stoichiometry, on IL2 treatment of the murine IL2-dependent T cell line CTLL-2 (Turner et al., 1991). This phosphorylation is associated with an approximately 5-fold increase in serine/threonine kinase activity associated Raf-1-containing immune complexes. Thus it appears likely that Raf-1 serves as a downstream effector for an IL2-regulated tyrosine kinase and could represent the mediator of IL2-induced phosphorylation of cellular substrates on serine and threonine.

Using western blotting techniques, the overall pattern of tyrosine phosphorylation induced by IL2 is distinct from that induced by ligation of the TCR/CD3 complex or CD4/CD8 antigens (Saltzman et al., 1990; Mills et al., 1990). It is probable, therefore, that the IL2R couples to a tyrosine kinase with different substrate specificity or substrate availability to CD4/CD8-associated p60^lck or TCR/CD3-associated p56/72^fyn. In support of this suggestion, a 50-
55kDa protein that is inducibly tyrosine phosphorylated on IL2 binding and may represent a p60c-src family kinase, can be immunoprecipitated with IL2Rβ (Merida and Gaulton, 1990).

1.16 TCR/CD3 coupling to intracellular signaling pathways -role of the TCR ζ and η subunits.

Although it has been established that the TCR/CD3 complex plays a critical role in the regulation of T cell activation and stimulates multiple intracellular signalling pathways, the role of individual components of the complex in coupling to these pathways is only just beginning to be appreciated. The invariant components of the TCR/CD3 complex, the γ, δ, ε, ζ and η subunits, are presumed to couple the receptor to signaling pathways but structural complexity of the receptor and its stringent requirements for assembly have made study of their function difficult. Since the TCR/CD3 complex can exist in either ζη- or ζη-containing isoforms, particular interest has focused upon the function of these molecules and the possibility that structural variants may demonstrate differential signalling function. Insight into potential roles for the ζ and η subunits has come from studies of variants of the murine antigen-specific hybridoma 2B4.11. These cells respond to antigen or mAbs directed against the TCR/CD3 complex by activation of PI-PLC and stimulation of tyrosine phosphorylation. In addition, the ability of antigen or immobilised TCR/CD3 mAbs to induce later biological events such as IL2 production can be measured.

Initial studies with variants that were deficient for expression of the η subunit, consequently demonstrating fewer surface ζη-containing TCR/CD3 complexes, suggested that ζη expression was the limiting factor for induction of inositol phosphate production by the receptor (Sussman et al., 1988; Mercep et al., 1988). Using a panel of variants that expressed levels of ζη heterodimer that ranged from 10-115% of that expressed by parental 2B4.11,
a linear relationship between ζη expression and ability to induce PI metabolism in response to either antigen or anti-CD3 mAb could be observed. In contrast, TCR/CD3-induced tyrosine phosphorylation appeared to be unimpaired. (Mercep et al., 1988). Although this data suggests that TCR/CD3 complexes containing ζη heterodimers may selectively couple to PI-PLC, while those containing ζζ might be linked to tyrosine phosphorylation, the fact that ζη-deficient variants responded poorly to a combination of phorbol esters and ionomycin for production of IL2 indicates that their functional impairment might have been of a more general nature. In addition, the 2B4.11 hybridoma has been observed to frequently lose expression of ζ in a spontaneous manner (Sussman et al., 1988). Thus it is possible that these cells were also heterogeneous for ζ expression.

Further studies employed 2B4.11 variants that were constitutively negative for detectable expression of both ζ and η. Reconstitution of these cells by transfection with ζ cDNA restored surface expression of TCR/CD3 complexes that exclusively contain ζζ homodimers (Mercep et al., 1989; Frank et al., 1990). In contrast to parental 2B4.11, ζη-negative cells did not respond to antigen with PI metabolism, while responses to anti-CD3 mAb were impaired but detectable. However, all variants induced tyrosine phosphorylation of ζ and produced similar amounts of IL2 to parental cells on stimulation with either agonist (Mercep et al., 1989).

The results provide evidence for functional heterogeneity between TCR/CD3 ζζ and ζη isoforms by suggesting that ζζ-containing TCR/CD3 complexes are capable of generating signals required for stimulation of tyrosine phosphorylation and IL2 production but not for the induction of PI metabolism. However, the ability of the TCR/CD3 complex to induce production of IL2 has also been reported to be qualitatively independent of whether the receptor complex contains ζ2 homodimers, η2 homodimers or ζη heterodimers (Clayton et al., 1990). Clearly, whether the combinatorial
possibilities provided by these TCR components provides potential for
diversity in signal transduction from the TCR/CD3 complex remains to be fully
investigated.

The signaling role of ζ was further analysed by transfection of
structurally altered ζ into ζ- and η-negative 2B4.11 variants. Truncation of the
cytoplasmic domain of ζ by up to 40% allowed apparently normal assembly
and surface expression of TCR/CD3 (Frank et al., 1990). Antigen-induced
responses of these cells were severely impaired; antigen-induced IL2
production was almost abolished while anti-CD3 or Ti mAbs induced 15-20% of
the IL2 production observed in cells transfected with full length ζ cDNA.
Interestingly, transfection with a ζ cDNA that contained a single point mutation
(Gly135→Val) generated a similar phenotype. This residue is in a region of the
ζ cytoplasmic domain that contains a possible consensus nucleotide binding
site (Gly135-X-Gly-X-Gly...Ala-X-Lys150; Weissman et al., 1988b). Although
this region has not been demonstrated to bind nucleotide, it appears likely that
it is important for signal transduction on antigen stimulation. One interpretation
of these data is that ζ does not directly couple to intracellular effectors but that
ζ expression is essential for translation of antigen binding to other components
of the TCR/CD3 complex that are responsible for signal transduction. This
model accounts for the differential impairment of antigen and CD3-induced
responses by inferring a signaling role for the CD3 complex such that direct
CD3-stimulation with mAbs bypasses the requirement for ζ.

A direct coupling role for the TCR/CDR ζ-subunit is suggested by a
recent study which elegantly addresses its function by creating a chimera
comprising the extracellular and transmembrane regions of the CD8 molecule
and the cytoplasmic domain of the ζ chain (Irving and Weiss, 1991). Removal
of the ζ transmembrane sequence, considered to be involved with receptor
assembly, allowed the chimeric molecule to be expressed independently of
the TCR/CD3 complex while the CD8 extracellular domain ensured that the
chimera would exist as a disulphide linked dimer. The construct was transfected into the human T cell leukaemic line Jurkat and a mutant which lacks surface TCR/CD3 expression as a consequence of deficient Tiβ expression. Stimulation of CD8/ζ in both chimera expressing cells resulted in inositol phosphate production, elevation of [Ca^{2+}]_i and tyrosine phosphorylation of an identical substrate pattern to that observed on TCR/CD3-triggering. CD8/ζ also proved capable of stimulating distal events such as IL2 production in the presence or absence of surface TCR/CD3 expression.

The fact that a chimeric CD8/ζ molecule can generate signals qualitatively indistinguishable from TCR/CD3 signaling strongly suggests that ζ directly couples the TCR/CD3 complex to intracellular signaling events. In the light of these findings, it appears likely that deletions or mutations in ζ disrupted antigen but not anti-CD3 responses in η-negative murine hybridomas (Frank et al., 1990) because these structural alterations were insufficient to block the ability of ζ to transduce signals in response to anti-CD3 mAb stimulation. A further implication from these observations is that the CD3γ, δ and ε chains do not serve a coupling role within the TCR/CD3 complex but might instead function to communicate ligand binding by the Ti components or other surface molecules that have been shown to interact with the TCR/CD3 complex, such as CD2, CD4 or CD8, to the ζ chain. The ability to independently study the function of a component of the TCR/CD3 complex in this manner should allow the relationship of ζ to intracellular effectors such as PI-PLC and tyrosine kinases such as p56^lyn to be investigated and should also enable the functions of components of the TCR/CD3 complex that are homologous to ζ to be studied.
1.17 Phosphatidylinositol specific phospholipase C.

Receptor-mediated activation of phosphatidylinositol-specific phosphodiesterase activity, resulting in hydrolysis of membrane inositol phospholipids, is a common mechanism for transduction of signals delivered by many diverse extracellular stimuli. The importance of Ca\(^{+2}\) and PKC in induction of IL2R expression and IL2 secretion indicates that activation of phosphatidylinositol-specific phospholipase C (PI-PLC) is an important event during T cell activation. The enzymes responsible for phosphatidylinositol hydrolysis, termed phosphatidylinositol-specific phospholipase C (PI-PLC), hydrolyze PI, PI(4)P and PI(4,5)P\(_2\) to yield 1,2 diacylglycerol and inositol mono-(Ins4P), bis- (Ins1,4P\(_2\)) and trisphosphates (Ins1,4,5P\(_2\)).

Protein purification and molecular cloning studies have revealed that multiple, immunologically distinct, PI-PLC enzymes are expressed in mammalian tissues. Nine isozymes of PI-PLC, varying in molecular weight from 65kDa to 150kDa, have been identified and categorized into four groups; PLC\(\alpha\), \(\beta\), \(\gamma\) and \(\delta\) (reviewed by Rhee et al., 1989 and Majerus et al., 1990) (See diagram 1.11).

PLC\(\alpha\) has a molecular weight of 65kDa and is the abundantly expressed in liver and many fibroblast cell lines. Although the cDNA for PI-PLC\(\alpha\) has been isolated, it demonstrates weak homology with other other members of the family and has not been formally demonstrated to encode a PI-PLC activity (Bennett et al., 1988). Three isoforms of PLC\(\beta\), ranging from 150-100kDa, have been identified (PLC\(\beta\)1, \(\beta\)2 and \(\beta\)3) (Suh et al., 1988; Katan et al., 1988; Kriz et al., 1990). Both share two conserved domains which are required for catalytic activity (domains I and II) with other members of the PI-PLC family (Rhee et al., 1989). Two PLC\(\gamma\) isoforms of molecular weight 130-150kDa have also been identified (PLC\(\gamma\)1 and \(\gamma\)2) (Suh et al., 1988; Stahl et al., 1988; Emori et al., 1989; Kriz et al., 1990). PLC\(\gamma\) contains regions that are related to sequences originally found in non-receptor tyrosine kinases of
the p60c-src family (src homology regions SH1 and SH2). Despite lying
between the catalytic domains, these regions are not required for catalytic
activity and are thought to serve a regulatory function. PLCδ has three isoforms
(Suh et al., 1988; Katan et al., 1988; Kriz et al., 1990) which are widely
expressed. These 85-88kDa enzymes do not possess extensive sequences in
addition to their catalytic domains (Rhee et al., 1989).

Despite being quite heterogeneous in terms of molecular size and
amino acid sequence, consistent with their lack of immunological cross-
reactivity, representatives of all four classes have demonstrated similar
catalytic properties in vitro (reviewed by Rhee et al., 1989). Specific for
phosphatidylinositols, they hydrolyse PI, PI(4)P and PI(4,5)P2 in a manner
dependent on the concentration of Ca2+. PI is generally hydrolysed best at
high Ca2+ concentrations with PI(4,5)P2 being the preferred substrate at
concentrations approximating the physiological (<1μM Ca2+) (Rhee et al.,
1989; Kriz et al., 1990). However, in vitro assay conditions have profound
effects on their enzymic properties that are often greater than any differences
observed between the isozymes. In particular, the lipid composition and
detergent content of substrate micelles can influence their specificity and
activity. All PI-PLC isoymes are inhibited by other phospholipids, particularly phosphatidylcholine and in vesicles containing PI, PI(4)P and PI(4,5)P2 the three phosphatidylinositols compete with each other. Although PI(4,5)P2 is the favoured substrate at equimolar concentrations of phosphatidyl inositols these conditions bias results against PI as a substrate since the concentration of PI(4,5)P2 in most cell membranes is only 1-10% that of PI (reviewed by Majerus et al., 1990).

Diagram 1.12 Inositol phospholipid hydrolysis by PI-PLC.

In addition, purified PI-PLC isoymes exhibit a high level of constitutive activity under optimum in vitro conditions, leading to the suggestion that PI-PLC
activity is negatively modulated by a specific repressor in vivo (Rhee et al., 1989). Despite these reservations, some reproducible differences between the isozymes have emerged, suggesting that they might fulfill distinct cellular roles. For example, the rates of hydrolysis of PI(4)P and PI(4,5)P₂ by PLCα in the absence of Ca²⁺ were 25-33% of the rate observed at Ca²⁺ concentrations in the µM range. In contrast, the activity of the other PI-PLC isozymes has been reported to be negligible in the absence of Ca²⁺ (Rhee et al., 1989).

1.1.8 Inositol phospholipid lipid metabolism and cellular signalling.

The phosphatidylinositols (PIs) are comprised of an sn-12-diaclylglycerol backbone linked to the D-1-hydroxyl of the inositol ring headgroup and constitute less than 10% of total membrane phospholipid in most mammalian cells with phosphatidylinositol (PI) being the most abundant form. Uniquely, PI can be phosphorylated at positions 4 and 5 of the inositol ring to give rise to the polyphosphoinositides phosphatidylinositol 4-monophosphate, PI(4)P, and phosphatidylinositol 4,5-bisphosphate, PI(4,5)P₂, which together account for 10% or less of cellular phosphatidylinositol. These sequential phosphorylations are catalysed by type II PI kinase and PIP kinase. Since PIP and PIP₂ can also be rapidly dephosphorylated by phosphomonoesterases, the various phospholipids are considered to be in a state of dynamic equilibrium (reviewed by Shears, 1989).

PI-PLC hydrolyzes PI, PI(4)P and PI(4,5)P₂ to yield 1,2 diacylglycerol and inositol mono- Ins(4)P, bis- Ins(1,4)P₂ and trisphosphate Ins(1,4,5)P₃. In addition to these inositol phosphates, many others have been isolated from mammalian cells as a consequence of the action of a complex metabolic network of kinases and phosphatases. Since the inositol moiety has six hydroxyl groups, a potential of 66 possible species of inositol phosphate exists. However, the functions of these inositol phosphates are unclear as only Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ have been convincingly demonstrated to
possess biological activity (reviewed by Berridge and Irvine, 1990). The inositol phosphates produced during agonist-induced metabolism of PIs are mainly removed by successive dephosphorylations to yield free inositol. Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ are specifically dephosphorylated by a 5-phosphomonoesterase. Although the pathway of dephosphorylation is not fully understood, the final dephosphorylating enzyme, Ins-1-monophosphatase is a 58kDa membrane-associated protein that is inhibited by Li$^+$ ions. Thus, Li$^+$ causes accumulation of inositol phosphates in cells and is used to prevent complete dephosphorylation of inositol phosphates during measurements of PI metabolism (Hallcher and Sherman, 1980).

1.18.1 The regulation of intracellular DAG levels.

Since PKC has been demonstrated to play a central role in T cell activation, an understanding of the mechanisms by which cellular levels of DAG are controlled is critical to an appreciation of the pathways by which PKC activity is regulated. sn-1,2-diacylglycerol, produced as a consequence of PI-PLC activity, is rapidly and preferentially phosphorylated by a membrane-bound DAG kinase to produce phosphatidic acid (PA) (MacDonald et al., 1988). This can be either reconverted to DAG by the action of a phosphohydrolase or enters the phosphoinositide cycle for resynthesis. In addition, DAG can be deacylated by lipases or reacylated by acetyltransferases. Together, these pathways ensure that DAG produced by transient agonist-induced PI-PLC activity is rapidly metabolised. Whether PA, which accumulates during agonist-stimulated PI breakdown, represents an inert metabolite of DAG or serves a cellular signalling function remains unclear. However, PA has been demonstrated to have multiple effects on cells including a potent mitogenic activity for fibroblasts (Yu et al., 1988; Van Corven et al., 1989).
Although PI-PLC-mediated hydrolysis of phosphatidylinositol is well established as a cellular source of receptor-induced diacylglycerol, several observations suggest that pathways involving the turnover of other membrane phospholipids can also contribute to cellular levels of DAG. Firstly, the magnitude and kinetics of agonist-induced increases in DAG and InsP₃ have been observed to differ. Secondly, the fatty acid composition of agonist-induced DAG often differs from that of PI lipids. Thirdly, the mass of DAG or PA produced can exceed the observed decrease in inositol phospholipids (reviewed by Exton, 1990). Several alternative pathways have been suggested to account for these inconsistencies including de novo synthesis of DAG, the action of PA phosphohydrolase and agonist-induced breakdown of phosphatidylethanolamine and phosphatidylcholine (PC) (reviewed by Pelech and Vance, 1989; Billah and Anthes, 1990).

Diagram 1.13 Regulation of cellular DAG levels.

Since PC comprises almost 50% of cellular phospholipid, it represents a potentially significant source of DAG. Breakdown of PC can either generate DAG and phosphocholine directly via a PC-specific PLC or indirectly by the
action of a PC-PLD to yield choline and PA which may be subsequently
cleaved to generate DAG by PA phosphohydrolase (Pelech and Vance, 1989;
Billah and Anthes, 1990). Candidate PLC activities have been purified from
myocardium and seminal plasma (Wolf and Gross, 1985; Sheikhnejad and
Srivastava, 1986) while a PC-specific PLD has been partially purified from rat
brain (Taki and Kanfer, 1979). Although receptor-induced PI-PLC activity
correlates with the initial rise in DAG production and Ca\(^{2+}\) mobilisation,
evidence is accumulating to suggest that a wide variety of stimuli are capable
of generating sustained DAG production by the actions of both PC-PLC and
PC-PLD in diverse cell types (reviewed by Exton, 1990). For example,
treatment of hepatocytes with vasopressin or angiotensin II results in a slow
and sustained DAG response that does not correlate with PI breakdown
stimulated by these agonists (Bocckino et al., 1987). In addition, the
composition of receptor-induced PA and DAG was consistent with significant
breakdown of PC occurring; DAG produced as a consequence of PC
breakdown contains more palmitic, oleic and linoleic acid than DAG resulting
from PI metabolism which is relatively enriched in stearic and arachidonic
acids. Production of choline and phosphocholine was also observed,
providing further indirect evidence for PC-PLC and PC-PLD stimulation.
Similar results have been obtained in fibroblasts stimulated with bombesin,
PDGF (Price et al., 1989; Larrodera et al., 1990), EGF (Wright et al., 1990), \(\alpha\)-thrombin (Pessin and Raban, 1989) or muscarinic agonists (Martinson et al.,
1989) and neutrophils stimulated with fMet-Leu-Phe (Cockcroft and Allen,
1984; Billah et al., 1989).

Interestingly, PKC has been demonstrated to promote PC turnover in
many cell types and phorbol esters have been demonstrated to induce the
appearance of choline and phosphocholine by a pathway that involves
stimulation of PC-PLD and possibly PC-PLD activity (reviewed by Exton,
1990). In this respect, the ability of an oncogenic p21\(^{ras}\) protein to induce
production of choline and phosphocholine, correlating with an increase in levels of cellular DAG, has been shown to require functional PKC in Swiss 3T3 fibroblasts (Lacal et al., 1987; Price et al., 1989).

The fact that PKC can stimulate PC metabolism suggests the possible existence of a pathway in which the initial transient production of DAG, as a consequence of agonist-induced PI hydrolysis, might activate PKC and give rise to a sustained increase in cellular DAG levels by promoting hydrolysis of PC (Exton, 1990). Although speculative, such a positive feedback pathway might facilitate the sustained activation of PKC required for mediation of cellular activation. The physiological relevance of PC-derived DAG species is suggested by their ability to regulate PKC activity in vitro (Nishizuka, 1988). However, a recent study casts doubt on their role by demonstrating that PKC activation in response to α-thrombin, measured by assessing the phosphorylation of the 80kDa cellular PKC substrate, correlates with the initial PI-derived DAG increase and not the later, more sustained, PC-derived rise (Leach et al., 1991). This result suggests that PC-derived DAG may not make a significant contribution to receptor stimulation of PKC activity in vivo. Clearly, the contribution made by alternative sources of DAG to PKC activity will require further investigation.

1.19 Regulation of PI-specific PLC.

The purpose of multiple PI-PLC isoforms remains unclear. Since multiple isozymes are differentially expressed in many cells, an explanation cannot be provided by simple tissue differences. One possible difference between PI-PLC isozymes concerns the mechanism by which the ligand binding signal is transduced from activated receptors to effect increased PI-PLC activity. Evidence has accumulated to indicate that many receptors employ a guanine nucleotide binding protein (G protein) to couple to PI-PLC. Recent studies in Swiss 3T3 fibroblasts have suggested that PI-PLC-γ1 and PI-
PLC-γ2 are phosphorylated on tyrosine residues in cells responding to PDGF or EGF and that this phosphorylation event is the mechanism by which PDGF and EGF receptors stimulate PI-PLC activity. Although these cells also express PLC-β and δ, growth factor treatment only results in phosphorylation of the PI-PLC-γ, suggesting that this mechanism may be specific for the γ isozymes (Nishibe et al., 1989). Bombesin, bradykinin or vasopressin, agonists which have been suggested to stimulate PI-PLC via a G protein, do not stimulate phosphorylation of PI-PLC-γ in these cells (Wahl et al., 1989; Meisenhelder et al., 1989). Thus, it appears possible that different receptors may couple to different PI-PLC isozymes and/or may use different mechanisms to transduce the ligand binding signal to PI-PLC. In this respect, northern and western blot analysis have recently provided evidence that the T leukaemic line Jurkat express PI-PLCα, PI-PLCβ1, γ1 and δ1 isozymes (Goldfien et al., 1991; Park et al., 1991).

1.19.1 Regulation of PI-PLC by growth factor receptor tyrosine kinases.

Since stimulation of PI-PLC activity is one of the earliest events induced by the binding of PDGF and EGF to their respective receptors, the observation that PI-PLCγ1 becomes physically associated with these receptors and is phosphorylated on tyrosine residues suggests that tyrosine phosphorylation might be the mechanism by which PI-PLC activity is regulated by these growth factor receptors (Nishibe et al., 1989; Wahl et al., 1989; Margolis et al., 1989; Meisenhelder et al., 1989; Kumjian et al., 1989). This theory is supported by the fact that mutant PDGF and EGF receptors that lack tyrosine kinase activity fail to stimulate PI hydrolysis (Moolenar et al., 1988; Escobedo et al., 1988; Margolis et al., 1990a). EGF-induced phosphorylation of PI-PLC-γ1 is of a high stoichiometry (>50% of cellular PI-PLC-γ1) and involves multiple sites. Purified EGF and PDGF receptors phosphorylate three major sites in vitro: Tyr771, 783 and 1254. These residues have also been identified to be phosphorylated in
intact cells (Kim et al., 1990; Margolis et al., 1989). Interestingly, Tyr771 and 783 are close to the conserved SH2 and SH3 domains of PI-PLC-γ1 which are thought to regulate its association with other tyrosine phosphoproteins (reviewed by Pawson 1991).

Although a strong correlation exists between the overall state of tyrosine phosphorylation of PI-PLC-γ1 and growth factor-induced inositol phosphate production, attempts to demonstrate a causal relationship between these two events have proven difficult. Amongst the strongest evidence has been the demonstration that overexpression of PI-PLC-γ1 or γ2 in fibroblasts results in enhanced tyrosine phosphorylation of these enzymes and the inositol phosphate response to PDGF (Margolis et al., 1990b; Sultzman et al., 1991).

A recent study has provided strong evidence that PDGF-induced PI-PLC activity is a direct consequence of tyrosine phosphorylation by expressing normal and mutant PI-PLC-γ1 isozymes in NIH 3T3 cells. Mutation of Tyr783 to phenylalanine, preventing its phosphorylation, completely blocked the activation by PDGF whereas mutation of Tyr1254 impaired and mutation of Tyr771 enhanced the response (Kim et al., 1991). Interestingly, the fact that basal PI metabolism was not enhanced in these overexpressing cells suggests that stimulation of PI-PLC-γ1 activity by PDGF may involve the removal of a negative modulatory effect. In this respect, profilin, a cytosolic actin and phosphatidylinositol binding protein has been observed to inhibit PI hydrolysis by unphosphorylated PI-PLC-γ1 but not enzyme that had been phosphorylated by purified EGF receptor (Goldshmidth-Claremont et al., 1991). Another report has demonstrated an increase in in vitro PI-PLC-γ1 catalytic activity on EGF treatment of A431 cells (Nishibe et al., 1990). In this study, PI-PLC activity was assayed in vitro and the stimulatory effect was only observed at certain concentrations of Triton X-100 detergent. Whether these in vitro conditions mimic the effect of the endogenous repressor activity, hypothesised
to exist *in vitro*, is unclear. Clearly, important questions concerning the regulation of PI-PLC activity by tyrosine phosphorylation remain unanswered.

### 1.19.2 Guanine nucleotide binding proteins and receptor-effector coupling.

Proteins which bind and hydrolyse GTP have been implicated in the regulation of a wide variety of biological processes in all organisms. These molecules can be grouped into several families according to structure and function: 1) Initiation and elongation factors which direct ribosomal protein synthesis. 2) Signal recognition particles which translocate nascent proteins to the endoplasmic reticulum (Bourne *et al.*, 1990). 3) Proteins such as dynein which guide vesicular traffic (Obar *et al.*, 1990). 4) Small GTP binding proteins of the ras superfamily which have diverse cellular functions including the regulation of cell growth and differentiation (reviewed by Hall, 1990; Downward, 1990). 5) Signal transduction G proteins which mediate transmembrane signalling to effector molecules by hormones and light (reviewed by Gilman, 1987; Bourne *et al.*, 1990; Birnbaumer, 1990). All of these proteins cycle from a GTP-bound state, in which they are active for their particular function, to an inactive GDP bound state via a hydrolytic GTPase step.

Analysis of the mechanism of activation of receptor-linked adenylate cyclase and light activated cyclic GMP phosphodiesterase has demonstrated a mediatory and mandatory role for G proteins in the transduction of signals to their respective effector enzyme. For example, β-adrenergic receptor regulation of adenylate cyclase is reciprocally mediated by stimulatory and inhibitory G proteins termed Gs and Gi (reviewed by Gilman, 1987).

Signal transducing G proteins are heterotrimers consisting of a guanine nucleotide binding α subunit which, when in the inactive GDP-bound state, is associated with β and γ subunits. To date, eight distinct α subunits have been biochemically identified and can be associated with the transmission of
specific signals. Since cDNAs encoding several more α subunits have been isolated, it appears likely that a multigene family of these proteins exists (Strathman and Simon, 1990). In addition to an intrinsic GTPase activity, the α subunits (ranging from 39-52kDa in molecular weight) are thought to provide the receptor and effector specificity required of such a signal transduction and amplification system. In contrast, the multiple β (35-36kDa) and γ (8kDa) subunits that have been identified are virtually identical and functionally interchangeable. Although it is clear that the α subunit regulates effector activity, βγ dimers are required for interaction with activated receptor and have been implicated in the regulation atrial K+ channel opening by muscarinic receptors (Okabe et al., 1990).

In systems where G protein involvement has been best characterized, the activation process mediated by these transducers can be considered to be cyclic (reviewed by Gilman, 1987). The α subunit of the inactive G protein trimer is occupied by GDP (αβγ.GDP). Interaction with the appropriate activated receptor stimulates the otherwise slow rate of dissociation of GDP. Because GTP is in considerable molar excess over GDP in the cytoplasm, exchange for GTP is invariably the consequence of dissociation of GDP. GTP binding to the α subunit then triggers dissociation of the αβγ complex from the activated receptor and separation of α.GTP from βγ. The α.GTP complex then interacts with and directly regulates the effector enzyme. Hydrolysis of bound GTP by the intrinsic GTPase activity of the α subunit subsequently terminates its interaction with the effector and induces reassociation of α.GDP with βγ. The resulting αβγ.GDP complex is available for activation by another activated receptor (see diagram 1.14).

Amplification is provided by the fact that each activated receptor can stimulate the formation of more than one α.GTP which may persist for longer than the agonist-receptor complex. For example, it has been estimated that in the adenylate cyclase system, the hormone-receptor and α.GTP complexes
Persist for less than one second and 10 seconds respectively. There is also evidence from several G protein coupled systems which suggests that βγ dimers act to improve the signal to noise ratio by selectively inhibiting coupling in the absence of agonist (Gilman, 1987).

It is now clear that many other G proteins are involved in cellular signal transduction. For example, transducin (Gt) mediates visual signal transduction from light-activate rhodopsin, GOff mediates olfactory signal transduction (Casey et al., 1990), Go is a G protein regulated by the growth cone protein GAP-43 in the tip of neurites in growing cells (Strittmatter et al., 1990) and multiple forms of Gs and Gi serve to regulate K+ channels in many tissues (reviewed by Birnbaumer, 1990). Evidence for G protein involvement in a particular receptor-regulated process can be derived from several
experimental approaches. In membrane preparations or permeabilised cells, receptor regulation of effector activity should be absolutely dependent on the presence of GTP and stable analogues of GTP should synergise with receptor triggering for activation of effector function (reviewed by Gilman, 1987). In addition, bacterial toxins which catalyse the transfer of ADP-ribose onto certain amino acid residues in the α subunit of many G proteins have proved invaluable tools for the study of G protein-mediated processes. For example, the A subunits of Pertussis toxin (PTX) ribosylates a cysteine residue in Giα, Goα and Giα (Katada and Ul, 1982; McLeish et al., 1989) while Cholera toxin (CTX) modifies Gsα (Cassel and Pfeuffer, 1978). Treatment with both toxins results in the persistent activation of activate adenylate cyclase by inhibiting receptor-induced nucleotide exchange on Giα and inhibiting the GTPase activity of Gsα. Reconstitution of purified receptor, G protein and effector components as a functional signal transduction system in an exogenous cell-free system, representing the definitive demonstration of G protein involvement, has only been achieved for β-adrenergic receptor regulation of adenylate cyclase by Gs (May et al., 1985; Feder et al., 1986).

1.19.3 Regulation of PI-PLC by G proteins.

The first evidence that PI-PLC activity could be regulated by a G protein came from studies which demonstrated that stable analogues of GTP could stimulate production of DAG in permeabilised platelets (Haslam and Davidson, 1984) and that receptor-stimulated inositol phosphate production in blowfly salivary gland membranes and human neutrophils (Litosh et al., 1985; Cockcroft and Gomperts, 1985). Since these initial discoveries, the use of bacterial toxins and guanine nucleotide analogues has provided evidence that G proteins mediate PI-PLC activation by adrenergic (Burch et al., 1986), purinergic (Stuchfield and Cockcroft, 1988; Boyer et al., 1989a; 1989b) and muscarinic receptors (Burch et al., 1986; Ashkenazi et al., 1989; Claro et al.,
1989) bombesin and vasopressin (Cattano and Vicentini, 1989), f-Met-Leu-Phe (Smith et al., 1986; Bradford and Rubin, 1986; Cockcroft and Stuchfield, 1989; McLeish et al., 1989) and thrombin (Paris and Pouyssegur, 1986; Brass et al., 1986; Huang and Ives, 1989).

Analysis of the activation of PI-PLC activity by guanine nucleotides and purinergic agonists in turkey erythrocyte membranes has provided the strongest evidence that a coupling mechanism exists for PI-PLC that is analogous to that for the regulation of adenylate cyclase (Boyer et al., 1989a; 1989b). Receptor stimulation accelerates the rate of activation of PI-PLC by stable GTP analogues, such as GTP[S], in exactly the same way as β-adrenoreceptor stimulation of adenylate cyclase in the same membranes, indicating that the receptor functions to stimulate guanine nucleotide exchange on the putative PI-PLC-regulatory G protein. Moreover, rapid decay of the activated G protein species occurs when GTP, but not its non-hydrolysable analogues, is used in the activation step. This suggests a role for GTP hydrolysis in the coupling process as for G protein regulation of adenylate cyclase.

Differential G protein coupling of receptors to PI-PLC in different systems is inferred by the ability of PTX to attenuate receptor coupling to PI hydrolysis in some systems but not others. In order to account for these results, it has been suggested that receptor-PI-PLC coupling in PTX-sensitive systems, such as is mediated by a G protein similar to G_i or G_o while a distinct G protein functions in PTX-insensitive systems, such as hepatocytes (reviewed by Cockcroft, 1987). In addition, distinct muscarinic acetylcholine receptor subtypes have been demonstrated to differentially couple to PI-PLC via PTX-sensitive and insensitive G_p proteins in the same cell, reinforcing the concept that multiple G_p s can exist in a single cell and can couple different receptors to the same effector (Ashkenazi et al., 1989). A single receptor subtype can also couple to multiple G proteins, stimulating both adenylate cyclase and PI-PLC.
(Ashkenazi et al., 1989). A further level of complexity is suggested by studies which have identified a permissive role for a pertussis toxin-sensitive G protein in receptor-activated G-protein-mediated regulation of PI-PLC (Okajima et al., 1989). Thus, in addition to a high degree of receptor and effector specificity, G protein mediated systems appear to possess a capacity for cross-talk between coupling systems.

Evidence is accumulating to suggest that other receptor-stimulated phospholipases in addition to PI-PLC, such as PC-PLC (Irving and Exton, 1987; Bocckino et al., 1987; Martin and Michaelis, 1990), PLD (Bocckino et al., 1987) and PLA2 (Burch et al., 1986), may be regulated by G proteins. Some receptors, such as the α1-adrenergic receptor of FRTL5 cells, have been demonstrated to couple to both PI-PLC and PLA2 by separate G proteins that can be distinguished by PTX sensitivity (Burch et al., 1986; Cockcroft and Stuchfield, 1989). Many G protein-coupled receptors appear to share a structural motif in that, within their single polypeptide, they possess seven α-helical transmembrane segments (reviewed by Gilman, 1987). Although at one time this feature was considered to be a requirement for G protein coupling, several receptors that do not conform to this model are now known to be G protein coupled.

A G protein α subunit has recently been purified from bovine brain membranes that were preactivated with GTP[S] by virtue of its ability to associate with and activate PI-PLC (Taylor et al., 1990). This 42kDa, PTX-insensitive, α subunit is not recognised by antibodies to known G proteins and has subsequently been classified as a member of the recently-identified Gq class (Taylor et al., 1991). Originally identified by homology cloning, studies have identified the existence of multiple related αq proteins with differential tissue distributions (Strathmann and Simon, 1990). Another G protein α subunit, purified from bovine liver by βγ-agarose chromatography, has also been demonstrated to regulate PI-PLC in response to AlF4⁻ (Smrcka et al.,
This 42kDa α subunit is also immunologically unique and PTX-insensitive. The relationship between these proteins remains to be determined but they clearly represent candidates for the G protein that mediates PTX-insensitive receptor-stimulated PI breakdown in these cells.

This idea has been strengthened by further analysis of the properties of the bovine liver-derived αq protein in mixed phospholipid vesicles containing purified PI-PLC-β1 (Taylor et al., 1991). Reconstitution of αq with PI-PLC-β1 stimulated a 2-3 fold increase in PI(4,5)P2 hydrolysis. This effect was unaffected by the concentration of Ca2+ suggesting stimulation by αq is not the result of an increase in the affinity of PI-PLC-β1 for Ca2+. Since maximal activation of PI(4,5)P2 hydrolysis was observed to require a molar ratio of 20:1 (αq:PLC-β1), it appears that αq is not a very efficient stimulator of PLC-β1 activity in this system. However, this analysis extended to an investigation of the PI-PLC isozyme specificity of αq-mediated stimulation. Interestingly, αq did not stimulate PI(4,5)P2 hydrolysis by either purified PI-PLC-γ1 or PI-PLC-δ1. Furthermore, preincubation of bovine liver membranes with antibodies against PI-PLC-β1, but not with antibodies against PI-PLC-γ1 or δ1, abolished αq stimulation of PI-PLC activity. Since these membranes contain multiple PI-PLC isozymes, including significant amounts of PI-PLC-α, it appears likely that the major target for regulation by αq in this system is the β1 isozyme of PI-PLC. A full appreciation of the contribution made by the Gq class of G protein to regulation of PI-PLC-β1 activity awaits expression of the relevant cDNAs in appropriate cells.

These studies suggest that several mechanisms may exist for regulation of PI-PLC activity. At this stage, all that can be clearly stated is that PI-PLC isozymes may be regulated by tyrosine phosphorylation or by a G protein mechanism. The possibility also exists that some isozymes may be regulated by both pathways or by another uncharacterised mechanism.
Evidence for PI-PLC regulation by tyrosine phosphorylation in T lymphocytes.

Evidence is accumulating to suggest that tyrosine phosphorylation may play a role in coupling the TCR/CD3 complex to PI-PLC. Analysis of the kinetics of induction of tyrosine phosphorylation on TCR/CD3 ligation has revealed that increased tyrosine phosphorylation of 100kDa and 135kDa cellular substrates can be observed within 5 seconds of stimulation of T lymphoblasts or Jurkat (June et al., 1990a). These phosphorylation events were observed to precede TCR/CD3-induced inositol phosphate production and Ca\(^{2+}\) mobilisation by about 10 seconds, raising the possibility of a causal linkage between tyrosine phosphorylation and activation of PI-PLC. Although the two substrates of interest were not identified and neither substrate could be precipitated by mAbs raised against PI-PLC isozymes expressed in bovine brain, the possibility that these substrates represents a PI-PLC or regulator of the enzyme remains an intriguing possibility.

