Analysis of the *Drosophila* insulin pathway using phosphorylation state-specific antibodies to *Drosophila* Akt; Identification and characterisation of an apoptosis-associated signal

Saif Alrubaie

A thesis submitted to the University of London for the degree of
Doctor of Philosophy

August 2002

Cancer Research UK
PO Box 123
44 Lincoln’s Inn Fields
London WC2A 3PX

Ludwig Institute for Cancer Research
91 Riding House Street
London W1W 7BS

Royal Free and University College Medical School
Gower Street
London WC1E 6BT
Abstract

Studies of components of the insulin/class IA phosphatidylinositol 3-kinase (PI3K) pathway in Drosophila, and more recently in mammals, have revealed a major function in the intrinsic control of cell size and organ growth during development. However, growth and cell size can be regulated through other signalling mechanisms such as those involving the small GTPase, Ras, and the transcription factor, Myc. The possibility that these systems are part of a common growth-regulating signalling network was investigated using an immunofluorescence-based assay to monitor insulin/PI3K pathway activity in vivo. This assay was based on the use of phosphorylation state-specific antibodies to detect phosphorylation and, hence, activation of a major transducer of this pathway, Drosophila Akt (dAkt). The phosphorylation and activation of dAkt by insulin was confirmed biochemically using Drosophila Schneider cells. The control of dAkt phosphorylation in vivo by Drosophila PI3K was analysed during development by in situ immunofluorescence of imaginal discs.

The phosphorylation of dAkt was then analysed in clones of cells that lacked Drosophila Ras1 (dRas1) or ectopically expressed constitutively active dRas1 (dRasV12) or Drosophila Myc (dMyc). Significantly, dAkt phosphorylation was increased in dRas1V12 clones, but was not altered in dRas1-null clones. However, the analysis was complicated by the presence of an intense 'pyknotic' cell staining pattern revealed by immunostaining with one of the phospho-specific dAkt antibodies. This unexpected and intriguing observation was characterised and found to originate from cells undergoing apoptosis.

Experiments in imaginal discs and in Schneider cells demonstrated that the pyknotic signal and the induction of apoptosis were accompanied by the appearance of an 80kDa signal on phospho-dAkt western blots that co-migrated with the 80kDa isoform of dAkt. Significantly, the appearance of this signal was caspase-dependent. However, further experiments ruled out the possibility that the 80kDa protein was dAkt.
Index of Contents

Abstract ................................................................................................................. 2
Index of Contents .................................................................................................. 3
Index of Figures ..................................................................................................... 8
Abbreviations ......................................................................................................... 9

Chapter 1: Introduction ......................................................................................... 11

1.1 Overview ........................................................................................................... 13
1.2 The insulin/PI3K signalling pathway ............................................................... 14
  1.2.1 Generation of phospholipid second messengers ........................................... 14
  1.2.2 Downstream targets of PI3K: the AGC Kinases ............................................ 15
  1.2.3 Activation of c-Akt ....................................................................................... 19
    1.2.3.1 Pleckstrin homology domain and phosphoinositide binding ................... 19
    1.2.3.2 Phosphorylation of Akt ......................................................................... 20
    1.2.3.3 PDK1 and phosphorylation of the activation loop of Akt ....................... 20
    1.2.3.4 Phosphorylation of Akt in the hydrophobic motif .................................. 21
    1.2.3.5 Summary of Akt activation ..................................................................... 22
  1.2.4 The Drosophila insulin/PI3K pathway .......................................................... 24
1.3 The regulation of growth during development ................................................. 27
  1.3.1 Imaginal disc development ......................................................................... 28
  1.3.2 Intrinsic and extrinsic regulation of imaginal disc growth ............................ 29
  1.3.3 Imaginal disc growth in relation to cell division, protein synthesis and patterning ................................................................. 30
    1.3.3.1 Relationship between growth and cell division ....................................... 31
    1.3.3.2 Relationship between growth and protein synthesis ............................ 32
    1.3.3.3 Relationship between growth and patterning ........................................ 33
  1.3.4 Molecular mechanisms of growth control ..................................................... 34
  1.3.5 Control of growth by the Drosophila insulin/PI3K pathway ........................ 35
  1.3.6 The effects of modulating insulin/PI3K signalling on cell number ............... 36
  1.3.7 The insulin/PI3K pathway and cell cycle progression .................................... 37
  1.3.8 Does insulin/PI3K signalling stimulate growth via effects on protein translation? ................................................................. 38
  1.3.9 Control of growth by Drosophila Myc and Ras1 ........................................... 40
  1.3.10 Control of growth by TSC1 and TSC2 ........................................................ 41
  1.3.11 Regulation of mammalian growth by the insulin/IGF-1/PI3K pathway ......... 42
# Index of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4 Apoptosis in <em>Drosophila</em></td>
<td>43</td>
</tr>
<tr>
<td>1.4.1 Apoptosis: the basics</td>
<td>44</td>
</tr>
<tr>
<td>1.4.2 Caspases: cellular executioners</td>
<td>47</td>
</tr>
<tr>
<td>1.4.3 Inhibitors of apoptosis proteins: key caspase inhibitors</td>
<td>49</td>
</tr>
<tr>
<td>1.4.4 Mechanisms of caspase activation</td>
<td>50</td>
</tr>
<tr>
<td>1.4.4.1 Death receptor pathway</td>
<td>50</td>
</tr>
<tr>
<td>1.4.4.2 <em>Drosophila</em> Apaf</td>
<td>51</td>
</tr>
<tr>
<td>1.4.4.3 Reaper, Grim and Hid</td>
<td>52</td>
</tr>
<tr>
<td>1.4.5 Mitochondrial regulation of cell death through the Bcl-2 protein family</td>
<td>54</td>
</tr>
<tr>
<td>1.4.6 Regulation of apoptosis by survival signal pathways</td>
<td>56</td>
</tr>
<tr>
<td>1.4.6.1 Control of cell death by <em>Drosophila</em> Ras/1/MAPK</td>
<td>57</td>
</tr>
<tr>
<td>1.4.6.2 Control of cell death by the <em>Drosophila</em> insulin/PI3K pathway</td>
<td>58</td>
</tr>
<tr>
<td>1.4.7 Mammalian PI3K pathway and the regulation of apoptosis</td>
<td>60</td>
</tr>
<tr>
<td>1.5 Cell growth and cell survival: two sides of the same coin?</td>
<td>63</td>
</tr>
<tr>
<td>1.5.1 Regulation of survival through the control of metabolic function</td>
<td>63</td>
</tr>
<tr>
<td>1.5.1.1 Regulation of mitochondrial hexokinases by Akt</td>
<td>64</td>
</tr>
<tr>
<td>1.5.1.2 Regulation of cell survival by Glycogen Synthase Kinase 3</td>
<td>66</td>
</tr>
<tr>
<td>1.5.2 Protein synthesis and cellular survival</td>
<td>67</td>
</tr>
<tr>
<td>1.6 Overview of thesis</td>
<td>69</td>
</tr>
</tbody>
</table>

## Chapter 2: Materials and Methods  

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Culture of cells and flies</td>
<td>72</td>
</tr>
<tr>
<td>2.1.1 Fly culture</td>
<td>72</td>
</tr>
<tr>
<td>2.1.2 Cell culture</td>
<td>72</td>
</tr>
<tr>
<td>2.1.2.1 Insulin, CuSO₄ and zVAD-fmk treatment</td>
<td>72</td>
</tr>
<tr>
<td>2.2 Generation and purification of dAkt antisera</td>
<td>72</td>
</tr>
<tr>
<td>2.2.1 Production of rabbit antisera against dAkt peptides</td>
<td>72</td>
</tr>
<tr>
<td>2.2.2 Affinity purification of dAkt antisera</td>
<td>73</td>
</tr>
<tr>
<td>2.3 Biochemical techniques</td>
<td>74</td>
</tr>
<tr>
<td>2.3.1 Preparation of lysates</td>
<td>74</td>
</tr>
<tr>
<td>2.3.2 Protein assays</td>
<td>74</td>
</tr>
<tr>
<td>2.3.3 Immunoprecipitation from cell lysates</td>
<td>74</td>
</tr>
<tr>
<td>2.3.4 Protein kinase assays</td>
<td>75</td>
</tr>
<tr>
<td>2.3.5 SDS polyacrylamide gel electrophoresis</td>
<td>75</td>
</tr>
<tr>
<td>2.3.6 Electroblotting PAGE-resolved proteins</td>
<td>75</td>
</tr>
<tr>
<td>2.3.7 Immunoprobing of western blots</td>
<td>76</td>
</tr>
<tr>
<td>2.3.8 Peptide competition assay</td>
<td>76</td>
</tr>
</tbody>
</table>
Chapter 3: Generation and Characterisation of Phosphorylation State-Specific Antibodies to Drosophila Akt

3.1 Generation and biochemical characterisation of phosphorylation-specific antibodies to Drosophila Akt

3.1.1 Strategy for the generation of phosphorylation-specific and phosphorylation state-independent antisera to dAkt ___________________________________________________________________________ 84

3.1.2 Immunoreactivity of antisera raised against dAkt peptides ___________________________________________________________________________ 86

3.1.3 Confirmation that antisera recognise dAkt in vitro in a phosphorylation state-dependent or - independent manner ___________________________________________________________________________ 90

3.1.3.1 Immunoprecipitation of p66- and p80-dAkt ___________________________________________________________________________ 90

3.1.3.2 Peptide competition removes dAkt signal ___________________________________________________________________________ 94

3.1.4 Affinity purification of dAkt antisera ___________________________________________________________________________ 95

3.1.5 Correlation between the phosphorylation state and kinase activity of dAkt ___________________________________________________________________________ 97

3.2 Cell biological characterisation of the phosphorylation-specific antibodies to Drosophila Akt

3.2.1 dAkt phosphorylation signal is reduced in dAkt null mitotic clones ___________________________________________________________________________ 102

3.2.2 Dp110 is required for dAkt phosphorylation at Serine 586 in vivo ___________________________________________________________________________ 105

3.2.3 Ectopic expression of Dp110 induces dAkt phosphorylation in vivo ___________________________________________________________________________ 105

3.2.4 Identification of a pyknotic signal with cprSer586 ___________________________________________________________________________ 112

3.2.5 Ectopic expression of Dp110 in the pouch region of the wing imaginal disc induces increased dAkt phosphorylation ___________________________________________________________________________ 115

3.3 Summary ___________________________________________________________________________ 119
Chapter 4: Investigation of Growth-Regulation Signalling and Characterisation of Pyknotic Signal

4.1 Investigating the relationship between PI3K, dRas1 and dMyc signalling in vivo
4.1.1 Investigating the role of dRas1 in PI3K signalling in vivo
4.1.1.1 Ectopic expression of dRas1 induces dAkt phosphorylation in vivo
4.1.1.2 Phosphorylation of dAkt is not reduced in dRas1 null clones
4.1.2 Ectopic expression of dMyc does not result in increased dAkt phosphorylation in vivo

4.2 Characterisation of αpSer586 pyknotic signal
4.2.1 Pyknotic signal is associated with the induction of apoptosis
4.2.2 Increased frequency of αpSer586 pyknotic signal in irradiated discs

4.3 Summary

Chapter 5: Characterisation of an 80kDa protein associated with Apoptosis in Drosophila

5.1 Introduction

5.2 Characterisation of an apoptosis-associated 80kDa protein
5.2.1 Pyknotic staining is associated with the appearance of an 80kDa protein detected by αpSer586
5.2.2 The 80kDa protein is also observed in S2 cells following Reaper expression
5.2.3 Timecourse of appearance of 80kDa protein following ectopic Reaper expression
5.3 The 80kDa protein is not p80-dAkt
5.3.1 Apoptosis is not associated with phosphorylation of dAkt at threonine 423
5.3.2 The 80kDa protein cannot be immunoprecipitated using antisera raised against dAkt
5.3.3 dAkt is cleaved following induction of apoptosis
5.3.4 Inhibition of dAkt expression does not affect the appearance of the 80kDa band
5.3.5 Detection of the 80kDa protein by αpSer586 is not phosphorylation-dependent

5.4 Summary
Chapter 6: Discussion

6.1 Overview

6.2 αpSer586: to use or not to use?