Possibly the most convincing evidence for the involvement of tyrosine phosphorylation in the regulation of PI-PLC activity by the TCR/CD3 complex comes from the study of variants of the human T leukaemic cell lines HPB-ALL and Jurkat which does not express the tyrosine phosphatase CD45 (Koretzky et al., 1990). These CD45-negative cells, unlike CD45 expressing variants, failed to generate inositol phosphates or mobilise Ca\(^{2+}\) in response to TCR/CD3 stimulation. Furthermore, reconstitution of CD45-negative HPB-ALL cells with cDNA encoding CD45 restored the ability of TCR/CD3 stimulation to induce PI-PLC activity. The fact that expression of this protein tyrosine phosphatase appears to be essential for coupling of the TCR/CD3 to PI-PLC strongly implicates tyrosine phosphorylation as a mediator of signal transduction from the antigen receptor complex and underlines the potential contribution that phosphatases might make to these pathways.

A number of recent studies have employed a variety of inhibitors to probe the function of tyrosine kinases in T cells. Herbimycin A is an antibiotic...
that was found to inhibit tyrosine phosphorylation and to reverse oncogenic transformation mediated by pp60\textsuperscript{v-src} (Uehara et al., 1989). Inhibition of cellular responses with herbimycin requires a period of 12-24 hours pre-incubation and appears to function by targetting tyrosine kinases for degradation rather than competitive inhibition of enzymic activity. T cells treated in this manner demonstrated impaired TCR/CD3-mediated tyrosine substrate phosphorylation, PI-PLC activity and IL2/IL2 receptor induction (June et al., 1990b). The ability of phorbol esters in conjunction with ionomycin to induce T cell proliferation and of AlF\textsubscript{4}\textsuperscript{-}, presumed to activate G proteins, to stimulate PI-PLC activity demonstrates some degree of specificity for these effects.

The isoflavone compound genistein has been shown to competitively inhibit EGF receptor- and pp60\textsuperscript{c-src}-mediated tyrosine phosphorylation (Akiyama et al., 1987). Two groups have reported the ability of this compound to inhibit TCR/CD3- or mitogen-stimulated tyrosine phosphorylation and IL2/IL2R induction in human peripheral blood-derived T cells (Mustelin et al., 1990; Trevillyan et al., 1990). Although these studies agree with respect to the inhibitory effect of genistein on the ability of the TCR/CD3 complex to induce IL2R expression and IL2 production, they report contradictory effects on TCR/CD3-induced PI-PLC activity. Thus, Mustelin and his colleagues demonstrate inhibition of TCR/CD3-mediated inositol phosphate production whilst Trevillyan and co-workers report no inhibitory effect on PI-PLC activity. A possible explanation for this disparity is suggested by observations made by both groups concerning the non-specific effects of genistein on cellular responses that are not considered to be mediated by tyrosine kinases. In this respect genistein was observed to inhibit phorbol ester-stimulated phosphorylation of the \(\gamma\) subunit of the CD3 antigen and T cell proliferation induced by phorbol esters and ionomycin (Mustelin et al., 1990; Trevillyan et al., 1990). Clearly, the biological effects of this compound cannot be
exclusively attributed to its inhibition of tyrosine kinases. The data derived from use of these various inhibitors suggests that tyrosine phosphorylation induced by ligation of TCR/CD3 plays an obligatory role in induction of IL2 production. The results are less conclusive concerning the role of tyrosine phosphorylation as a mechanism for regulation of PI-PLC by the TCR/CD3 complex.

In the light of evidence that PI-PLC-γ1 and γ2 activity is modulated by tyrosine phosphorylation, the strongest evidence for the involvement tyrosine phosphorylation in the regulation of PI-PLC activity can be considered to be the observation of a ligand-induced increase in enzymic activity on tyrosine phosphorylation of the enzyme. Technically, this requires the reciprocal immunoprecipitation of increased PI-PLC activity with mAbs specific for phosphotyrosine and increased phosphotyrosine content of specific PI-PLC-γ1 immunoprecipitates. Despite the accumulation of evidence suggesting a regulatory role for tyrosine phosphorylation, these rigorous criteria have not yet been fulfilled for TCR/CD3-induced activation of PI-PLC (June et al., 1990a).

1.21 Evidence for PI-PLC regulation by G proteins in T lymphocytes.

Much of the evidence suggesting that the TCR/CD3 complex is coupled to PI-PLC by a G protein has been derived from studies using the T leukaemic cell line Jurkat in conjunction with bacterial toxins such as cholera and pertussis toxins (CTX and PTX). In T lymphocytes, CTX and PTX catalyse the ADP ribosylation of a 43kDa Gsα-like and 41kDa Gα-like membrane-associated proteins respectively (Moingeon et al., 1990). Initial studies showing that pretreatment of Jurkat with CTX inhibited TCR/CD3-induced Ca2+ mobilisation, inositol phosphate production and IL2 production were interpreted as evidence that Gsα or a similar G protein was the active coupling agent (Imboden et al., 1986; Aussel et al., 1988). However, interpretation of
these results is complicated by the fact that modification of Gsα by CTX also results in the activation of adenylate cyclase with consequent production of cyclic AMP. Reports that address the effect on TCR/CD3-mediated inositol phosphate production of agents which elevate cyclic AMP concentrations are confused in their conclusions, suggesting that cyclic AMP completely (Patel et al., 1987), partially (Stewart et al., 1989) or does not (Imboden et al., 1986) inhibit inositol phosphate production in Jurkat. Thus elevation of cyclic AMP could partially explain the observed effects of CTX on TCR/CD3 signaling.

A recent study has demonstrated that CTX treatment of Jurkat cells results in a loss of the TCR/CD3 complex from the cell surface. This TCR/CD3 complex down-regulation could not be mimicked by elevation of cyclic AMP with forskolin, suggesting that CTX mediates it's inhibitory effect on TCR/CD3 signaling by acting at the receptor level (Sommermeyer et al., 1990). Pertussis toxin, which is mitogenic for T cells, also appears to mediate it's effects by interaction with and modification of a cell surface receptor rather than by the ADP-ribosylation (Rosoff et al., 1987). Thus, the inhibition of TCR/CD3-induced PI-PLC activity observed after pre-treatment with PTX holotoxin is mediated by the mitogenic B subunit of the toxin and does not require ADP-ribosylation catalysed by the A protomer (Gray et al., 1989). These characteristics of CTX and PTX render them of limited use in the investigation of the role of G proteins in coupling TCR/CD3 to PI-PLC in T cells.

More direct evidence for G protein involvement in TCR/CD3 signaling comes from studies which employ permeabilised cell or membrane systems in conjunction with poorly hydrolysable analogues of GTP such as GTP[S]. The ability of guanine nucleotide analogues to regulate the activity of PI-PLC in murine thymocytes and Jurkat membranes suggests the presence of a regulatory Gp activity which is coupled to PI-PLC in T cells (Zilberman et al., 1987; Sommermeyer et al., 1990). In addition, the fluoroaluminate ion AlF₄⁻, can mimic GTP occupancy of the guanine nucleotide binding site of G protein
α subunits in conjunction with bound GDP. This pharmacological agent has been demonstrated to induce phosphatidylinositol metabolism, the elevation of [Ca\(^{2+}\)], and phosphorylation of the γ subunit of the CD3 antigen in murine T cells (O'Shea et al., 1987). Although these data are suggestive of G protein involvement in TCR/CD3 coupling to PI-PLC, AlF\(_4^-\) mimics γ-phosphate in other cellular circumstances to influence the activity of many kinases and phosphatases, complicating interpretation of results obtained (O'Shea et al., 1987). Transfection of a functional muscarinic receptor into TCR/CD3 signaling-deficient Jurkat mutants provides further evidence that T cells contain the G protein required for coupling this heterologous receptor to PI-PLC (Goldsmith et al., 1989).

Although these lines of evidence support a role for a G protein in the regulation of PI-PLC, the relationship of this G protein to the TCR/CD3 complex remains unclear. Elevation in the activity of the enzyme ornithine decarboxylase (ODC) is an early event associated with mitogenesis in many cells including T cells. Increased ODC activity can be stimulated by mAbs directed against the TCR/CD3 complex and has been demonstrated to correlate with TCR/CD3-induced PI-PLC activity (Mustelin et al., 1986). The requirement for GTP demonstrated by the TCR/CD3 complex for the induction of ODC activity and the ability of GTP[S] to stimulate ODC activity in a permeabilised cell system has been interpreted as evidence for direct G protein coupling of the TCR/CD3 complex to PI-PLC (Mustelin, 1987).

A second study has provided evidence for indirect G protein coupling of the TCR/CD3 complex to exocytotic secretion of serine esterase by a cytotoxic T cell line (Schrezenmeier et al., 1988). In these cells, exocytotic secretion can be triggered by TCR/CD3 stimulation or by agents which mimic PI-PLC activity, such as phorbol esters and Ca\(^{2+}\). Introduction of GTP[S] into permeabilised cells was observed to trigger exocytotic secretion, suggesting the involvement of a G protein. Furthermore, the ability of GDP[S] to antagonise TCR/CD3-
induced exocytosis but not that stimulated by phorbol esters implies that this G protein is downstream of TCR/CD3 but upstream of PI-PLC. Downregulation of PKC by chronic treatment with phorbol esters inhibited GTP[S]-induced exocytosis, as did downregulation of surface expression of the TCR/CD3 complex by prolonged incubation with mAbs specific for the receptor itself (Pantaleo et al., 1987). The authors conclude that a G protein, which is physically associated with the TCR/CD3 complex, couples this receptor to PI-PLC and thence to secretion (Schrezenmeier et al., 1988). Since neither of these studies directly address the question of G protein coupling of the TCR/CD3 complex to PI-PLC, the contribution made by G proteins to the regulation of PI-PLC by the TCR/CD3 complex remains to be investigated.

1.22 The p21ras guanine nucleotide binding proteins and growth regulatory pathways.

As a consequence of their oncogenic potential and membrane localization, the guanine nucleotide binding proteins encoded by the ras genes have been suggested to play a critical role as signal transduction components of cellular growth regulatory pathways. Ras genes were first identified as oncogenes carried by the transforming Harvey and Kirsten rat sarcoma viruses (v-Ha-ras and v-Ki-ras). A third closely related oncogene was subsequently detected in a human neuroblastoma (N-ras). The mammalian cellular homologues of these, the ras proto-oncogenes (Ha-ras, Ki-ras and N-ras), have been identified in all vertebrates (reviewed by Barbacid, 1987). The ras proteins are the prototypes of a class of small (20-30kDa) monomeric guanine nucleotide binding proteins that appear to be involved in diverse cellular activities (reviewed by Hall, 1990; Downward, 1990). In addition, close homologues have been detected in lower eukaryotes such as saccharomyces cerevisiae (reviewed by Tamanoi, 1988), Schizosaccharomyces pombi (Fukui and Kaziro, 1985) and drosophila (Schejter and Shilo, 1985).
The mammalian ras genes encode closely related 21kDa molecular weight proteins (p21ras) that bind guanosine triphosphate (GTP) and catalyse its hydrolysis to guanosine diphosphate (GDP) (reviewed by barbacid, 1987; Bourne et al., 1990). Comprising 189 amino acids (Ha-ras and N-ras) or 188 amino acids (Ki-ras), a maximum of 13 amino acid differences exist between them over their first 165 amino acids. The only region where there is significant diversity between the p21ras proteins lies between amino acids 164 and 185 where they are only 10% homologous. A conserved CAAX consensus sequence, comprising the last four C-terminal amino acids (C=cysteine, A=any aliphatic amino acid and X=any uncharged amino acid), undergoes post-translational modification consisting of the attachment of a lipid polyisoprenyl group to the cysteine (Hancock et al., 1989), cleavage of the three C-terminal amino acids and carboxy methylation of the isoprenylated terminal cysteine (Clarke et al., 1988; Gutierrez et al., 1989). Ha-ras and N-ras subsequently undergo palmitoylation of upstream cysteines that are lacking in Ki-ras (Gutierrez et al., 1989; Hancock et al., 1989; 1990). The function of all these modifications is to localize p21ras to the plasma membrane. In this respect, isoprenylation is required but not sufficient for membrane localization of ras proteins (Hancock et al., 1990). The palmitoylation of Ha-ras and N-ras ensures their membrane localization whereas a string of basic residues close to the C terminal of Ki-ras appears to be necessary for its targeting to the membrane. For all forms of p21ras, membrane localization is essential for biological function (Hancock et al., 1990).

1.22.1 Oncogenic mutations and the biological activity of p21ras.

Activated ras oncogenes, derived from the cellular proto-oncogenes by point mutations at codons 12, 13 and 61, have been found in around 30% of human tumours (reviewed by Bos, 1988). In addition, mutations generated at 59, 63, 116, 117 and 119 also result in oncogenically active ras proteins as
determined by their capability to transform mammalian cells in culture (Walter et al., 1986; Sigal et al., 1986; Reynolds et al., 1987). Oncogenic mutations all act to retain p21\textit{ras} in the GTP-bound state but can be devided into two groups according to the apparent mechanism by which they achieve this result. Mutations at residues 12, 13, 59, 61 and 63 lead to a reduction in the GTPase activity of the protein by up to 10-fold (Sweet et al., 1984; McGrath et al., 1984; Seeburg et al., 1984; Barbacid, 1987). However, a discrepancy was observed between the reduction in \textit{in vitro} GTPase activity and transforming ability of the mutants (Lacal et al., 1986; Trahey et al., 1987). This paradox was resolved by discovery of the GTPase activating protein (GAP) for p21\textit{ras} and the observation that although oncogenic proteins bind (\textit{ras})GAP, they are insensitive to its GTPase-stimulating activity (Trahey and McCormick, 1987; Vogel et al., 1988). Thus, the difference between GTPase activity of normal and oncogenic \textit{ras} proteins \textit{in vivo} is over 1000-fold.

In contrast, mutations at codons 116, 117, 119 and 146 increase the rate of exchange of bound GDP for GTP (Der et al., 1986; Walter et al., 1986; Sigal et al., 1986; Reynolds et al., 1987). The fact that oncogenic mutations in \textit{ras} serve to induce it's accumulation in the GTP-bound state supports the hypothesis that, by analogy with other GTP binding proteins, p21\textit{ras}.GTP is the active state for generation of biological signals. More direct support for this hypothesis has been derived from studies in which the microinjection of GTP-but not GDP-bound p21\textit{ras} results in the induction of effects associated with the oncogenic protein (Trahey and McCormick, 1987; Satoh et al., 1988).

Furthermore, mutant Ras proteins that preferentially bind GDP have a dominant negative effect on the growth of mammalian cells and \textit{s. cerevisiae} (Feig and Cooper, 1988; Powers et al., 1989).

Introduction of oncogenic \textit{ras} into fibroblast cell lines stimulates morphological transformation and, in the presence of insulin-like growth factor (IGF-1), can induce complete malignant transformation as determined by
The acquisition of the ability to grow in low serum conditions, to high saturation densities and in an anchorage-independent manner (Morris et al., 1989; reviewed by Barbacid, 1987; Marshall, 1991). These changes can be induced by levels of oncogenic p21\textsuperscript{ras} that are equivalent to endogenous levels of normal Ras. When over-expressed at levels corresponding to 20 to 100-fold higher than endogenous levels, normal p21\textsuperscript{ras} can also induce transformation in some cell types (Rickets and Levinson, 1988). Microinjection of oncogenic p21\textsuperscript{ras} into \textit{xenopus} oocytes induces maturation, characterised by progression from prophase to metaphase of meiosis and accompanied by germinal vesicle breakdown (Birchmeier et al., 1985). While it is clear that microinjection of oncogenic \textit{ras} can induce DNA synthesis and proliferation in fibroblast lines, introduction of p21\textsuperscript{ras} into PC12 pheochromocytoma and Schwann cells blocks proliferation and promotes cellular differentiation (Bar-Sagi and Feramisco, 1985; Sugimoto et al., 1988; Ridley et al., 1988). Thus it is possible that although p21\textsuperscript{ras} plays a role in the regulation of cell growth, mammalian cells may be programmed to respond differently to the signals generated.

1.2.2.2 Cellular factors which regulate p21\textsuperscript{ras}.

By analogy with the effects of oncogenic mutations and other guanine nucleotide binding proteins, the ratio of GTP:GDP on p21\textsuperscript{ras} can be regulated in a cyclic fashion by altering the rate of guanine nucleotide exchange onto p21\textsuperscript{ras} or the rate of hydrolysis of p21\textsuperscript{ras}-bound GTP (reviewed by Bourne et al., 1990).

The intrinsic rate of GTP hydrolysis by normal p21\textsuperscript{ras}, measured \textit{in vitro}, is in the order of 10^{-2} \text{ min}^{-1} (McGrath et al., 1984; Cales et al., 1988). However, analysis of nucleotides bound to p21\textsuperscript{ras} that had been microinjected into \textit{Xenopus} oocytes suggested that hydrolysis of GTP to GDP occurred very much faster \textit{in vivo} than \textit{in vitro}. This observation led to the identification of a predominantly cytosolic protein (GTPase activating protein; GAP) which binds
both normal and oncogenic p21\textsuperscript{ras} but only activates the GTPase activity of the normal protein (Trahey and McCormick, 1987). The 116kDa (ras)GAP protein has been purified and cloned from bovine brain and human placenta and appears to consist of an N-terminal hydrophobic domain and a C-terminal catalytic domain (Gibbs \textit{et al.}, 1988; Vogel \textit{et al.}, 1988; Trahey \textit{et al.}, 1988). In this respect, a fragment consisting of the 343 C-terminal amino acids is sufficient to stimulate p21\textsuperscript{ras} GTPase activity with with an efficiency similar to that of the intact protein (Marshall \textit{et al.}, 1989). The affinity of (ras)GAP for p21\textsuperscript{ras} is quite low (binding constants are in the order of 0.1 mM for p21\textsuperscript{ras}.GTP and >1 mM for p21\textsuperscript{ras}.GDP). Although the (ras)GAP.p21\textsuperscript{ras}.GTP complex is likely to be short-lived, (ras)GAP accelerates the GTPase activity of p21\textsuperscript{ras} by up to 20,000-fold and maintains cellular p21\textsuperscript{ras} in a predominantly GDP-bound state. (ras)GAP has subsequently been found in all higher eukaryotes, but not in yeast (Adhari \textit{et al.}, 1988). Sequence analysis reveals no striking homology to other proteins apart from two SH2 domains and one SH3 domain, motifs found in \textit{src} family kinases and several other proteins, in the N-terminal region (Vogel \textit{et al.}, 1988).

The recent cloning of the gene at the locus responsible for the inherited malignancy neurofibromatosis type 1 (NF1) revealed an open reading frame of around 2500 amino acids that was found to contain a region (amino acids 840-1200) that displayed striking homology to the catalytic domain of mammalian (ras)GAP and the protein products of the IRA1 and IRA2 genes of \textit{S.cerevisiae} (Xu \textit{et al.}, 1990a; Tanaka \textit{et al.}, 1990a; 1990b; Buchberg \textit{et al.}, 1990). Since inactivation the ubiquitously expressed NF1 gene by deletion or point mutation is associated with malignant transformation, it probably represents a tumour repressor or anti-oncogene (Viskochil \textit{et al.}, 1990; Wallace \textit{et al.}, 1990). The region of NF1 with homology to (ras)GAP were subsequently demonstrated to functionally replace the IRA genes when expressed in \textit{S.cerevisiae} and stimulate the GTPase activity of normal but not
oncogenic mammalian Val-12 p21ras (Xu et al., 1990b; Ballester et al., 1990; Martin et al., 1990). The biological implications of the existence of multiple cellular proteins with p21ras-GTPase stimulating activity are unclear.

Diagram 1.15 Structure of (ras)GAP.

The rate of exchange of guanine nucleotides onto purified p21ras or p21ras in membranes corresponds to a half-life of around 60 minutes for bound nucleotide (Hall and Self, 1986; Cales et al., 1988). Several recent studies have identified cellular proteins that stimulate this otherwise slow rate of nucleotide exchange (Guanine nucleotide exchange proteins; GEPs). Clarification of the relationship between these activities, associated with 35kDa membrane-associated (West et al., 1990; Huang et al., 1990), 100-160kDa cytosolic (Wolfman and Macara, 1990) and 60kDa cytosolic proteins (Downward et al., 1990a), awaits their cloning.

The identification of proteins which interact with p21ras to modulate the GTPase activity and rate of guanine nucleotide exchange onto p21ras presents the possibility that these proteins are upstream regulatory components of the ras pathway. However, neither the nature of the upstream cellular signals nor the contribution made by these activities to the regulation of ras proteins is clearly understood. In addition, definitive identification of a downstream effector of ras action has been complicated by the pleiotropic nature of ras transformation. Although it is clear that mammalian ras proteins can
Diagram 1.16 The p21 ras cycle of GTP binding and hydrolysis.

functionally substitute for the structurally homologous proteins encoded by the RAS1 and RAS2 genes of saccharomyces cerevisiae it is not clear how far analogies between these systems extend (Broek et al., 1985; Kataoka et al., 1984; Tatchell et al., 1984). The activation states of RAS1 and RAS2 are regulated by the product of the CDC25 gene which is probably a guanine nucleotide-exchange factor that responds to the availability of nitrogen in the medium (Broek et al., 1987; Crechet et al., 1990). GTP-bound RAS proteins stimulate adenylate cyclase activity via a cyclase-associated protein (CAP) (Field et al., 1990). RAS protein function is antagonised by the (ras)GAP
homologues encoded by the \textit{IRA1} and \textit{IRA2} genes, which stimulate the GTPase activity of RAS1 and RAS2 (Tanaka \textit{et al.}, 1990). Thus, in \textit{Saccharomyces cerevisiae}, it appears that the upstream regulator is a nucleotide exchange factor, the downstream effector is CAP/adenylate cyclase and the (ras)GAP homologues act as downregulators. (reviewed by Broach, 1991). However, it has been clearly demonstrated that ras proteins do not regulate either mammalian or \textit{Xenopus} oocyte adenylate cyclase activity (Birchmeier \textit{et al.}, 1985; Beckner \textit{et al.}, 1985).

\textbf{1.22.3 The cellular effector of p21\textsubscript{ras} action.}

Genetic analysis has revealed the existence of a region of p21\textsubscript{ras} that is essential for normal and oncogenic biological activity. Mutations within the region 32-40 neutralize the transforming ability of oncogenic p21\textsubscript{ras} without altering cellular localization, nucleotide binding or intrinsic GTPase activity (Sigal \textit{et al.}, 1986a, Willumsen \textit{et al.}, 1986). These residues are completely conserved between the three mammalian ras proteins and form part of an exposed loop which is predicted to undergo significant conformational change upon GTP hydrolysis (Pai \textit{et al.}, 1989; 1990; Milburn \textit{et al.}, 1990). It has been suggested that these residues form an "effector loop" by which active p21\textsubscript{ras}.GTP interacts with the elusive downstream mediator of it's biological activity. However, mutations within this region also prevent GAP from stimulating the GTPase activity of normal ras (Cales \textit{et al.}, 1988; Adari \textit{et al.}, 1988; Vogel \textit{et al.}, 1988). An analogous effector region is highly conserved in \textit{S. cerevisiae} RAS proteins whose GTPase activity can be stimulated by mammalian GAP (Tanaka \textit{et al.}, 1990). By these criteria, in addition to being an upstream regulator of p21\textsubscript{ras}, GAP is a candidate for a downstream target of ras and may be part of an effector complex. In support of this suggestion p21\textsubscript{ras}, in conjunction with (ras)GAP, has been demonstrated to inhibit the coupling of muscarinic receptors to the G protein Gk which regulates the
opening of atrial potassium channels in isolated membranes (Yanati et al., 1990).

A prediction from a model in which GAP functioned as a downstream target for ras would be that mutant p21\textsuperscript{ras} proteins unable to bind GAP would be biologically inactive. This hypothesis is supported by studies in which the ability of various mammalian ras effector mutants to complement the deletion of \textit{S. cerevisiae} RAS proteins and regulate adenylate cyclase activity was found to correlate with (ras)GAP binding by these mutants (Scaber et al., 1989). In addition, the Ala38 p21\textsuperscript{ras} effector mutant, which is biologically inactive and insensitive to GAP, is unable to bind (ras)GAP (Vogel et al., 1988). Further supportive evidence can be derived from the observation that a cytosolic Leu68 p21\textsuperscript{ras} mutant, which binds GAP but is locked in the GTP-bound state, blocks p21\textsuperscript{ras} action in a manner that can be overcome by transfection of additional (ras)GAP (Gibbs et al., 1989). Similarly, the Krev-1 gene encodes a member of the ras family of guanine nucleotide binding proteins (rap1A) that binds (ras)GAP more tightly than does p21\textsuperscript{ras} and can suppress ras transformation (Hata et al., 1990; Frech et al., 1990). It is possible that both of these proteins antagonise ras function by sequestering (ras)GAP from normal p21\textsuperscript{ras}.

The correlation between (ras)GAP binding and biological activity is not perfect. For example, a p21\textsuperscript{ras} mutant with a glutamine substituted at codon 38, a mutation which renders p21\textsuperscript{ras} biologically inactive and unresponsive to GAP, binds (ras)GAP with an affinity that is similar to the normal protein (Krengel et al., 1990). The significance of this result is that it suggests that neither constitutive maintenance of p21\textsuperscript{ras} in the GTP-bound state nor association of GAP with p21\textsuperscript{ras}.GTP is sufficient for the delivery of an oncogenic signal. One possibility is that in addition to (ras)GAP, other components of a p21\textsuperscript{ras}-effector complex are required for transmission of a growth regulatory signal. Overexpression of (ras)GAP has been reported to
suppress transformation of NIH 3T3 cells induced by normal but not oncogenic p21\textsuperscript{ras} and src (Zhang et al., 1990a; DeClue, 1991; Nori et al. 1991). A fragment comprising the C-terminal catalytic domain of (ras)GAP was sufficient to cause reversion of cells transformed by overexpression of normal ras. (Zhang et al., 1990a). Thus it appears likely that this effect is mediated by the ability of GAP to induce the GTPase activity of normal but not oncogenic ras protein. These results are not compatible with a simple upstream regulator or downstream effector relationship existing between (ras)GAP and p21\textsuperscript{ras} in growth-regulatory pathways.

1.22.4 The role of p21\textsuperscript{ras} in signal transduction.

Although the identity of the effector component of the ras pathway is unclear, some features of the downstream events that underly normal cellular p21\textsuperscript{ras} function and transformation by oncogenic p21\textsuperscript{ras} have been elucidated. Microinjection of a neutralising monoclonal antibody raised against p21\textsuperscript{ras} has proved to be a useful approach for analysis of the role played by p21\textsuperscript{ras} in signal transduction pathways involved in the regulation of cell growth. Injection of mAb Y13-259 was originally observed to block serum-induced cell division and cause reversion of the p21\textsuperscript{ras}-transformed phenotype of NIH 3T3 fibroblasts (Mulcahy et al., 1985; Feramisco et al., 1985; Kung et al., 1986). Microinjection of Y13-259 has subsequently been demonstrated to block the induction of DNA synthesis induced by such diverse stimuli as PDGF, phosphatidic acid, phorbol esters and transformation mediated by growth-factor receptor-like oncogenes such as \textit{fms}, \textit{fes} and src (Smith et al., 1986). These results strongly implicate p21\textsuperscript{ras} in the signal transduction pathways by which growth factor receptors regulate cell growth and differentiation in fibroblasts (Mulcahy et al., 1985). Similar experiments in rat PC12 cells and in \textit{xenopus} oocytes suggest a comparable role for p21\textsuperscript{ras} in nerve growth factor-
induced cellular differentiation and maturation in response to insulin respectively (Hagag et al., 1986; Korn et al., 1987).

The possibility that p21ras might function to control growth-factor receptor-induced activation of phospholipases such as PI-PLC and PLA2 in an analogous way to the heterotrimeric signal transducin G proteins has been extensively investigated. Microinjection of Y13-259 with PI-PLC-γ1 or p21ras proteins resulted in inhibition of p21ras- but not PI-PLC-γ1-induced DNA synthesis and proliferation (Smith et al., 1990). The simplest interpretation of these results is that p21ras is an upstream effector component of PI-PLC-regulatory signal transduction pathways and that phosphatidylinositol metabolism is required for ras transformation.

Even when expressed at high levels, normal p21ras does not appear to alter basal levels of PI metabolism (Hancock et al., 1988; Wakelam et al., 1986). However, in an Swiss 3T3-derived clone, which contains N-ras under the control of an inducible promoter, enhanced stimulation of inositol phosphate production by bombesin was observed on induction of p21ras expression. Because no effect of p21ras-induction on bombesin receptor number or affinity was detected, this result was originally interpreted to suggest that p21ras could synergise with bombesin for the stimulation of PI metabolism and may therefore couple or enhance coupling of the bombesin receptor to PI-PLC (Wakelam et al., 1986, Lloyd et al., 1989). This effect, the mechanism of which remains unclear, proved impossible to reproduce in other cell lines and is probably a unique property of the original clone.

Other workers have reported that ras-transformed 3T3 cells demonstrate enhanced bradykinin-induced PI hydrolysis and are desensitized with respect to PDGF, bombesin and serum (Parries et al., 1987; Benjamin et al., 1987). However, transfection of both normal and oncogenic p21ras results in up to 40-fold higher expression of bradykinin receptors than parental cells, suggesting that the enhanced response to bombesin in ras-transformed cells
is a function of receptor number (Downward et al., 1988). Another study has demonstrated that microinjection of Y13-259 inhibited proliferation of induced by phorbol esters in conjunction with calcium ionophore and by prostaglandin F2\(\alpha\). Since these conditions were intended to broadly mimic the activation of PI-PLC and PLA\(_2\) respectively, the results infer that p21\(^{ras}\) functions downstream of these phospholipases and is required for mitogenesis in response to their activation (Yu et al., 1988).

Although the accumulated evidence suggests that p21\(^{ras}\) does not directly couple receptors to PI-PLC activity, elevated cellular levels of DAG and activation of PKC have been reported on introduction of oncogenic p21\(^{ras}\) (Wolfman and Macara, 1987; Lacal et al., 1987a). Since basal inositol phosphate production had been observed to be enhanced in cells transformed with oncogenic p21\(^{ras}\), it appeared possible that DAG produced as a consequence of PI metabolism might account for this effect (Fleischman et al., 1986; Lacal et al., 1987a; Hancock et al., 1988). Subsequent studies revealed that p21\(^{ras}\)-induced PI-PLC activity could be both quantitatively and temporally dissociated from DAG production and PKC activation, indicating that DAG produced under these circumstances is probably derived from alternative cellular sources (Lacal et al., 1987b, Seuwen et al., 1988). Since production of choline and phosphocholine has been identified as an early consequence of the introduction of oncogenic p21\(^{ras}\) into fibroblasts, this source has been suggested to be breakdown of phosphatidylcholine (Price et al., 1989b;1989c; Lopez-Barahona et al., 1990). In support of this idea, microinjection of p21\(^{ras}\) into Xenopus oocytes causes a increase in DAG and phosphocholine (Lacal et al., 1987b; 1990). However, since a rapid increase in choline kinase activity is also observed under these circumstances it is not clear that this reflects phosphatidylcholine breakdown (Macara, 1989). The mechanism by which oncogenic p21\(^{ras}\) induces the rapid activation of PKC is controversial and will require further clarification.
Evidence that PKC may function as an important downstream effector of p21\(^{ras}\) action in Swiss 3T3 cells is largely derived from studies in which PKC has been down-regulated by prolonged prior exposure to phorbol esters. By this method, elevation of cellular DAG levels, arachidonic acid production and DNA synthesis in response to oncogenic Val12 p21\(^{ras}\) has been demonstrated to require functional PKC (Price \textit{et al}., 1989a; Morris \textit{et al}., 1989; Lacal \textit{et al}., 1987c). However, morphological transformation and induction of c-myc expression by this \textit{ras} mutant were unaffected by downregulation of PKC (Lloyd \textit{et al}., 1989). Interestingly, addition of growth factors to Swiss 3T3 cells also causes Ras-dependent DNA synthesis but is not blocked by removal of PKC (Morris \textit{et al}., 1989; Yu \textit{et al}., 1988). In addition, expression of inhibitory Asn17 p21\(^{ras}\) inhibited DNA synthesis induced by phorbol esters, serum and growth factors without affecting the ability of phorbol esters and EGF to induce expression of c-fos, c-jun and c-myc (Cai \textit{et al}., 1990). These studies collectively suggest that \textit{ras}-induced activation of PKC is an early event in the pleiotropic transformation process initiated by oncogenic p21\(^{ras}\) but that parallel, PKC-independent, mitogenic signal transduction pathways are also initiated.

1.22.5 Possible links between growth regulatory pathways and p21\(^{ras}\).

A potential link between growth factor receptors and \textit{ras}-mediated pathways has been suggested by the observation that (ras)GAP is tyrosine phosphorylated on stimulation of cells with certain growth factors and in cells transformed by tyrosine kinase oncogenes (Molloy \textit{et al}., 1989; Ellis \textit{et al}., 1990 and reviewed by McCormick, 1990). PDGF-stimulation of NIH 3T3 fibroblasts induces the tyrosine phosphorylation of a number of proteins, including (ras)GAP (Molloy \textit{et al}., 1989). This phosphorylation event appears to be accompanied by the translocation of (ras)GAP from the cytoplasm to the membrane. Further studies have revealed that approximately 10% of cellular
(ras)GAP forms part of a complex with activated PDGF receptors (Kazlauskas et al., 1990) and that this complex also includes the Raf-1 serine/threonine protein kinase and PI-PLC-γ1 (Kaplan et al., 1990). A correlation between the ability of mutant receptors to bind and phosphorylate (ras)GAP and induce mitogenesis suggests that (ras)GAP binding might be an important event for PDGF-R signalling (Kazlauskas et al., 1990). Activation of the colony-stimulating factor 1 receptor (CSF1-R, c-fms) in NIH-3T3 cells has also been reported to result in the tyrosine phosphorylation of (ras)GAP and a 62kDa protein (Reedijk et al., 1990).

Transformation of Rat-2 fibroblasts with both receptor and cytoplasmic tyrosine kinase oncogenes, such as v-fps, v-src, v-abl and v-fms, also stimulates tyrosine phosphorylation of (ras)GAP (Ellis et al., 1990). In this study, tyrosine phosphorylated GAP was observed to co-immunoprecipitate with two proteins of 62kDa (p62) and 190kDa (p190) whose phosphorylation on tyrosine also correlated with the induction of oncogenic transformation. Stimulation of the EGF receptor in these cells also induced tyrosine phosphorylation of (ras)GAP, p62 and p190 (Ellis et al., 1990). The interaction between (ras)GAP and tyrosine phosphoproteins including autophosphorylated growth factor receptors, oncogenic tyrosine kinases and cellular tyrosine phosphoproteins is probably mediated by the two SH2 domains located in the non-catalytic C-terminal region of GAP (Moran et al., 1990; 1991). Other proteins known to share these sequences, including src family tyrosine kinases, PI-PLC-γ and the v-crk oncoprotein, have been implicated in signal transduction or cellular transformation.

Phosphorylation of (ras)GAP and its association with other cellular phosphoproteins on treatment of cells with growth factors or transformation with tyrosine kinase oncogenes provides a possible link between p21ras and these growth-regulatory pathways. Stimulation of Rat-2 fibroblasts with EGF or transformation by v-src has been observed to induce changes in the
subcellular localisation (ras)GAP and the formation of two distinct complexes with p62 and p190 (Moran et al., 1991). One possible functional connection would be if tyrosine phosphorylation of (ras)GAP or the interaction of GAP with other phosphotyrosine containing proteins, such as p62 and p190, were to alter the ability of GAP to stimulate p21\textsuperscript{ras} GTPase activity. Inhibition of (ras)GAP activity under these circumstances would be predicted to cause ras to accumulate in the biologically active GTP-bound state. Although attractive, no experimental evidence currently exists to support this model.

In Swiss 3T3 fibroblasts, which overexpress c-H-ras by about 30 fold, serum treatment of quiescent cells was observed to induce a 2-fold accumulation of p21\textsuperscript{ras} in the GTP-bound state (Satoh et al., 1990). Another recent report has demonstrated small, approximately 2-3 fold, increases in the basal amount of GTP complexed to normal cellular ras in NIH-3T3 cells treated with PDGF or transformed with the tyrosine kinase oncogenes v-src or v-abl (Gibbs et al., 1990). PDGF-treatment of these cells caused the proportion of p21\textsuperscript{ras} in the GTP-bound state to rise from 7% to 15% of total cellular ras. Transformation by v-src or v-abl caused the proportion of cellular p21\textsuperscript{ras}.GTP to rise to 22%. Although these changes are proportionately small, the authors quote studies in S.cerevisiae which have shown that a 3-4 fold increase in the basal level of GTP bound to RAS is sufficient for the activation of biological activity (Tanaka et al., 1990). Thus, it is possible that a small range of variation in the gross cellular amount of p21\textsuperscript{ras}.GTP might be involved in regulating ras function in mammalian cells.
Aims of this study.