6.3 Does dRas1 regulate the Drosophila PI3K pathway?

6.4 dMyc - a regulator of cell size and cell death

6.5 p80 and the caspase connection

6.5.1 Caspase substrates

6.5.2 Modulating activity through caspase cleavage

6.5.3 p80: how and why?

6.5.4 The biological significance of p80 in apoptosis

6.5.5 Breaking down a cell's defences

6.6 Summary

Acknowledgements

References
Index of Figures

Figure 1.1  Class IA PI3K signalling ____________________________ 16
Figure 1.2  Mammalian and Drosophila Akt ________________________ 18
Figure 1.3  Model of the activation mechanism of Akt by PI3K and PDK1 ___________ 23
Figure 1.4  The insulin/PI3K pathway in mammals and Drosophila ______________ 25
Figure 1.5A The apoptotic system in Drosophila ___________________ 45
Figure 1.5B The apoptotic system in mammals _____________________ 46
Figure 3.1   Amino acid sequence comparison of Drosophila Akt with human Akt1 _______ 85
Figure 3.2  Immunoreactivity of antisera raised against dAkt peptides ____________ 89
Figure 3.3  Immunoprecipitation of p66- and p80-dAkt ____________________ 91
Figure 3.4  Immunoprecipitation of phosphorylated dAkt _________________ 93
Figure 3.5  Peptide competition of dAkt signal ______________________ 96
Figure 3.6  Affinity purification of the dAkt antisera ________________ 98
Figure 3.7  Correlation between phosphorylation state of dAkt and kinase activity 101
Figure 3.8  The flp/FRT system ___________________________________ 103
Figure 3.9  dAkt phosphorylation is reduced in mitotic clones of dAkt null and Dp110 null cells ___________ 107
Figure 3.10 The 'flp-out' system _________________________________ 108
Figure 3.11A Ectopic expression of dAkt in cell clones induces increased αCT signal_________ 110
Figure 3.11B Ectopic expression of dAkt in cell clones induces increased αpSer586 signal_________ 111
Figure 3.12 Ectopic expression of Dp110 in cell clones induces phosphorylation of dAkt in vivo ___________ 113
Figure 3.13 Pyknotic signal ____________________________________ 114
Figure 3.14 Ectopic expression of Dp110 in the wing disc pouch induces dAkt phosphorylation ___________ 116
Figure 3.15 Membrane localisation of phosphorylated dAkt ____________ 117
Figure 3.16 Pyknotic staining cells in MS1096>dpp10 wing discs ____________ 118
Figure 4.1  Ectopic expression of dRas1V12 in cell clones induces an increase in αpSer586 signal ___________ 125
Figure 4.2  dAkt phosphorylation is not reduced in mitotic clones of dRas1 null cells ___________ 127
Figure 4.3  Ectopic expression of dMyc induces increased number of pyknotic staining cells ___________ 129
Figure 4.4  Pyknotic signal in apoptosing cells from clones ectopically expressing dMyc ___________ 132
Figure 4.5  Irradiation of wing discs induces an increase in the pyknotic signal ___________ 135
Figure 5.1  Detection of an 80kDa protein in irradiated wing imaginal discs ___________ 140
Figure 5.2  Detection of the 80kDa protein during apoptosis induced by Reaper expression ___________ 143
Figure 5.3  αpSer586 specifically detects increased signal at 80kDa in lysates from apoptosing cells ___________ 144
Figure 5.4  Timecourse of appearance of the 80kDa protein during apoptosis _______ 146
Figure 5.5  The appearance of the 80kDa protein is a caspase-dependent event ______________________ 148
Figure 5.6  Phosphorylation of dAkt at threonine 423 is not increased during apoptosis ___________ 150
Figure 5.7  The 80kDa protein cannot be immunoprecipitated using antisera raised against dAkt _______ 153
Figure 5.8  dAkt is cleaved following induction of apoptosis ___________ 154
Figure 5.9  Double-stranded RNA-mediated interference of dAkt expression _______ 157
Figure 5.10 Inhibition of dAkt expression does not affect the appearance of the 80kDa protein _______ 158
Figure 5.11 Detection of the 80kDa protein by αpSer586 is not phosphorylation-dependent _______ 160
Figure 6.1  Caspase substrates ______________________________________ 170
Abbreviations

AEL: after egg laying
AIF: apoptosis-inducing factor
ANT: adenine-nucleotides translocator
Apaf-1: apoptotic protease activating factor-1
arm: armadillo promoter
ATP: adenosine triphosphate
Bcl-2: B-cell lymphoma 2 (protein)
BHI-4: Bcl-2 homology domain 1-4
BIR: baculovirus IAP repeat
CAD: caspase-activated deoxyribonuclease
CARD: caspase recruitment domain
Cbl: Casitas B-lineage lymphoma
Cdc(2/27): cell division control (2/27) (protein)
Cdk(1/2): cyclin dependent kinase (1/2)
CNS: central nervous system
cpm: counts per minute
DAP-kinase: death-associated protein kinase
dBorg-(1/2): Drosophila Bcl-2 ortholog-(1/2)
DCP-1: Drosophila caspase-1
DED: death effector domain
DER: Drosophila epidermal growth factor receptor
Df: deficiency chromosome
DFF(40/45): DNA-fragmentation factor (40/45) kDa subunit
DILP: Drosophila insulin-like peptide
Dpp: Decapentaplegic
drICE: Drosophila interleukin 1 β-converting enzyme
Drob-1: Drosophila ortholog of the Bcl-2 family-1
DRONC: Drosophila Nedd2-like caspase
dsRNA: double-stranded RNA
dsRNAi: dsRNA-mediated interference (of gene expression)
4E-BP: eIF4E-binding protein
eIF: eukaryotic initiation factor
EGF: epidermal growth factor
ERK: extracellular-signal regulated kinase
ey: eyeless promoter
FACS: fluorescence-activated cell sorting
FADD: Fas-associated death domain
FLP: flipase (recombinase)
FRT: flipase recombination target
β-gal: β-galactosidase
GDP: guanosine diphosphate
GTP: guanosine triphosphate
GFP: green fluorescent protein
GMR: glass multimer reporter
GSK-3: glycogen synthase kinase-3
Hid: head involution defective
HK-(I/II): hexokinase-(I/II)
hs: heat shock
HtrA2: high temperature requirement A2
IAP: inhibitor of apoptosis protein
ICAD: inhibitor of CAD
IDGF: imaginal disc-derived growth factors
Abbreviations