The major technical goal of this study was to establish a permeabilised cell system that would allow access to otherwise membrane-impermeant nucleotide and peptide reagents while preserving receptor-mediated signal transduction pathways such as stimulation of PI-specific PLC activity and PKC activity. This would enable extensive manipulation of these signalling pathways and provide an opportunity to investigate the following questions:

1) The role of guanine nucleotide binding proteins in coupling the TCR/CD3 complex to PI-specific phospholipase C (see section 3).

2) The role of guanine nucleotide binding proteins in the regulation of protein kinase C activity (see section 4).

3) The regulation of endogenous p21ras by T cell surface receptors that control cellular activation and proliferation (see section 5).
Section 2: Materials and Methods.

2.1 Materials.

Purified PHA and reduced streptolysin O and were obtained from Wellcome diagnostics (Dartford, Kent, UK).

*Myo-[3H]inositol* (3.05 TBq/mmol), [methyl-3H] thymidine (37 MBq/ml), 32P orthophosphate (3.7 MBq/ml), [γ-32P]ATP (370 MBq/ml), [γ-32P]GTP (370 MBq/ml) and [adenylate-32P]NAD (74MBq/ml) were from Amersham International (Amersham, Bucks, UK).

All unlabelled nucleotides were from Boehringer Mannheim (Lewes, Sussex, UK).

Ionomycin and Phorbol 12,13-dibutyrate were from Calbiochem (Cambridge Biosciences, Cambridge, UK).

Forskolin, Dibutyryladenosine 3′:5′-cyclic monophosphoric acid, bacterial toxins and other biochemicals were obtained from Sigma Chemical Co. (Poole, Dorset, UK) and were of the highest available purity.

The PKC pseudosubstrate prototope, peptide PS (sequence; Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gly-Gln-Lys-Asn-Val), is derived from a sequence in the regulatory domain of PKC and has been described as a selective inhibitor of PKC *in vitro* and in permeabilised cells (House and Kemp, 1987; Alexander et al., 1989).

Peptide GS (sequence; Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys) was derived from a sequence in glycogen synthase kinase and has been used as a PKC substrate *in vitro* and in permeabilised cells (House and Kemp, 1987; Alexander et al., 1990). Both peptides were synthesized as previously described (Davies, 1987).

Protein A agarose "Affi-Gel" was obtained from Bio Rad Laboratories (Hemel Hempstead, Herts., UK.).
2.2 Antibodies.

The UCHT1 (IgG1) murine monoclonal antibody against the ε subunit of the CD3 antigen (Beverley and Callard, 1981) was purified from hybridoma supernatants by protein A affinity chromatography as previously described (Davies, 1987).

The rat monoclonal antibodies Y13-259 and Y13-258, raised against oncogenic p21v-H-ras, have previously been described (Furth et al., 1982). The murine monoclonal antibodies OKT11 and .GT2 (Huet et al., 1986), specific for distinct epitopes on the extracellular domain of the CD2 antigen, were purified as above.

T lymphocyte activation via the CD2 antigen requires cross-linking of two CD2 epitopes. In order to achieve effective crosslinking of the GT2 and OKT11-defined epitopes, bispecific GT2/OKT11 F(Ab')3 comprising two OKT11 Fab' arms and one GT2 Fab' arm was prepared (Glennie et al., 1987). Briefly, F(Ab')2 from the GT2 and OKT11 antibodies, prepared by pepsin digestion of intact antibody, were purified on Ultragel ACA44 (LKB, Milton Keynes, UK) and reduced to provide Fab' with free hinge region SH groups (Fab'(SH)). The SH groups of the GT2 Fab'(SH) were then fully alkylated with an excess of the crosslinker O-phenylenedimaleimide to provide free maleimide groups. The bispecific reagent was prepared by mixing OKT11 Fab'(mal) and GT2 Fab'(SH) at a ratio of 2:1 under conditions that allowed crosslinking of the maleimide and SH groups but avoided reoxidation of the SH groups. The products of the reaction mixture were then fractionated on Ultragel ACA44 and stored at 4°C. HPLC fractionation of final preparations showed that F(Ab')3 derivatives were >95% homogeneous.

2.3 Cell culture.

Human T lymphoblasts were prepared as described (Cantrell et al., 1985). Briefly, peripheral blood mononuclear cells (10^6/ml) in RPMI 1640
supplemented with 10% (v/v) foetal calf serum and a 5% CO₂ atmosphere at 37°C were stimulated with 5μg/ml of PHA for 72 h in order to induce expression of receptors for the T cell growth factor interleukin-2 (IL2). After washing, cells were maintained in exponential growth for 10-20 days in RPMI 1640/10% foetal calf serum supplemented with 0.1nM recombinant IL2. Before use cells were deprived of IL2 and allowed to quiesce in the G₀/G₁ stage of the cell cycle.

The Jurkat 6 human T leukaemic cell line (Robb et al., 1981) was cultured in RPMI 1640/10% (v/v) foetal calf serum in a 5% CO₂ atmosphere at 37°C. The CTLL-2, IL2-dependent murine T leukaemic cell line (Robb et al., 1981) was cultured in RPMI 1640/10% (v/v) foetal calf serum supplemented with 10⁻⁵M 2-mercaptoethanol in a 5% CO₂ atmosphere at 37°C. Swiss 3T3 and Rat-1 fibroblasts were maintained and subcultured in 30mm Petri dishes using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum in a 5% CO₂ atmosphere at 37°C. Quiescent cells were deprived of serum for 2 days prior to use (Downward et al., 1988).

All cell culture materials were obtained from Gibco (Paisley, Scotland, UK).

2.4 Cell permeabilisation.

The bacterial cytotoxin streptolysin O was chosen to permeabilise cells. Preliminary experiments established an optimum streptolysin O concentration of 0.4i.u./ml, at which concentration >95% of T lymphoblasts became permeable to Trypan Blue within 1 min of addition of the toxin. Typically, T lymphoblasts were washed twice and resuspended at 5×10⁷ cells/ml in permeabilisation medium containing 150mM-KCl, 12.5mM-HEPES (pH7.4) and 12.5mM-EGTA. Streptolysin O (reduced), obtained as a freeze-dried preparation of a partially purified culture filtrate from a strain of haemolytic streptococcus, was reconstituted with the appropriate volume of distilled water.
to 2i.u./ml in 10mM-sodium phosphate/150mM-NaCl pH6.5. Addition of this preparation to permeabilisation medium, in a ratio of 1:4 (v/v), resulted in a final permeabilisation mixture comprising 120mM-KCl, 30mM-NaCl, 10mM-HEPES and 10mM-EGTA at a final pH of 7.2. Unless otherwise stated, 100μM-NaATP was also added.

The concentrations of CaCl$_2$ and MgCl$_2$ required to give free Ca$^{2+}$ in the range of nominal 0-1μM and Mg$^{2+}$ in the range of 1-10mM (37°C and pH7.2) were predicted by the computer program CHELATE using appropriate dissociation constants for Ca$^{2+}$, Mg$^{2+}$ and H$^+$ (Howell and Gomperts, 1987).

2.5 Phosphorylation and immunoprecipitation of the γ subunit of the CD3 antigen.

Phosphorylation of proteins in permeabilised cells was carried out by permeabilising 2.5×10$^7$ cells in the presence of 100μM-[γ-$^{32}$P] ATP (1μCi/pmol) and the specified stimulant or inhibitor in a final volume of 500μl. After incubation at 37°C for the indicated time, cells were pelleted at 8000g for 5 s and lysed on ice in a buffer containing 10mM-Tris HCl (pH7.4), 1% Nonidet P-40, 150mM-NaCl, 1mM-EDTA, 1mM-EGTA, 20mM-NaP$_2$O$_5$, 50mM-NaF, 4μg of Leupeptin/ml and 1 mM-benzamide. The CD3 antigen was immunoprecipitated using the anti-CD3 antibody UCHT1 as described (Cantrell et al., 1985; Cantrell et al., 1987). CD3 antigens were separated by 12%(w/v) SDS/PAGE under reducing conditions. Gel autoradiographs were scanned with an LKB Ultroscan XL densitometer, and $^{32}$P incorporation into the 26kDa CD3 γ chain quantified by using the Ultroscan XL internal digital integrator.

2.6 [$^3$H]inositol labelling and inositol phosphate determination.

T lymphoblasts were washed three times in inositol-free DMEM with intervening incubations of 15min in order to deprive the cells of free inositol. The cells were then labelled by incubation at 2×10$^7$/ml in inositol-free DMEM
containing 10mM-HEPES (pH 7.4) and 1mg of bovine serum albumin/ml with
myo-[3H] inositol (15μCi/10^7 cells) for 4 h at 37°C in a 5% CO₂ atmosphere.
Cells were subsequently washed three times in inositol-free DMEM and once
in permeabilisation buffer before resuspension at 5x10^7 cells/ml and
permeabilisation in a final volume of 500μl with the addition of 10mM-LiCl,
100μM-ATP and the appropriate stimulant. After incubation for the indicated
time at 37°C the reaction was terminated by addition of 800μl of ice cold PBSA
(10mM-sodium phosphate/150mM-NaCl). The cells were pelleted by
centrifugation at 8000g for 10 s and the supernatant loaded directly on to
Dowex columns (formate form) for analysis by anion exchange
chromatography as described previously (Berridge et al., 1983). The loaded
columns were washed with 10mls of distilled water, to elute free myo-[3H]
inositol and each fraction eluted with 6mls of the following solution:
Glycerophosphatidylinositol; 5mM borax, 60mM ammonium formate. InsP₁;
0.2M borax, 0.1mM ammonium formate. InsP₂; 0.4M borax, 0.1mM ammonium
formate. InsP₃; 0.6M borax, 0.1mM ammonium formate. InsP₄/total inositol
phosphates; 0.2M borax, 0.1mM ammonium formate.

2.7 Peptide assay for PKC activity in permeabilised cells.

An assay for PKC activity, involving the introduction of a peptide
substrate for PKC (peptide GS) into permeabilised cells in the presence of [γ-
32P]ATP, was as performed according to a previously described method
(Alexander et al., 1990) with some slight variations. T lymphoblasts (2-
3x10^6/point) were permeabilised in a final reaction volume of 250μl containing
100μM-[γ-32P]ATP (1μCi/pmol), 250μM-peptide GS and the appropriate
stimuli. Reactions were continued for the times indicated and stopped by
addition of 100μl of 25% (w/v) trichloroacetic acid in 2M-acetic acid. The
samples were kept on ice for at least 10 min prior to centrifugation at 8000g for
5 min. Aliquots of the supernatant were spotted onto squares of P81 ion-
exchange chromatography paper (Whatman, Maidstone, Kent, UK) which were then washed 3× in 30% (v/v) acetic acid containing 1% H₃PO₄ for 10 min and once with ethanol. The P81 squares were dried, immersed in Ecolmole liquid scintillation cocktail (ICN biochemicals, Irvine, CA, USA) and the bound radioactivity measured in a scintillation counter. Data are presented as means of duplicate values, expressed as pmol of ³²P incorporated into peptide/time (min) of the assay for the number of cells used. The value obtained in the absence of peptide GS (normally 3-10% of that observed in the presence of GS) were subtracted in all cases.

2.8 Phospholipid labelling and analysis in permeabilised T lymphoblasts.
T lymphoblasts (2-3×10⁶/point) were permeabilised in a final reaction volume of 250µl containing 100µM-[γ³²P]ATP (1µCi/pmol). Reactions were continued for the times indicated and stopped by addition of 600µl of 1:2 (v/v) chloroform:methanol with 1% (v/v) concentrated HCl. The mixture was vortexed thoroughly and a phase split achieved by the addition of 150µl of 10mM HCl and 150µl of chloroform followed by centrifugation at 8000g for 5 min. Aliquots of the chloroform (lower) phase were loaded onto silica gel 60A thin layer chromatography plates (Whatman, Maidstone, Kent, UK) that had been previously sprayed with a freshly prepared solution of 1% (w/v) potassium oxalate and allowed to dry for at least an hour. A chromatography tank, lined with Whatman 3mm paper, was allowed to equilibrate for approximately 30 min with a solvent comprising 80ml chloroform, 26ml methanol, 30ml acetone, 24ml acetic acid and 16ml distilled water. The chromatography plate was placed in the tank and allowed to run until the solvent front was within 1cm of the top of the plate. The plate was removed, allowed to dry for 1 hour and exposed to Kodak X-Omat S autoradiography film for 72 h. Phospholipid species and phosphatidic acid were identified by
comparison with unlabelled standards that were visualized by exposure to iodine vapour.

2.9 Determination of p21\textsuperscript{ras} activity in T lymphocytes.

p21\textsuperscript{ras} proteins were immunoprecipitated with antibody Y13-259 from appropriately activated or quiescent T cells in which guanine nucleotides were labelled with \textsuperscript{32}P by one of two methods; either cells were metabolically labelled with \textsuperscript{32}P-orthophosphate or were permeabilised in cytosolic buffer containing [\(\alpha\)-\textsuperscript{32}P]GTP. Metabolic labelling with \textsuperscript{32}P-orthophosphate was performed as described previously (Cantrell \textit{et al.}, 1985). Briefly, the cells were washed 3 times in phosphate-free DMEM with intervening incubation periods of 15 min to deplete the cells of phosphate. The cells were then labelled by incubation at 2x10\textsuperscript{7}/ml in phosphate-free DMEM containing 10mM-HEPES (pH 7.4) and 1mg/ml of bovine serum albumin with \textsuperscript{32}P orthophosphate (100\textmu Ci/ml) for 4 h at 37\textdegree C in a 5% CO\textsubscript{2} atmosphere. Cells were subsequently washed three times in phosphate-free DMEM prior to aliquoting and addition of the appropriate stimulant. Alternatively, the cells were permeabilised in a permeabilisation buffer containing 5mM Mg\textsuperscript{2+} and 100nM Ca\textsuperscript{2+} in the presence of 10\textmu Ci/ml of [\(\alpha\)-\textsuperscript{32}P]GTP.

Cell lysis was performed in 50mM-HEPES pH7.4, containing 1% Triton X-100, 100mM-NaCl, 5mM MgCl\textsubscript{2}, 1mg of BSA/ml, 10mM-benzamidine, 10\mu g of leupeptin/ml, 10\mu g of aprotinin/ml, 10\mu g of soybean trypsin inhibitor/ml. Nuclei were removed by centrifugation at 15,000g for 2 min followed by addition of 0.5M-NaCl, 0.5% deoxy cholate and 0.05% sodium dodecyl sulphate to the supernatant lysate. Immunoprecipitation of p21\textsuperscript{ras} was for 40 min with antibody Y13-259 precoupled to protein A agarose via rabbit anti-rat immunoglobulin. Immunoprecipitates were washed with 8x1ml of 50mM-HEPES pH7.4, 500mM-NaCl, 5mM-MgCl\textsubscript{2}, 0.1% Triton X-100 and 0.005% SDS. Nucleotide bound to immunoprecipitated p21\textsuperscript{ras} was eluted with 2mM-
EDTA, 2mM-DTT, 0.2% SDS, 0.5mM GTP and 0.5mM GDP at 68°C for 20 min. Thin layer chromatographic separation of eluted nucleotide was performed on PEI-cellulose plates run in 1.2M ammonium formate and 0.8M HCl. Labelled nucleotides, separated by t.l.c., were quantitated by direct scanning for β radiation using an Ambis β Scanner.

2.10 Cellular activity of p21\textsuperscript{ras} GTPase activating protein.

Lysates were made from 5x10\textsuperscript{8} T lymphoblasts that were either quiescent or activated as indicated. Okadaic acid (100nM) was included in all incubations. Lysates were made by disrupting cells in 0.5ml of homogenization buffer, 10mM-pipes pH7.2, containing 120mM-KCl, 30mM-NaCl, 5mM free Mg\textsuperscript{2+}, 100nM free Ca\textsuperscript{2+}, 1% Triton X-100, 10% glycerol and 100nM-okadaic acid. The lysate was centrifuged at 300,000g for 10 min and the supernatants assayed for ability to stimulate p21\textsuperscript{ras} GTPase activity. 50ng of pure, bacterially-expressed wild-type human p21\textsuperscript{c-H-ras} was allowed to bind 25μCi of [α-\textsuperscript{32}P]GTP in the presence of 5mM-EDTA at 37°C for 5 minutes. 10mM-MgCl\textsubscript{2}, 1mM-GTP and 1mM-GDP were then added and 0.75 μCi aliquots added to varying amounts of GAP extract made up to the same volume with homogenization buffer. The mixtures were incubated for 10 min at 37°C, the reaction stopped by addition of 10 volumes of cold lysis buffer containing 0.5M NaCl and p21\textsuperscript{ras} immunoprecipitated using antibody Y13-259. The proportion of [α-\textsuperscript{32}P]GTP to total labelled nucleotide on p21\textsuperscript{ras} was calculated following separation of GTP from GDP by thin layer chromatography and direct scanning for β radiation.

2.11 Proliferation assay (\textsuperscript{3}H-thymidine incorporation).

Recombinant IL2 was titrated by serial 3-fold dilutions over a range from 100μg of IL2/ml to 500ng/ml in a flat-bottomed 96 well microtitre plate. T lymphoblasts were plated out in RPMI/10% FCS at a concentration of 2x10\textsuperscript{5}
cells/well and with a final volume of 200μl. After an incubation period of 24hr, the cells were pulsed for 4 hours with 1 mCi \([^3\text{H}]\) thymidine/well and were precipitated onto glass filters using a Titertek semi-automated cell harvester. \([^3\text{H}]\) thymidine incorporation was determined by liquid scintillation counting. The data are presented as the mean of triplicate cultures, the standard errors were usually < 15% of the mean.
Section 3.
The role of G proteins in coupling the TCR/CD3 complex to phospholipase C: Inositol phosphate production in permeabilised T cells.

Introduction.

T lymphocyte activation via the TCR/CD3 complex results in stimulation of phosphatidylinositol-specific phospholipase C (PI-PLC) (Weiss and Imboden, 1987). The consequences of inositol phospholipid hydrolysis by PI-PLC are the generation of inositol phosphates (InsPs) which regulate intracellular Ca\(^{2+}\) concentration and production of diacylglycerol which activates PKC (Berridge and Irvine, 1989; Nishizuka, 1988; Bell, 1986). The mechanism that couples TCR/CD3 to PLC, although the subject of intense investigation, has not been established. However, by analogy with other cellular systems, two likely coupling mechanisms can be considered. In fibroblasts it has been demonstrated that binding of peptide growth factors such as platelet-derived growth factor (PDGF) or epidermal growth factor (EGF) to their receptors stimulates the intrinsic tyrosine kinase activity of these receptors and promotes tyrosine phosphorylation of the \(\gamma\)-isoenzyme of PI-PLC (Wahl et al., 1989; Margolis et al., 1989; Meisenhelder et al., 1989). Several lines of experimental evidence support the hypothesis that tyrosine phosphorylation of PI-PLC-\(\gamma\) is the mechanism whereby PDGF and EGF receptors stimulate PLC activity (Nishibe et al., 1990; Margolis et al., 1990b; Kim et al., 1991; Sultzman et al., 1991). In contrast, receptors such as those for bombesin, vasopressin, f-Met-Leu-Phe or thrombin are proposed to use guanine nucleotide binding regulatory (G) proteins to regulate PI-PLC in a manner analogous to the coupling of receptors to adenylate cyclase (Cattano and Vicentini, 1989; Smith et al., 1986; Bradford and Rubin, 1986; Cockcroft and Stuchfield, 1989; McLeish et al., 1989; Paris and Pouyssegur, 1986; Brass et al., 1986; Huang and Ives, 1989).
Several studies have implicated tyrosine phosphorylation in TCR/CD3 coupling to PI-PLC. Firstly, activation via TCR/CD3 molecules is associated with tyrosine phosphorylation of a number of cellular substrates (Hsi et al., 1989; June et al., 1990a). This observation raises the possibility that one such substrate might be PLC. Secondly, tyrosine kinase inhibitors can inhibit TCR/CD3-mediated regulation of PLC (June et al., 1990a; Mustelin et al., 1990). Thirdly, in T cells that lack surface expression of the tyrosine phosphatase CD45, the Ti/CD3 complex is uncoupled from PLC (Koretzky et al., 1990). Although these data demonstrate a role for tyrosine phosphorylation in the coupling of the TCR/CD3 complex to PI-PLC they do not exclude a role for G proteins in this process. For example, it has been suggested that the TCR/CD3 complex is coupled to the regulation of exocytotic secretion by a G protein (Schrezenmeier et al., 1988a; 1988b). Since exocytotic secretion in many cells has been shown to correlate with activation of PKC and subsequent PI hydrolysis, it has been proposed that the same G protein that couples TCR/CD3 to secretion might couple TCR/CD3 to a PI-specific PLC. However, although thymocytes and Jurkat have been demonstrated to express a PI-PLC activity that can be regulated by guanine nucleotides, it has not been directly established that this G protein-regulated PI-PLC is coupled to the TCR/CD3 complex (Zilberman et al., 1987; Sommermeyer et al., 1990). The relative contribution of G proteins and tyrosine phosphorylation to TCR/CD3 regulation of PLC remains to be established.

Several predictions concerning the behaviour of receptor-effector systems that employ mediatory G proteins can be made and experimentally tested (Gilman, 1987; Boyer et al., 1989). Firstly, the presence of both the relevant ligand and GTP is required for initiation of the appropriate response. A productive approach, derived from this prediction, employs non-hydrolysable analogues of GTP such as GTP[S]. In conjunction with a ligand
for the receptor in question, and under appropriate conditions, reagents such as these should synergistically and persistently activate the effector response of G protein mediated pathways. At higher concentrations, GTP[S] bypasses receptor stimulation by inducing spontaneous G protein subunit dissociation and effector activation. In addition, GDP and stable analogues, such as GDP[S], can maintain G proteins in their inactive state and antagonise G protein-mediated events. The use of reagents such as these, in membrane and permeabilised cell systems where they are allowed access to the intracellular environment, has allowed G protein involvement in receptor signal transduction to be investigated in many systems.

Experimental strategies that have been employed to render cells permeable for the analysis of receptor-mediated events include electroporation, the use of detergents and channel-forming bacterial exotoxins (reviewed by Gomperts and Fernandez, 1985). Ideally, a permeabilising agent should selectively generate stable and homogeneous plasma membrane lesions that allow investigations to be performed over a period of minutes. The holes generated by high voltage discharge do not fulfill these requirements since they are subject to resealing. Detergents such as saponin and digitonin are difficult to use because their effects are not confined to the plasma membrane and are likely to perturb a wide variety of cellular processes (Ahnert-Hilger et al., 1985). In contrast, the transmembrane protein channels formed by bacterial toxins such as Streptolysin O (SLO) are both stable and homogeneous. Haemolytically active in its reduced form, monomeric SLO is a 35kDa protein which binds to cholesterol in the outer leaflet of the plasma membrane and polymerises into ring-like structures that describe membrane lesions of approximately 12nM in diameter (Smythe and Duncan, 1978). Although these pores are large enough to allow egress of many cytoplasmic proteins, this system has allowed access to the cytoplasm for guanine nucleotides and investigation of the regulation of PI-PLC activity and
exocytotic secretion in HL60 cells (Howell and Gomperts, 1987; Stuchfield and Cockcroft, 1988).

Transmembrane and membrane-bound proteins retained Eg. T/CD3 and signal transduction machinery.

Entry of membrane impermeant reagents Eg. nucleotides and peptides.

Some cytoplasmic proteins lost Eg. LDH and Phosphatase type 2A (90% loss by 5 minutes).

Streptolysin-O binds to cholesterol in outer leaflet of plasma membrane and forms pores in excess of 12nm diameter.
Results.

3.1-3.3 Establishment of conditions for permeabilisation of T lymphoblasts with SLO and characterisation of signal transduction pathways in permeabilised cells.

3.1 Optimisation of conditions for permeabilisation of T lymphoblasts.

Streptolysin O was obtained as a partially purified culture filtrate that was pre-calibrated for haemolytic activity in international units (I.U.)/ml. In order to determine the concentration of SLO that would result in the rapid permeabilisation of a high proportion of T lymphoblasts, cells were exposed to various concentrations of SLO for 5 minutes in the presence of the nuclear stain trypan blue. Figure 3.1a shows that although >97% of untreated T lymphoblasts excluded the dye, the effects of the toxin were apparent at concentrations as low as 0.05 iU/ml and approximately 100% of cells were permeabilised by treatment with 0.35iU/ml of SLO. A SLO concentration of 0.4 I.U./ml was chosen and used in further experiments designed to estimate the time course of cellular permeabilisation with SLO. T lymphoblasts, in the presence of the fluorescent nuclear stain ethidium bromide, were treated with 0.4 I.U./ml of SLO in a spectrofluorimeter cuvette and the fluorescent emission at a wavelength of 485nM monitored with time. The intensity of fluorescent emission increased within seconds of SLO addition and plateaued after 30 seconds, indicating that cellular permeabilisation was rapid and essentially complete after this time (Fig 3.1b). Thus, a concentration of 0.4I.U./ml of SLO was found to be sufficient to permeabilise effectively 100% of T lymphoblasts within seconds of addition.
Percentage permeabilised cells

Fluorescence (arbitrary units)

Streptolysin O concentration (IU/ml)

Time (mins)

Figure 3.1

Permeabilisation of T lymphoblasts with the bacterial cytolysin Streptolysin O.

Specrophotometer and is plotted against time.

The fluorescence optical at 485nm was measured in a Perkin Elmer.

(a) T lymphoblasts at a concentration of 2x10^7/ml were treated with the indicated concentration of Streptolysin O in the presence of 0.2% trypan blue. After 5 minutes exposure, the percentage of stained cells was determined. The dye was excluded by <97% of untreated cells. (b) Streptolysin O at a final concentration of 0.4IU/ml was added to T lymphoblasts (2x10^7/ml) in the presence of 0.05% ethidium bromide at the indicated time. The fluorescence optical at 485nm was measured in a Perkin Elmer.

Figure 3.1

Permeabilisation of T lymphoblasts with the bacterial cytolysin Streptolysin O.
3.2 Inositol phospholipid metabolism in SLO-permeabilised T lymphoblasts.

For signal transduction studies in permeabilised T lymphoblasts, the intracellular concentration of divalent cations was controlled by Ca\(^{2+}\)/Mg\(^{2+}\)-EGTA buffers which allowed the concentration of free Ca\(^{2+}\) and Mg\(^{2+}\) to be predictably buffered in approximately physiological micromolar and millimolar ranges respectively (see materials and methods section 2.4). This buffer system also allows the effect of variations in the concentration of free Ca\(^{2+}\) and Mg\(^{2+}\) on signalling events to be investigated. Although permeabilisation of T lymphoblasts with SLO leads to the rapid loss of cytoplasmic proteins such as lactate dehydrogenase, the membrane-associated components of cellular signal transduction pathways should remain intact (Alexander et al., 1989).

In order to determine that permeabilisation of T lymphoblasts by SLO would allow access to exogenous nucleotides while maintaining signal transduction pathways, such as TCR/CD3-induced PI-PLC activity, T lymphoblasts were permeabilised with 0.4I.U./ml of SLO in the presence of \([\gamma-^{32}P]ATP\) and the incorporation of \(^{32}P\) into inositol phospholipids examined. Receptor triggering was achieved with the polyclonal T cell mitogen PHA and the stable GTP analogue GTP[S] was used to stimulate G protein-regulated pathways. Since diacylglycerol (DAG) produced as a consequence of PI-PLC mediated phosphatidylinositol metabolism is phosphorylated to form phosphatidic acid (PA) by endogenous DAG kinase activities, the incorporation of \(^{32}P\) into PA can serve as an indication of PI-PLC activity. During the subsequent re-generation of substrate, PA may be incorporated into phosphatidylinositol (PI) which is sequentially phosphorylated to give phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)). Thus, incorporation of \(^{32}P\) into PI is indicative of cyclic re-synthesis of PI while \(^{32}P\)-labelling of PIP and PIP\(_2\) may arise from both
phosphorylated PI and directly as a consequence of phosphorylation of their phosphatidylinositol precursors.

Figure 3.2 shows the result of an experiment in which this technique was employed. T lymphoblasts were exposed to SLO in permeabilisation buffer containing either nominally zero [Ca^{2+}], 100nM [Ca^{2+}] or 500nM [Ca^{2+}] in the presence or absence of either 10μg/ml PHA or 100μM GTP[S]. The phospholipid phase was extracted and analysed by thin layer chromatography to allow separation of PI, PIP, PIP_2 and PA, which were identified by visualisation of phospholipid standards with iodine vapour. In the absence of Ca^{2+} no breakdown of inositol phospholipids was detected as determined by the failure of either PHA or GTP[S] to stimulate production of PA. This is unsurprising since most isozymes of PI-PLC demonstrate an absolute requirement for Ca^{2+} in vitro. Under these conditions, incorporation of ^{32}P into PIP and PIP_2 was observed with some detectable labelling of PI probably occurring as a consequence of the low basal levels of PA that were observed in both stimulated and unstimulated cells.

In the presence of either 100nM or 500nM Ca^{2+}, both PHA and GTP[S] induced an approximately 10 fold increase in production of PA with respect to unstimulated cells (Fig 3.2). At 100nM Ca^{2+} this enhanced PA production was reflected as an increase in labelled PI. Since it is not possible to discriminate between phosphatidylinositol labelling and breakdown under these conditions, it is unclear whether the reduced labelling of PI, PIP and PIP_2 observed on stimulation with PHA or GTP[S] at 500nM Ca^{2+} relative to 100nM Ca^{2+} is a result of enhanced breakdown or reduced labelling. However, the reduction of labelling observed in the PIP and PIP_2 fractions upon addition of PHA or GTP[S] suggests that breakdown of one or both of these inositol phospholipids is occurring.

These results demonstrate that under conditions where T lymphoblasts are rendered permeable to exogenous [γ-^{32}P]ATP and GTP[S], both T cell
Figure 3.2
Lipid phosphorylation and metabolism in T lymphoblasts permeabilised with Streptolysin O.

T lymphoblasts were permeabilised in the presence of 5mM Mg\(^{2+}\), [\(\gamma\)-\(^{32}\)P]ATP and the indicated concentration of Ca\(^{2+}\). Cells were either unstimulated or stimulated with 10\(\mu\)g of PHA/ml or 100\(\mu\)M-GTP[S]. Phospholipids were extracted at 5 min by addition of an acidified chloroform/ methanol mixture, were separated by thin layer chromatography in parallel with lipid standards and were subjected to autoradiography.
surface receptor and G protein-mediated stimulation of PI-PLC activity is supported. Furthermore, a high level of cellular integrity is suggested by the observation that re-generation and phosphorylation of phosphatidylinositol substrates also occurs.

3.3 Phosphorylation of an exogenous peptide substrate for PKC in SLO-permeabilised T lymphoblasts.

To further characterise the ability of permeabilised T lymphoblasts to allow access to the cytoplasm for exogenous material and support receptor-mediated signal transduction pathways, a synthetic peptide derived from a sequence within glycogen synthase kinase (peptide GS) was introduced into SLO-treated T lymphoblasts. This peptide has been demonstrated, both in vitro and in permeabilised cells, to act as a highly specific substrate for PKC. Introduction of peptide GS in conjunction with $[\gamma\textsuperscript{32}P]$ATP allows receptor-mediated activation of PKC to be simply and reproducibly assayed (Alexander et al., 1988).

Figure 3.3 shows the results of an experiment in which T lymphoblasts were permeabilised in the presence of $[\gamma\textsuperscript{32}P]$ATP, peptide GS and various agonists. The time course of GS peptide phosphorylation in response to the PKC activator phorbol 12, 13-dibutyrate (PDBu), the anti-CD3 mAb UCHT1, the anti-CD2 GT2/OKT11 F(Ab')\textsuperscript{3} hybrid or the stable GTP analogue GTP[S] was determined. The data demonstrate that PDBu induced rapid phosphorylation of peptide GS in SLO permeabilised T cells, detectable within 1 minute of stimulation. Maximum stimulation, of a 8 fold increase in GS phosphorylation, was induced by PDBu after 5 minutes of incubation with a slight decrease detectable at 10 minutes. UCHT1 and GT2/OKT11 F(Ab')\textsuperscript{3} -induced GS phosphorylation were of comparable magnitude and demonstrated similar kinetics with phosphorylation detectable by 1-3 minutes.
Figure 3.3
Time course of phosphorylation of a PKC substrate peptide induced by PKC activation, the mitogen PHA and triggering of TCR/CD3 or CD2 in SLO-permeabilised T lymphoblasts.

T lymphoblasts were permeabilised in the presence of 5mM Mg$^{2+}$, 150nM Ca$^{2+}$, [γ-$^{32}$P]ATP and 250μM-peptide GS. Cells were either unstimulated (△) or stimulated with either 20ng of PDBu/ml (▲), 1μg/ml of GT2/OKT11 F(Ab')₃ (□), 10μg/ml of UCHT1 (○) or 100μM-GTP[S] (●). Peptide GS was recovered at the indicated time, washed and $^{32}$P incorporation quantified by scintillation counting. Data are expressed as pmol of $^{32}$P incorporated into peptide/time (min) of the assay for the number of cells used.
and maximal (6 fold) by 5 minutes. GTP[S] was also observed to induce phosphorylation of the GS peptide in permeabilised T cells but was observed to be slightly less effective than either UCHT1 or GT2/OKT11 F(\text{Ab}')3. GTP[S]-induced GS phosphorylation was detectable by 1 minute and reached a maximal 3-4 fold, with respect to unstimulated controls, by 5 minutes. Thus, under circumstances where T lymphoblasts had been rendered permeable to a peptide substrate for PKC and exogenous $^{32}$P-labelled ATP, activation of PKC with phorbol dibutyrate, triggering of the TCR/CD3 complex and CD2 antigen or stimulation of G proteins with GTP[S] could induce phosphorylation of the peptide.

Collectively, these results demonstrate that receptor and G protein agonist-induced phosphatidylinositol metabolism and PKC-mediated phosphorylation are facilitated by SLO permeabilisation of T lymphoblasts. These properties make the SLO permeabilisation system suitable for the investigation of signal transduction events such as TCR/CD3 regulation of PI-specific PLC and PKC.
3.4-3.6 Effect of guanine nucleotides and triggering of the TCR/CD3 complex or CD2 antigen on inositol phosphate production in SLO-permeabilised T lymphoblasts.

3.4 Time course of production of individual inositol phosphates in response to the anti-CD3 mAb UCHT1 or the G protein agonist GTP[S].

Another method for measurement of PI-PLC activity involves analysis of the soluble inositol phosphate products of phosphatidylinositol metabolism from cells labelled with myo-[³H]inositol. In order to further establish that G protein and TCR/CD3-mediated stimulation of PI-PLC activity could be detected in permeabilised T cells, myo-[³H]inositol-labelled T lymphoblasts were permeabilised in the presence of either GTP[S] (Fig 3.4a) or the anti-CD3 mAb UCHT1 (Fig 3.4b). [³H]inositol phosphates were extracted and separated by anion exchange chromatography. This technique allows separation of InsP₁, InsP₂, InsP₃ and InsP₄ but does not allow the different isomers of these inositol phosphate fractions to be discriminated.

Both GTP[S] and UCHT1 induced a time-dependent accumulation of InsP₁, InsP₂ and a fraction comprising InsP₃+InsP₄ that was linear for at least 7 minutes. The inositol phosphates produced by both agonists, in the presence of 10mM LiCl, comprised mainly InsP₁ and InsP₂ (Fig 3.4a and Fig 3.4b). Previous studies in intact T lymphoblasts and the T leukaemic line Jurkat, employing chloroform/methanol extraction techniques, have also identified InsP₁ and InsP₂ as the major inositol phosphate species induced by triggering the TCR/CD3 complex (King et al., 1989; Ward and Cantrell, 1990). One possible explanation is provided by the fact that InsP₃ is subject to the action of cellular phosphatases and may be rapidly dephosphorylated in both intact and permeabilised T cells. However, since no clear difference in the time course of accumulation of inositol phosphate fractions was observed, as might be expected if InsP₁ and InsP₂ were produced by the sequential
Permeabilised, myo-[3H]inositol-labelled, T lymphoblasts were stimulated with (a) 100nM GTP[S] or (b) 10pg of UCHT1/ml.

Inositol phosphates were extracted at the indicated time and separated by anion exchange chromatography. Data show radioactivity, expressed as dpm, incorporated into released InsP₁ (○/▪), InsP₂ (□/■), InsP₃+InsP₄ (▼/▲) and are normalised with respect to controls in the absence of stimulation.