IGF-1: insulin-like growth factor-1
IkB: Inhibitor of κB
IKK(α/β): IκB kinase (α/β)
ILK: integrin linked kinase
IP: immunoprecipitate
IRES: internal ribosome entry segment
IRS: insulin receptor substrate
JNK: c-Jun N-terminal kinase
KLH: keyhole limpet haemocyanin
MAPK: mitogen-activated protein kinase
MDM2: mouse double minute 2
MEF: mouse embryonic fibroblast
MEKK1: MAPK/ERK kinase kinase 1
MMP: mitochondrial membrane permeabilisation
MS1096-dp110: MS1096-GAL4; UAS-dp110
MST (1/2): mammalian STE20-like kinase (1/2)
NFκB: nuclear factor κB
NGF: nerve growth factor
ORF: open reading frame
PAGE: polyacrylamide gel electrophoresis
PAK2: p21-activated kinase 2
PARP: poly (ADP-ribose) polymerase
PBS: phosphate buffered saline
PBST: PBS with 0.1% (v/v) Tween-20
PDGF: platelet derived growth factor
PDK(1/2): 3′phosphoinositide-dependent kinase (1/2)
PH: pleckstrin homology domain
3′-PI: 3′phosphorylated-phosphoinositides
PI3K: phosphoinositide-3 kinase
PIF: PDK1 interacting fragment
PKB: protein kinase B
PKC: protein kinase C
PRK2: PKC-related kinase 2
PT: permeability transition
PtdIns: phosphatidylinositol
PTEN: phosphatase and tensin homolog deleted on chromosome ten
RBD: Ras-binding domain
ROCK I: Rho-activated serine/threonine kinase I
rpr: reaper
RTK: receptor tyrosine kinase
S6K: S6 kinase
SAPK: stress-activated protein kinase
TBS: tris-buffered saline
TBST: TBS with 0.1% (v/v) Tween-20
5′TOP: 5′-terminal oligopyrimidine tract
(d/m)TOR: (Drosophila / mammalian) target of rapamycin
TSC(1/2): Tuberosus Sclerosis complex (1/2)
TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling
UAS: upstream activating sequences
UV: ultraviolet
VDAC: voltage dependent anion channel
WB: western blot
Wg: Wingless
zVAD-fmk: N-benzyloxy carbonyl-Val-Ala-Asp-fluromethylketone
Chapter 1: Introduction

1.1 Overview

1.2 The insulin/PI3K signalling pathway
   1.2.1 Generation of phospholipid second messengers
   1.2.2 Downstream targets of PI3K: the AGC kinases
   1.2.3 Activation of c-Akt
      1.2.3.1 Pleckstrin homology domain and phosphoinositide binding
      1.2.3.2 Phosphorylation of Akt
      1.2.3.3 PDK1 and phosphorylation of the activation loop of Akt
      1.2.3.4 Phosphorylation of Akt in the hydrophobic motif
      1.2.3.5 Summary of Akt activation
   1.2.4 The Drosophila insulin pathway

1.3 The regulation of growth during development
   1.3.1 Imaginal disc development
   1.3.2 Intrinsic and extrinsic regulation of imaginal disc growth
   1.3.3 Imaginal disc growth in relation to cell division, protein synthesis and patterning
      1.3.3.1 Relationship between growth and cell division
      1.3.3.2 Relationship between growth and protein synthesis
      1.3.3.3 Relationship between growth and patterning
   1.3.4 Molecular mechanisms of growth control
   1.3.5 Control of growth by the Drosophila insulin pathway
   1.3.6 The effects of modulating insulin/PI3K signalling on cell number
   1.3.7 The insulin/PI3K pathway and cell cycle progression
   1.3.8 Does insulin/PI3K signalling stimulate growth via effects on protein translation?
   1.3.9 Control of growth by Drosophila Myc and Ras1
   1.3.10 Control of growth by TSC1 and TSC2
   1.3.11 Regulation of mammalian growth by the insulin/IGF-1/PI3K pathway

1.4 Apoptosis in Drosophila
   1.4.1 Apoptosis: the basics
   1.4.2 Caspases: cellular executioners
   1.4.3 Inhibitors of apoptosis proteins: key caspase inhibitors
   1.4.4 Mechanisms of caspase activation
      1.4.4.1 Death receptor pathway
      1.4.4.2 Drosophila Apaf

11
Chapter 1: Introduction

1.4.4.3 Reaper, Grim and Hid .................................................. 52
1.4.5 Mitochondrial regulation of cell death through the Bcl-2 protein family ......................... 54
1.4.6 Regulation of apoptosis by survival signal pathways ............................................. 56
   1.4.6.1 Control of cell death by Drosophila Ras1/MAPK .......................................... 57
   1.4.6.2 Control of cell death by the Drosophila insulin/PI3K pathway ......................... 58
1.4.7 Mammalian PI3K pathway and the regulation of apoptosis ................................... 60

1.5 Cell growth and cell survival: two sides of the same coin? .................................... 63
   1.5.1 Regulation of survival through the control of metabolic function ....................... 63
      1.5.1.1 Regulation of mitochondrial hexokinases by Akt ................................... 64
      1.5.1.2 Regulation of cell survival by Glycogen Synthase Kinase 3 ......................... 66
   1.5.2 Protein synthesis and cellular survival ............................................................ 67

1.6 Overview of thesis ............................................................................. 69
1.1 Overview

The development of a multicellular organism involves a myriad of complex cellular and extracellular processes that must integrate in order to achieve the necessary functional outcome. The intricate co-ordination of these molecular processes within defined spatial and temporal parameters is fundamental to the generation of properly developed tissues, organs, and organisms. However, as will be discussed in later sections, eukaryotic development, despite its intricacies and complexities, is also remarkably robust and resistant to perturbation. This is perhaps testament to the power of the intrinsic control mechanisms that regulate development, which are able to sense and compensate for such perturbations.

The cellular and extracellular processes that underlie eukaryotic development can be grouped into several major categories that are involved in the regulation of cell size, cell death, cell division, and cell differentiation. Communication between cells, as well as signalling within a cell, is essential for the stimulation, regulation, and co-ordination of these general cellular activities. A common way in which intercellular communication is achieved is through the generation of extracellular factors that activate receptors on the surface of cells, resulting in the stimulation of intracellular signalling pathways. One such class of receptors are the receptor tyrosine kinases (RTKs), which, upon activation by extracellular ligands, have been shown to stimulate many cellular processes including differentiation, mitogenesis, metabolism, and cytoskeletal rearrangement (van der Geer et al., 1994). Signals from activated RTK complexes can be transduced to the intracellular machinery through the activities of enzymes, such as kinases, phosphatases and phospholipases, which are recruited to RTKs directly or indirectly through interactions with other proteins. Several signalling mechanisms triggered by activated RTKs have been well characterised and one key example is the class IA phosphoinositide 3-kinase (PI3K) pathway, which is proposed to represent a major branch of RTK-mediated signalling (Pawson, 1995).

The evolutionarily conserved insulin receptor pathway is one such RTK signal transduction system that is in part mediated by the class IA PI3K signalling cassette. Studies in mammals, the nematode Caenorhabditis elegans and the fruit fly Drosophila
melanogaster have shown that the insulin/PI3K pathway is an essential regulator of development that co-ordinates a number of intracellular processes with extracellular cues. One such developmental process that has become evident through studies initially performed in Drosophila is the regulation of growth (i.e. increase in mass) during organogenesis. The sections in this introduction will introduce insulin/PI3K signalling in mammals and Drosophila and then describe the role of this pathway in the control of growth.

A second developmental process that is proposed to be controlled by class IA PI3K signalling is the promotion of cell survival through the inhibition of apoptosis. However, PI3K signalling impacts on a number of anabolic processes that are crucial for the maintenance of cellular homeostasis. Thus, it is not clear whether PI3K-mediated promotion of cellular survival is a result of the direct regulation of apoptosis or an indirect consequence of its other cellular functions such as the control of protein synthesis and metabolism. The second part of this introduction will therefore describe the regulation of apoptosis in Drosophila during development and the role of the Drosophila insulin/PI3K pathway in cell survival.

1.2 The Insulin/PI3K signalling pathway

1.2.1 Generation of phospholipid second messengers

Phosphoinositide 3-kinases are an evolutionary conserved family of lipid kinases that catalyse the phosphorylation of inositol phospholipids on the 3'-OH position of the inositol ring (Stephens et al., 1993). The lipid products, 3'-phosphorylated phosphoinositides (3'-PI), are important second messengers that have been implicated in a range of cellular functions including growth factor receptor signalling, cell survival, cytoskeletal organisation, chemotaxis, protein trafficking and, more recently, tissue growth (as reviewed by Katso et al., 2001). PI3Ks are grouped into three main classes with two additional sub-classes (IA, IB, II and III) according to their in vitro lipid substrate specificity, structure and mode of regulation (Vanhaesebroeck et al., 1997a). Both class IA and class II PI3Ks are involved in insulin signalling. However, the role of class IA PI3K has been best characterised and will be the focus of discussion below.
Chapter 1: Introduction

Class IA PI3Ks form heterodimeric complexes with adaptor proteins that link them to upstream signalling events (see figure 1.1). Binding of growth factors such as insulin and PDGF to their cognate receptor tyrosine kinases results in the transphosphorylation of the receptor complexes on key tyrosine residues and, in the cases of the insulin receptor, the phosphorylation of insulin receptor substrate proteins (IRS). The generation of phosphotyrosine residues in specific consensus motifs promotes the recruitment of PI3K heterodimers to the plasma membrane where they preferentially phosphorylate the membrane phospholipids phosphatidylinositol 4-phosphate (PtdIns(4)P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) to generate PtdIns(3,4)P2 and PtdIns(3,4,5)P3, respectively (reviewed by Katso et al., 2001).

Class IA PI3K activity is physiologically antagonised by the lipid phosphatase and tumour suppressor PTEN (phosphatase and tensin homolog deleted on chromosome ten). The gene encoding PTEN is mutated or deleted in a large fraction of sporadic cancers as well as autosomal dominant cancer-predisposing syndromes (Li et al., 1997a). Despite the fact that PTEN shares homology with protein tyrosine phosphatases, the recombinant protein is a poor catalyst towards phospho-peptide/protein substrates (Maehama and Dixon, 1998). Rather, PTEN can dephosphorylate PtdIns(3,4)P2 and PtdIns(3,4,5)P3 at the 3'-OH position of the inositol ring in vitro and therefore acts as a negative regulator of class IA PI3K signalling (Maehama and Dixon, 1998). This conclusion has been further supported by studies in vivo using pten deficient transgenic mice (see for example Stambolic et al., 1998).

Class IA PI3K activity can also be antagonised by the cell permeable low molecular mass compound, wortmannin, which irreversibly inhibits class IA PI3Ks by Schiff base formation with a lysine in the kinase domain (Wymann et al., 1996). An unrelated compound, LY294002, is also inhibitory but through a reversible mechanism (Vlahos et al., 1994). Both these compounds have been used extensively to investigate the role of PI3K signalling in cell biology.

1.2.2 Downstream targets of PI3K: the AGC Kinases

Once generated, PtdIns(3,4)P2 and PtdIns(3,4,5)P3 function as signal transduction intermediates, influencing the activity of a number of downstream signalling molecules.
Figure 1.1 Class IA PI3K Signalling