Figure 3.4

Time course of inositol phosphate production in response to UCHT1 and GTP[S] in permeabilised T lymphoblasts.
dephosphorylation of InsP$_3$, it is possible that breakdown of PI, and PIP contributed to the accumulation of InsP$_1$ and InsP$_2$. Inositol phosphate fractions were pooled for further experiments and the accumulation of total inositol phosphates used as an indication of PI-PLC activity stimulated by the appropriate agonists.

3.5 Dose-response of UCHT1, hybrid anti-CD2 F(\Ab'\gamma)\_3 and polyclonal mitogen PHA for inositol phosphate production.

Stimulation of production of total inositol phosphate accumulation in response to TCR/CD3 triggering by UCHT1 was dose-dependent, demonstrating half-maximal stimulation at an antibody concentration of ~3 \mu g/ml. At the optimal UCHT1 concentration of 20\mu g/ml, a 12-fold stimulation of inositol phosphate accumulation above basal levels was typically observed to occur (Fig 3.5a). The lectin PHA, a polyclonal T cell mitogen that is considered to interact with multiple cell surface molecules, was also found to induce inositol phosphate accumulation in permeabilised T lymphoblasts (fig 3.5b). Accumulation of inositol phosphates in response to PHA was dose-dependent with half-maximal stimulation occurring at a concentration of 3 \mu g/ml and 15-fold stimulation with respect to basal at the optimum concentration of 20\mu g/ml. It has been shown previously that T cells can be activated by crosslinking of the anti-CD2 antibodies, GT2 and OKT11 (Huet et al., 1986). Figure 3.5b also shows that triggering of the CD2 antigen with a bispecific GT2/OKT11 hybrid F(\Ab'\gamma)\_3 induced a dose-dependent accumulation of inositol phosphates. The half maximal concentration for this reagent was ~1\mu g/ml and maximal induction of inositol phosphates, with fold stimulation comparable to that achieved by PHA, was at a concentration of 2\mu g GT2/OKT11 F(\Ab'\gamma)\_3 /ml.
Permeabilised, myo-[3H]inositol-labelled, T lymphoblasts were stimulated with the indicated concentration of either (a) UCHT1/mIL (•) or (b) PHA (○), and GT2/OKT11 F(ab')3 (□). Inositol phosphates were extracted and separated by anion exchange chromatography. Data show radioactivity, expressed as dpm, incorporated into total InsPs released after 7 minutes of stimulation. Data show radioactivity, expressed as dpm, incorporated into total InsPs released after 7 minutes of stimulation.

**Figure 3.5**

Dose-response of UCHT1, PHA and GT2/OKT11 hybrid F(ab')3-induced inositol phosphate production in permeabilised T lymphoblasts. Data show radioactivity, expressed as dpm, incorporated into total InsPs released after 7 minutes of stimulation.
3.6 Dose-response and guanine nucleotide specificity for inositol phosphate production.

The GTP[S] dose-response curve for inositol phosphate production (Fig 3.6) showed a half-maximal effect at a concentration of 0.7-1.0μM GTP[S]. At maximal concentrations, between 1μM and 10μM, GTP[S] induced an 8-10 fold increase in production of total inositol phosphates with respect to unstimulated cells. Inositol phosphate production in response to p[NH]ppG was only observed at nucleotide concentrations greater than 1μM. This weakly hydrolysable imido analogue of GTP was observed to induce a 2-3 fold accumulation of inositol phosphates at a p[NH]ppG concentration of 1mM (Fig 3.6). No inositol phosphate production was detected in response to GTP at concentrations ranging up to 1mM. Thus, only hydrolysis-resistant analogues of GTP, such as GTP[S] and p[NH]ppG, were observed to stimulate phosphatidylinositol metabolism. The rank order of potency for inositol phosphate production by these nucleotides reflected their reported ability to stimulate G protein-mediated events in other systems (GTP[S] > p[NH]ppG) (Barrowman et al., 1986; Turkey papers; reviewed by Gilman, 1987). The lack of inositol phosphate production observed in response to GTP is unsurprising since, unlike GTP[S] and to a lesser extent p[NH]ppG, this nucleotide does not induce spontaneous G protein subunit dissociation in the absence of appropriate receptor stimulation. Under conditions where T cells were stimulated in the presence of the respective agonists, TCR/CD3 triggering by UCHT1 was consistently observed to be more effective than GTP[S] in the induction of inositol phosphate production. Whereas UCHT1 maximally induced 12-13-fold increases in inositol phosphate production, GTP[S] was typically observed to induce a 8-10 fold increase (Fig 3.5 and 3.6).

Taken together with the previous data demonstrating TCR/CD3- and G protein-stimulated production of PA and phosphatidylinositol breakdown, the ability to regulate inositol phosphate production by guanine nucleotide
Permeabilised, myo-[^3]H]inositol-labelled, T lymphoblasts were stimulated with the indicated concentration of GTP[S] (●), p[NH]ppG (■) or GTP (▲). Inositol phosphates were extracted and separated by anion exchange chromatography. Data show radioactivity, expressed as dpm, incorporated into total InsPs released after 7 minutes of stimulation.
analogues, the mitogen PHA and mAb directed against the CD3 and CD2 antigens indicates that both G protein and receptor-mediated pathways of PI-PLC regulation are supported in cells permeabilised with SLO.

3.7-3.10 Effect of variations in the concentration of free Ca\(^{2+}\) and Mg\(^{2+}\) on TCR/CD3- and G protein-mediated inositol phosphate production.

The concentration of free Ca\(^{2+}\) and Mg\(^{2+}\), is of critical importance to numerous biochemical processes since these divalent cations can influence the enzymic activity of many proteins. In particular, PI-PLC activity is dependent on the [Ca\(^{2+}\)] over a nM range (reviewed by Rhee), whilst Mg\(^{2+}\) is required by protein kinases and at high mM concentrations promotes guanine nucleotide exchange and subunit dissociation of heterotrimeric G proteins (Higashijima et al., 1987; reviewed by Gilman, 1987). Thus, both [Mg\(^{2+}\)] and [Ca\(^{2+}\)] can potentially affect signal transduction events at multiple levels. The [Ca\(^{2+}\)] and [Mg\(^{2+}\)] in the permeabilisation buffer can be predictably varied using EGTA buffers.

3.7 Effect of varying [Ca\(^{2+}\)] on UCHT1 and GTP[S]-induced inositol phosphate production.

In order to determine the effect of variations in the [Mg\(^{2+}\)] and [Ca\(^{2+}\)] on agonist-induced inositol phosphate production, T lymphoblasts were permeabilised in the presence of various free magnesium or calcium concentrations and were either unstimulated or stimulated with GTP[S] or the anti-CD3 mAb UCHT1. The effect of varying the [Ca\(^{2+}\)] within a range from nominally zero to 1\(\mu\)M on TCR/CD3 and G protein-mediated stimulation of phosphatidylinositol metabolism in SLO-permeabilised T lymphoblasts is shown in figure 3.7. Both anti-CD3 mAb and GTP[S] were observed to synergise with [Ca\(^{2+}\)] for the production of \([^{3}\text{H}]\)-labelled inositol phosphates. Maximum synergy for both agonists occurred at 150nM Ca\(^{2+}\). While GTP[S]
Figure 3.7
Effect of variations in the concentration of free Ca$^{2+}$ on UCHT1- and GTP[S]-induced inositol phosphate production in SLO-permeabilised T lymphoblasts.

Permeabilised, myo-$[^3]$H]inositol-labelled, T lymphoblasts were unstimulated (□) or stimulated with 10μg of UCHT1/ml (○) or 100μM-GTP[S] (●) in the presence of 3mM-Mg$^{2+}$ and the indicated concentration of Ca$^{2+}$. Inositol phosphates were extracted and separated by anion exchange chromatography. Data show radioactivity, expressed as dpm, incorporated into total InsPs released after 7 minutes of stimulation.
included 30% of maximal inositol phosphate accumulation at nominally zero [Ca$^{2+}$], UCHT1 failed to induce significant phosphatidylinositol hydrolysis in the absence of Ca$^{2+}$. In the absence of any other stimulus, Ca$^{2+}$ did not induce significant inositol phosphate production over the range of concentrations employed. This range was restricted by the innability of EGTA to predictably buffer Ca$^{2+}$ at concentrations higher than around 1$\mu$M.

3.8 Effect of varying [Mg$^{2+}$] on UCHT1 and GTP[S]-induced inositol phosphate production.

Since free Mg$^{2+}$ concentrations in the mM range have been reported to influence G protein subunit stability, variations in [Mg$^{2+}$] over this range might be expected to affect G protein-mediated processes. Figure 3.8 shows the effect of varying [Mg$^{2+}$] from 1mM to 10mM on inositol phosphate accumulation induced by anti-CD3 mAb or GTP[S]. Induction of inositol phosphate production by UCHT1 demonstrated a strict requirement for Mg$^{2+}$ with little phosphatidylinositol hydrolysis (20% of maximum) at 1mM [Mg$^{2+}$] and synergy with [Mg$^{2+}$] up to a maximum at 5mM. GTP[S]-induced inositol phosphate production was 65% of maximum at 1mM [Mg$^{2+}$] with the optimum [Mg$^{2+}$] occurring at between 3 and 5mM. There was a small but reproducible inhibition of the induction of inositol phosphate production by both agonists at [Mg$^{2+}$] above 5mM. Mg$^{2+}$ in the absence of any other stimulus had no detectable effect on inositol phosphate production over the concentration range used. Although stimulation of PI-PLC activity by UCHT1 and GTP[S] demonstrated a similar dependency on [Mg$^{2+}$], a consistent difference in the abilities of these two agonists to induce inositol phosphate production at 1mM Mg$^{2+}$ was observed.
Figure 3.8
Effect of variations in the concentration of free Mg$^{2+}$ on UCHT1- and GTP[S]-induced inositol phosphate production in permeabilised T lymphoblasts.

Permeabilised, myo-$[^3]$H]inositol-labelled, T lymphoblasts were unstimulated (□) or stimulated with 10μg of UCHT1/ml (○) or 100μM-GTP[S] (●) in the presence of the indicated concentration of Mg$^{2+}$. Inositol phosphates were extracted and separated by anion exchange chromatography. Data show radioactivity, expressed as dpm, incorporated into total InsPs released after 7 minutes of stimulation.
3.9 Effect of varying [Mg^{2+}] on the dose-response of UCHT1 and GTP[S] for inositol phosphate production.

In order to assess whether [Mg^{2+}] influenced the dose-response of UCHT1- and GTP[S]-induced inositol phosphate production, both agonists were titrated in the presence of either 1mM, 3mM or 10mM Mg^{2+}. Consistent with the previous results, optimum UCHT1-induced inositol phosphate production, a maximal 10 fold increase with respect to unstimulated cells, was observed at a [Mg^{2+}] of 3mM (fig 3.9a). At a [Mg^{2+}] of 10mM and 1mM, maximal inositol phosphate accumulation in response to UCHT1 was 7 fold and 4 fold respectively. Although the magnitude of the response to TCR/CD3 triggering by UCHT1 was significantly affected by variations in the [Mg^{2+}] over this range, the dose-response of UCHT1 for inositol phosphate production (approximately 3μg/ml) was unaffected. Figure 3.9b shows the results obtained for variation in [Mg^{2+}] on GTP[S]-induced inositol phosphate accumulation. The half-maximal dose for induction of phosphatidylinositol hydrolysis by GTP[S] (0.7-1.0μM) was also observed to be independent of the [Mg^{2+}] over this range. However, optimum stimulation of inositol phosphate accumulation by GTP[S] was again achieved at 3mM Mg^{2+} (9-10 fold) with both 10mM and 1mM Mg^{2+} being sub-optimal for the induction of inositol phosphates by GTP[S] (8 and 6 fold respectively).

3.10 Effect of varying [Mg^{2+}] on the time course of UCHT1 and GTP[S]-induced inositol phosphate production.

The time course for inositol phosphate accumulation induced by UCHT1 and GTP[S] in the presence of either 1mM, 3mM or 10mM Mg^{2+} were investigated (figures 3.10a and 3.10b). As previously demonstrated (fig 3.4a and 3.4b), the rate of inositol phosphate accumulation induced by both UCHT1 and GTP[S] at a Mg^{2+} concentration of 3mM was linear for at least 10 minutes. Although the response to UCHT1 and GTP[S] in the presence of
Permeabilised, myc-[3H]inositol-labelled, T lymphoblasts were stimulated with the indicated concentration of (a) UCHT1 (open symbols) or (b) GTP[S] (closed symbols) in the presence of either 1mM- (□/■), 3mM- (□/■) or 10mM-Mg2+ (○/●). Inositol phosphates were extracted and separated by anion exchange chromatography. Data show radioactivity, expressed as dpm, incorporated into total InsPs released after 7 minutes of stimulation.

Figure 3.9 Effect of varying [Mg2+] on the dose-response of UCHT1 and GTP[S] for inositol phosphate production in permeabilised T lymphoblasts.
Permeabilised, myo-[3H]inositol-labelled, T lymphoblasts were stimulated with (a) 10 pg of UCHT1/ml (open symbols) or (b) 100 pM GTP[S] (closed symbols) in the presence of either 1 mM (A/A), 3 mM (□/■) or 10 mM Mg2+ (○/●) inositol phosphates. Data show radioactivity, expressed as dpm, incorporated into total InsPs. Inositol phosphates were extracted at the indicated time and separated by anion exchange chromatography. Data show radioactivity, expressed as dpm incorporated into total InsPs.

**Figure 3.9**: Effect of varying [Mg2+] on the time course of UCHT1 and GTP[S]-induced inositol phosphate production in permeabilised T lymphoblasts.
10mM Mg\(^{2+}\) was also observed to be linear over this time period, the rate of accumulation of inositol phosphates in response to both agonists was less than that observed at 3mM Mg\(^{2+}\). However, in the presence of 1mM Mg\(^{2+}\), there was a lag period of 3 minutes before either UCHT1 or GTP[S] induced significant inositol phosphate accumulation (figures 3.10a and 3.10b).

To summarise the effects of variations in the concentration of free Mg\(^{2+}\) on TCR/CD3 and G protein-mediated inositol phosphate production, it appears probable that [Mg\(^{2+}\)] regulates the ability of G protein (GTP[S]) and TCR/CD3 (UCHT1) agonists to stimulate inositol phosphate production by influencing the rate at which both agonists induce PI-PLC stimulation.

3.11-3.14 Effect of exogenous nucleotides, including GDP and analogues of GDP, on G protein and TCR/CD3-mediated inositol phosphate production.

The ability of GDP and analogues of GDP, such as GDP[S], to antagonise G protein functions has allowed G protein involvement in receptor coupling to PI-PLC activity to be investigated in other cell types (Cockcroft, 1987; Boyer et al., 1989a). Although the previous results demonstrate that PI-PLC activity can be regulated by both TCR/CD3- and G protein stimulation in SLO-permeabilised T lymphoblasts, they do not directly address the relationship between these two pathways. In particular, whether the G protein that regulates PI-PLC activity in this system is coupled to the TCR/CD3 complex remains to be established. In order to investigate the mechanism of Ti/CD3-coupling to phosphatidylinositol hydrolysis in permeabilised T lymphoblasts, the effect of various exogenous nucleotides on UCHT1 and GTP[S]-induced inositol phosphate accumulation was examined.
3.11 Effect of GDP and analogues of GDP on GTP[S]-induced inositol phosphate production.

Figure 3.11 depicts the effect of various concentrations of CDP, IDP, GTP, GDP, p[NH]ppG and GDP[S] on [3H]inositol phosphate accumulation induced by 10μM GTP[S]. The data show that CDP, at concentrations up to 1mM, did not significantly influence the magnitude of GTP[S]-induced phosphatidylinositol hydrolysis. IDP also demonstrated little influence on inositol phosphate accumulation in response to GTP[S] and was observed to cause a 20% inhibition at a nucleotide concentration of 1mM. In contrast, both GDP[S] and GDP had a marked antagonistic effect on GTP[S]-induced inositol phosphate production, with GDP proving a slightly more potent inhibitor than GDP[S], such that the half-maximal doses for inhibition were 100μM and 400μM for GDP and GDP[S] respectively. This result, although initially surprising, may be attributed to the fact that the major synthetic contaminant in preparations of GDP[S], comprising up to 30% in some batches, is GTP[S]. GTP and, to a lesser extent, p[NH]ppG also inhibited GTP[S]-induced inositol phosphate production in a dose-dependent manner. p[NH]ppG exhibited 50% inhibition at around 1mM whilst the ability of GTP to antagonise GTP[S], with a half-maximal concentration of 400μM, was comparable to that of GDP[S]. Later experiments established that a likely explanation for the inhibitory effect of GTP was its rapid hydrolysis to GDP in permeabilised cells (see Results Section 5.7).

3.12 Effect of GDP and analogues of GDP on UCHT1-induced inositol phosphate production.

The relative abilities of these nucleotides to inhibit G protein-mediated inositol phosphate production were also observed when their effect on TCR/CD3-induced phosphatidylinositol metabolism was investigated (Fig 3.12). Thus, GDP proved most effective, with a concentration for half-
Figure 3.11
Dose-response and nucleotide specificity for inhibition of GTP[S]-induced inositol phosphate production by exogenous nucleotides in permeabilised T lymphoblasts.

Permeabilised, myo-[3H]inositol-labelled, T lymphoblasts were stimulated with 10μM-GTP[S] in the presence of the indicated concentration of either CDP (○), IDP (∆), GDP (●), GTP (▲), p[NH]ppG (□) or GDP[S] (■). Inositol phosphates were extracted and separated by anion exchange chromatography. Data show percentage of maximum stimulation of radioactivity, expressed as dpm, incorporated into total InsPs released after 7 minutes of stimulation. Maximum values for UCHT1 and GTP[S] stimulation were 4128 dpm and 3554 dpm respectively.
maximal dose for inositol phosphate accumulation of 250μM. The concentrations for half maximal inhibition of UCHT1-induced phosphatidylinositol metabolism by GDP[S], GTP and p[NH]ppG, were 600μM, 700μM and 1mM respectively.

Although stimulation of PI-PLC activity in response to both agonists was inhibited by GDP and GDP[S], the concentration of guanine nucleotide required for half maximal inhibition of UCHT1-induced phosphatidylinositol metabolism was greater than that required for inhibition of inositol phosphate accumulation induced by GTP[S]. However, UCHT1-induced inositol phosphate accumulation was more sensitive to inhibition by the irrelevant nucleotides CDP and IDP than was the response to GTP[S], particularly at higher nucleotide concentrations. Thus, a nucleotide concentration at which both TCR/CD3 and G protein responses demonstrated significant inhibition by GDP and GDP[S], with an acceptably low non-specific inhibitory effect, was chosen for further experiments.

Table 3.1 summarises the results of experiments in which the effect of addition of various exogenous nucleotides, at a concentration of 100μM, on GTP[S]- and UCHT1-induced inositol phosphate accumulation was investigated.

Table 3.1 Inhibition of GTP[S]- and UCHT1-induced inositol phosphates by exogenous nucleotides.

<table>
<thead>
<tr>
<th>Percentage inhibition on addition of nucleotide (100μM)</th>
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<tr>
<td>Stimulus</td>
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<tr>
<td>10μM GTP[S]</td>
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<td>10μg UCHT1/ml</td>
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Permeabilised, myo-[³H]inositol-labelled, T lymphoblasts were stimulated with 10μg of UCHT1/ml in the presence of the indicated concentration of either CDP (○), IDP (△), GDP (●), GTP (▲), p[NH]ppG (□) or GDP[S] (■). Inositol phosphates were extracted and separated by anion exchange chromatography. Data show percentage of maximum stimulation of radioactivity, expressed as dpm, incorporated into total InsPs released after 7 minutes of stimulation. Maximum values for UCHT1 and GTP[S] stimulation were 4128 dpm and 3554 dpm respectively.
The results show that addition of 100μM GDP inhibited PI-PLC activity induced by 10μM GTP[S] by 55% and UCHT1-induced PI-PLC activity by 39% under conditions where inhibition of these responses by CDP was 4% and 8% respectively. Although 100μM GDP[S] also demonstrated a stronger inhibitory effect on inositol phosphate production induced by GTP[S] (35% inhibition) than that stimulated by UCHT1 (27% inhibition) the overall effect of GDP[S] was consistently weaker than that achieved by addition of GDP. Once again, GTP was found to reproducibly inhibit PI-PLC activity induced by both agonists. These results reinforce the conclusion that TCR/CD3-mediated regulation of PI-PLC activity, although significantly antagonised by inhibitory guanine nucleotides, is less profoundly inhibited than direct G protein stimulation of phosphatidylinositol metabolism by GTP[S]. In the light of the finding that GDP was a consistently more effective inhibitor of responses to both agonists than was GDP[S], GDP was employed in preference to GDP[S] as an inhibitory guanine nucleotide in subsequent experiments.

3.13 Effect of GDP on the dose-response of GTP[S] and UCHT1 for induction of inositol phosphate production.

To further clarify the inhibitory effect of GDP on inositol phosphate production induced by GTP[S] and UCHT1, both agonists were titrated against several concentrations of GDP. The effect of the presence or absence of either 50μM, 100μM or 500μM GDP on the accumulation of inositol phosphates induced by various concentrations of GTP[S] or UCHT1 is shown in Figure 3.13. GDP clearly competed with GTP[S] shifting the GTP[S] dose-response curve to the right (Fig 3.13a). Thus, 100μM GDP increased the half-maximal GTP[S] concentration for inositol phosphate production from 0.75μM to 75μM. GDP also strongly inhibited a dose-response of UCHT1, but without any apparent competitiveness, irrespective of the GDP concentration used (Fig 3.13b). The lack of competitive inhibition of UCHT1-induced inositol
Permeabilised, myo-[^3H]inositol-labelled, T lymphoblasts were stimulated with the indicated concentration of (a) GTP[S] (closed symbols) or (b) UCHT1/ml (open symbols) in the presence of either 50pM- (•/○), 100pM- (■/□) or 500pM-GDP (▲/▲). Inositol phosphates were extracted and separated by anion exchange chromatography. Data show radioactivity, expressed as dpm, incorporated into total InsPs released after 7 minutes of stimulation.

Figure 3.13: Effect of GDP on the dose-response for GTP[S] and UCHT1-induced inositol phosphate production in permeabilised T lymphoblasts.
phosphate accumulation by GDP under these circumstances could reflect that, unlike GTP[S], insufficient anti-CD3 mAb was added to the permeabilised system to enable the inhibitory effect of these concentrations to be overcome.


Another approach that has been widely employed to investigate the role of G proteins in receptor coupling to effector molecules exploits the requirement of G protein-mediated processes for GTP. In permeabilised cells and membrane preparations, where endogenous guanine nucleotide levels have been depleted, G protein-mediated receptors become uncoupled from effector activity. Under such conditions, exogenous analogues of GTP such as p[NH]ppG and GTP[S] have been used to reconstitute receptor coupling. Since triggering of G protein-coupled receptors promotes guanine nucleotide exchange on the G protein pool coupled to the receptor, exogenous guanine nucleotides such as GTP[S] would be predicted to synergise with receptor agonists for the induction of effector responses (Stuchfield and Cockcroft, 1988; Boyer et al. 1989a).

3.14 Guanine nucleotide dependency of UCHT1-induced inositol phosphate production.

In order to gain further insight into the role of G proteins in the coupling of TCR/CD3 complexes to PLC, the guanine nucleotide dependency of UCHT1-induced inositol phosphate accumulation was examined in an extensive series of experiments. In an effort to allow depletion of endogenous guanine nucleotides, the effect of GTP[S] and TCR/CD3 triggering with UCHT1 either singly or in combination, was examined in cells that were permeabilised for 1 minute, 10 minutes or were pelleted and resuspended in permeabilisation
buffer following 10 minutes of permeabilisation, prior to stimulation with the appropriate agonist or combination of agonists (Fig 3.14).

In T lymphoblasts that had been pre-permeabilised for 1 minute, an optimal (10μg/ml) concentration of UCHT1 induced a 13-fold increase in \([^{3}H]\)inositol phosphate accumulation relative to unstimulated cells while an 8-fold accumulation was observed in response to a sub-optimal UCHT1 concentration (2μg/ml). Optimal and sub-optimal concentrations of GTP[S] induced a 9-fold and 4-fold stimulation of PI metabolism respectively (Fig 3.14a). The effect of combining these various concentrations of agonists revealed that no synergy or additivity could be observed with respect to induction of inositol phosphate accumulation. For example, the effect an optimal concentration of UCHT1 in combination with a sub-optimal concentration of GTP[S] was similar to that achieved with UCHT1 alone and visa versa. Combination of sub-optimal agonist concentrations produced similar results (Fig 3.14a).

In order to exclude the possibility that TCR/CD3 coupling, as a consequence of incomplete depletion of endogenous guanine nucleotides, prevented any synergistic interaction between TCR/CD3 and G protein agonists from being observed, the experiment was repeated in cells that had been pre-permeabilised for 10 minutes prior to stimulation (Fig 3.14b). Compared to pre-permeabilisation for 1 minute, the effect of pre-permeabilising T lymphoblasts for 10 minutes was to reduce inositol phosphate accumulation induced by 10μg UCHT1/ml from 13- to 4-fold with respect to unstimulated cells. Similarly, 100μM GTP[S]-stimulated inositol phosphate production was reduced from 9- to almost 6-fold by pre-permeabilisation for 10 minutes. The effects of sub-optimal concentrations of both UCHT1 (1μg/ml) and GTP[S] (1μM) were correspondingly reduced (Fig 3.14a and b). When optimal and sub-optimal concentrations of GTP[S] were combined with optimal and sub-optimal concentrations of UCHT1, there was
Myo-[3H]inositol-labelled, T lymphoblasts were permeabilised for 1 minute, 10 minutes or were pelleted and resuspended prior to addition of the indicated concentrations of either GTP[S], UCHT1, GTP[S] plus UCHT1 or no addition. (see key above). Inositol phosphates were extracted and separated by anion exchange chromatography. Data show radioactivity, expressed as dpm, incorporated into total InsPs released after 7 minutes of stimulation.
no evidence of any additive or synergistic reconstitution of the lost coupling (Fig 3.14b). Removing cytosol from permeabilised cells by spinning-down and resuspending, did not cause further significant reduction in either UCHT1- or GTP[S]-induced inositol phosphate accumulation beyond those observed on pre-permeabilisation for 10 minutes. Under these "washed" conditions GTP[S] together with UCHT1, in any combination, again failed to reconstitute responsiveness lost due to depletion of cytosolic nucleotides (Fig 3.14c).

The loss of GTP[S]- and UCHT1-induced inositol phosphate accumulation observed on pre-permeabilisation suggests that some signal transduction components such as PLC or it's substrates are being lost from the system. However, the observation that UCHT1-mediated induction of inositol phosphate production was more significantly reduced than GTP[S] suggests that some uncoupling of Ti/CD3 complexes from PLC was taking place under these conditions. The observed innability of these conditions to completely uncouple Ti/CD3 from PLC may indicate that a component of Ti/CD3 coupling to PLC in permeabilised cells is not dependent on exogenous guanine nucleotides. No additive or synergistic interaction between optimal and sub-optimal concentrations of TCR/CD3 and G protein agonists (UCHT1 and GTP[S] respectively) was observed under any of the endogenous nucleotide-depletion regimes employed. Further experiments were performed in an attempt to clarify the relationship between G protein- and TCR/CD3-mediated pathways for the regulation of PI-PLC activity.

3.15-3.16 Effect of TCR/CD3 triggering on the dose-response and timecourse of GTP[S]-induced inositol phosphate production.

According to theories concerning receptor-effector coupling by heterotrimeric G proteins, receptor triggering should affect the kinetics and dose-dependency of guanine nucleotide-stimulated effector responses by making more of the receptor-coupled G protein pool accessible to GTP[S].
This effect should be most evident at sub-optimal GTP[S] concentrations. In the light of these predictions, the effect of addition of UCHT1 on the time course and dose-response for GTP[S]-induced inositol phosphate accumulation was studied.

3.15 Effect of UCHT1 on the dose-response of GTP[S]-induced inositol phosphate production.

Figure 3.15 shows the effect of either 10pg or 1pg of UCHT1/ml on the dose-response of GTP[S] for induction of inositol phosphates in T lymphoblasts that had been pre-permeabilised for 10 minutes prior to stimulation. Neither concentration of UCHT1 was observed to significantly alter the dose-response of GTP[S] for inositol phosphate production, which was approximately 1μM. Furthermore, at no concentration of GTP[S] was inositol phosphate accumulation observed to be additive or synergistic with either concentration of UCHT1.

3.16 Effect of UCHT1 on the timecourse of GTP[S]-induced inositol phosphate production.

Further evidence for the lack of synergy or additivity between TCR/CD3 triggering by UCHT1 and G protein stimulation with GTP[S] was obtained from kinetic experiments. Figure 3.16 shows the results of an experiment in which the effect of UCHT1 addition on the time course of inositol phosphate production induced by optimal and sub-optimal concentrations of GTP[S] in T lymphoblasts that had been pre-permeabilised for 10 minutes was investigated. The results show that the time course of inositol phosphate accumulation observed in response to addition of 10μg of UCHT1/ml in combination with 10μM GTP[S] was almost identical to that observed in response to 10μM GTP[S] alone. Moreover, the result of combining 10μg of
Figure 3.15
Effect of UCHT1 on the dose-response for GTP[S]-induced inositol phosphate production in permeabilised T lymphoblasts.

Myo-[^3H]inositol-labelled T lymphoblasts were permeabilised for 10 min prior to the addition of the indicated concentration of GTP[S] (●) or GTP[S] plus either 1μg (○) or 10μg (□) of UCHT1/ml. Inositol phosphates were extracted and separated by anion exchange chromatography. Data show radioactivity, expressed as dpm, incorporated into total InsPs released after 7 minutes of stimulation.
Figure 3.16
Effect of UCHT1 on the time course of GTP[S]-induced inositol phosphate production in permeabilised T lymphoblasts.

Myo-[3H]inositol-labelled T lymphoblasts were permeabilised for 10 min prior to the addition of either 10μg/ml of UCHT1 (O), 1μM-GTP[S] (■), 10μM-GTP[S] (●), 10μg/ml of UCHT1 plus 1μM-GTP[S] (Δ) or 10μg/ml of UCHT1 plus 10μM-GTP[S] (▲). Inositol phosphates were extracted at the indicated time and separated by anion exchange chromatography. Data show radioactivity, expressed as dpm, incorporated into total InsPs.
UCHT1/ml and 1μM GTP[S] closely resembled the time course of inositol phosphate accumulation in response to UCHT1 alone (Fig 3.16).

These results demonstrate that TCR/CD3-mediated regulation of PI-PLC activity in permeabilised T lymphoblasts does not appear to be dependent on exogenous guanine nucleotides. Furthermore, the effect of TCR/CD3 triggering by UCHT1 was neither additive nor synergistic with GTP[S] for the induction of phosphatidylinositol metabolism, failing to alter the kinetics or dose-response of UCHT1-induced accumulation of inositol phosphates under any of the circumstances under which these agonists were combined.


In reciprocal experiments, UCHT1 was titrated in the presence or absence of various concentrations of GTP[S]. (Fig 3.17). Comparison of the effect of UCHT1 titration in combination with either 100μM, 10μM or 3μM GTP[S] to the dose-response of UCHT1 or GTP[S] alone revealed that inositol phosphate accumulation in response to 100μM or 10μM GTP[S] was unaffected by the combinatorial titration of UCHT1. However, inositol phosphate accumulation induced by these concentrations of GTP[S] was greater than the maximal response induced by UCHT1. When UCHT1 was titrated in the presence of 1μM GTP[S], which induced less inositol phosphate accumulation than maximal UCHT1 concentrations, the two agonists were again neither additive nor synergistic (Fig 3.17).
Figure 3.17
Effect of GTP[S] on the dose-response for UCHT1-induced inositol phosphate production in permeabilised T lymphoblasts.

Myo-[³H]inositol-labelled T lymphoblasts were permeabilised for 10 min prior to the addition of the indicated concentration of either UCHT1 (○), GTP[S] (●) or UCHT1 plus 100μM- (●), 10μM- (■) or 3μM-GTP[S] (▲). Inositol phosphates were extracted and separated by anion exchange chromatography. Data show radioactivity, expressed as dpm, incorporated into total InsPs released after 7 minutes of stimulation.
3.18 Effect of analogues of cyclic AMP on TCR/CD3- and G protein-induced phosphatidylinositol hydrolysis in SLO-permeabilised T lymphoblasts.

In some, but not all, T cell populations, elevated cyclic AMP levels can inhibit TCR/CD3-mediated PI hydrolysis (Patel et al., 1987; Stewart et al., 1989; Imboden et al., 1986). Since GTP[S] could activate the stimulatory G protein coupled to adenylate cyclase and increase production of cyclic AMP, interactions between TCR/CD3 and G protein agonists could be complicated by the inhibitory effect of elevated cyclic AMP. However, in permeabilised human peripheral blood-derived T cells, the data show that pre-treatment of T lymphoblasts with the adenylate cyclase stimulator Forskolin prior to permeabilisation did not influence the dose-response of UCHT1- or GTP[S] induced inositol phosphate production. Although these conditions might be expected to result in elevation of cellular cAMP levels prior to permeabilisation, they do not closely mimic those in which GTP[S] might be expected to simultaneously stimulate cAMP accumulation and production of inositol phosphates. In order to account for this fact, T lymphoblasts were pre-treated and permeabilised in the continued presence of the cyclic AMP analogue dibutyryl cyclic AMP. Once more, no effect was observed on the dose-response of either UCHT1 or GTP[S] for the production of inositol phosphates (Fig 3.18). These findings argue against an antagonistic role for cyclic AMP in this system and suggest that the failure to observe synergistic induction of inositol phosphates by a combination of receptor and G protein agonists is not a consequence of elevation of endogenous cyclic AMP concentrations.
Myo-[3H]-inositol labelled T lymphoblasts were either untreated or pre-treated for 30 min with either 10μM-dibutyryl-cyclic AMP (□/■) or 10μM-Forskolin (▲/▼) prior to permeabilisation and stimulation with the indicated concentration of (a) UCHT1 and (b) GTP[S] (closed symbols). Data show radioactivity, expressed as dpm, incorporated into total InsPs after 7 min of stimulation. Chromatography. Data show radioactivity, expressed as dpm, incorporated into total InsPs after 7 min of stimulation. (open symbols) or (d) GTP[S] (closed symbols). Insoluble phosphates were extracted and separated by anion exchange chromatography. Total inositol phosphates (dpm)

**Figure 3.18**

Effect of pre-treatment with Forskolin or dibutyryl-cyclic AMP on the dose-response for UCHT1 and GTP[S]-induced inositol phosphate production in permeabilised T lymphoblasts.
3.19-3.20 Effect of TCR/CD3 down-regulation on GTP[S]-induced inositol phosphate accumulation in SLO-permeabilised T lymphoblasts.

Down-regulation of the TCR/CD3 complex has been shown to inhibit the functions of a G protein involved in the control of exocytic secretion (Schrezenmeier, 1988a; 1988b). The effect of TCR/CD3 down-regulation on the ability of guanine nucleotides to induce phosphatidylinositol hydrolysis was therefore explored. Exposure of T cells to 2μg UCHT1/ml for 3 days modulates the CD3 complex from the cell surface as determined by fluorescent staining (data not shown).

TCR/CD3 down-regulated cells are refactory to stimulation of inositol phosphate production via TCR/CD3 as shown by a comparison of the UCHT1 dose-response for inositol phosphate accumulation in control versus down-regulated cells (Fig 3.19a). Similarly, in a kinetic experiment, 10μg UCHT1/ml induced a time-dependent accumulation of inositol phosphates in untreated cells that was abolished in TCR/CD3 downregulated cells (Fig 3.20a). In contrast, there was no difference in the kinetics or dose-dependency of GTP[S]-induced inositol phosphate production between control and TCR/CD3 modulated cells (Fig 3.19b and 3.20b). These results indicate that down-regulation of the TCR/CD3 complex does not result in co-modulation of GTP[S]-induced phosphatidylinositol hydrolysis in SLO-permeabilised T lymphoblasts.
Myo-[3H]inositol-labelled T lymphoblasts were either untreated (□) or pre-treated for 72 h with 2μg of UCHT1/ml (○) before permeabilisation and stimulation with the indicated concentration of either (a) UCHT1 (open symbols) or (b) GTP[S] (closed symbols). Inositol phosphates were extracted and separated by anion exchange chromatography. Data show radioactivity expressed as dpm, incorporated into total InsPs after 7 min of stimulation.
Myo-[3H]inositol-labelled T lymphoblasts were either untreated (○) or pre-treated for 72 h with 2 μg of UCHT1/ml (□) before permeabilisation and stimulation with either (a) 10 μg of UCHT1/ml (open symbols) or (b) 10 μM-γTP[S] (closed symbols). Inositol phosphates were extracted at the indicated time and separated by anion exchange chromatography. Data show radioactivity, expressed as dpm, incorporated into total InsPs.

Figure 3.20

Effect of down-regulation of the TCR/CD3 complex on the time course for UCHT1 and GTP[S]-induced inositol phosphate production in permeabilised T lymphoblasts.
Permeabilisation of human peripheral blood-derived T lymphoblasts with streptolysin O has allowed the effect of exogenous guanine nucleotides and triggering of the TCR/CD3 complex or CD2 antigen on phosphatidylinositol metabolism to be investigated. In SLO-permeabilised T lymphoblasts, production of inositol phosphates can be stimulated by triggering the TCR/CD3 complex with the anti-CD3 mAb UCHT1, the anti-CD2 GT2/OKT11 hybrid or the polyclonal mitogen PHA. In addition, phosphatidylinositol metabolism can be stimulated by the hydrolysis-resistant GTP analogues GTP[S] and p[NH]ppG in permeabilised T cells. The order of potency of these nucleotides is consistent with the involvement of a G protein in the induction of inositol phosphate production. Thus, peripheral blood-derived human T cells contain PI-specific PLC activity that can be regulated by both the TCR/CD3 complex or CD2 antigen and G proteins. These findings are consistent with previous studies which have demonstrated the presence of G protein- and TCR/CD3-regulated PI-PLC activities in lysed rabbit thymocytes and membrane preparations from the T leukaemic line Jurkat (Zilberman et al., 1987; Sommermeyer et al., 1990).

In permeabilised T lymphoblasts, variations in the concentration of free Ca$^{2+}$ over a physiological range (50-500nM) revealed that both TCR/CD3 and G protein-mediated stimulation of phosphatidylinositol metabolism was dependent on [Ca$^{2+}$]. The synergy observed between TCR/CD3 triggering or G protein stimulation and [Ca$^{2+}$] is consistent with previous studies which have demonstrated that PI-PLC activity is dependent on Ca$^{2+}$ and that G protein regulation serves to lower the enzyme's affinity for Ca$^{2+}$ (Bradford and Rubin, 1986; Stuchfield and Cockcroft, 1988). The concentration of free Mg$^{2+}$
was also observed to profoundly affect TCR/CD3 and G protein-induced phosphatidylinositol metabolism. Although [Mg$^{2+}$] appeared to influence the rate at which both stimuli induced PI-PLC activity, at 1mM [Mg$^{2+}$] TCR/CD3 triggering induced 20% of maximal inositol phosphate production while GTP[S]-induced inositol phosphate production was 65% of maximal. Although it is unclear from these studies how Mg$^{2+}$ might mediate these effects, possibilities include modulation of G protein subunit stability and the activity of protein kinases. The differential effect of [Mg$^{2+}$] on TCR/CD3 and G protein induced activation of PI-PLC activity may reflect mechanistic differences between these pathways.

These observations demonstrate the existence of both receptor and G protein mediated pathways for the regulation of PI-PLC activity in T lymphoblasts but do not directly address the relationship between these regulatory pathways. One possible role for a G protein in the regulation of PI-PLC activity would be as a coupling protein linking the TCR/CD3 complex to PI-PLC. Studies of G protein-mediated receptor coupling to PI-PLC in other systems, such as purinergic receptors in turkey erythrocyte membranes and f-Met-Leu-Phe receptors in permeabilised neutrophils or HL60 cells (Boyer et al., 1989a; Cockcroft and Stuchfield, 1989), has revealed that the regulatory cycle for activation of PI-PLC is similar to that observed for G protein-dependent activation of adenylate cyclase. Thus, interaction of agonist with receptors coupled to both the adenylate cyclase or PI-PLC effector systems stimulates dissociation of GDP from the $\alpha$ subunit of the appropriate G protein heterotrimer. Since the concentration of cellular GTP is very much greater than that of GDP, the result of this event is to promote the overall exchange of bound GDP for GTP. The GTP-bound $\alpha$ subunit then dissociates from the $\beta\gamma$ subunits to directly regulate the effector enzyme. The intrinsic GTPase activity of the G protein $\alpha$ subunit subsequently terminates this cycle and induces
reformation of the inactive αβγ heterotrimer by hydrolysing bound GTP to GDP (reviewed by Gilman, 1987).

By analogy with the adenylate cyclase system and on the basis of the experimental characteristics demonstrated by receptors that are coupled to PI-PLC activity via a G protein in other cell systems, several predictions can be made concerning the experimental characteristics of a G protein-coupled receptor in cells which have been rendered permeable to exogenous guanine nucleotides (summarised in diagram 3.1).

Diagram 3.1 Predicted experimental characteristics of a G protein-coupled effector system in permeabilised cells.
Firstly, receptor coupling to PI-PLC activity should demonstrate an absolute requirement for the presence of guanine nucleotides. For example, in membrane preparations from turkey erythrocytes, purinergic receptor regulation of PI-PLC activity is dependent on the addition of exogenous GTP or stable GTP analogues (Boyer et al., 1989a). Similarly, permeabilisation of HL60 cells with SLO has been observed to uncouple the G protein-linked f-Met-Leu-Phe receptor from PI-PLC (Cockcroft and Stuchfield, 1989). However, in SLO-permeabilised T lymphoblasts, it was not possible to render TCR/CD3-induced phosphatidylinositol metabolism dependent on exogenous guanine nucleotides. Under permeabilisation conditions that would be expected to deplete endogenous guanine nucleotides, the ability of both TCR/CD3 triggering and G protein stimulation to induce inositol phosphate production was observed to be reduced but not abrogated. Furthermore, addition of GTP[S] in combination with TCR/CD3 triggering did not reconstitute receptor coupling that was lost under these circumstances. One explanation for the reduction in both receptor and G protein coupling is that PI-PLC is lost from permeabilised cells or that its activity is otherwise impaired. In this fundamental respect, the TCR/CD3 complex does not function as a classical G protein coupled receptor.

A second criterion that has been used to establish a functional linkage between receptors and G proteins is that the receptor should regulate guanine nucleotide exchange on the pool of G protein that is coupled to it. In practical terms, receptor triggering would be expected to potentiate the ability of GTP[S] to stimulate PI-PLC activity by making a larger pool of receptor-coupled G protein more quickly accessible to GTP[S] (Boyer et al., 1989a; Cockcroft and Stuchfield, 1989). However, in an extensive series of experiments, TCR/CD3 stimulation was not observed to alter the kinetics or dose-response of GTP[S]-induced phosphatidylinositol hydrolysis. The fact that TCR/CD3 triggering does not regulate guanine nucleotide exchange on the GTP[S]-sensitive G
protein pool that can regulate PI-PLC activity strongly suggests that the TCR/CD3 complex is not coupled to PI-PLC via this pool of G proteins.

Another criterion that has been used to establish whether a G protein pool is linked to the TCR/CD3 complex is the dependence of G protein function on surface expression of the TCR/CD3 complex. For example, antibody-mediated down-regulation of the TCR/CD3 complex has been shown to correlate with a decrease in the ability of aluminium fluoride to elevate [Ca\(^{2+}\)] (Schrezenmeier, 1988a) and of GTP[S] to stimulate a G protein involved in the regulation of exocytotic secretion in T cells (Schrezenmeier, 1988b). However, in this study, although TCR/CD3-induced phosphatidylinositol metabolism was abolished in TCR/CD3 down-regulated T lymphoblasts, the ability of GTP[S] to induce PI-PLC activity was unimpaired.

The ability of GDP and stable analogues such as GDP[S] to antagonise G protein function has also been used as an experimental indication that receptor regulation of effector activity is mediated by a G protein. Although this data appears discrepant with the hypothesis that the TCR/CD3 complex is not directly coupled to PI-PLC activity by a G protein, several alternative interpretations are possible. In particular, the inhibitory effect of GDP on TCR/CD3-induced phosphatidylinositol metabolism could reflect that G proteins indirectly contribute to receptor regulation of PI-PLC activity. Thus, the fact that GDP proved to be a more effective inhibitor of GTP[S]-induced inositol phosphate production than of inositol phosphate production induced by triggering the TCR/CD3 complex could be explained by the possibility that a G protein exerts a modulatory influence on phosphatidylinositol metabolism but is not directly involved in directly coupling the TCR/CD3 complex to PI-PLC. Although G proteins could potentially influence receptor regulation of inositol phospholipid turnover at multiple points, one possibility is that guanine nucleotides may influence the phosphatidylinositol cycle and exert a regulatory influence on the availability of substrates to PI-PLC. Although the
lack of additivity or synergy between GTP[S] and TCR/CD3 triggering would appear to argue against this possibility, the hypothesis remains to be fully investigated.

In the light of these findings, several possible models for the mechanism by which the TCR/CD3 complex is coupled to PI-PLC can be considered. A model in which the TCR/CD3 complex is directly coupled to PI-PLC could account for the ability of GDP to inhibit both TCR/CD3- and G protein-mediated phosphatidylinositol metabolism (see diagram 3.2; model 1). However, this model is incompatible with the observation that the TCR/CD3 complex does not appear to regulate guanine nucleotide exchange on the G protein pool that is stimulated by GTP[S]. The simplest model that can be proposed to account for both of these findings proposes the existence of multiple pools of G protein capable of stimulating PI-PLC. According to this model, the TCR/CD3 complex is coupled to the PI-PLC via a G protein that is insensitive to GTP[S]. However, a distinct G protein pool that is stimulated by GTP[S] but is not coupled to the TCR/CD3 complex is also capable of regulating PI-PLC activity (see diagram 3.2; model 2). A variation on this model is that the G protein coupled to the TCR/CD3 complex is not regulated by receptor stimulation of guanine nucleotide exchange.

Although both of these G protein-mediated models represent theoretical possibilities, they fail to account for the lack of dependence on guanine nucleotides demonstrated by the TCR/CD3 complex in coupling to PI-PLC. Therefore, an alternative hypothesis is that the TCR/CD3 complex is coupled to PI-PLC via a mechanism that does not directly involve guanine nucleotide binding proteins (see diagram 3.2; model 3). According to this non-G protein coupled but G protein modulated model a distinct G protein pathway that is not coupled to the TCR/CD3 complex also exists for the regulation of PI-PLC activity in T lymphocytes.
Diagram 3.2 Proposed models for G protein involvement in coupling of the TCR/CD3 complex to PI-specific PLC.

1) G Protein-coupled model.

2) G Protein-coupled separate pool model.

3) Non-G Protein-coupled G protein-modulated model.

For explanation of proposed models see supporting text.
This scheme affords an explanation for most of the experimental findings made in this study but also raises several important questions. Both the identity of the GTP[S]-responsive G protein which regulates PI-PLC activity in T lymphoblasts and the receptor to which it is coupled are not known. In this respect, the identity of the pertussis toxin-insensitive G protein which regulates PI-PLC activity in other cell types has not been clearly established. However, the recent purification from bovine brain and liver of a G protein α subunit capable of activating PI-PLC-β \textit{in vitro} has provided one candidate (Taylor \textit{et al.}, 1991). This protein belongs to the Gq class of G protein and may be a member of a family of proteins which serve to couple different receptors to PI-PLC in a variety of tissues.

In the light of accumulated evidence in T lymphocytes and by analogy with the mechanism by which PDGF and EGF receptors are thought to induce PI-PLC activity in fibroblasts, a likely non-G protein-mediated coupling mechanism for the regulation of PI-PLC activity by the TCR/CD3 complex involves tyrosine phosphorylation of PI-PLC-γ. In this respect, the T leukaemic cell line Jurkat has been demonstrated to express PI-PLCα, PI-PLCβ1, γ1 and δ1 isozymes (Goldfien \textit{et al.}, 1991; Park \textit{et al.}, 1991). The recent observation of PI-PLC-γ phosphorylation on stimulation of the TCR/CD3 complex in Jurkat strengthens the hypothesis that T cells might possess a tyrosine kinase-mediated mechanism for regulation of PI-PLC activity (Park \textit{et al.}, 1991). Consequently, T cells may possess both G protein and tyrosine kinase pathways for the regulation of PI-PLC activity. Interestingly, UCHT1 and GTP[S] were not found to be additive for induction of PI-PLC activity, as might be predicted if TCR/CD3 and G protein agonists acted via completely independent regulatory pathways. This observation suggests that these pathways interact at some point distal to both the TCR/CD3 complex and G protein.
One possibility is that the signals generated by TCR/CD3 triggering and G protein stimulation act in parallel to regulate a common pool of PI-PLC. Although this hypothesis could explain the current data, preliminary evidence suggests that tyrosine kinase and G protein-mediated mechanisms might regulate distinct isozymes of PI-PLC. Thus, PDGF or EGF receptors induce tyrosine phosphorylation of PI-PLC-γ but not of the α or β isozymes while triggering of bradykinin, bombesin or vasopressin receptors, which are thought to couple to PI-PLC via a G protein, does not result in phosphorylation of PI-PLC-γ (Nishibe et al., 1989; Wahl et al., 1989; Meisenhelder et al., 1989). Conversely, the candidate PI-PLC-regulatory G protein α subunit (Gqα) can stimulate PI-PLC-β \textit{in vitro} but does not activate the γ or δ isozymes (Taylor et al., 1991). Although these results suggest that PI-PLC-γ and β are regulated by distinct mechanisms, it is not known how other PI-PLC isozymes are regulated. Thus, it remains possible that T cells could express a pool of PI-PLC that is regulated by both G protein and non-G protein pathways.
Section 4.
Phosphorylation of the γ-subunit of the CD3 antigen: Regulation of protein kinase C activity in SLO-permeabilised T lymphoblasts.

Introduction.

The serine/threonine protein kinase C (PKC) plays an central role in T lymphocyte activation and has been implicated in diverse early cellular responses (reviewed by Cantrell and Alexander, 1989; Berry and Nishizuka, 1990). Evidence to suggest that PKC is involved in T cell activation is largely derived from the use of phorbol esters which directly activate PKC and can mimic aspects of TCR/CD3 or CD2 triggering. By this criterion PKC is thought to mediate TCR/CD3- or CD2-induced phosphorylation of T cell surface molecules such as CD4, CD8, (Acres et al., 1986; 1987; Minami et al., 1987; Blue et al., 1987) CD45 (Autero and Gahmberg, 1987; Schackleford and Trowbridge) and the γ-subunit of the CD3 antigen (Cantrell et al., 1985; 1987, Samelson et al., 1987). Two cytoplasmic proteins, of 19 and 80kDa, have also been documented as being rapidly phosphorylated (Friedrich et al., 1988).

Since these receptors are involved in the physiological activation process, their phosphorylation suggests that one role of PKC in T cells might be to regulate receptor function (Cantrell et al., 1985). A negative feedback role for PKC in regulation of intracellular calcium concentrations is suggested by the observation that activation of PKC inhibits TCR/CD3-induced elevation of [Ca²⁺] in the T leukaemic cell line Jurkat (Ward and Cantrell, 1990). In addition, the ability of PKC in conjunction with Ca²⁺ ionophores to induce expression of the IL2 receptor, production of IL2 and therefore T cell proliferation, indicates that PKC is an important component of the signal transduction pathways by which the TCR/CD3 complex regulates T cell growth (reviewed by Berry and Nishizuka, 1990).
The PKC family comprises multiple isozymes which share a basic functional requirement for Ca\(^{2+}\), phospholipid and diacylglycerol (DAG). By increasing the enzyme's affinity for Ca\(^{2+}\), DAG is thought to function as the physiological regulator of PKC (Nishizuka, 1988; Bell, 1986). Although it is established that DAG is produced as a consequence of PI-PLC-mediated metabolism of phosphatidylinositols, examples of receptor stimulation of PKC activity in the absence of phosphatidylinositol hydrolysis can be explained by the fact that DAG can arise from alternative cellular sources. In particular, phosphatidylcholine-specific phospholipase C (PC-PLC) and phospholipase D (PC-PLD) activities have demonstrated the potential to generate DAG in other cells (Pelech and Vance, 1989; Billah and Anthes, 1990; Exton, 1990). These alternative pathways present the possibility that multiple receptor regulated mechanisms for the control of cellular PKC activity may exist.

Further complexity is suggested by the observation that multiple PKC isozymes exist and display subtly different requirements for activation in vitro. In particular, the \(\beta\) isozyme but not the \(\alpha\) isozyme of PKC can be significantly activated in the nominal absence of Ca\(^{2+}\) (Shearman et al., 1988) and the \(\gamma\) isozyme, unlike other PKC subtypes, can be activated \textit{in vitro} by arachidonic acid (Nishizuka, 1988). These findings suggest that the molecular heterogeneity of PKC may provide functional diversity and that distinct PKC isozymes might be activated under different cellular circumstances.

Although G proteins can regulate the activity of PI-PLC in T lymphocytes, the role of G proteins in the regulation of cellular PKC activity is not clearly understood. \(\text{AlF}_4^-\), a pharmacological stimulator of G proteins in intact cells, can induce phosphatidylinositol metabolism and PKC activation in murine T cell hybridomas (O'Shea et al., 1987). Guanine nucleotides have also been demonstrated to stimulate phosphorylation of the CD3 antigen in T cell membranes (Davies et al., 1988). However, the ability of G proteins to regulate PI-PLC activity does not necessarily indicate that this is the only
mechanism by which G proteins might regulate PKC. The fact that G proteins have also been implicated in the regulation of PC-PLC, PC-PLD and PLA$_2$ activities in other cells suggests that G proteins could affect PKC activity by multiple pathways (Irving and Exton, 1987; Bocckino et al., 1987; Martin and Michaelis, 1990; Burch et al., 1986).

In order to investigate the role of G proteins in the regulation of PKC activity, the ability of both receptor and G protein agonists to induce phosphorylation of the $\gamma$-subunit of the CD3 antigen in SLO-permeabilised T lymphoblasts was examined. The CD3 $\gamma$-subunit has previously been demonstrated to be rapidly phosphorylated in response to antigen, mitogenic lectin or phorbol ester stimulation of human T cells and is considered to be a substrate for PKC (Cantrell et al., 1985; 1987). Thus, the transmembrane CD3 $\gamma$-subunit represents an ideal endogenous PKC substrate for permeabilised cell studies.
Results.

4.1-4.2 Effect of PDBu, GTP[S] and PHA on phosphorylation of the γ-subunit of the CD3 antigen in permeabilised T lymphoblasts.

To establish that PKC-mediated phosphorylation of CD3 γ chains could be detected in SLO-permeabilised T lymphoblasts, cells were permeabilised in the presence of [γ-32P]ATP and were stimulated with the phorbol ester phorbol 12, 13-dibutyrate (PDBu), which activates protein kinase C, or the polyclonal T cell mitogen PHA. These stimuli have previously been shown to induce CD3 γ chain phosphorylation in intact T cells (Cantrell et al., 1985; Davies et al., 1985; Cantrell et al., 1987). The anti-CD3 mAb UCHT1 was used to immunoprecipitate the CD3 antigen for analysis by SDS-PAGE and autoradiography. Since immunoprecipitation of CD3 with UCHT1 proved impossible after using this anti-CD3 mAb to trigger the TCR/CD3 complex, the polyclonal T cell mitogen PHA was used as a receptor stimulant in these studies. Figure 4.1 shows that a weak but detectable phosphorylation of the 26kDa CD3 γ polypeptide was observed in the absence of any stimulation. However, PDBu was observed to induce a rapid and stable phosphorylation of the 26kDa CD3 γ polypeptide, detectable as early as 1 minute after stimulation and increasing for at least 10 minutes. PHA also induced rapid and stable phosphorylation of CD3 γ subunits in permeabilised T lymphoblasts (Fig 4.1). Figure 4.2 shows that phosphorylation of the CD3 γ-subunit in response to PHA was dose-dependent. The half maximal concentration of PHA for induction of CD3 γ phosphorylation was 1-2μg/ml and maximal phosphorylation was observed to occur at a concentration of between 10 and 20μg/ml. In agreement with previous studies performed on intact T cells, PHA was a consistently less potent stimulator of CD3 γ chain phosphorylation than PDBu. PHA stimulated a 2 to 4-fold increase in 32P incorporation into the CD3 γ chain, PDBu induced a 6 to 8-fold increase in phosphorylation (Fig 4.1).
T lymphoblasts were permeabilised in the presence of 500 nM Ca2+, [γ-32P]ATP and either 20 ng of PDBu/ml (▲), 100 pM-GTP[S] (■) or 1000 nM-β-mercaptoethanol (●) for the time indicated. The CD3 antigen was immunoprecipitated, analysed by SDS/PAGE (left panel) and 32P incorporation quantified by densitometric scan of gel autoradiographs. The level of CD3γ phosphorylation, normalised with respect to controls and expressed in arbitrary units, is shown in the right panel.
T lymphoblasts were permeabilised in the presence of 500nM-Ca²⁺, [γ-32P]ATP and were either unstimulated or stimulated with 20ng/ml PdBu (▲) or the indicated concentration of PHA (■) for 5 min. The CD3 antigen was immunoprecipitated, analysed by SDS/PAGE (left panel) and 32P incorporation quantified by densitometric scan of gel autoradiographs. The level of CD3γ phosphotyrosine, normalised with respect to controls and expressed in arbitrary units, is shown in the right panel.

Figure 4.2

Dose-response of PHA for induction of CD3γ-subunit phosphorylation in permeabilised T lymphoblasts.
To establish whether G protein regulation of CD3 γ phosphorylation could be detected in permeabilised T lymphoblasts, cells were permeabilised in the presence of the stable GTP analogue GTP[S]. Figure 4.1 shows that 100μM GTP[S] induced rapid incorporation of 32P into the CD3γ chain which was maximal by 5 minutes. GTP[S] proved to be as effective as PDBu in inducing CD3γ phosphorylation in permeabilised T cells and was consistently observed to be 2-3 times more potent than PHA. These results indicate that direct activation of PKC by PDBu, receptor stimulation by PHA and G protein stimulation by GTP[S] can stimulate phosphorylation of the CD3γ chain in T lymphoblasts permeabilised with SLO.

4.3 Dose response and specificity of guanine nucleotide-induced CD3 γ-subunit phosphorylation.

The specificity of guanine nucleotide-induced phosphorylation of the CD3 γ chain was examined by permeabilising T lymphoblasts in the presence of a panel of exogenous nucleotides at various concentrations in the range 10μM-1mM. Phosphorylation of the CD3 γ chain in response to GTP[S] was dose-dependent, with half-maximal phosphorylation at a nucleotide concentration of around 75μM and maximal CD3γ chain phosphorylation was observed at 200μM GTP[S] (Fig 4.3). The weakly hydrolysed GTP analogue p[NH]ppG was a less effective stimulus for induction of 32P incorporation into the CD3 γ chain. The ability of these two agonists to induce phosphorylation of the CD3 γ chain reflected their relative ability to stimulate inositol phosphate accumulation in permeabilised T lymphoblasts (GTP[S] > p[NH]ppG; see figure 3.5). Figure 4.3 demonstrates that only hydrolysis-resistant analogues of GTP, such as GTP[S] and p[NH]ppG, induced significant phosphorylation of the CD3 γ chain. Thus, GTP, GDP[S], CTP and ITP were ineffective at inducing phosphorylation of the CD3 antigen.
T lymphoblasts were permeabilised in the presence of 500nM-Ca2+, [\( \gamma \)-32P]ATP and the indicated concentration of GTP[S] (○), GTP (■), GTP[S] (□), CTP (▼), GTP [NH]ppG (○), GTP[S] (□), CTP [NH]ppG (○), or IPT (▲) for 5 min. The CD3 antigen was immunoprecipitated, analysed by SDS/PAGE (left panel) and 32P incorporation quantified by densitometric scan of gel autoradiographs. The level of CD3\( \gamma \) phosphorylation, normalised with respect to controls and expressed in arbitrary units, is shown in the right panel.

**Figure 4.3** Nucleotide specificity of CD3\( \gamma \)-subunit phosphorylation in permeabilised T lymphoblasts.
4.4 Inhibition of CD3 $\gamma$-subunit phosphorylation by a pseudo-substrate inhibitor of PKC.

The identification of PKC as the mediator of CD3-antigen phosphorylation in permeabilised cells rests heavily on the use of a pseudo-substrate inhibitor of PKC. This 13-amino acid peptide inhibitor, based on a sequence in the regulatory domain of the enzyme itself, acts as a competitive inhibitor of PKC with high efficiency and specificity. Application of this inhibitor to T lymphocytes, including a demonstration that the peptide specifically inhibits PDBU- and PHA-induced CD3 antigen phosphorylation, has provided strong evidence for the involvement of PKC in CD3 $\gamma$ phosphorylation by these stimuli (Alexander et al., 1989). Although the ability of both PDBu and GTP[S] to induce phosphorylation the $\gamma$ chain of the CD3 antigen is consistent with GTP[S]-induced phosphorylation of this substrate being mediated by PKC, use of the pseudo-substrate PKC inhibitor enables the involvement of PKC in G protein-mediated CD3 $\gamma$ phosphorylation to be investigated in permeabilised cells.

T lymphoblasts were permeabilised in the presence of the indicated concentration of pseudo-substrate inhibitor peptide and were stimulated with either PDBu or GTP[S]. Figure 4.4 shows that both PDBu- and GTP[S]-induced CD3 $\gamma$ phosphorylation were inhibited by the pseudo-substrate peptide in a dose-dependent manner, with half-maximal inhibition for both agonists at a peptide concentration of 10-20$\mu$M. In agreement with previous studies (Alexander et al., 1989), the phosphorylation level of non-PKC substrate bands, observed in total lysates after immunoprecipitation of the CD3 antigen, was not affected by the pseudo-substrate inhibitor (data not shown).
with 20 ng of PDBu/ml (o) or 100 pM-GTP[S] (•) for 5 min in the presence of the indicated concentration of pseudo-substrate peptide. The CD3 antigen was immunoprecipitated, analysed by SDS/PAGE (left panel) and 32P incorporation quantified by densitometric scan of gel autoradiographs. The level of CD3γ phospho-tyrosine, normalised with respect to controls and expressed in arbitrary units, is shown in the right panel.

**Figure 4.4**

Effect of a PKC pseudo-substrate peptide on PDBu- and GTP[S]-induced CD3γ-subunit phosphorylation in permeabilised T lymphoblasts.
4.5-4.6 Effect of variations in the concentration of free Ca$^{2+}$ and Mg$^{2+}$ on PDBu, PHA and G protein-mediated CD3γ-subunit phosphorylation.

The [Ca$^{2+}$] and [Mg$^{2+}$] in the permeabilised cell system might be expected to affect PKC activity since the in vitro activity of this kinase is dependent on the [Ca$^{2+}$] over a nM range, while Mg$^{2+}$ is a cofactor required by many protein kinases including PKC (reviewed by Nishizuka). In addition, variation in the [Ca$^{2+}$] and [Mg$^{2+}$] have been demonstrated to influence PI-PLC activity in permeabilised T lymphoblasts (see figures 3.7-3.9). Using EGTA buffers, the [Ca$^{2+}$] and [Mg$^{2+}$] in the permeabilisation buffer was varied in order to determine the effect of these variations on agonist-induced phosphorylation of the γ-subunit of the CD3 antigen.

The effect of varying the [Ca$^{2+}$] within a range from nominally zero to 1μM is shown in figure 4.5. In the absence of other stimuli, Ca$^{2+}$ was only observed to induce detectable phosphorylation of the CD3 γ chain at Ca$^{2+}$ concentrations greater than 150nM. PHA did not induce detectable phosphorylation of CD3 γ chains at nominally zero [Ca$^{2+}$], but was synergistic with [Ca$^{2+}$] up to 150nM Ca$^{2+}$, after which point the effects of PHA and [Ca$^{2+}$] were additive. The Ca$^{2+}$-dependency curve for GTP[S]-induced CD3 γ phosphorylation correlated closely with that of PHA-stimulated phosphorylation, with no response at zero and maximum synergy at a [Ca$^{2+}$] of 150nM. In contrast to GTP[S] and PHA, a substantial effect on CD3 γ phosphorylation in response to PDBu was observed at nominally zero [Ca$^{2+}$]. This effect was approximately 60% of the maximal PDBu-induced CD3 γ phosphorylation, which occurred at a [Ca$^{2+}$] of 500nM and represented a 10 fold enhancement over CD3γ phosphorylation induced by Ca$^{2+}$ alone. A slight but reproducible inhibition of PDBu-induced CD3γ phosphorylation was observed at a [Ca$^{2+}$] of 1μM.
Figure 4.5

Effect of variations in the concentration of free Ca²⁺ on CD3γ-subunit phosphorylation in permeabilised T lymphoblasts.
T lymphoblasts were permeabilised in the presence of the indicated concentration of Mg$^{2+}$, 500nM-Ca$^{2+}$, [$\gamma^{32}$P]ATP and either 20ng/ml of PDBu (●), 10μg/ml of PHA (■) or 100μM-GTP[S] (▲) for 5 min. The CD3 antigen was immunoprecipitated, analysed by SDS/PAGE and $^{32}$P incorporation quantified by densitometric scan of gel autoradiographs. The level of CD3 γ phosphorylation was normalised with respect to controls and expressed in arbitrary units.
Figure 4.6 shows the effect on agonist-induced CD3 γ phosphorylation of permeabilising T lymphoblasts in the presence of 500nM Ca\textsuperscript{2+} and various concentrations of Mg\textsuperscript{2+} over a range from nominally zero to 10mM. Neither PDBu, PHA or GTP[S] induced significant phosphorylation of the CD3 γ chain at nominally zero [Mg\textsuperscript{2+}] and displayed a linear dependency up to the maximum [Mg\textsuperscript{2+}] used in this experiment of 10mM. The fact that PDBu-, PHA- and GTP[S]-induced phosphorylation demonstrated a similar dependency on [Mg\textsuperscript{2+}] suggests that the predominant effect of Mg\textsuperscript{2+} on this system is at the level of kinase or phosphatase activity.

4.7 Comparison of the dose-response for CD3γ-subunit phosphorylation and inositol phosphate production by GTP[S] and PHA.

The accumulated data suggests that both GTP[S] and PHA stimulate PKC activity and thereby induce phosphorylation of the CD3 γ chain in permeabilised T lymphoblasts. Since both GTP[S] and PHA also stimulate inositol phosphate production in permeabilised cells, agonist-induced phosphatidylinositol metabolism with consequent production of DAG represents a likely pathway by which GTP[S] and PHA regulate PKC activity. To investigate the relationship between PKC activation and phosphatidylinositol metabolism by PHA and GTP[S], the dose-response of both agonists for CD3 γ phosphorylation and inositol phosphate production in permeabilised cells were compared.

The results (Fig 4.7) demonstrate several interesting points. Firstly, the dose-response curves of PHA for inositol phosphate production and CD3 γ chain phosphorylation showed a half-maximal effect for both responses at 2μg/ml (Fig 4.7a). However, the concentration of GTP[S] required to induce CD3 γ phosphorylation was markedly different from that required to initiate inositol phosphate production (Fig 4.7b). For example, at a GTP[S] concentration of 10μM, inositol phosphate production was maximal whereas
The presence of 500nM Ca²⁺, 10mM Mg²⁺ and the indicated concentration of (a) PHA or (b) GTP[S] for 10 mins. Data are expressed as fold stimulation of inositol phosphate production or CD3γ phosphorylation with respect to unstimulated cells. The lymphoblasts were permeabilised in the presence of 500nM Ca²⁺, 10mM Mg²⁺ and the indicated concentration of (a) PHA or (b) GTP[S]. Total inositol phosphates were extracted and separated at 10 mins. For CD3γ phosphorylation (closed symbols), lymphoblasts were permeabilised in the presence of 500nM Ca²⁺, 10mM Mg²⁺ and the indicated concentration of GTP[S]. (a) or PHA (b). Total inositol phosphates were permeabilised in the

Figure 4.7

Comparison of the dose-response of PHA and GTP[S] for induction of CD3-γ subunit phosphorylation and inositol phosphate production.
CD3 γ chain phosphorylation was 10% of maximal. The half maximal concentrations for inositol phosphate accumulation and CD3 γ phosphorylation were 0.7μM and 75μM respectively. Secondly, PHA was consistently observed to be more effective than GTP[S] in the induction of inositol phosphate production. This is in direct contrast to the relative abilities of these two agonists with respect to CD3 γ phosphorylation, where GTP[S] was more potent than PHA (Fig 4.7a and 4.7b). To summarise this point, PHA induced approximately 8-10 fold increases in inositol polyphosphates, but 3-4 fold increases in CD3 γ chain phosphorylation. GTP[S], however, induced 4-5 fold increases in phosphatidylinositol metabolism but 7-10 fold stimulation of CD3 γ phosphorylation (Fig 4.7a and 4.7b).

The ability to dissociate GTP[S]-induced inositol phosphate production from CD3 γ phosphorylation in permeabilised T lymphoblasts suggests that GTP[S] may regulate PKC activity by a mechanism distinct from the induction of PI-PLC activity and phosphatidylinositol-derived DAG production.

4.8 Comparison of the Ca^{2+}-dependence of CD3 γ-subunit phosphorylation and inositol phosphate production in permeabilised T lymphoblasts and Jurkat.

Studies have revealed the existence of some heterogeneity in the signalling pathways by which the TCR/CD3 complex regulates PI-PLC activity in T lymphocytes. For example, agents that elevate cyclic AMP concentrations have been reported to inhibit TCR/CD3-induced inositol phosphate production and Ca^{2+} mobilisation in murine hybridomas and the human T leukaemic cell line Jurkat (Patel et al., 1987; Stewart et al., 1989). In addition, activation of PKC has been demonstrated to inhibit TCR/CD3-induced inositol phosphate production and Ca^{2+} mobilisation in Jurkat but not in T lymphoblasts (Ward and Cantrell, 1990). Since activation of PKC is associated with downregulation of TCR/CD3 expression in both Jurkat and T lymphoblasts, the differential effect of PKC activation suggests that some heterogeneity exists in
the signalling pathways by which the TCR/CD3 complex regulates PI-PLC activity in these cells.

The molecular basis of the difference between PI-PLC regulation in Jurkat and T blasts is unclear. Although there is no evidence to suggest that Jurkat and T lymphoblasts express different PI-PLC isozymes, differential expression of components of the signalling pathway, such as PKC or PI-PLC, could explain the regulatory differences observed. In this respect, Jurkat have been demonstrated to express high levels of the α isozyme of PKC and relatively low levels of PKC β whereas the relative ratios of these isozymes are reversed in T lymphoblasts (Lucas et al., 1990). Furthermore, studies in fibroblasts have revealed that the receptor for bombesin is coupled to PI-PLC via a G protein and that bombesin-induced PI-PLC activity is sensitive to inhibition by PKC. In contrast, PDGF-induced PI-PLC activity is considered to be mediated by tyrosine phosphorylation and has been demonstrated to be insensitive to PKC inhibition. Thus the pathway by which the TCR/CD3 complex is coupled to PI-PLC may be different in Jurkat and T lymphoblasts.

To investigate differences in the regulation of PI-PLC activity in Jurkat and T lymphoblasts, both cell types were permeabilised with SLO. When Jurkat cells were permeabilised in the presence of 10mM Mg2+ and 500mM Ca2+, without additional stimulation, a high level of CD3 γ phosphorylation was observed in comparison to T lymphoblasts permeabilised under the same conditions. This observation prompted a comparative analysis of the effect of variations in the [Ca2+] on induction of CD3 γ phosphorylation in the two cell types. Figure 4.8a shows the effect of varying [Ca2+] on CD3 γ phosphorylation in T lymphoblasts and Jurkat in the presence or absence of PDBu. Whereas almost 60% of maximal PDBU-induced CD3 γ phosphorylation occured in the nominal absence of Ca2+ in T lymphoblasts, the effect of variations in [Ca2+] on PDBu-induced CD3 γ phosphorylation in Jurkat was very different. Thus, in Jurkat, only 16% of maximal PDBu-induced phosphorylation occured at
10 mins. Data are expressed as percentage of maximum stimulation of inositol phosphate production or CD3γ phosphorylation.

For CD3γ phosphorylation (a), T lymphoblasts (○) or Jurkat (□) were permeabilised in the presence of 10 mM Mg2+ and the indicated concentration of Ca2+. Total inositol phosphates were extracted and separated at 10 mins. For inositol phosphate determinations (b), myo-[3H]inositol-labelled T lymphoblasts (○) or Jurkat (□) were permeabilised in the presence of 20 ng/ml of PDBu (closed) or absence (open symbols) or presence of 20 ng/ml of PDBu (closed) for 10 mins. Data are expressed as percentage of maximum stimulation of inositol phosphate production or CD3γ phosphorylation.

Figure 4.8

Production in permeabilised T lymphoblasts and the T-leukemic cell line Jurkat of the effect of variations in the concentration of the free Ca2+ on CD3γ-subunit phosphorylation and inositol phosphate.
nominally zero [Ca\(^{2+}\)] with maximum synergy observed with Ca\(^{2+}\) observed at 150nM. The observation of differential effects of Ca\(^{2+}\) on PDBu-induced phosphorylation between Jurkat and T blasts suggests that PKC may exhibit differential sensitivity to [Ca\(^{2+}\)] in these two cells.