Class IA PI3Ks form heterodimeric complexes with adaptor proteins that link them to upstream signalling events. Binding of insulin ligands to their cognate receptor tyrosine kinase (RTK) results in transphosphorylation of the receptor complex (not shown). This leads to the binding of insulin receptor substrate (IRS) proteins, which in turn also become phosphorylated. The phosphorylation of tyrosine residues in a specific consensus motif stimulates the recruitment of PI3K heterodimers to the plasma membrane where they phosphorylate inositol phospholipids on the 3'-OH position of the inositol ring. This process is antagonised by the lipid phosphatase, PTEN. Generation of PI(3,4)P₂ and PI(3,4,5)P₃ promotes the translocation and/or activation of PH-domain containing proteins.
These 3'-PI targets are proposed to include several guanine-nucleotide exchange-factors and GTPase-activating proteins such as GRP-1, ARNO and cytohesin-1, as well as members of the Tec family of intracellular tyrosine kinases, and phospholipase C γ (reviewed in Vanhaesebroeck et al., 2001). However, much of the work on class IA PI3Ks targets has focused on members of the AGC class of protein kinases, a broad sub-family of sequence-related serine/threonine kinases. A large number of studies have indicated that many of the downstream events controlled by class IA PI3Ks are mediated by the AGC kinase, Akt/protein kinase B (PKB), the cellular homolog of the protein encoded by the AKT8 retrovirus oncogene, v-akt (Staal, 1987; Bellacosa et al., 1991). Mammals have three closely related akt/pkb genes termed akt1/pkbα, akt2/pkbβ, and akt3/pkbγ. These genes encode structurally similar proteins with predicted molecular weights of approximately 57 kDa. All three isoforms show a broad tissue distribution pattern, although their specific levels of expression may vary: for example, akt3 is expressed at higher levels in the brain and testis than in other tissues. Each isoform is composed of an amino-terminal pleckstrin homology (PH) domain and a carboxy-terminal kinase catalytic domain, both of which are evolutionarily conserved in Akt from different species (see figures 1.2 and 3.1). Furthermore, all Akt proteins identified to date, with the exception of C. elegans Akt2 (Paradis and Ruvkun, 1998) and, possibly, a splice variant of mammalian Akt3 (Walker et al., 1998), also possess a carboxy-terminal regulatory tail.

Another AGC kinase that is regulated by PI3K signalling is S6 kinase (S6K). This protein has been shown to be involved in the selective translation of a subset of mRNAs that are characterised by an oligopyrimidine tract at their 5' transcriptional start site, or 5'TOP (Jefferies et al., 1994; Jefferies et al., 1997). S6K is proposed to control 5'TOP mRNA translation through the phosphorylation of the 40S ribosomal protein, S6. The 5'TOP family members constitute 20-30% of the total cellular mRNA and encode for components of the translational apparatus, including ribosomal proteins and translational elongation factors, whose increased expression is essential for growth and proliferation. In addition to being regulated by pathways which involve PI3Ks, the S6 kinases are also regulated by the PIK-related kinase, mTOR (for mammalian-target of rapamycin), which may serve an additional function as a checkpoint for amino acid availability (Dufner and Thomas, 1999).
Figure 1.2 Mammalian and Drosophila Akt

A schematic comparison of the major domains and phosphorylation sites in mammalian Akt1 and Drosophila Akt. Note that Drosophila cells express two Akt isoforms generated through the use of alternative translation initiator codons. This results in the expression of a 66kDa isoform and a longer 80kDa variant, the latter possessing an additional amino-terminal extension that is rich in serine, threonine, and proline residues (see also figure 3.1).
Both Akt and S6K are phosphorylated and regulated by a third AGC kinase, 3-phosphoinositide-dependent kinase 1 (PDK1). This serine/threonine kinase was first identified by its ability to phosphorylate threonine 308 of Akt1 in vitro in a manner dependent on the presence of lipid vesicles containing a low molar fraction of PtdIns(3,4)P\(_2\) or PtdIns(3,4,5)P\(_3\) (Alessi et al., 1997a; Alessi et al., 1997b). PDK1 is a ubiquitously expressed 63kDa protein that consists of an amino-terminal kinase domain and a carboxy-terminal PH domain (Stephens et al., 1998; Currie et al., 1999). The role of PDK1 in PI3K-mediated Akt activation is discussed below.

1.2.3 Activation of c-Akt

1.2.3.1 Pleckstrin homology domain and phosphoinositide binding

The PH domain is an approximately 100 amino acid globular protein motif that was originally identified in pleckstrin, the major phosphorylation target for protein kinase C (PKC) in platelets (Mayer et al., 1993). Since then, more than 150 PH domains have been identified to date in numerous eukaryotic proteins. Although the primary amino acid sequence of PH domains is not highly conserved, their tertiary structure is, forming a hydrophobic pocket that is capped by a carboxy-terminal amphipathic helix (Lemmon et al., 1996). Some PH domains have been shown to bind specific phospholipids with varying affinities. This binding of phospholipids to PH domains provides a mechanism by which membrane-bound lipids convey signals to the cytosol (Isakoff et al., 1998; Fruman et al., 1999). The PH domains of both Akt1 and PDK1 were found to bind preferentially to PtdIns(3,4)P\(_2\) or PtdIns(3,4,5)P\(_3\) over other PIs in vitro (James et al., 1996; Stephens et al., 1998; Currie et al., 1999). Interestingly, surface-plasmon-resonance-based binding assays have shown that PDK1 has a high affinity for PtdIns(4,5)P\(_2\), a constitutive component of the plasma membrane (Currie et al., 1999). However, other studies employing either lipid-vesicle binding assays or protein/lipid overlay assays failed to observe significant interaction between PDK1 and PtdIns(4,5)P\(_2\) (Stephens et al., 1998; Dowler et al., 1999).
1.2.3.2 **Phosphorylation of Akt**

The binding of phosphoinositides to PH domains can influence the activity of target molecules by different mechanisms, for example, by changing the protein’s conformation, subcellular localisation, activation state, and/or interaction with other proteins. Franke and colleagues found that mutation of the PH domain in Akt could block activation of this kinase by PDGF in cultured NIH3T3 cells (Franke et al., 1995). Furthermore, initial observations suggested that synthetic or PI3K-generated 3’-Pis could activate Akt1 in vitro, indicating that Akt activation may be regulated through the direct binding of these phospholipids to its PH domain (Franke et al., 1995; Frech et al., 1997; Klippel et al., 1997; Stokoe et al., 1997). However, other studies have failed to observe direct activation of Akt1 by 3’-Pis (James et al., 1996). Moreover, previous studies had shown that activated Akt1 was inhibited by serine/threonine-specific phosphatases, but not tyrosine-specific protein phosphatases, suggesting that Akt activity may also be dependent on phosphorylation on serine/threonine residues (Burgering and Coffer, 1995; Cross et al., 1995; Andjelkovic et al., 1996). Consistent with this observation, Alessi and colleagues demonstrated by phosphoamino acid analysis that the activation of Akt1 by insulin or insulin-like growth factor-1 (IGF-1) in cultured cells is accompanied by increased phosphorylation on serine/threonine residues (Alessi et al., 1996a). Two major phosphorylation sites were mapped: threonine 308 in the activation- or T-loop of the kinase domain, and serine 473 in a hydrophobic motif of the carboxy-terminal regulatory domain. Activation of Akt1 and phosphorylation of both these sites was inhibited in cells incubated with wortmannin prior to insulin or IGF-1 treatment. Moreover, phosphorylation of both residues was shown to be required for maximal activation of Akt1. Interestingly, mutation of either site to alanine did not prevent the phosphorylation of the other suggesting that they could be phosphorylated independently of each other (Alessi et al., 1996a).