In contrast to T lymphoblasts, where significant Ca\(^{2+}\)-induced phosphorylation of CD3\(\gamma\) (around 20% of maximal) was observed at a [Ca\(^{2+}\)] of 500nM, 55% of maximal CD3 phosphorylation was observed at 500nM Ca\(^{2+}\) in Jurkat and almost 30% of maximal CD3\(\gamma\) phosphorylation was induced by 150nM Ca\(^{2+}\) (Fig 4.6a). One possible explanation for the comparatively strong Ca\(^{2+}\)-induced CD3\(\gamma\) phosphorylation observed in Jurkat is that inositol phospholipid metabolism, with consequent production of DAG, may be more sensitive to stimulation by Ca\(^{2+}\) in Jurkat than T blasts. To investigate this possibility myo-[3H]inositol labelled Jurkat and T lymphoblasts were permeabilised in the presence of Ca\(^{2+}\) at a range of concentrations. The results (figure 4.8b) show that in T lymphoblasts, 500nM Ca\(^{2+}\) alone induced a two fold increase in inositol phosphate accumulation. However, in Jurkat cells, inositol phosphate production was markedly more sensitive to [Ca\(^{2+}\)] with a 7 fold increase at 150nM [Ca\(^{2+}\)] and a significant (5 fold) increase observed at 50nM. These data indicate that accumulation of inositol phosphates is more sensitive to [Ca\(^{2+}\)] in Jurkat cells than T lymphoblasts and are consistent with the hypothesis that the strong stimulatory effect of [Ca\(^{2+}\)] on PKC activity in Jurkat may be explained by [Ca\(^{2+}\)] stimulation of PI-PLC activity.
Discussion.
Phosphorylation of the \(\gamma\)-subunit of the CD3 antigen: Regulation of protein kinase C activity in SLO-permeabilised T lymphoblasts.

This study demonstrates that phosphorylation of the \(\gamma\)-subunit of the CD3 antigen can be induced in response to PDBu, PHA and GTP[S] in SLO-permeabilised T lymphoblasts. The specificity of guanine nucleotide effects are consistent with the hypothesis that phosphorylation of the CD3\(\gamma\)-subunit in permeabilised T cells is regulated by a G protein. The data also indicate that GTP[S] regulates CD3 antigen phosphorylation via PKC. This conclusion is based on experiments with a specific peptide inhibitor which has previously been used to demonstrate that PHA-induced CD3 \(\gamma\) phosphorylation is mediated by PKC (Alexander et al., 1989). Of particular relevance to the identity of the GTP[S]-regulated kinase is the observation that the inhibition dose/response curves for the PKC pseudosubstrate peptide are identical for PDBu- and GTP[S]-induced phosphorylation. Since this peptide is a competitive inhibitor of PKC (House and Kemp, 1987; Alexander et al., 1989) the data imply that PDBu and GTP[S] regulate CD3 \(\gamma\) phosphorylation via a common kinase, PKC.

As a consequence of the fact that PKC-mediated CD3 antigen phosphorylation can be stimulated by agonists such as PHA and GTP[S], which stimulate phosphatidylinositol metabolism, it has been proposed that CD3 antigen phosphorylation is regulated via agonist-induced diacylglycerol production resulting from metabolism of inositol phospholipids. However, it is now clear that the production of diacylglycerol from phosphatidylinositol breakdown does not always correlate with stimulation of PKC (reviewed by Pelech and Vance, 1989; Exton, 1990). In permeabilised T cells, the timecourse, \(Ca^{2+}\)-dependency and dose/response of PHA effects on inositol phosphate production correlate with PHA induced stimulation of CD3\(\gamma\) chain
phosphorylation. These results are consistent with PHA regulation of PKC-mediated CD3 phosphorylation via diacylglycerol production resulting from inositol phospholipid turnover (see diagram 4.1). However, the observation that PHA induces higher levels (>200%) of phosphatidylinositol turnover than GTP[S] but is much weaker (<50%) than GTP[S] with respect to the induction of CD3 antigen phosphorylation suggests that some discrepancy exists between the abilities of these agonists to stimulate phosphatidylinositol metabolism and PKC activity.

The lack of a correlation between inositol lipid hydrolysis and PKC stimulation is demonstrated further by the observation that GTP[S] induced inositol phosphate production was maximal at 10μM whereas CD3γ phosphorylation was only 10% of maximum. As GTP[S] concentrations are increased above 10μM, PKC mediated phosphorylation increases but there are no further increases in inositol phospholipid metabolism. It therefore appears that GTP[S] regulation of CD3γ chain phosphorylation is not regulated solely by the products of inositol phosphate hydrolysis. These results suggest that there is more than one G protein regulated mechanism for the control of PKC in T lymphocytes. Whether the differences in the GTP[S] dose/response curves for phosphatidylinositol turnover and PKC activation reflects that these pathways are controlled by guanine nucleotide binding proteins with different affinities for GTP[S] remains to be determined.

Because there are several points at which agonists could influence the phosphorylation level of PKC substrates, several possible explanations for the discrepancy between GTP[S] and PHA effects on phosphatidylinositol hydrolysis and CD3γ chain phosphorylation must be considered. One possibility is that PKC may be regulated by diacylglycerol produced as a result of the turnover of other membrane phospholipids such as phosphatidylcholine. Since both PC-specific PLC and PLD activities have been demonstrated to be under G protein control in other cell systems, phosphodiesteric breakdown of
phosphatidylcholine may contribute to GTP[S]-induced DAG production (Pelech and Vance, 1989; Exton, 1990). It should also be emphasized that T cells express at least two isoenzymes of PKC, the α and β forms (Beyers et al., 1988; Lucas et al., 1990). The physiological relevance of PKC heterogeneity is not known, but it is possible that these isoenzymes are differentially stimulated by PHA and GTP[S]. In this respect, phospholipid metabolites such as arachidonic acid, which could be regulated by G proteins or PHA, may also stimulate PKC (Nishizuka, 1988).

The possibility should also be considered that these agonists may influence the level of substrate phosphorylation by mechanisms that are distinct from regulation of kinase activity. Thus, the relative effects of PHA and GTP[S] on CD3 γ phosphorylation could reflect that they differentially regulate cellular phosphatase activity. Finally, the hypothesis that GTP[S] might promote PKC-mediated phosphorylation of CD3 γ cannot be excluded. A speculative model for such a mechanism might involve the GTP[S]-induced dissociation of a guanine nucleotide binding protein from the CD3 cytoplasmic domains of the CD3 chains or a GTP[S]-induced conformational change in the CD3 γ chain itself. In conclusion, these data are consistent with the model that stimulation of PKC is explained in part by agonist effects on phosphatidylinositol metabolism. It is probable, however, that GTP[S] does not regulate PKC and CD3 phosphorylation by phosphatidylinositol turnover alone but may use alternative mechanisms or a combination of different signals (see diagram 4.1).

T lymphocytes have been demonstrated to vary in their expression of PKC isozymes. Thus, the T leukaemic line Jurkat express high levels of the α isozyme of PKC and relatively low levels of PKC β whereas T lymphoblasts express low levels of α and relatively more PKC β (Lucas et al., 1990). Although the physiological implications of PKC heterogeneity are unclear, in vitro assays have revealed several differences between isozymes. For
Diagram 4.1 Proposed models for the regulation of protein kinase C activity in permeabilised T lymphoblasts.

For explanation of proposed models see supporting text.
example, in the absence of Ca\(^{2+}\) PKC \(\alpha\) and \(\beta\) can be stimulated \textit{in vitro} to 18 and 65% of maximal activity respectively (Nishizuka, 1988). Comparison of SLO-permeabilised T lymphoblasts and Jurkat has revealed that these cells differ with respect to the \([\text{Ca}^{2+}]\) sensitivity of CD3 \(\gamma\) chain phosphorylation. One major difference is that a nominally zero \([\text{Ca}^{2+}]\), PDBu induces 60% of maximal CD3 \(\gamma\) chain phosphorylation in T lymphoblasts but only 16% of the maximal response in Jurkat. Thus, the \textit{in vitro} calcium sensitivity of PKC \(\alpha\) and \(\beta\) isotypes, in conjunction with their relative levels of expression in T lymphoblasts and Jurkat, could explain the differences that are observed in the \([\text{Ca}^{2+}]\) sensitivity of PDBu-induced CD3 \(\gamma\) chain phosphorylation. However, T cells might also express other PKC isozymes that differ in their sensitivity to \([\text{Ca}^{2+}]\). Although T cells do not express the \(\gamma\) isozyme of PKC, expression of PKC \(\delta\) and \(\epsilon\) in T lymphocytes has not been examined (Beyers \textit{et al.}, 1988; Lucas \textit{et al.}, 1990).

The ability of Ca\(^{2+}\), in the absence of PDBu, to induce CD3 \(\gamma\) phosphorylation was also found to differ between these two cells. Thus, Ca\(^{2+}\) was observed to induce significant CD3\(\gamma\) phosphorylation in Jurkat but not in T lymphoblasts. It is not known how this effect might relate to the pattern of PKC isozyme expression in these cells. One possible explanation can be inferred from the observation that production of inositol phosphates was found to be markedly stimulated in Jurkat by concentrations of Ca\(^{2+}\) that did not induce significant phosphatidylinositol metabolism in T lymphoblasts. This suggests that Ca\(^{2+}\)-induced levels of DAG and therefore PKC activity will be higher in permeabilised Jurkat than T lymphoblasts under these conditions. In addition, the possibility that a Ca\(^{2+}\)-regulated kinase distinct from PKC may differentially contribute to CD3\(\gamma\) phosphorylation in these permeabilised cells cannot be excluded.

Although the differential Ca\(^{2+}\) sensitivity of inositol phosphate production between Jurkat and T lymphoblasts could be explained by
differences in the expression of PI-PLC isozymes, there is no evidence to suggest that these cells differ in their pattern of expression of PI-PLC subtypes. However, clear differences between these cells have been observed with respect to regulation of PI-PLC activity. In particular, activation of PKC has been demonstrated to inhibit TCR/CD3-induced inositol phosphate production and Ca$^{2+}$ mobilisation in Jurkat but not in T lymphoblasts (Ward and Cantrell, 1990). These results may provide a basis for the differences between PI-PLC regulation in Jurkat and T blasts.
Section 5.
The regulation of p21ras during T cell activation: Analysis in SLO-permeabilised T lymphoblasts.

Introduction

The products of the ras proto-oncogenes (p21\textsuperscript{ras}) are 21kDa membrane-associated guanine nucleotide-binding proteins that are thought to play an important and ubiquitous role in the transduction of growth-regulatory signals (reviewed by Barbacid, 1987; Hall, 1990; Downward 1990). Evidence to suggest that p21\textsuperscript{ras} functions in pathways associated with the regulation of cell growth is largely derived from the transforming properties of mutant or overexpressed normal ras proteins and the ability of neutralising anti-p21\textsuperscript{ras} mAbs to block proliferation in response to a wide range of cellular stimuli (reviewed by Marshall, 1991).

In common with other guanine nucleotide-binding proteins, such as the signal-transducing G proteins, p21\textsuperscript{ras} proteins are in the 'active' state when bound to GTP and are converted to the 'inactive' GDP-bound state by their intrinsic GTPase activity (reviewed by Bourne et al., 1990). Point mutations that result in oncogenic p21\textsuperscript{ras} serve to cause an accumulation of the GTP-bound protein by either increasing the rate of exchange of GTP for GDP on p21\textsuperscript{ras} or by reducing the rate of hydrolysis of bound GTP (Sweet et al., 1984; Der et al., 1986). Thus, under normal cellular circumstances, the proportion of cellular p21\textsuperscript{ras} in the GTP-bound state could be reciprocally regulated by modulation of the rate of nucleotide exchange or GTPase activity (Bourne et al., 1990). In this respect, cellular proteins which interact with p21\textsuperscript{ras}.GTP and stimulate the protein's intrinsic GTPase activity (Trahey and McCormick, 1987; Xu et al., 1990b; Ballester et al., 1990; Martin et al., 1990) and rate of nucleotide exchange have been identified (West et al., 1990; Huang et al., 1990; Wolfman and Macara, 1990; Downward et al., 1990a). Despite intensive investigation,
the contribution these activities might make to regulation of cellular \( p21^{ras} \) activity or the cellular signals to which \( p21^{ras} \) might respond are unclear.

Although proteins which interact with \( p21^{ras} \) and stimulate its GTPase activity, such as \((ras)\text{GAP}\) (Trahey and McCormick, 1987) and the protein encoded by the neurofibromatosis type-1 locus (NF1) (Xu et al., 1990b; Ballester et al., 1990; Martin et al., 1990), represent candidates for upstream regulatory factors, there is also evidence to suggest that they may participate in the poorly characterised downstream signalling pathways from \( p21^{ras} \) (reviewed by McCormick, 1989; Hall, 1990a). A regulatory role for \((ras)\text{GAP}\) can be inferred from studies in which transfection of GAP into \( \text{ras} \) or \( \text{src} \)-transformed fibroblasts causes reversion of the transformed phenotype (Zhang et al., 1990a; DeClue, 1991; Nori et al. 1991). In addition, a link between growth factor signalling and \( p21^{ras} \) has been suggested by the observation that \((ras)\text{GAP}\) is phosphorylated by activated PDGF and EGF receptors or certain tyrosine kinase oncogenes (Molloy et al., 1989; Ellis et al., 1990 and reviewed by McCormick, 1990). Further studies will be required to clarify the role of these proteins in \( p21^{ras} \)-mediated cellular growth regulatory pathways.

By analogy with the heterotrimeric signal transducing \( G \) proteins, it has been suggested that if the Ras proteins function as intermediates in growth factor-stimulated signalling pathways then \( p21^{ras} \) would accumulate in the GTP-bound form on growth factor treatment (McCormick, 1990). In view of the accumulated evidence that \( p21^{ras} \) functions in such pathways, the effect of triggering surface receptors that regulate T cell activation and proliferation was investigated. In T lymphocytes, progression to the \( G_1 \) stage of the cell cycle, secretion of growth factor IL2 and expression of its high affinity receptor is controlled by signals generated by T cell surface molecules such as the TCR/CD3 complex (reviewed by Weiss and Imboden, 1987). Interaction of IL2 with its receptor is required for entry of T cells into the S phase of the cell cycle (reviewed by Smith, 1988).
One method that has been applied to the analysis of cellular Ras activity involves immunoprecipitation of p21\textsuperscript{ras} from cells whose endogenous GTP pools have been labelled with \textsuperscript{32}P-orthophosphate (Satoh \textit{et al.}, 1988). This approach enables p21\textsuperscript{ras} activity to be quantitated by measurement of the proportion of cellular Ras in the GTP-bound state. In addition, permeabilisation of T cells should allow direct access to the cytoplasm for \textsuperscript{32}P-labelled GTP and enable investigation of the kinetics of nucleotide binding and hydrolysis on p21\textsuperscript{ras} in T lymphocytes. These techniques were employed to investigate the effect of triggering T cell surface receptors, such as the TCR/CD3 complex and IL2R, on the guanine nucleotide bound to endogenous p21\textsuperscript{ras} in human T lymphocytes.
Results.

5.1 Effect of TCR/CD3 or CD2 triggering on guanine nucleotide bound to p21\textsuperscript{ras} in T lymphoblasts.

In order to determine the effect of signalling via the TCR/CD3 complex or CD2 antigen on the activation state of cellular p21\textsuperscript{ras}, [\textsuperscript{32}P]orthophosphate-labelled T lymphoblasts were either unstimulated or stimulated with the anti-CD3 mAb UCHT1, the polyclonal mitogen PHA or the CD2 agonist GT2/OKT11 hybrid F(\textsuperscript{Ab'}\textsuperscript{y})\textsubscript{3} prior to lysis and immunoprecipitation of p21\textsuperscript{ras}. \textsuperscript{32}P-labelled nucleotides bound to p21\textsuperscript{ras} were eluted from the immunoprecipitates, separated by thin layer chromatography and subjected to autoradiography.

The results (Fig 5.1) show that p21\textsuperscript{ras} from unstimulated cells is 90-95% GDP-bound. Stimulation with UCHT1, PHA or GT2/OKT11 F(\textsuperscript{Ab'}\textsuperscript{y})\textsubscript{3} induced the accumulation of GTP on p21\textsuperscript{ras} such that GTP constituted approximately 40% of the guanine nucleotide eluted from p21\textsuperscript{ras} immunoprecipitates after 10 minutes of stimulation. Activation of T cells via the CD2 antigen has been demonstrated to require cross linking of a combination of antibodies, such as the anti-CD2 mAbs GT2 and OKT11, that are specific for distinct epitopes on the CD2 molecule (Huet et al., 1986). Figure 5.1 shows that stimulation of T lymphoblasts with a bispecific GT2/OKT11 hybrid F(\textsuperscript{Ab'}\textsuperscript{y})\textsubscript{3} agonist stimulates the accumulation of GTP on p21\textsuperscript{ras} but that treatment of cells with a combination of soluble intact GT2 and OKT11 antibodies does not stimulate p21\textsuperscript{ras}. These results demonstrate that triggering of the TCR/CD3 complex, ligation of the CD2 antigen and treatment with the mitogen PHA stimulates an approximately 8-fold accumulation of GTP on cellular p21\textsuperscript{ras} in normal human peripheral blood-derived T lymphoblasts.
Thin layer chromatogram of the guanine nucleotides eluted from immunoprecipitates of P21 ras from 32P-orthophosphate-labelled T lymphoblasts. Immunoprecipitation was with P21 ras mAb Y13-259 or with a non-specific control antibody as indicated. Cells were unstimulated or stimulated for 10 mins with 10μg of CD3 mAb UCHT1/ml, 10μg of PHA/ml, 10μg of both CD2 mAbs OKT1 and OKT2/ml, or 1μg of CD2 bispecific F(Ab')3/ml. The position at which GTP and GDP standards ran is indicated.

Effect of TCR/CD3 or CD2 triggering on guanine nucleotide bound to P21 ras in T lymphoblasts.

Figure 5.1
5.2-5.3 Effect of phorbol dibutyrate and ionomycin on guanine nucleotide bound to p21\textsuperscript{ras} in T lymphoblasts and the T-leukaemic line Jurkat.

Since triggering of the TCR/CD3 complex or CD2 antigen stimulates multiple intracellular signalling pathways, including elevation of [Ca\textsuperscript{2+}]\textsubscript{i}, activation of PKC and stimulation of tyrosine kinase activity, it is unclear which receptor-regulated cellular pathway might be responsible for stimulation of p21\textsuperscript{ras} in these cells. Pharmacological agents such as ionomycin which elevates the [Ca\textsuperscript{2+}]\textsubscript{i} and the phorbol 12,13-dibutyrate (PDBu) which activates PKC have previously been used to mimic these aspects of receptor stimulation. To investigate the mechanism by which TCR/CD3 and CD2 stimulation regulate p21\textsuperscript{ras}, \textsuperscript{32}P orthophosphate-labelled T lymphoblasts and Jurkat cells were treated with either PDBu or ionomycin prior to analysis of the guanine nucleotides bound to p21\textsuperscript{ras}.

The results shown in figure 5.2 whereas PDBu induced the accumulation of GTP on p21\textsuperscript{ras} in both T lymphoblasts and the T-leukaemic cell line Jurkat, treatment with ionomycin did not stimulate p21\textsuperscript{ras} in either cell type. In both T lymphoblasts and Jurkat, the proportion of GTP bound to p21\textsuperscript{ras} was observed to increase from approximately 5% in unstimulated cells to 70% of total p21\textsuperscript{ras}-associated nucleotide in PDBu-stimulated cells.

Figure 5.3 summarises the results from a number of experiments in which the effect of various agonists on the activation state of p21\textsuperscript{ras} in T lymphoblasts was investigated. The results are presented as the amount of GTP as a proportion of total nucleotide, as determined by direct scanning of thin layer chromatograms for \(\beta\) radiation. In unstimulated cells GTP represented 5-10% of total nucleotide. Stimulation with either UCHT1, PHA or GT2/OKT11 hybrid F(\text{Ab'\gamma})\textsubscript{3} induced from 35-45% of p21\textsuperscript{ras} to accumulate in the GTP-bound state while PDBu treatment stimulated a 70-80% accumulation.
Figure 5.2
Effect of the calcium ionophore ionomycin or phorbol ester PDBu on guanine nucleotide bound to p21\textsuperscript{ras} in T lymphoblasts and the T-leukaemic cell line Jurkat.

Thin layer chromatogram of the guanine nucleotides eluted from immunoprecipitates of p21\textsuperscript{ras} from $^{32}$P-orthophosphate-labelled T lymphoblasts and Jurkat. Immunoprecipitation was with p21\textsuperscript{ras} mAb Y13-259. Cells were unstimulated or stimulated for 30 mins with 50ng of PDBu/ml, or 5\mu g of ionomycin/ml. The position at which GTP and GDP standards ran is indicated.
Figure 5.3
Effect of TCR/CD3 or CD2 triggering, the mitogen PHA or activation of PKC on guanine nucleotide bound to p21\textsuperscript{ras} in T lymphoblasts.

Ratio of [\alpha-\textsuperscript{32P}]GTP as a percentage of total [\alpha-\textsuperscript{32P}]-labelled nucleotide eluted from immunoprecipitates of p21\textsuperscript{ras} from \textsuperscript{32P}-orthophosphate-labelled T lymphoblasts. Immunoprecipitation was with p21\textsuperscript{ras} mAb Y13-259. Cells were unstimulated or stimulated for 30 mins with 10\mu g of UCHT1/ml, 10\mu g of PHA/ml, 1\mu g of GT2/OKT11 F(\text{Ab'})\textsubscript{3}/ml, 50ng of PDBu/ml, or 5\mu g of ionomycin/ml. Data shows mean±S.E. and is representative of at least 5 separate determinations.
of GTP. PDBu was consistently found to be a more potent stimulator of p21\textsuperscript{ras} than any of the receptor agonists employed. Since these cells contain about 40,000 molecules of p21\textsuperscript{ras} per cell, the number of GTP bound p21 molecules rises from about 2,000 to 20,000 upon TCR/CD3 or CD2 stimulation and to around 35,000 in cells treated with PDBu. Taken together, these results suggest that a major intracellular pathway for regulation of p21\textsuperscript{ras} via the TCR/CD3 complex or CD2 antigen is mediated by activation of PKC.

5.4 Time course of activation of p21\textsuperscript{ras} on stimulation of PKC and triggering the TCR/CD3 complex or CD2 antigen.

The time course for activation of p21\textsuperscript{ras} by UCHT1, GT2/OKT11 F(\text{Ab'\gamma})\text{3} and PDBu in \textsuperscript{[32P]}orthophosphate-labelled T lymphoblasts is shown in figure 5.4. The proportion of cellular p21\textsuperscript{ras} in the GTP-bound state rises to 80% within 2 minutes of PDBu treatment of T lymphoblasts. TCR/CD3 triggering with UCHT1 or CD2 ligation with GT2/OKT11 F(\text{Ab'\gamma})\text{3} also induced the rapid accumulation of approximately 50% of cellular p21\textsuperscript{ras} in the GTP-bound state. The response to these receptor agonists, reproducibly slower than that in response to direct PKC stimulation with PDBu, was detectable within 2 minutes and was maximal after 10 minutes of stimulation. These results demonstrate that the increases in GTP levels on p21\textsuperscript{ras} following activation by these agonists are very rapid and are consistent with TCR/CD3 and CD2-induced activation of p21\textsuperscript{ras} being mediated by PKC rather than a direct interaction with p21\textsuperscript{ras}.

5.5 Effect of PKC activation or TCR/CD3 triggering on p21\textsuperscript{ras} in the T leukaemic cell line HPBALL.

The T cell line HPBALL is an interesting model for the study of signal transduction in T cells. Recent work has demonstrated that the TCR/CD3 complex is uncoupled from regulation of PI-PLC activity in this cell type and
Figure 5.4
Time course of activation of p21\textsuperscript{ras} on stimulation of PKC or triggering the TCR/CD3 complex and CD2 antigen in T lymphoblasts.

Ratio of [\alpha-\textsuperscript{32}P]GTP as a percentage of total [\alpha-\textsuperscript{32}P]-labelled nucleotide eluted from immunoprecipitates of p21\textsuperscript{ras} from \textsuperscript{32}P-orthophosphate-labelled T lymphoblasts. Immunoprecipitation was with p21\textsuperscript{ras} mAb Y13-259. Cells were stimulated for the indicated time with PDBu (\textbullet{}), UCHT1 (■) or GT2/OKT11 F(\text{Ab'})\textsubscript{3} (●). Immunoprecipitation was with p21\textsuperscript{ras} mAb Y13-259.
Figure 5.5
Effect of PKC activation or TCR/CD3 triggering on guanine nucleotide bound to p21^{ras} in the T cell line HPB-ALL.

Thin layer chromatogram of the guanine nucleotides eluted from immunoprecipitates of p21^{ras} from ^{32}P-orthophosphate-labelled HPB-ALL cells. Immunoprecipitation was with p21^{ras} mAb Y13-259. Cells were unstimulated or stimulated for 30 mins with 10\mu g of UCHT/ml, 10\mu g of PHA/ml or 50ng of PDBu/ml. The position at which GTP and GDP standards ran is indicated.
that this disfunction correlates with it’s lack of expression of the tyrosine phosphatase CD45 (Koretzky et al., 1990). This cell therefore presents an opportunity to gain further insight into the mechanism by which receptor agonists regulate the level of GTP on p21\(^{ras}\) in T cells. Figure 5.5 is an autoradiogram of the nucleotide bound to p21\(^{ras}\) in \([^{32}P]\)orthophosphate-labelled HPBALL cells that were either unstimulated or stimulated with PDBu, PHA or UCHT1. Whereas PDBu induced the accumulation of GTP on p21\(^{ras}\) both PHA and UCHT1 failed to stimulate p21\(^{ras}\) in these cells. Although the precise nature of the signalling deficiency demonstrated by HPBALL cells is not understood, the inability of receptor agonists to stimulate p21\(^{ras}\) in these cells demonstrates that expression of CD45 is required for receptor-mediated regulation of p21\(^{ras}\) in T cells.

5.6 Stimulation of p21\(^{ras}\) in SLO-permeabilised T lymphoblasts.

Previous data has demonstrated that TCR/CD3 and CD2-induced regulation of PI-PLC activity and both TCR/CD3 and PDBu-induced PKC activity can be studied in SLO-permeabilised T lymphoblasts (see Section 3 and Section 4). This system offers considerable potential for the investigation and manipulation of the intracellular mechanism by which these stimuli regulate p21\(^{ras}\). In order to determine whether receptor and PDBu-induced activation of p21\(^{ras}\) were supported in SLO-permeabilised T lymphoblasts, cells were permeabilised in the presence of [\(\alpha-^{32}P\)]GTP and were stimulated with either PDBu, PHA, UCHT1 or GT2/OKT11 F(\(\text{Ab'\'y})_3\).

Figure 5.6 is an autoradiogram of the nucleotide eluted from p21\(^{ras}\) immunoprecipitated from permeabilised cells after 5 minutes of stimulation. Since no nucleotide is detected in the absence of specific antibody, p21\(^{ras}\) must rapidly bind labelled nucleotide on exposure to [\(\alpha-^{32}P\)]GTP in permeabilised cells. In agreement with results obtained in intact cells, p21\(^{ras}\) is
stimulated with either 50 ng of PDBu/ml, 10 pg of UCHT1/ml, 10 pg of PHA/ml or 1 pg of GT2/OKT11 Fab' for 5 min. Guanine nucleotides were eluted from p21ras immunoprecipitates and separated by thin layer chromatography. Left panel shows an autoradiograph of the chromatogram. The ratio of [α-32P]GTP as a percentage of total [α-32P]-labelled nucleotide is shown in the right panel as a % of total [α-32P]-labelled nucleotide bound to p21ras in p21ras immunoprecipitates. Data is mean±S.E. and is representative of at least 5 separate determinations.

Figure 5.6

Effect of PKC activation and TCR/CD3 or CD2 triggering on guanine nucleotide bound to p21ras in SLO-permeabilised T lymphoblasts.
Lymphoblasts were permeabilised in the presence of 150mM-Ca²⁺, 5mM-Mg²⁺, and [γ-32P]GTP (■) in the indicated time prior to centrifugation in a microcentrifuge and removal of a small aliquot of supernatant. Guanine nucleotides were separated by thin layer chromatography. Left panel shows an autoradiograph of the chromatogram. The amount of GTP (○), GDP (●) and GMP (■) present at the indicated time, as a percentage of total [γ-32P]-labelled nucleotide, is shown in the right panel.

**Figure 5.7**

**Time course of hydrolysis of GTP in SLO-permeabilised lymphoblasts.**
bound almost exclusively to GDP in permeabilised unstimulated T lymphoblasts; GTP constitutes around 5% of total labelled nucleotide bound to p21\textsuperscript{ras} under these circumstances. However, on PDBu treatment, p21\textsuperscript{ras} was observed to accumulate in the GTP-bound state such that GTP accounted for 20% of p21\textsuperscript{ras}-bound nucleotide. Similarly, stimulation with PHA, UCHT1 or GT2/OKT11 F(\text{Ab}'\gamma)_3 induced GTP, as a proportion of total p21\textsuperscript{ras}-bound nucleotide, to rise to 15%.

It is important to make the point that some p21\textsuperscript{ras} will bind GDP directly as a result of the action of other cellular GTPases. However, the ratio of labelled GTP to GDP in permeabilised cells 5 minutes after permeabilisation is roughly 1:1 (Fig. 5.7) and is not significantly altered by agonist treatment (data not shown).

5.8-5.11 An analysis of p21\textsuperscript{ras} activation in permeabilised T lymphoblasts.

The increase in GTP-bound p21\textsuperscript{ras} observed upon stimulation of protein kinase C or triggering of the TCR/CD3 complex and CD2 antigen could occur by an increase in the rate at which which GDP dissociates from p21\textsuperscript{ras} and GTP subsequently binds or by a decrease in the rate of GTP hydrolysis on p21\textsuperscript{ras} (reviewed by Bourne et al., 1990). In order to address these possibilities, the kinetics of [\alpha-\text{32P}]GTP exchange and hydrolysis on p21\textsuperscript{ras} in permeabilised T lymphoblasts that were unstimulated, stimulated with PDBu, the anti-CD3 mAb UCHT1 or the anti-CD2 GT2/OKT11 F(\text{Ab}'\gamma)_3 hybrid was studied.

5.8-5.9 Effect of PKC activation and triggering of the TCR/CD3 complex or CD2 antigen on the guanine nucleotides bound to p21\textsuperscript{ras} in permeabilised T lymphoblasts.

Figure 5.8 shows the \text{32P}-labelled guanine nucleotides associated with immunoprecipitates of p21\textsuperscript{ras} from permeabilised cells that were unstimulated or stimulated and exposed to [\alpha-\text{32P}]GTP for the indicated time. The results
show that the rate at which total nucleotide bound to \( p21^{ras} \) in permeabilised cells was very rapid and that stimulation with either PDBu, UCHT1 or GT2/OKT11 F(\( Ab^\gamma \))\_3 induced accumulation of GTP on \( p21^{ras} \) that was detectable at the earliest time point of 1 minute. Since the rate limiting step for exchange of guanine nucleotides on \( p21^{ras} \) is GDP dissociation (Hall and Self, 1986; Cales et al., 1988), the rate of binding of total \( ^{32}P \)-labelled guanine nucleotide can be considered to reflect the nucleotide exchange rate. Figure 5.9 is a graphical representation of these results as rate of total \( ^{32}P \)-labelled nucleotide binding (Fig 5.9a) and ratio of GTP to total nucleotide bound to \( p21^{ras} \) (Fig 5.9b). The rate at which total \( ^{32}P \)-labelled guanine nucleotide bound to \( p21^{ras} \) was found to be identical in unstimulated cells or cells that had been stimulated with PDBu, UCHT1 or GT2/OKT11 F(\( Ab^\gamma \))\_3 (Fig 5.9a). However, the ratio of GTP to total nucleotide bound to \( p21^{ras} \) was higher in stimulated cells than in unstimulated cells at all time points (Fig 5.9b). These results suggest that while the exchange rate of nucleotides onto \( p21^{ras} \) in permeabilised cells is unaffected, the increased proportion of GTP-bound \( p21^{ras} \) observed upon activation of PKC or receptor triggering is probably a consequence of decreased hydrolysis of GTP on \( p21^{ras} \).

In order to slow the otherwise rapid rate of exchange of \( ^{32}P \)-labelled nucleotide onto \( p21^{ras} \), T lymphoblasts were permeabilised at 27°C and were either unstimulated or stimulated with PDBu. At this temperature, PDBu treatment clearly did not influence the rate of guanine nucleotide binding to \( p21^{ras} \) (Fig 5.10a). Figure 5.10b shows the ratio of GTP to total nucleotide on \( p21^{ras} \) during this timecourse and demonstrates the accumulation of GTP on \( p21^{ras} \) on phorbol ester treatment. Collectively, these results support the conclusion that the difference in the level of GTP bound is due to a decrease in the rate of hydrolysis of GTP bound to \( p21^{ras} \) in T lymphoblasts on activation of PKC or triggering of the TCR/CD3 complex or CD2 antigen.
Figure 5.8
Time course of p21ras activation on stimulation of PKC and triggering the TCR/CD3 complex or CD2 antigen in SLO-permeabilised T lymphoblasts.

Thin layer chromatogram of the guanine nucleotides eluted from immunoprecipitates of p21ras from T lymphoblasts permeabilised in the presence of 150nM Ca²⁺, 5mM Mg²⁺ and [γ-³²P]GTP. Cells were unstimulated or stimulated with either 50ng of PDBu/ml, 10µg of UCHT1/ml, 10µg of PHA/ml or 1µg of GT2/OKT11 F(AB')₃ for the time indicated.
stimulated with either 50ng of PDBu/ml (•), 10pg of UCHT1/ml (□), or 1pg of GT2/OKT11 F(ab')3 (■) for the time indicated. Guanine nucleotides were eluted from p21ras immunoprecipitates and separated by thin layer chromatography. Guanine nucleotides were eluted from p21ras immunoprecipitates and separated by thin layer chromatography. Analysis of the effect of PKC activation or triggering of the TCR/CD3 complex and CD2 antigen on the guanine nucleotide bound to p21ras in permeabilised T lymphoblasts. (a) Time course for accumulation of total [α-32P]-labelled nucleotide (b) Time course of the ratio of [α-32P]-labeled nucleotide to total [α-32P]-labelled nucleotide. (a) Time course for accumulation of total [α-32P]-labelled nucleotide (b) Time course of the ratio of [α-32P]-labeled nucleotide to total [α-32P]-labelled nucleotide. (a) Time course for accumulation of total [α-32P]-labelled nucleotide (b) Time course of the ratio of [α-32P]-labeled nucleotide to total [α-32P]-labelled nucleotide. (a) Time course for accumulation of total [α-32P]-labelled nucleotide (b) Time course of the ratio of [α-32P]-labeled nucleotide to total [α-32P]-labelled nucleotide.
Analysis of the effect of PKC activation on P21ras in permeabilised T lymphoblasts at 27°C.

Figure 5.10
5.11 Specificity of nucleotide exchange onto p21\textsuperscript{ras} in permeabilised T lymphoblasts.

The previous studies have suggested that guanine nucleotides bind to p21\textsuperscript{ras} in permeabilised cells with a very rapid half time (consistent with an effective $k_{\text{diss-GDP}}$ of $\approx 1\text{min}^{-1}$ at 37°C). This rate is very much faster than the intrinsic rate of exchange on p21\textsuperscript{ras} would allow (Hall and Self, 1986; Cales et al., 1988). To illustrate this point, under identical conditions (0.4 I.U. of streptolysin O/ml and permeabilisation buffer containing 100nM Ca\textsuperscript{2+} and 5mM Mg\textsuperscript{2+}), baculovirus expressed p21\textsuperscript{ras} and p21\textsuperscript{ras} in T cell membrane preparations demonstrate a $k_{\text{diss-GDP}}$ of approximately $60\text{min}^{-1}$ (data not shown).

To further investigate and validate the rapid exchange rate of nucleotide onto p21\textsuperscript{ras} observed in permeabilised T cells, the effect of addition of various 'cold' nucleotides on the timecourse of $^{32}$P-labelled guanine nucleotide binding to p21\textsuperscript{ras} in permeabilised cells was investigated at 27°C. T lymphoblasts were permeabilised in the presence of [$\alpha$-$^{32}$P]GTP for various times up to 15 minutes when either no addition or 100μM unlabelled GTP, ATP, CTP, ITP were added for various times up to a further 15 minutes. The results (Figure 5.11) show that whereas ATP, CTP and ITP did not influence the amount of $^{32}$P-labelled guanine nucleotide bound to p21\textsuperscript{ras}, addition of GTP caused a time dependent decrease in labelled nucleotide recovery. The timecourse of this specific and competitive displacement of $^{32}$P-labelled guanine nucleotide from p21\textsuperscript{ras} is identical to that of nucleotide binding, providing confirmation that in permeabilised T cells the rate of nucleotide binding is equal to the rate of GDP dissociation. These data support the observation that the $k_{\text{diss-GDP}}$ of p21\textsuperscript{ras} at 37°C is $\approx 1\text{min}^{-1}$, roughly 60 times faster than the intrinsic $k_{\text{diss-GDP}}$ of p21\textsuperscript{ras} or the rate of nucleotide binding to p21\textsuperscript{ras} in membrane preparations (data not shown).
Time course for accumulation of total $[\alpha-^{32}P]$-labelled nucleotide on p21$\text{ras}$ in permeabilised T lymphoblasts. T lymphoblasts were permeabilised at 27°C in the presence of 150nM Ca$^{2+}$, 5mM Mg$^{2+}$ and $[\gamma-^{32}P]$GTP for 10 min prior to the addition of 100μM GTP (●), ITP (■), CTP (△) or no addition (□). Guanine nucleotides were eluted from p21$\text{ras}$ immunoprecipitates and separated by thin layer chromatography.
5.12 Inhibition of (p21\textsuperscript{ras})GAP activity by agents that stimulate p21\textsuperscript{ras} in T lymphoblasts.

A possible mechanism for the observed inhibition of p21\textsuperscript{ras} GTPase activity could be inhibition of the activity of a p21\textsuperscript{ras} GTPase activating protein upon stimulation of protein kinase C or triggering of the TCR/CD3 complex or CD2 antigen. Since cells contain multiple proteins with p21\textsuperscript{ras} GTPase stimulating activity, this effect could be mediated by the p21\textsuperscript{ras} GTPase activating protein (GAP) (Trahey and McCormick, 1987) and the protein encoded by the neurofibromatosis type-1 locus (NF1), the product of the NF1 gene which has been shown to possess GAP-like activity (Xu \textit{et al.}, 1990b; Ballester \textit{et al.}, 1990; Martin \textit{et al.}, 1990), or possibly by the activity of another as yet uncharacterised protein.

In order to investigate this hypothesis, the p21\textsuperscript{ras} GTPase-stimulating activity in a Triton X-100 lysate of T lymphoblasts that had been treated for 10 minutes with either PDBu, UCHT1 or GT2/OKT11 hybrid F(Ab\textsuperscript{'y})\textsubscript{3} was compared to the activity in lysates from untreated cells. Varying amounts of lysate were mixed with pure bacterially-expressed p21\textsuperscript{c-H-ras} that had been preloaded with [\textalpha-\textsuperscript{32}P]GTP. After incubation, the p21\textsuperscript{ras} was recovered by immunoprecipitation and the guanine nucleotide bound to it analyzed by thin layer chromatography.

Figure 5.12 shows that the ability of an extract from phorbol ester treated T lymphoblasts to induce hydrolysis of GTP on p21\textsuperscript{ras} is reduced relative to a lysate from treated cells. In this experiment p21\textsuperscript{ras} GTPase-stimulating activity was depressed by 5 fold relative to lysate from untreated cells. Treatment of T lymphoblasts with UCHT1 or GT2/OKT11 hybrid F(Ab\textsuperscript{'y})\textsubscript{3} was also observed to reduce the ability of a lysate from activated cells to induce the hydrolysis of GTP on p21\textsuperscript{ras}. Both of these agonists demonstrated a 2 fold inhibition of GTPase-stimulating activity in cell extract. The magnitude of these inhibitory effects on p21\textsuperscript{ras} GTPase-stimulating activity correspond to the
T lymphoblasts were incubated for 10 min with 100nM-okadaic acid and either 50ng of PDBu/ml (●), 10μg of UCHT1/ml (■), 1μg/ml of GT2/OKT11 F(Ab')₃ (□) or no addition (○) Activity of GAP in a Triton X-100 lysate was determined by mixing various dilutions of lysate with p21ras that had been preloaded with [α-32P]GTP. After incubation, the p21ras was recovered by immunoprecipitation and the guanine nucleotide bound to it analyzed by thin layer chromatography.
relative abilities of these stimuli to induce the accumulation of GTP on p21 ras
(PDBu > UCHT1 = GT2/OKT11 hybrid F(\text{Ab}^\gamma)_3).

Since this inhibition of p21 ras GTPase-stimulating activity is observed in whole cell lysates, it appears likely that the effect is on the specific activity of GAP/NF1, or possibly the affinity of such a protein for p21 ras, rather than its subcellular localisation. In this experiment okadaic acid, a potent inhibitor of phosphatases 1a and 2, was included in the 10 minute incubation of cells with or without stimuli and was also present in the lysis buffer. While treatment of cells with okadaic acid alone had no effect on the level of GAP-like activity in cell lysates, it significantly enhanced the level of inhibition of p21 ras GTPase-stimulating activity observed upon PDBu or receptor stimulation. This is compatible with the inhibitory effect on GAP-like activity being mediated by protein kinase C.

5.13-5.15 Effect of a PKC pseudosubstrate peptide inhibitor on PDBu, CD2 and TCR/CD3-induced stimulation of p21 ras in permeabilised T lymphoblasts.

In order to further elucidate the role of PKC in phorbol ester and receptor-stimulated activation of p21 ras, the effect of a pseudosubstrate inhibitor of PKC on p21 ras activation induced by PDBu and the mitogen PHA was investigated in permeabilised cells. This inhibitor peptide has been demonstrated to act as a highly specific and potent inhibitor of PKC activity in permeabilised T lymphoblasts (Alexander et al., 1990) and to inhibit phosphorylation of the y chain of the CD3 antigen in response to these agonists.

Figure 5.13 shows the effect of addition of 100\mu M pseudosubstrate peptide on $^{32}$P-labelled guanine nucleotide bound to p21 ras in permeabilised T lymphoblasts that were either unstimulated, stimulated with PDBu or triggered via the TCR/CD3 complex for various times. Comparison of the rate of total nucleotide binding indicates that addition of PKC inhibitor peptide to
permeabilised cells did influence the rate of exchange of nucleotide onto p21\text{ras}. Figure 5.14 shows the proportion of GTP as a percentage of total $^{32}$P-labelled guanine nucleotide bound to p21\text{ras} in permeabilised T lymphoblasts that were either unstimulated, stimulated with PDBu or triggered via the TCR/CD3 complex or CD2 antigen in the presence or absence of this inhibitor. In the presence of pseudosubstrate peptide, the proportion of p21\text{ras} bound to GTP in unstimulated cells was only slightly decreased from 10% to ~7% (Fig 5.14). The accumulation of p21\text{ras}-GTP induced by PDBu (~30% of total nucleotide) was reduced to between 3 and 5% in the presence of pseudosubstrate inhibitor. In contrast, activation of p21\text{ras} in response to UCHT1 or GT2/OKT11 F(\text{Ab}')\text{3} was only marginally affected by addition of the pseudosubstrate.

In comparison, the phosphorylation of a PKC substrate peptide induced by PDBu, UCHT1 or GT2/OKT11 F(\text{Ab}')\text{3} in permeabilised cells was abolished by the same concentration of pseudosubstrate over an identical time course (Fig 5.15). Taken together with the complete inhibition of PDBu-induced GTP accumulation on p21\text{ras} observed on addition of the PKC pseudosubstrate peptide, these results are consistent with the effects of PDBu on p21\text{ras} being mediated via the activation of PKC. However the insensitivity of both UCHT1 and GT2/OKT11 F(\text{Ab}')\text{3} -induced p21\text{ras} activation to pseudosubstrate addition, under circumstances where PKC-mediated phosphorylation induced by these agonists is completely inhibited, suggests that the effects of these stimuli on p21\text{ras} cannot be completely explained by their effects on PKC activity.
Figure 5.13
Effect of a pseudosubstrate peptide inhibitor of PKC on PDBu and TCR/CD3-induced stimulation of p21ras in permeabilised T lymphoblasts.

Thin layer chromatogram of the guanine nucleotides eluted from immunoprecipitates of p21ras from T lymphoblasts permeabilised in the presence of 150nM Ca2+, 5mM Mg2+ and [γ-32P]GTP. Cells were unstimulated or stimulated with 50ng of PDBu/ml or 10μg of UCHT1/ml in the presence or absence of 100μM-PS peptide for the time indicated. Immunoprecipitation was with p21ras mAb Y13-259. The position at which GTP and GDP standards ran is indicated.
stimulated with (a) 50 ng of PDBu/ml (■/□), (b) 1 pg of GT2/OKT11 F(ab')3/ml (△/□) or (c) 10 pg of UCHT1/ml (○/□) either with (closed symbols) or without (open symbols) the addition of 100 nM-P5 peptide for the time indicated. Guanine nucleotides were eluted from p21ras immunoprecipitates and separated by thin layer chromatography. The figures show a time course of the ratio of [γ-32P]GTP as a percentage of total [γ-32P]-labelled nucleotide.

Figure 5.14

Effect of a pseudosubstrate peptide inhibitor of PKC on stimulation of p21ras in permeabilised T lymphoblasts.
Effect of a pseudosubstrate peptide inhibitor of PKC on stimulation of PKC substrate peptide phosphorylation in permeabilised T lymphoblasts.

Figure 5.15

GS peptide phosphorylation (32P pmoles/5 x 10^6 cells)

- Either unstimulated (○) or stimulated with (a) 20 ng of PDBu/ml, (b) 194 of GET2/OKT11 Fab', (c) 194 of OKT3/UCHT1/ml, or (d) 10 ng of either unstimulated or stimulated with (e) 20 ng of PDBu/ml, or (f) 194 of GET2/OKT11 Fab', (g) 194 of OKT3/UCHT1/ml.

Data are presented as means of duplicate values, expressed as pmol of 32P incorporated into peptide/time (min) of the assay for the number of cells used.

The peptide was recovered, washed and 32P incorporation quantified by scintillation counting. Data are presented as means of duplicate values.
5.16 Stimulation of p21\textsuperscript{ras} by the T cell growth factor IL2.

In T lymphocytes the growth factor Interleukin 2 (IL2) is required for entry into the S phase of the cell cycle (Smith \textit{et al.}, 1988). To investigate the possibility that p21\textsuperscript{ras} functions in the intracellular signalling pathways initiated by triggering the IL2R, the effect of IL2 treatment on the activation state of endogenous p21\textsuperscript{ras} was measured in intact T cells. For these studies, IL2R-positive human T lymphoblasts, the murine IL2-dependent T cell clone CTLL-2 and a population of IL2 receptor negative cells, the human T leukaemic cell line Jurkat were used (Robb \textit{et al.}, 1981). T cells were metabolically labelled with $[^{32}\text{P}]$ orthophosphate, p21\textsuperscript{ras} immunoprecipitated from cell lysates and the bound guanine nucleotides analysed by thin layer chromatography.

Figure 5.16 is an autoradiogram showing the nucleotides bound to p21\textsuperscript{ras} immunoprecipitated from either T lymphoblasts, CTLL or Jurkat that were either untreated or stimulated with PDBu or IL2. In the absence of stimulation, p21\textsuperscript{ras} was almost entirely in the GDP bound state (~ 95%) in all three cell types. However, stimulation of T lymphoblasts with IL2 induced the accumulation of GTP on p21\textsuperscript{ras}, increasing the amount of GTP bound from 5% to ~ 35% of total labelled nucleotide. Treatment of T lymphoblasts with PDBu induced a greater stimulation of p21\textsuperscript{ras} than IL2 (~ 80% accumulation of p21\textsuperscript{ras}-GTP complexes). Both PDBu and IL2 treatment was also observed to stimulate an increase in the level of GTP on p21\textsuperscript{ras} in murine CTLL-2 cells such that the proportion of GTP-bound cellular p21\textsuperscript{ras} rose to ~ 40% and 60% respectively (Fig 5.16). In contrast, IL2 did not influence GTP levels on p21\textsuperscript{ras} in the IL2R-negative human T leukaemic line Jurkat. In these cells, Pdbu was able to increase the amount of GTP bound to p21\textsuperscript{ras} from ~ 5% to ~ 90% of labelled p21\textsuperscript{ras}-associated nucleotide.
Thin layer chromatography. The position at which GTP and GDP standards ran is indicated.

![Thin layer chromatogram of the guanine nucleotide bound to p21ras in T lymphocytes.](image)

**Figure 5.16**

Effect of IL2 treatment on the guanine nucleotide bound to p21ras in T lymphocytes.
5.17 Kinetics and persistence of p21\textsuperscript{ras} stimulation by IL2 in T lymphoblasts.

IL2 binds rapidly, within minutes, to its physiologically active high affinity receptor and yet a minimum 4-6 h duration of IL-2R occupancy is necessary to commit T cells to progression into S phase of the cell cycle and hence mitosis (Cantrell and Smith, 1986). To determine whether IL2 induced activation of p21\textsuperscript{ras} was an immediate response to the occupation of IL2 receptors, or whether a prolonged period of IL-2R occupancy was necessary for p21\textsuperscript{ras} stimulation, the kinetics and duration of IL2-mediated p21\textsuperscript{ras} activation was investigated.

Figure 5.17a shows the variation with time of the ratio of GTP, as a percentage of total nucleotide, bound to p21\textsuperscript{ras} following stimulation of [\textsuperscript{32P}] orthophosphate-labelled T lymphoblasts with IL2. Accumulation of p21\textsuperscript{ras}-GTP complexes in response to IL2 treatment of T lymphoblasts was detectable within 1 min, reached a maximum of 40% by 3 min and was maintained over a period of 20 min. The persistence of IL2-induced p21\textsuperscript{ras} activation was investigated by treating [\textsuperscript{32P}] orthophosphate-labelled T lymphoblasts with IL2 for various times from 15 minutes to 4 hours prior to lysis and immunoprecipitation of p21\textsuperscript{ras}. Figure 5.17b shows that, over this more prolonged time course, maximal IL2-induced accumulation of p21\textsuperscript{ras}-GTP complexes was observed to be maintained over a period of 30-60 minutes. Moreover, in IL2 stimulated T lymphoblasts, \~15% of cellular p21\textsuperscript{ras} was in an active GTP-bound state 4 hours after exposure to IL2 (Fig 5.17b).

Taken together, these findings suggest that IL2-mediated activation of p21\textsuperscript{ras} occurs rapidly, possibly as a consequence of early intracellular changes induced by IL2 binding to it's cellular receptor. The prolonged activation of p21\textsuperscript{ras} observed in the presence of IL2 is consistent with p21\textsuperscript{ras}-GTP playing a role in transducing the growth-regulatory signal delivered by IL2.
Indicated time with 20 ng of IL2/ml orophosphate-labelled T lymphoblasts. Immunoprecipitation was with p21ras MAAb Y13-259. Cells were stimulated for the indicated time with 20ng of IL2/ml.

Figure 5.17

Time course and persistence of IL2-induced activation of p21ras in T lymphoblasts.
5.18-5.19 Dose-response of IL2 for stimulation of p21^{ras} and DNA synthesis in T lymphoblasts.

T cells express at least two IL2 binding molecules, the IL2Rα or p55 subunit which has a relatively low affinity (K_{d} \approx 10^{-8} M) and the IL-2Rβ or p75 subunit which has a K_{d} of \approx 10^{-9} M. These two subunits physically associate in the cell membrane to yield the high affinity (K_{d} \approx 10^{-11} M) IL2R complex that mediates the proliferative response to IL2 (reviewed by Smith, 1988). To determine whether the binding of IL2 to its high affinity receptor was responsible for inducing the intracellular signals that activate p21^{ras}, the concentration of IL2 necessary to induce proliferation in T cells was compared to the concentration of IL2 required to reduce p21^{ras} activation.

For the measurement of p21^{ras} activity, T lymphoblasts were labelled with [^{32}P]orthophosphate for four hours prior to stimulation with various concentrations of IL2 for a further hour. Figure 5.18 is an autoradiogram of chromatographically separated nucleotides eluted from p21^{ras} immunoprecipitates. IL2-induced stimulation of p21^{ras} was dose-dependent with stimulatory effects of IL2 on p21^{ras} detectable at a concentration of \approx 0.7 ng/ml recombinant human IL2 (Fig 5.18). The half-maximal dose of IL2 for inducing the accumulation of p21^{ras}-GTP was 2.5ng/ml.

Figure 5.19 demonstrates that the dose-response of IL2 for activation of p21^{ras} coincided with the IL2 dose-response for the initiation of T cell proliferation, as judged by examination of the IL2 dose-response for incorporation of [^{3}H]thymidine into cellular DNA. These data indicate that levels of IL2 that occupy the high affinity IL2R activate p21^{ras} and suggest that there is a signalling pathway in T cells that couples the high affinity IL-2R to p21^{ras} proteins.
Cells were either unstimulated or stimulated with 50ng of PDBu/ml or the indicated concentration of IL-2 for 15 min. Permeabilization in the presence of 150nM Ca²⁺, 5mM Mg²⁺ and [γ-32P]GTP. Immunoprecipitation was with p21ras MAb Y13-259. Thin layer chromatogram of the guanine nucleotides eluted from immunoprecipitates of p21ras from T lymphoblasts were performed in the presence of 150nM Ca²⁺, 5mM Mg²⁺ and [γ-32P]GTP. Immunoprecipitation was with p21ras MAb Y13-259.

**Figure 5.18**

Dose-response of IL2 for stimulation of p21ras in T lymphoblasts.
Figure 5.19
Dose-response of IL2 for stimulation of p21\textsuperscript{ras} and DNA synthesis in T lymphoblasts.

Comparison of IL2-induced accumulation of α\textsuperscript{-32P}GTP on p21\textsuperscript{ras} (●) with [\textsuperscript{3}H] thymidine incorporation (○), expressed as percentage of maximal response. For p21\textsuperscript{ras} assay, T lymphoblasts were stimulated with the indicated concentration of IL2/ml for 15 min. Guanine nucleotides were eluted from p21\textsuperscript{ras} immunoprecipitates and separated by thin layer chromatography. For proliferation assay, T lymphoblasts were stimulated with the indicated concentration of IL2 for 24hr prior to a 4 h pulse with [\textsuperscript{3}H] thymidine. [\textsuperscript{3}H] thymidine incorporation was determined by liquid scintillation counting.
5.20 Effect of IL2 on guanine nucleotide bound to p21\textsuperscript{ras} in permeabilised T lymphoblasts.

The ability to measure the kinetics of [\alpha-\textsuperscript{32}P]GTP exchange and hydrolysis on p21\textsuperscript{ras} in SLO-permeabilised T lymphoblasts has allowed dissociation of the rate of exchange of nucleotide onto p21\textsuperscript{ras} from the rate of hydrolysis of GTP on p21\textsuperscript{ras} and demonstrated that the stimulatory effect of PKC activation and TCR/CD3 or CD2 agonists is mediated by inhibition of p21\textsuperscript{ras} GTPase activity (Fig 5.8-5.10). Application of this technique to investigate the mechanism by which IL2 induced accumulation of GTP on p21\textsuperscript{ras} revealed that IL2 treatment did not alter the ratio of GTP to total nucleotide in permeabilised T lymphoblasts (data not shown).

To investigate the possibility that the recombinant material used in these studies might have a deleterious effect on the regulation of GTPase activity in permeabilised cells, permeabilised T lymphoblasts were stimulated with various concentrations of IL2 in the presence or absence of PDBu or PHA. Figure 5.20 shows that, in permeabilised T lymphoblasts, IL2 did not stimulate p21\textsuperscript{ras} at any of the concentrations employed. Furthermore, the presence of these concentrations of IL2 did not interfere with the ability of PDBu or PHA to induce accumulation of GTP on p21\textsuperscript{ras}, demonstrating that IL2 itself is not inhibitory in this system.

The observation that IL2 does not alter the ratio of GTP to total nucleotide in permeabilised cells suggests that the IL2 receptor is uncoupled from regulation of p21\textsuperscript{ras} activity under these conditions. Thus, it was not possible to analyse the mechanism by which IL2 induces the accumulation of GTP on p21\textsuperscript{ras} in intact cells by this method.
Thin layer chromatogram of the guanine nucleotides eluted from p21ras immunoprecipitates of T lymphoblasts were permeabilised in the presence of 150nM Ca^{2+}, 5mM Mg^{2+} and [γ-32P]GTP. Cells were either unstimulated or stimulated with the indicated concentration of IL2/ml in the presence or absence of 10μg of PHA/ml or 50ng of PDBu/ml for 5 minutes. Guanine nucleotides were eluted from p21ras immunoprecipitates and separated by thin layer chromatography.

**Figure 5.20**

Effect of IL2 treatment on p21ras in permeabilised T lymphoblasts.
5.21 Effect of IL2 on (p21ras)GAP activity in lysates from T lymphoblasts.

To directly investigate the possibility that the mechanism for IL2-mediated accumulation of GTP on p21ras involves inhibition of a p21ras GTPase activating protein, Triton X-100 lysate from T lymphoblasts that had been treated for 10 minutes with either PDBu or IL2 were compared to lysates from untreated cells for their ability to stimulate the GTPase activity of p21ras, as previously described.

After incubation, the p21ras was recovered by immunoprecipitation and the bound guanine nucleotide analyzed by thin layer chromatography. Figure 5.21 shows that the ability of an extract from phorbol ester treated cells to induce hydrolysis of GTP on p21ras is reduced relative to a lysate from untreated cells. In contrast, IL2 treatment did not alter the ability of cellular extract to induce p21ras GTPase activity. In repeated experiments, where T lymphoblasts were responsive to IL2 in terms of proliferation and p21ras activation (data not shown), no effect of IL2 treatment on GAP activity in total lysate was observed.

5.22 Relationship between IL2 stimulation and other agents that activate p21ras in T lymphoblasts.

One possible explanation of the previous results is that the IL2R employs a distinct mechanism to that employed by PKC and TCR/CD3 or CD2 for the regulation of p21ras, such as regulation of the kinetics of nucleotide exchange. A prediction from such a model is that these stimuli might be expected to interact in an additive or synergistic manner on treatment in combination. To investigate the mechanism by which IL2 receptor occupancy stimulates accumulation of GTP on p21ras the effect of TCR/CD3 triggering or PHA stimulation in combination with IL2 treatment was investigated. Stimulation of T lymphoblasts with IL2, the mitogenic lectin PHA or via the TCR/CD3 complex induced the accumulation of GTP on p21ras, increasing the
Figure 5.21
Comparison of (p21^{ras})GAP activity in lysates from quiescent and IL2-stimulated T lymphoblasts.

T lymphoblasts were incubated for 10 min with 100nM-okadaic acid and either 20ng of IL2/ml (Δ), 50ng of PDBu/ml (●) or no addition (○). Activity of GAP in a Triton X-100 lysate was determined by mixing various dilutions of lysate with p21^{ras} that had been preloaded with [α-^{32}P]GTP. After incubation, the p21^{ras} was recovered by immunoprecipitation and the guanine nucleotide bound to it analyzed by thin layer chromatography.
Figure 3.22
Effect of IL2 stimulation in combination with PKC activation or receptor stimulation on guanine nucleotide bound to p21\textsuperscript{ras} in T lymphoblasts.

Ratio of [\alpha-\textsuperscript{32}P]GTP as a percentage of total [\alpha-\textsuperscript{32}P]-labelled nucleotide eluted from immunoprecipitates of p21\textsuperscript{ras} from \textsuperscript{32}P-orthophosphate-labelled T lymphoblasts. Immunoprecipitation was with p21\textsuperscript{ras} mAb Y13-259. Cells were unstimulated or stimulated for 5 mins with 20ng of IL2/ml, 50ng of PDBu/ml, 10\mu g of UCHT1/ml, 10\mu g of PHA/ml, 1\mu g of GT2/OKT11 F(\textsuperscript{Ab'})\textsubscript{3}/ml or 20ng of IL2/ml in combination with each of the other stimuli.
amount of GTP bound from 5% to between 30 to 40% of total labelled nucleotide (Fig 5.22). Pdbu treatment increased the amount of GTP bound to p21\textsuperscript{ras} from ~ 5% to ~ 70% of p21\textsuperscript{ras}-associated nucleotide (Fig.5.22). In combination, the effects of PHA treatment or TCR/CD3 triggering and IL2 on p21\textsuperscript{ras} were neither synergistic or additive and did not combine together to allow an accumulation of p21\textsuperscript{ras}.GTP to the maximum levels observed in response to Pdbu (Fig 5.22).

5.23-5.24 Exchange rate of guanine nucleotide onto p21\textsuperscript{ras} in permeabilised Rat-1 and NIH-3T3 fibroblasts.

Rat-1 and NIH-3T3 fibroblasts are frequently used as models of cellular growth regulation and have been extensively employed for the study of p21\textsuperscript{ras} function. To determine whether the observation of enhanced guanine nucleotide exchange onto p21\textsuperscript{ras} in permeabilised T cells could be extended to fibroblast lines, Rat-1 and NIH-3T3 fibroblasts were permeabilised in the presence of [\(\alpha\)-\(^{32}\)P]GTP for various times. Parallel experiments were performed with cells in different growth states, to investigate p21\textsuperscript{ras} regulation in actively growing or quiescent cells. In an initial set of experiments, the kinetics of streptolysin O permeabilisation of fibroblasts was found to be slower than for T lymphoblasts. In order to correct for this permeabilisation lag, possibly a consequence of restricted accessibility for streptolysin O or mobility within the cell membrane, both Rat-1 and NIH-3T3 fibroblasts were exposed to streptolysin O for 5 minutes prior to the addition of [\(\alpha\)-\(^{32}\)P]GTP.

Under these conditions \(^{32}\)P-labelled nucleotide bound rapidly to p21\textsuperscript{ras} in both subconfluent (growing) and confluent (quiescent) Rat-1 and NIH-3T3 cells (Figure 5.23). The rate of nucleotide binding to p21\textsuperscript{ras}, with a half time for binding of 3 minutes (equivalent to a \(k_{diss}.GDP\) of \(\sim\)3min\(^{-1}\)), was not influenced by the growth state of the cells and was almost identical for both cell types. Figure 5.24 shows a comparison between the rate of nucleotide binding,
Chromatography.

Immunoprecipitation was with p21ras MAbs Y13-259. Guanine nucleotides were eluted from p21ras immunoprecipitates and separated by thin layer chromatography.

Figure 5.23

Rate of guanine nucleotide exchange on p21ras in permeabilised Rat1 and Swiss 3T3 fibroblasts.
Figure 5.24
Comparison of the rate of guanine nucleotide exchange onto p21\textsuperscript{ras} in permeabilised Rat-1, Swiss 3T3 fibroblasts and T lymphoblasts.

Unstimulated T lymphoblasts (○), confluent Rat-1 fibroblasts (△) or confluent Swiss 3T3 fibroblasts (□) were permeabilised for 5 min in the presence of 150nM \(\text{Ca}^{2+}\), 5mM \(\text{Mg}^{2+}\) prior to the addition of \([\gamma^{32}\text{P}]\text{GTP}\) for the time indicated. Immunoprecipitation was with p21\textsuperscript{ras} mAb Y13-259. Guanine nucleotides were eluted from p21\textsuperscript{ras} immunoprecipitates, separated by thin layer chromatography and quantitated by direct scanning for β radiation. Data are presented as percentage of maximum \(32\text{P}\)-labelled nucleotide bound to p21\textsuperscript{ras}. 
expressed as percentage of maximum, for p21ras in permeabilised T lymphoblasts, Rat-1 and NIH-3T3 fibroblasts. The rate of nucleotide exchange onto p21ras in permeabilised fibroblasts was consistently slower than that observed in T lymphoblasts but is still many times faster than the constitutive rate for p21ras.

5.25 Effect of PKC activation on the activation state of p21ras in permeabilised Rat-1 fibroblasts.

Since p21ras in T lymphocytes appears to be subject to regulation by PKC, the effect of PDBu treatment on the ratio of GTP to total nucleotide on p21ras in Rat-1 fibroblasts was examined. PDBu treatment was observed to have no effect on the activation state of p21ras in intact Rat-1 cells. In further experiments, Rat-1 cells were either treated or untreated with PDBu and were permeabilised for 5 minutes prior to the addition of [α-32P]GTP. As in previous experiments, the rate at which nucleotide bound to p21ras was found to be very rapid and unaffected by treatment with PDBu (Fig 5.25). However PDBu treatment did not alter the ratio of GTP to total nucleotide over the timecourse of the experiment.

Thus, under conditions where PDBu induced a significant proportion of cellular p21ras to move to the GTP bound state in permeabilised T lymphoblasts, activation of PKC did not stimulate the accumulation of GTP on p21ras in permeabilised Rat-1 fibroblasts.
Thin layer chromatogram of the guanine nucleotide eluted from immunoprecipitates of p21ras from Rat-1 fibroblasts were permeabilised in the presence of 150mM Ca2+, 5mM Mg2+ and [γ-32P]GTP. Immunoprecipitation was with p21ras MAb Y13-259. Cells were unstimulated or stimulated with 50ng of PDBu/ml for the indicated time.

Figure 5.25

Effect of PKC activation on guanine nucleotide bound to p21ras in permeabilised Rat-1 fibroblasts.
Discussion.
The regulation of p21\(\text{ras}\) during T cell activation: Analysis in SLO-permeabilised T lymphoblasts.

The results presented demonstrate that, in T lymphocytes, the activation state of p21\(\text{ras}\) is regulated by triggering of the TCR/CD3 complex, the CD2 antigen or the binding of the growth factor IL2 to its high affinity cellular receptor. All three stimuli were observed to induce an 8-10 fold increase in the proportion of endogenous p21\(\text{ras}\) bound to GTP within minutes of stimulation. Since these cells contain about 40,000 molecules of p21\(\text{ras}\) per cell, the number of GTP-bound p21 molecules rises from about 2,000 to approximately 20,000 upon activation via these pathways. Thus, stimulation of a significant proportion of cellular p21\(\text{ras}\) is an early consequence of triggering the TCR/CD3 complex, CD2 antigen or IL2 receptor in T lymphocytes. These observations strongly suggest the involvement of p21\(\text{ras}\) in the signal transduction pathways by which these receptors mediate their cellular effects.

A major question concerns the identity of the cellular signalling pathways by which these receptors regulate p21\(\text{ras}\). Amongst the intracellular biochemical changes induced by TCR/CD3 or CD2 agonists are increases in the cytoplasmic Ca\(^{2+}\) concentration, activation of PKC and changes in tyrosine phosphorylation of cellular substrates. Since activation of PKC by the phorbol ester PDBu induces a rapid 15-fold accumulation of GTP on p21\(\text{ras}\) in T lymphoblasts or the T leukaemic cell line Jurkat, receptor-mediated stimulation of PKC represents a candidate for the pathway by which the TCR/CD3 complex and CD2 antigen control the activation state of ras proteins in these cells. This hypothesis is consistent with the observation that triggering of the TCR/CD3 complex in the CD45-negative T cell line HPBALL does not result in activation of p21\(\text{ras}\) under conditions where activation of PKC stimulates a significant proportion of p21\(\text{ras}\) to accumulate in the GTP-bound
Although the absence of surface expression of CD45 in this cell line was originally demonstrated to correlate with uncoupling of the TCR/CD3 complex from stimulation of phosphatidylinositol hydrolysis and hence activation of PKC (Koretzky et al., 1990), subsequent reports have revealed that the TCR/CD3 complex is also unable to regulate tyrosine phosphorylation in these cells (Koretzky et al., 1991). In view of this fact and in consideration of the possibility that TCR/CD3-induced tyrosine phosphorylation may also regulate PI-PLC activity in T cells, these experiments do not enable the relative contributions made by PKC and tyrosine phosphorylation pathways to regulation of p21\textsuperscript{ras} via the TCR/CD3 complex to be assessed.

Permeabilisation of T lymphoblasts with streptolysin O has enabled regulation of PI-PLC activity in response to TCR/CD3 or CD2 triggering and PKC activation in response to receptor agonists or phorbol ester treatment to be examined (see Sections 3 and 4). Since the use of a pseudosubstrate inhibitor of PKC has allowed the role of PKC in phosphorylation of both endogenous substrates and exogenous peptides to be investigated, the SLO-permeabilised cell system represents an attractive approach for analysis of the mechanism by which the TCR/CD3 complex and CD2 antigen regulate p21\textsuperscript{ras} in T lymphoblasts. In agreement with the results obtained in intact cells, triggering of the TCR/CD3 complex or CD2 antigen and activation of PKC by treatment with phorbol ester induced the accumulation of p21\textsuperscript{ras},GTP in cells that had been permeabilised in the presence of \textsuperscript{32}P-labelled GTP. In permeabilised cells, the pseudosubstrate inhibitor was observed to block phorbol ester-induced p21\textsuperscript{ras} activation but the ability of TCR/CD3 triggering to stimulate p21\textsuperscript{ras} was only slightly impaired. Although activation of PKC is likely to contribute to the regulation of p21\textsuperscript{ras} by the TCR/CD3 complex, the differential effect of the pseudosubstrate inhibitor suggest that whereas phorbol ester-induced activation of p21\textsuperscript{ras} is mediated by stimulation of PKC
activity, the TCR/CD3 and CD2 antigens may also regulate p21\textsuperscript{ras} by a distinct pathway.

One possibility is that, in addition to a PKC-dependent pathway, the TCR/CD3 complex and CD2 antigen regulate p21\textsuperscript{ras} via a pathway that involves tyrosine phosphorylation. Support for this idea may be derived from the relationship that has been demonstrated to exist between tyrosine kinases and p21\textsuperscript{ras} activity in fibroblasts. For example, considerable evidence exists to suggest that p21\textsuperscript{ras} functions in the pathways by which growth factor receptor tyrosine kinases such as PDGF and EGF receptors or tyrosine kinase oncogenes such as v-src and v-fms regulate cellular growth (Smith \textit{et al.}, 1986; reviewed by Marshall, 1991). Furthermore, in a fibroblast cell line which overexpresses p21\textsuperscript{ras}, triggering of the PDGF and EGF receptors or introduction of the v-src or \textit{erbB-2}/\textit{neu} oncogenes has been demonstrated to induce a 3-4 fold increase in p21\textsuperscript{ras}.GTP (Satoh \textit{et al.}, 1990a; 1990b). Recently accumulated evidence indicates that the \textit{src}-family tyrosine kinase p59\textsubscript{fyn} may play an important role in signalling via the TCR/CD3 complex (Samelson \textit{et al.}, 1990; Cooke \textit{et al.}, 1991). TCR/CD3 and CD2-mediated activation of p59\textsubscript{fyn} could represent a primary event in a PKC-independent pathway for regulation of p21\textsuperscript{ras} activity in T cells.

In contrast to the TCR/CD3 complex, the signalling pathways by which the IL2 receptor mediates its growth regulatory effects are poorly understood. Although IL2 rapidly stimulates phosphorylation of cellular substrates on both serine/threonine and tyrosine, analysis of receptor signalling function and the cellular consequences of receptor triggering suggest that the signal transduction pathways employed by the TCR/CD3 complex and IL2R are different. While it is clear that PKC plays a crucial role in signal transduction via the TCR/CD3 complex, PKC is not required for the cellular growth response to IL2 (Mills \textit{et al.}, 1988; Valge \textit{et al.}, 1988) and IL2 receptor triggering does not appear to activate PKC (Mills \textit{et al.}, 1985; 1986). Therefore,
the simplest hypothesis is that IL2 does not regulate $p21^{ras}$ via PKC but employs an alternative mechanism.

Unlike the TCR/CD3 complex and CD2 antigens, the IL2R appeared to be unable to regulate $p21^{ras}$ in permeabilised cells. The reason for this uncoupling is unclear but could reflect the fact that the IL2R activates $p21^{ras}$ via an intracellular pathway that is distinct from that induced by the TCR/CD3 complex and that IL2R coupling to this pathway is not maintained in permeabilised cells. Alternatively, the IL2R may use a pathway in common with the TCR/CD3 complex but may be selectively uncoupled upon permeabilisation. The relationship between the PKC-independent pathway which may contribute to TCR/CD3 or CD2-mediated regulation of $p21^{ras}$ and that initiated by IL2R triggering is unclear. However, the lack of additivity observed between TCR/CD3 or CD2 agonists and IL2 for activation of $p21^{ras}$ suggests that these pathways may converge at some point. Although this study presents no direct evidence in its support, an attractive hypothesis is that the PKC-independent pathway by which both the TCR/CD3 complex or CD2 antigen and the IL2 receptor regulate $p21^{ras}$ is mediated by receptor-induced tyrosine kinase activity.