1.2.3.3 **PDK1 and phosphorylation of the activation loop of Akt**

Subsequent studies revealed that threonine 308 on Akt1 and the equivalent sites on Akt2 and Akt3 were phosphorylated by PDK1 (Alessi et al., 1997b; Stokoe et al., 1997; Stephens et al., 1998; Walker et al., 1998). As mentioned above, this phosphorylation was
enhanced 1000-fold in the presence of vesicles containing PtdIns(3,4)P$_2$ or PtdIns(3,4,5)P$_3$. These phospholipids, which bind the PH domains of both Akt and PDK1, are suggested to allow PDK1 and Akt to co-localise and/or to allow a conformational change in Akt such that threonine 308 becomes accessible to PDK1. This hypothesis is supported by three main observations. First, full length Akt is not phosphorylated by PDK1 in the absence of PtdIns(3,4,5)P$_3$, whereas a truncated Akt mutant lacking the PH domain is phosphorylated and activated by PDK1 (Alessi et al., 1997a). Second, Akt carrying point mutations in the PH domain that abolish its ability to bind PtdIns(3,4,5)P$_3$ is not phosphorylated by PDK1 (Stokoe et al., 1997; Stephens et al., 1998). Third, a truncated PDK1 mutant that lacks the PH domain still requires PtdIns(3,4,5)P$_3$ in order to phosphorylate and activate Akt (Alessi et al., 1997a; Stephens et al., 1998).

1.2.3.4 Phosphorylation of Akt in the hydrophobic motif

The mechanism of phosphorylation of serine 473 on Akt1 and equivalent sites in other Akt isoforms is far from clear. Several candidate proteins tentatively referred to as ‘PDK2’ have been proposed to be responsible for serine 473 phosphorylation in vivo, based on their ability to specifically phosphorylate Akt1 on this residue in vitro and in cell culture. For example, ectopically expressed MAPKAP kinase-2 was shown to phosphorylate Akt1 on serine 473 in a p38- and PtdIns(3,4,5)P$_3$-dependent manner, which in turn was suggested to promote the dissociation of Akt1 from an inhibitory complex with Hsp27 (Rane et al., 2001). Similarly, insulin-stimulated integrin-linked kinase (ILK) was also proposed to phosphorylate the hydrophobic motif of Akt1 in a 3’-PI dependent manner, consistent with the reported observation that both ILK and Akt activity is constitutively elevated in PTEN deficient prostate carcinoma cells (Delcommenne et al., 1998; Persad et al., 2001). Moreover, inhibition of ILK in this cell line was suggested to suppress phosphorylation of Akt on serine 473 but not threonine 308.

However, the physiological relevance of these observations is unclear. For example, MAPKAP kinase-2 activation and phosphorylation of Akt1 on serine 473 can occur independently of each other in HEK 293 cells (Alessi et al., 1996a). Similarly, wortmannin can inhibit Akt1 phosphorylation and activation but not activation of MAPKAP kinase-2 by arsenite in L6 myotube or HEK293 cells. Furthermore, it is not evident whether ILK is a
true kinase since it lacks both the canonical HRDLXXN motif, containing the catalytic aspartate residue, and the DFG motif, involved in the co-ordination of Mg\(^{2+}\) (Lynch et al., 1999). Indeed, ILK immunoprecipitates do not show any significant kinase activity against a variety of substrates including Akt.

PDK1 was also suggested to be able to phosphorylate Akt1 on serine 473 following interaction with PIF, a carboxy-terminal fragment of the AGC kinase, PRK2 (Balendran et al., 1999). Paradoxically, a subsequent study by the same group revealed that PDK1 in complex with PIF could only phosphorylate catalytically inactive Akt1 on threonine 308 and not serine 473. Nevertheless, the authors suggested that the kinase dead Akt mutant they used may have been misfolded and therefore not recognised by PDK1 complexed to PIF. Alternatively, serine 473 may be an autophosphorylation site (Toker and Newton, 2000). According to this model, phosphorylation of threonine 308 by PDK1 partially activates Akt and allows it to phosphorylate itself on the hydrophobic site, thereby becoming fully activated. Intriguingly however, Akt is still phosphorylated on serine 473 following IGF-1 stimulation of embryonic stem cells deficient in PDK1, despite being neither phosphorylated on threonine 308 or catalytically active (Williams et al., 2000). Thus, it is likely that, in these cells at least, a separate kinase phosphorylates Akt on serine 473.

1.2.3.5 Summary of Akt activation

The mechanism of Akt activation is subject to continuous revision, expansion and debate. Nevertheless, the following basic model has been proposed. In unstimulated cells, Akt is believed to reside in the cytosol in a low activity conformation (see figure 1.3). Upon activation of PI3Ks, PtdIns(3,4)P\(_2\) and PtdIns(3,4,5)P\(_3\) are generated at the plasma membrane, which in turn interact with the PH domain of Akt. The increased concentration of these phospholipids is thought to induce the translocation of Akt from the cytosol to the inner leaflet of the plasma membrane, thereby promoting a conformational change in Akt such that it can become phosphorylated by PDK1 and, possibly, PDK2. It should also be noted that a fraction of the active Akt is then proposed to detach from the plasma membrane and to translocate to the cytosol or the nucleus (Andjelkovic et al., 1997; Meier
Figure 1.3 Model of the activation mechanism of Akt by PI3K and PDK1

See text for description (based on Vanhaesebroeck and Alessi, 2000)

PI = phosphatidylinositol
et al., 1997). The mechanism of this second translocation is unclear but may involve binding to chaperone proteins such as HSP-90 (Sato et al., 2000).