The observation that activation of PKC does not induce significant accumulation of GTP on $p21^{ras}$ in Swiss 3T3 or Rat-1 fibroblasts suggests that some cellular heterogeneity may exist for the regulation of $p21^{ras}$ activity. Although it might be expected that the principal components of a $p21^{ras}$ regulatory pathway would be shared by both T cells and fibroblasts it is possible the differential effects of phorbol esters reflect that these cells express different subtypes of PKC. However, this explanation appears unlikely as the T leukaemic cell line Jurkat expresses the same major PKC isozymes as most fibroblasts (Lucas et al., 1990). Another possibility is that an intermediary regulator or kinase, expressed in a cell lineage-specific manner, is activated by PKC and determines the T cell response of $p21^{ras}$ to phorbol esters. In this
respect it remains unclear whether PKC regulation of Ras is confined to T cells or is a more general phenomenon. Interestingly, about 20% of acute lymphocytic malignancies contain mutationally activated ras oncogenes, a value that is close to the average figure for all tumour types (Neri et al., 1988). The fact that the proportional increases in p21ras.GTP observed in fibroblasts in response to activated tyrosine kinases are significantly smaller than those observed on TCR/CD3 or IL2 receptor stimulation of T lymphoblasts may also reflect that regulatory differences exist between these cell types (Satoh et al., 1990a; 1990b; Gibbs et al., 1990).

In common with other guanine nucleotide binding proteins and by analogy with the effect of oncogenic mutations, the proportion of cellular p21ras in the GTP-bound state may be reciprocally regulated by the rate of exchange of GTP onto p21ras and the rate of hydrolysis of bound GTP. Thus, stimuli which induce accumulation of GTP on p21ras could act by increasing the rate of binding of GTP to p21ras, by reducing the rate of GTP hydrolysis or by a combination of these mechanisms (reviewed by Bourne et al., 1990). To investigate the contribution made by these pathways to stimulation of p21ras by activation of PKC or triggering of the TCR/CD3 complex and CD2 antigen, the dynamics of guanine nucleotide binding and hydrolysis was analysed in permeabilised T cells. Although the rate at which total 32P-labelled guanine nucleotide bound to p21ras in permeabilised cells was unaffected by these stimuli, the ratio of GTP to total labelled nucleotide was found to be increased over the same timecourse. These results suggest that both activation of PKC and triggering of the TCR/CD3 complex or CD2 antigen stimulate accumulation of GTP on p21ras by reducing the rate of hydrolysis of bound GTP by p21ras and do not appear to promote exchange of guanine nucleotides on p21ras.

One possible mechanism by which these stimuli might bring about a reduction in the rate of GTP hydrolysis by p21ras is by inhibition of the activity
of cellular factors which activate p21\textsuperscript{ras} GTPase activity. The observation that activation of PKC and triggering of the TCR/CD3 complex or CD2 antigen reduces the ability of cell extract to stimulate the GTPase activity of purified p21\textsuperscript{ras} supports this conclusion. Since cells contain multiple proteins that have been demonstrated to possess p21\textsuperscript{ras} GTPase-stimulating activity, including the p21\textsuperscript{ras} GTPase activating protein (GAP) and the product of the NF1 gene, the identity of the cellular factor responsible for this effect is unclear. The 3-5 fold inhibition of GTPase stimulating activity observed under these circumstances might appear to be insufficient to mediate the 8-14 fold accumulation of p21\textsuperscript{ras} GTP. However, the changes in p21\textsuperscript{ras} GTPase-stimulating activity measured under the assay conditions presumably reflect the whole cellular population of p21\textsuperscript{ras} GTPase stimulating activity and do not allow for the possibility that, in intact cells, a subpopulation of p21\textsuperscript{ras} GAP-like activity may be involved in regulation of cellular p21\textsuperscript{ras} activity in response to external stimuli.

Study of the characteristics of the NF1 protein are at an early stage and it is not known how the cellular activity of this protein might be regulated. However, recent observations in fibroblasts suggest several possible mechanisms by which the activity of GAP may be regulated. Firstly, GAP has been demonstrated to associate with activated PDGF and EGF receptors as part of a putative signalling complex that also includes PLC\textgamma, PI-3 kinase and the serine/threonine kinase c-raf (Kazlauskas \textit{et al}., 1990; Kaplan \textit{et al}., 1990). Secondly, in cells stimulated with PDGF and EGF or expressing an activated cytoplasmic tyrosine kinase, GAP is observed to be phosphorylated on both tyrosine and serine residues (Molloy \textit{et al}., 1989; Ellis \textit{et al}., 1990). Thirdly, under these circumstances, GAP associates with a 62kDa tyrosine phosphoprotein and a 190kDa protein that is phosphorylated mainly on serine (Ellis \textit{et al}., 1990; Moran \textit{et al}., 1991). Thus it has been suggested that GAP activity may be modulated by either direct modification, by association with
other cellular phosphoproteins and/or by changes in subcellular localisation in response to growth factors (Moran et al., 1991). In addition, it has been reported that high concentrations of certain mitogenic lipids can inhibit the activity of GAP *in vitro* (Tsai et al., 1989; Yu et al., 1990). The physiological relevance and regulatory potential of these observations are unclear and the contribution that they might make to regulation of cellular GAP activity in response to TCR/CD3, CD2 or IL2R triggering remains to be determined.

Since the effects of IL2 on the dynamics of guanine nucleotide binding and hydrolysis could not be directly investigated in permeabilised cells, a mechanism for IL2-induced p21\textit{ras} activation cannot be proposed. The observation that, unlike activation of PKC and TCR/CD3 or CD2 triggering, IL2 treatment did not alter the ability of cell extract to stimulate p21\textit{ras} GTPase activity can be interpreted to suggest IL2 does not regulate p21\textit{ras} by modulation of GTPase activity. However, if IL2 regulated guanine nucleotide exchange on p21\textit{ras} proteins it would be predicted that IL2 would synergise to induce the accumulation of GTP on p21\textit{ras} in intact cells with agonists that controlled p21\textit{ras} GTPase activity. The fact that TCR/CD3 agonists and IL2 were neither synergistic nor additive for activation of p21\textit{ras} supports the hypothesis that both agonists exert their regulatory effect by a common pathway. One possibility that might account for these observations is that TCR/CD3 or CD2 agonists and IL2 modulate the activity of distinct cellular factors which stimulate p21\textit{ras} GTPase activity and that IL2R-regulated factor is inactivated by cell permeabilisation or lysate preparation.

As a consequence of the analogies between heterotrimeric G proteins and p21\textit{ras}, growth factor-stimulated guanine nucleotide exchange has been considered a likely mechanism for upstream regulation of cellular p21\textit{ras} activity. In addition, the activation state of the homologous RAS proteins in *S. cerevisiae* is thought to be regulated by modulation of the guanine nucleotide exchange activity of the \textit{CDC25} gene product in response to nutrient...
concentration (Broek et al., 1987; Crechet et al., 1990). Thus, the recent identification and partial purification of cellular factors which promote guanine nucleotide exchange on p21\textsuperscript{ras} has provided candidate factors with characteristics that would be expected of physiological activators of p21\textsuperscript{ras} in cellular growth signalling pathways (West et al., 1990; Huang et al., 1990; Wolfman and Macara, 1990; Downward et al., 1990).

The rate of guanine nucleotide binding to p21\textsuperscript{ras} in permeabilised T lymphocytes, corresponding to a $k_{\text{diss,GDP}}$ of about 1 minute, was very much faster than that on purified p21\textsuperscript{ras} or Ras in membrane preparations where the exchange rate is equivalent to a $k_{\text{diss,GDP}}$ of about 60 minutes (Hall and Self, 1986; Cales et al., 1988). This observation suggests that, in permeabilised T cells, an activity exists which enhances the constitutive rate of guanine nucleotide exchange on p21\textsuperscript{ras}. The relationship of this activity to those identified \textit{in vitro} is unclear. A rapid rate of turnover of guanine nucleotide on p21\textsuperscript{ras} is consistent with the proportionally large and kinetically rapid changes in the proportion of GTP bound to cellular p21\textsuperscript{ras} that are observed in intact T cells on activation of PKC or stimulation via the TCR/CD3 complex, CD2 antigen and IL2R. Although it appears from experiments in permeabilised cells that p21\textsuperscript{ras} GTPase activity is the major target of for regulation of p21\textsuperscript{ras} in T lymphocytes, a role for regulation of exchange activity cannot be excluded.

From these experimental findings, a model for the regulation of cellular p21\textsuperscript{ras} activity in T lymphocytes can be postulated (see diagram 5.1). Under the prevailing cellular conditions, where GTP is in approximately 20-fold excess over GDP, GTP from cellular pools will rapidly exchange onto p21\textsuperscript{ras} due the activity of cellular factors which promote dissociation of bound GDP. In quiescent cells, cellular GTPase-stimulating activity ensures that bound GTP is rapidly hydrolysed and p21\textsuperscript{ras} is maintained in a predominantly GDP-bound state. Upon activation of PKC or triggering of the TCR/CD3 complex and CD2
antigen, the activity of cellular GTPase-stimulating factors is reduced and GTP rapidly accumulates on p21\textsuperscript{ras}. It appears likely that IL2 binding to its high affinity cellular receptor also leads to a reduction in p21\textsuperscript{ras} GTPase activity. Thus, the activation state of p21\textsuperscript{ras} appears to be the result of a dynamic balance between the activities of cellular factors which stimulate the rate of guanine nucleotide exchange and factors which respond to growth regulatory signals to regulate the rate of hydrolysis of GTP on p21\textsuperscript{ras}. In this respect, the regulatory cycle of p21\textsuperscript{ras} appears to resemble that of the elongation factor EF-Tu, where guanine nucleotide exchange is constitutive and GTPase activity conditional, rather than heterotrimeric G proteins, where regulatory signal input occurs at the level of receptor-stimulated guanine nucleotide exchange (reviewed by Bourne et al., 1990).

**Diagram 5.1 Regulation of p21\textsuperscript{ras} in T lymphocytes.**
Microinjection of neutralising anti-p21\textsuperscript{ras} antibodies into normal quiescent murine fibroblasts has revealed that endogenous p21\textsuperscript{ras} function is required for the proliferative response of these cells to a wide variety of growth factors. Time course experiments show that p21\textsuperscript{ras} is required during the first eight hours following growth factor stimulation after which time the cells are committed to mitosis (Mulchahy \textit{et al.}, 1985; Yu \textit{et al.}, 1988). Thus p21\textsuperscript{ras} appears to play a general role in the proliferative response of fibroblasts to growth factors during progression from G\textsubscript{0} through G\textsubscript{1} to the S phase of the cell cycle. In normal human T lymphocytes, endogenous p21\textsuperscript{ras} is responsive to triggering the TCR/CD3 complex and CD2 antigen that promote movement from G\textsubscript{0} to G\textsubscript{1} phase of the cell cycle and signals generated by the IL2R that regulate progression from G\textsubscript{1} to S phase. In this respect, p21\textsuperscript{ras} appears to play a similar role to that which has been suggested in fibroblasts, in signal transduction pathways from receptors which regulate the movement of cells through the cell cycle and commit them to mitosis. This idea is strengthened by the observation that p21\textsuperscript{ras} remains activated for at least 4 hours in the continuous presence of IL2 and that exposure to IL2 for a period of 4-6 hours is required for commitment of T cells to mitosis (Cantrell and Smith, 1986).

Since the cellular consequences of triggering the TCR/CD3 complex and IL2 receptors are clearly different, several possibilities concerning the role of p21\textsuperscript{ras} in the pathways stimulated by these receptors may be considered. Firstly, p21\textsuperscript{ras} may serve as a specific mediator in both pathways by interacting with distinct cellular effectors to mediate different cellular responses. The idea that alternative effector pathways may exist for p21\textsuperscript{ras} is supported by the existence of multiple cellular proteins with GAP-like activity for p21\textsuperscript{ras} that also represent candidates for effectors of Ras action. Secondly, p21\textsuperscript{ras} may be required for TCR/CD3 complex and IL2R signalling function in addition to parallel receptor-specific pathways that ultimately determine the cellular
response to these stimuli. In this respect, not all cells respond similarly to the introduction of oncogenic Ras. For example, although oncogenic p21\textsuperscript{ras} induces proliferation in fibroblast lines, in the PC12 cell line Ras blocks proliferation inducing differentiation to neurone-like cells and mimicks the action of nerve growth factor (Bar-Sagi and Feramisco, 1985). The fact that different cells appear to be able to "read" the Ras signal differently raises the possibility that the cellular consequences of p21\textsuperscript{ras} activation in a single cell type may vary according to cellular circumstances. It is interesting to speculate that if these results reflect that the TCR/CD3 complex and IL2R regulate p21\textsuperscript{ras} by modulating the activity of distinct GAP-like molecules that they may also participate in the formation of distinct p21\textsuperscript{ras}.GTP-effector complexes (See diagram 5.2).

Diagram 5.2 Possible involvement of p21\textsuperscript{ras} in signalling pathways from the TCR/CD3 complex and IL2R.
Despite the fact that the nature of the p21ras-effector complex is unclear, some of the features of the downstream signalling pathway mediated by p21ras have been elucidated. In particular, introduction of oncogenic p21ras into fibroblasts rapidly stimulates activation of PKC (Morris et al., 1989). The mechanism by which oncogenic Ras stimulates PKC activity is controversial but appears to be the result of diacylglycerol production resulting from increased breakdown of phosphatidylcholine (Price et al., 1989b; 1989c). Interestingly, induction of PC metabolism by oncogenic p21ras is blocked by downregulation of functional PKC by prolonged treatment with phorbol esters, suggesting that it is in turn dependent upon PKC. An important role for PKC in the signalling pathways stimulated by p21ras is inferred by the fact that the ability of oncogenic p21ras to induce DNA synthesis is also blocked by downregulation of functional PKC (Lacal et al., 1987; Morris et al., 1989). However, since growth factor-induced DNA synthesis is independent of PKC but requires p21ras, it appears likely that oncogenic Ras also employs PKC-independent cellular pathways to mediate its effects (Yu et al., 1988; Morris et al., 1989). In support of this idea, morphological transformation and induction of c-myc transcription by oncogenic p21ras can occur in the absence of PKC (Lloyd et al., 1989). The nature of this PKC-independent pathway is unclear but one possibility is a kinase cascade involving the protooncogenic c-raf and c-mos gene products. These serine/threonine kinases represent candidates for a role downstream of p21ras by virtue of the fact that blocking ras function does not prevent their oncogenic effects (Stacey et al., 1988; Feig and Cooper, 1988). However, these results could also be interpreted to suggest that c-Raf and c-Mos function in a distinct pathway that acts in parallel to p21ras.

Although investigations of the relationship between p21ras and PKC have suggested that PKC can act downstream of p21ras in fibroblasts, they rely heavily upon phorbol ester-mediated down-regulation of PKC, a process that is poorly understood. Furthermore, since most of this work was performed with
oncogenic p21\textsuperscript{ras} which is insensitive to the action of GAP, an upstream regulatory role for PKC would not have been apparent in these studies. Although activation of PKC does not induce significant accumulation of GTP on p21\textsuperscript{ras} in fibroblasts, the fact that the growth response of these cells to phorbol esters is inhibited by functionally blocking p21\textsuperscript{ras} with specific antibodies suggests that p21\textsuperscript{ras} may also function downstream of PKC in fibroblasts (Yu et al., 1988). In this respect, an additional role for PKC downstream of p21\textsuperscript{ras} in T lymphocytes cannot be excluded since analogous studies have not been performed. Thus, it is possible that in the case of normal T cell growth, a positive feedback loop exists in which PKC and p21\textsuperscript{ras} reciprocally stimulate one another. This putative pathway could serve to prolong the effect of transient TCR/CD3-mediated induction of PI metabolism by stimulating sustained activation of PKC as a result of p21\textsuperscript{ras}-induced production of diacylglycerol from other cellular sources.

Another possible role for p21\textsuperscript{ras} in TCR/CD3 and IL2R signalling pathways is in the transmission of signals from these receptors to mediate transcriptional regulation of genes which encode proteins that are important for cell cycle regulation. The ability of oncogenic p21\textsuperscript{ras} to induce expression of c-myc (Lloyd et al., 1989) raises the possibility that p21\textsuperscript{ras} may be involved in TCR/CD3 and IL2R-mediated induction of c-myc expression (Stern and Smith, 1986; Reed et al., 1986; reviewed by Crabtree, 1989). Interestingly, ras oncogenes have been demonstrated to cooperate with c-myc for the complete transformation of normal cells (Land et al., 1983). These results suggest that, in addition to the triggering of mitogenic pathways by p21\textsuperscript{ras}, genetic changes that include the induction of other nuclear oncogenes and possibly the inactivation of tumour supressor genes conspire to generate a cellular environment in which p21\textsuperscript{ras} can promote normal cell growth and oncogenic transformation.
In support of this hypothesis, oncogenic p21\textsuperscript{ras} has been demonstrated to activate transcription from promoters that contain binding sites for the AP-1 family of transactivating factors (Imler \textit{et al.}, 1988; reviewed by Karin, 1991) which include the proto-oncogene products c-Fos and c-Jun (Imler \textit{et al.}, 1988; Curran and Franza, 1988). Since genes containing AP-1 sites are also induced by growth factors such as PDGF and NGF, oncogenes such as c-src and phorbol esters, p21\textsuperscript{ras} may be a key regulator of genes that are essential for normal proliferation and malignant transformation (reviewed by Marshall, 1991). The findings that \textit{ras} cooperates with \textit{c-jun} for cellular transformation (Schutte \textit{et al.}, 1989) and that cells resistant to transformation by \textit{v-fos} are also resistant to \textit{ras} and \textit{c-jun} (Zarbl \textit{et al.}, 1987; Wisdom and Verma, 1990) indicate that these nuclear factors may play an important role downstream of p21\textit{ras} (reviewed by Herrlich and Ponta, 1989).

Although Fos and Jun expression do not appear to be constitutively activated in \textit{ras}-transformed cells, p21\textit{ras} has been demonstrated to induce transient expression of c-Fos (Mulcahy \textit{et al.}, 1985; Stacey \textit{et al.}, 1987). Induction of \textit{c-fos} expression by p21\textit{ras} involves the serum response element in the \textit{c-fos} enhancer. However, since Ras appears to be able to induce \textit{c-fos} by both PKC-dependent and independent pathways, it is unclear whether activation of the serum response factor is involved in a manner analogous to the mechanism employed by growth factors and phorbol esters (Gauthier-Rouviere \textit{et al.}, 1990; Fukumoto \textit{et al.}, 1990) The importance of c-Fos for p21\textit{ras}-mediated growth and transformation is demonstrated by the fact that inhibition of \textit{c-fos} expression by microinjection of anti-sense \textit{c-fos} causes phenotypic reversion of \textit{ras}-transformed cells (Ledwith \textit{et al.}, 1990).

An explanation for the apparent discrepancy between the effects of oncogenic \textit{ras} on AP-1 activity and induction of \textit{c-fos} and has been provided by recent observations which suggest that that p21\textit{ras} may also activate AP-1 in the absence of changes in c-Fos levels by stimulating c-Jun activity.
(Binetruy et al. 1991; Baichwal et al. 1991). Induction of c-jun is thought to result from a positive autoregulatory mechanism as a consequence of posttranslational modification of pre-existing c-Jun but the precise mechanism by which growth factors, transforming oncogenes and phorbol esters stimulate c-Jun is unclear (reviewed by Karin, 1991). One possibility is that these stimuli relieve c-Jun of the effects of an endogenous repressor activity. A recent study has provided evidence to suggest that oncogenic p21\textit{ras} stimulates c-Jun via this mechanism (Baichwal et al. 1991). However, other recent studies have suggested that PKC-mediated activation of c-Jun results in the reduced phosphorylation of residues that negatively regulate its DNA-binding activity (Boyle et al., 1991) while p21\textit{ras} has been reported to induce phosphorylation at positive regulatory sites (Binetruy et al. 1991). The relationship between these pathways and the identity of the Ras-stimulated kinase involved in phosphorylation of c-Jun are not known.

Amongst the genes in T lymphocytes whose enhancer sequences contain sites for AP-1 are those that encode the α and β subunits of the IL2R and the IL2 gene itself (Crabtree, 1989). Thus, these genes represent potential targets for TCR/CD3 and IL2R regulation by a pathway involving p21\textit{ras}. This hypothesis is consistent with the fact that expression of high affinity IL2R heterodimers can be stimulated by PKC activation and by TCR/CD3 or IL2R agonists (Smith, 1988). Since p21\textit{ras} may also play an important role in the induction of IL2 production it is possible that Ras functions as a key regulator of this autocrine growth regulatory pathway in T lymphocytes. The induction of autocrine growth factor production has been suggested to contribute to transformation by oncogenic Ras in other cell types and may play a role in T cell lymphomas that involve mutant p21\textit{ras}.

It is interesting to speculate how p21\textit{ras} might fit into the model that has evolved to explain signalling requirements for IL2 production. Studies have revealed that multiple cellular signals are required for IL2 production; one
critical signal appears to be activation of PKC, the second signal can be provided by T cell agonists that elevate the [Ca²⁺] or those that do not. Since evidence presented in this study suggests that the TCR/CD3 complex may regulate p21ras by both PKC-dependent and independent mechanisms it is possible that Ras may act to integrate signals from these TCR/CD3-induced pathways for the induction of IL2 transcription. In this respect, p21ras could also influence the activity of T cell-specific transactivating factors such as NF-AT which bind to sites in the IL2 enhancer that are responsive to TCR/CD3 triggering (Crabtree, 1989). It should be emphasized, however, that TCR/CD3 triggering or PKC activation alone are insufficient for the induction of IL2 production and signals in addition to those which activate p21ras are clearly required. The identity of these distinct receptor-induced pathways is unclear but one possibility is that the p21ras pathway acts in parallel with the Ca²⁺ signal to stimulate IL2 production and T lymphocyte proliferation.
Section 6: Conclusions and perspectives.

Analysis of the role of guanine nucleotide binding proteins in T lymphocyte activation.

Permeabilisation of T lymphoblasts with streptolysin O has proven to be an effective method for gaining access to the cell interior for otherwise membrane-impermeant nucleotide and peptide reagents. Because the integrity of receptor-mediated signalling events appear to be maintained in this system it has been possible to investigate the effect of receptor agonists, guanine nucleotides and peptide inhibitors of PKC on TCR/CD3 coupling to PI-PLC activity, regulation of PKC and the activation state of endogenous p21ras in SLO-permeabilised cells.

Analysis of the effects of guanine nucleotides on TCR/CD3 coupling to PI-specific phospholipase C in permeabilised T lymphoblasts is an experimental approach that is fundamental to understanding the role of G proteins in TCR/CD3 regulation of PI-PLC. The data show that the TCR/CD3 complex and a population of G proteins can regulate PI-PLC in T cells but that the effects of guanine nucleotides on TCR/CD3 coupling are not compatible with a simple receptor→G protein→PI-PLC model. Although TCR/CD3-mediated regulation of PI-PLC activity can be inhibited by the G protein antagonist GDP, this result most likely reflects that a guanine nucleotide binding protein can indirectly modulate TCR/CD3 coupling to PI-PLC.

An alternative TCR/CD3 coupling mechanism could involve tyrosine phosphorylation of the γ isozymes of PI-PLC as has been suggested for PDGF receptor regulation of phosphatidylinositol metabolism in fibroblasts. In this respect, G protein agonists have been demonstrated to potentiate PDGF-induced phosphatidylinositol metabolism in permeabilised cells, suggesting that G proteins can indirectly modulate the activity of PI-PLC activity stimulated in response to a distinct receptor coupling mechanism (Huang and Ives,
One point at which a G protein could influence TCR/CD3 coupling to PI-PLC might be by regulating the availability of substrate. Although preliminary results indicate that G protein agonists do not specifically stimulate the production of phosphatidylinositols in permeabilised T cells, further investigation will be required to clarify this possibility.

Since one interpretation of the data presented is that the TCR/CD3 complex is coupled to PI-PLC activity via tyrosine phosphorylation, future studies will investigate TCR/CD3-mediated induction of tyrosine kinase activity, the contribution made by tyrosine phosphorylation to the regulation of PI-PLC activity and the possible influence of guanine nucleotides on induction of tyrosine phosphorylation in permeabilised T cells. In addition, if these results do reflect that the TCR/CD3 complex is not coupled to PI-PLC by the G protein that can regulate phosphatidylinositol metabolism, then it will be interesting to attempt to identify the receptor that is coupled to this pathway. Since this study did not address the mechanism by which the CD2 antigen regulates inositol phosphate production, it will be interesting to investigate G protein involvement in CD2 coupling to PI-PLC.

Receptor and G protein agonists were also observed to induce PKC-mediated phosphorylation of the CD3 γ subunit in permeabilised T lymphoblasts. These results are consistent with stimulation of PKC activity being explained by agonist effects on phosphatidylinositol metabolism. However, the ability dissociate G protein agonist effects on PI-PLC activity and phosphorylation of CD3 suggest that guanine nucleotide binding proteins also exert a regulatory effect on PKC activity by distinct mechanisms. One mechanism by which G protein agonists could promote phosphorylation of CD3 is by increasing the accessibility of the γ subunit to PKC. Since a G protein has been demonstrated to be associated with the TCR/CD3 complex, by virtue of the fact that downregulation of the TCR/CD3 complex also downregulates the ability of G protein agonists to stimulate exocytosis and
elevate [Ca\textsuperscript{2+}], it is possible that G protein agonists could stimulate the
dissociation of a guanine nucleotide binding protein from the TCR/CD3
complex (Schrezenmeier, 1988a; 1988b). This hypothesis could provide an
explanation for the presented data and remains to be tested.

Another possibility is that G protein agonists stimulate production of
DAG from cellular sources other than the metabolism of phosphatidylinositols.
In particular, agonist stimulation of phosphatidylcholine breakdown by PLC or
PLD activities can generate DAG and has been implicated in regulation of
PKC activity in other cell systems (Pelech and Vance, 1989; Billah and Anthes,
1990; Exton, 1990). Furthermore, in membrane preparations, both PC-PLC
and PC-PLD can be regulated by G proteins (Irving and Exton, 1987; Bocckino
\textit{et al}., 1987; Martin and Michaelis, 1990; Burch \textit{et al}., 1986). It is possible,
therefore, that breakdown of PC could represent an additional source of G
protein and receptor-responsive DAG in T lymphocytes. The potential
importance of a PC-degradative pathway is emphasised by the fact that, in
permeabilised cells, significant PKC activity could be induced by phorbol
esters in the absence of free Ca\textsuperscript{2+}. Thus, cellular pathways which result in
generation of DAG in the absence of inositol phosphate production and
therefore mobilisation of intracellular Ca\textsuperscript{2+} may be efficient stimulators of PKC
activity in T lymphobasts.

Several experimental approaches, such as measurement of
phosphocholine production and differential fatty acid labelling, can be
employed to investigate the involvement of PC breakdown in agonist-
stimulated responses (Billah and Anthes, 1990). Application of techniques
such as these would enable the contribution of phospholipids to agonist
induced DAG production in T lymphocytes to be assessed.

This study also demonstrates that the activation state of endogenous
p21\textsuperscript{ras} can be regulated by triggering the TCR/CD3 complex, CD2 antigen or
high affinity receptor for the T cell growth factor IL2. These results infer that
normal p21^ras functions in the signalling pathways by which these receptors regulate T lymphocyte activation and proliferation. The ability of phorbol esters to induce the accumulation of p21^ras.GTP suggests that one pathway by which the TCR/CD3 complex and CD2 antigen regulate Ras is by stimulation of PKC activity. However, analysis of p21^ras activation in permeabilised cells with a PKC inhibitor peptide suggests that these receptors might also stimulate p21^ras activity via a PKC-independent pathway. Since accumulated evidence suggests that IL2 signalling does not involve PKC it appears likely that the high affinity IL2R also regulates p21^ras by a PKC-independent mechanism. Although the identity of these pathways is currently unknown, a candidate PKC-independent signalling mechanism might involve tyrosine phosphorylation. The relationship between TCR/CD3 and IL2R-induced pathways for regulation of p21^ras and the possible involvement of tyrosine protein kinase activity are likely areas of future study.

Studies in permeabilised T lymphoblasts also suggest that activation of PKC or triggering of the TCR/CD3 complex and CD2 antigen increase the proportion of cellular Ras in the GTP-bound state by inhibiting the GTPase activity of p21^ras rather than by promoting nucleotide exchange. This conclusion is supported by the observation that the ability of cell lysate to stimulate the GTPase activity of p21^ras in vitro is inhibited in cells treated with these stimuli. Interestingly, IL2 failed to stimulate p21^ras in permeabilised T cells and did not appear to influence p21^ras GTPase activity in the in vitro assay system. One possible interpretation of these results is that IL2 induces the accumulation of p21^ras.GTP by stimulating exchange of guanine nucleotide onto p21^ras rather than by inhibiting p21^ras GTPase activity. However, the lack of synergy or additivity observed between IL2 and TCR/CD3 or CD2 agonists infers that these stimuli employ a common mechanism for regulation of Ras. Although these data argue that, in T lymphocytes, p21^ras GTPase activity appears to be the target for regulation by cellular stimuli which
activate p21\text{ras}, a regulatory role for nucleotide exchange cannot be completely excluded. Further studies will be required to conclusively determine the contribution made by these two pathways to regulation of p21\text{ras} by the TCR/CD3 complex and IL2 receptor.

Another goal of future research must be the elucidation of the mechanisms by which cellular GAP-like activities are modulated by PKC activation, TCR/CD3 triggering and possibly IL2R stimulation. Activated tyrosine kinases have been suggested to regulate cellular GAP activity in fibroblasts by direct modification of GAP, altering its subcellular distribution or inducing its association with other cellular phosphoproteins such as p62 and p190 (Moran \textit{et al.}, 1991). Since no evidence exists in T lymphocytes to suggest that GAP is directly modified in response to external stimuli, it is likely that additional cellular components are involved. The identification of additional regulatory components, which may or may not be protein kinases themselves, will make a significant contribution to understanding the function of p21\text{ras} in these cells.

That cellular signals which activate p21\text{ras} do so by inhibiting the activity of cellular factors which stimulate p21\text{ras} GTPase activity is consistent with these factor serving as upstream regulators of the ras pathway. Since cells contain at least two proteins, GAP and NF1, which have been demonstrated to stimulate the GTPase activity of p21\text{ras} multiple potential targets exist for regulation by signals which impinge upon cellular Ras. Although it is clear that purified GAP can function under \textit{in vitro} assay and permeabilised cell conditions similar to those employed in these studies, the characteristics of the NF1 protein are poorly defined. In this respect, the innability to detect an inhibitory effect of IL2 on \textit{in vitro} p21\text{ras} GTPase-stimulating activity could reflect that the TCR/CD3 complex regulates GAP activity while IL2 regulates the activity of a different factor. An alternative interpretation is that the
regulatory modification stimulated by the IL2R is labile or requires association with other cellular proteins and does not survive preparation of cell lysates.

Considerable evidence exists to suggest that, in addition to a role as upstream regulators, GAP and possibly NF1 may also participate in downstream signal transduction from p21\textsuperscript{ras} (reviewed by McCormick, 1990; Hall, 1990). The possibility that TCR/CD3 and IL2R pathways interact with distinct proteins possessing GAP-like activity may therefore have implications for the p21\textsuperscript{ras}-mediated signalling pathways that are stimulated by these receptors. Clearly, it will be important to clarify whether these pathways employ different regulatory mechanisms, whether they modulate the activity of distinct GAP-like proteins and whether these potential differences are manifested in the regulation of downstream events.

The observation that PKC can regulate the activity of p21\textsuperscript{ras} in T lymphocytes suggests that the Ras proteins may function as downstream effectors of PKC action in these cells. However, in fibroblasts and xenopus oocytes, a significant body of evidence exists to suggest that PKC does not regulate p21\textsuperscript{ras} but that oncogenic p21\textsuperscript{ras} stimulates PKC activity (reviewed by Marshall, 1991). Whether these results indicate the existence of a T cell-specific mechanism and reflect lineage-specific differences in the functional relationship between PKC and p21\textsuperscript{ras} will require further investigation. In this respect it will be important to determine whether the Ras proteins can also stimulate PKC activity in T cells by introducing oncogenic p21\textsuperscript{ras} into T lymphocytes.

The pathway by which p21\textsuperscript{ras} activates PKC appears to involve generation of DAG as a consequence of phosphatidylcholine metabolism (Price \textit{et al.}, 1989b;1989c; Lopez-Barahona \textit{et al.}, 1990; Lacal \textit{et al.}, 1990; Macara, 1989). However, as p21\textsuperscript{ras}-induced PC metabolism appears to require PKC activity, it is still unclear how p21\textsuperscript{ras} initially activates PKC. Since this study also demonstrates that a guanine nucleotides can regulate PKC
activity in permeabilised T cells, it appears that guanine nucleotide binding proteins function both upstream and downstream of PKC in these cells. One possibility that could explain these results is that the pathway by which guanine nucleotides regulate PKC activity is via stimulation of p21\textsuperscript{ras} with consequent production of DAG from cellular sources other than phosphatidylinositol metabolism. Ras proteins may indeed function both upstream and downstream of PKC in T lymphocytes in a positive feedback circuit that serves to amplify and perpetuate PKC-mediated responses.

In addition, several observations suggest that p21\textsuperscript{ras} may exert an modulatory influence on G protein coupling mechanisms. The most direct evidence for such a role has been obtained in patch clamp studies where p21\textsuperscript{ras} in conjunction with GAP were found to uncouple muscarinic receptors from the G protein (G\textsubscript{k}) that regulates atrial potassium channel opening (Yatani \textit{et al.}, 1990). This effect appears to be a consequence of p21\textsuperscript{ras}-GAP preventing association of G\textsubscript{k}\alpha with the βγ subunits of the G protein and thereby blocking receptor interaction. Since G protein βγ subunits are functionally interchangeable it is possible that p21\textsuperscript{ras} may regulate other G protein coupled systems in a similar manner. In particular p21\textsuperscript{ras} may modulate receptor coupling to the G protein pool that regulates PI-PLC activity in T lymphocytes. It is intriguing to speculate that this effect might explain the innability to observe TCR/CD3-induced exchange of GTP[S] on this G protein in permeabilised cells.

Analysis of the effects of oncogenic p21\textsuperscript{ras} on receptor-induced PI-PLC activity have yielded confusing and often contradictory results. However, several studies have observed uncoupling of bombesin and thrombin receptors from phosphatidylinositol metabolism and potentiation of bradykinin responses in \textit{ras}-transformed fibroblasts (Parries \textit{et al.}, 1987; Alonso \textit{et al.}, 1988; Suewen \textit{et al.}, 1988; Kamata \textit{et al.}, 1988). The fact that regulation of PI-PLC activity by the pharmacological G protein activator AIF\textsubscript{4}\textsuperscript{-} is also impaired
in these cells may indicate that the effects of p21\textsuperscript{ras} on bombesin and thrombin may indeed be explained by effects on G protein coupling to PI-PLC (Alonso et al., 1990). However, the fact that PDGF receptors, which are not thought to regulate PI-PLC activity via a G protein, are also uncoupled under these circumstances suggests that a mechanism in addition to p21\textsuperscript{ras} effects on G protein coupling may be involved. In this respect, introduction of oncogenic p21\textsuperscript{ras} into Swiss-3T3 fibroblasts inhibits PDGF and bombesin-induced phosphatidylinositol metabolism by a mechanism that involves PKC (Price et al., 1989). Interestingly, PKC activation does not down-regulate TCR/CD3-induced PI-PLC activity in T lymphoblasts (Ward and Cantrell, 1990).

Diagram 6.1 A speculative model for the involvement of guanine nucleotide-binding proteins in T cell activation.

Although this observation indicates that p21\textsuperscript{ras} may not modulate TCR/CD3 coupling to PI-PLC it is possible that another receptor which couples to PI-PLC via a G protein may be affected by p21\textsuperscript{ras}. In this case, the TCR/CD3 complex could, by induction of p21\textsuperscript{ras} activity, regulate the ability of other T cell
surface receptors to stimulate PI-PLC activity. The possibility that \( p21^{ras} \) may participate in a heterologous desensitization pathway of this sort remains to be investigated. The SLO-permeabilised cell system described in this study offers the opportunity to answer many of these questions.

Of the other potential downstream roles of \( p21^{ras} \) in T cells, the possibility that Ras proteins contribute to the transcriptional regulation of genes that are critical for cell cycle progression and are responsive to TCR/CD3 or IL2 signalling is the most intriguing. In particular, the possibility that \( p21^{ras} \) may contribute to regulation of the genes that encode IL2 and its cellular receptor is a promising area for future study. Transfection of T cells with oncogenic \( p21^{ras} \) should enable the contribution of \( p21^{ras} \) to the regulation of endogenous IL2 and IL2R genes. Alternatively, the effect of \( ras \) transfection on the ability of IL2 and IL2R gene enhancer regions to direct transcription and translation of an indicator gene, such as chloramphenicol acetyltransferase, linked to a ubiquitously-expressed promoter could be assessed.

Further analysis of the mechanisms by which the TCR/CD3 complex and IL2R regulate \( p21^{ras} \) and of the cellular consequences of Ras activation, should provide valuable insight into \( ras \) function in T lymphocytes.
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This thesis has been brought to you by the letter E...
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