In summary, one way in which growth factors such as insulin can transduce intracellular signals is through the activation of class IA PI3K and Akt, both of which have multiple targets involved in diverse cellular processes.

1.2.4 The Drosophila insulin/PI3K pathway

Various genetic and biochemical studies have established a pathway in Drosophila that shares a high degree of homology and evolutionary conservation with the mammalian insulin/PI3K signalling pathway (see figure 1.4). A family of genes was recently identified that encodes seven Drosophila insulin like peptides (DILPs, (Brogiolo et al., 2001). As with mammalian insulin, the predicted amino acid sequences of these genes contains a signal peptide, an α- and β-chain, and a C peptide with a consensus proteolytic processing site. The different dilp genes have distinct expression patterns, suggesting distinct functions (Brogiolo et al., 2001; Cao and Brown, 2001; Rulifson et al., 2002). The mammalian and Drosophila insulin ligands activate homologous receptors, comprised of two α and two β subunits, with a cytoplasmic tyrosine kinase domain (Fernandez et al., 1995). These receptors, in turn, are likely to activate a number of pathways in both mammals and Drosophila, respectively. Analogous to mammalian insulin/PI3K pathway signalling, activation of the Drosophila class IA PI3K homolog, Dp110, by the Drosophila insulin receptor, Inr, is mediated through the Drosophila class IA PI3K adaptor subunit, p60, and an insulin receptor substrate (IRS) homolog, termed Chico (Weinkove et al., 1997; Bohni et al., 1999). However, in contrast to mammalian insulin receptors, Inr has a carboxy-terminal extension that also possesses three potential tyrosine phosphorylation sites within YXXM motifs involved in PI3K binding and activation (Fernandez et al., 1995; Yenush et al., 1996; Marin-Hincapie and Garofalo, 1999; Poltilove et al., 2000). Thus, this extension may represent an alternative, direct mechanism for the binding and activation of p60/Dp110. Indeed, chico null mutants are viable, indicating that Inr can signal in the absence of an IRS protein, given that Drosophila with null mutations in p60 or dp110 are not viable (see later sections).
Figure 1.4 The insulin/PI3K pathway in mammals and Drosophila

See text for description
Mammalian class IA PI3Ks are also proposed to be activated by the small GTPase, Ras (see section 4.1). However, this model is largely based on experiments employing constitutively-active or dominant-negative Ras mutants, which may not correctly reflect the endogenous, physiological situation (see later sections). Moreover, although *Drosophila* possesses a Ras homolog, dRas1, the role of Ras signalling in Dp110 activation is uncertain (see Chapters 4 and 6). As in mammals, *Drosophila* PI3K activity is negatively regulated by a homolog of the lipid phosphatase PTEN, termed DPTEN (Goberdhan *et al.*, 1999; Huang *et al.*, 1999; Smith *et al.*, 1999; Gao *et al.*, 2000; Scanga *et al.*, 2000). Dp110 can activate the *Drosophila* Akt homolog, dAkt, in concert with signals from the *Drosophila* PDK1 homolog, dPDK1/Dstpk61, and possibly a third, as yet unidentified, kinase tentatively termed PDK2/dPDK2 (Scanga *et al.*, 2000; Rintelen *et al.*, 2001; Radimerski *et al.*, 2002; Stocker *et al.*, 2002). Analogous to mammals, activation of the *Drosophila* S6K homolog, dS6K, is thought to be mediated through a complex phosphorylation process involving dPDK1 and the *Drosophila* homolog of mTOR, termed dTOR, as well as the putative kinase, PDK2 (Rintelen *et al.*, 2001; Radimerski *et al.*, 2002). Both mTOR and dTOR are suggested to activate S6K and dS6K, respectively, in response to levels of nutrients, such as amino acids (AA; Thomas and Hall, 1997; Oldham *et al.*, 2000; Zhang *et al.*, 2000c; Miron and Sonenberg, 2001). Akt has also been proposed to activate S6K through phosphorylation and activation of mTOR (Nave *et al.*, 1999; Sekulic *et al.*, 2000). However, the putative Akt phosphorylation site on mTOR is not conserved in other species, including *Drosophila* (Oldham *et al.*, 2000; Zhang *et al.*, 2000c). Furthermore, this model has not been supported by other studies (for example (Radimerski *et al.*, 2002). A number of downstream targets of Akt/dAkt and S6K/dS6K will be discussed in later sections.

With the exception of the ligands, each component of the *Drosophila* insulin/PI3K pathway is encoded by a single gene, reducing the possibility of redundancy in functional analysis. However, it should be noted that several of the *Drosophila* genes encode additional transcripts as a consequence of alternative splicing and/or the use of additional initiation codons. For example, the *Drosophila pten* gene homolog, dpten, is alternatively spliced and is predicted to encode three proteins, which vary in length at their carboxy-terminus (Smith *et al.*, 1999). Similarly, the dakt and, possibly, ds6k genes each encode two isoforms as a result of transcription at an additional upstream initiation codon or alternative
splicing, respectively (Andjelkovic et al., 1995; Stewart et al., 1996; Watson et al., 1996). Additionally, the Drosophila pdk1 gene homolog, dpdk1, is suggested to encode four protein isoforms through the combination of alternative initiation and splicing (Clyde and Bownes, 2000). Remarkably, the predicted dpdk1 isoforms differ significantly in their amino termini and vary in length from 539 to 836 amino acid residues.

The existence of these various alternative isoforms raises the question of whether some of these proteins each possess distinct activities or subtle differences in function as discussed in subsequent sections.

1.3 The regulation of growth during development

Studies in Drosophila have demonstrated that the class IA PI3K signalling pathway is involved in the intrinsic control of growth (increase in mass) during the development of epithelial organs, termed the imaginal discs. Consistent with this observation, class IA PI3K function has been implicated in a number of anabolic processes necessary for maintaining cellular homeostasis and promoting tissue growth. These include the regulation of cell proliferation, differentiation, metabolism, protein synthesis and survival. The following discussion, therefore, will initially focus on the control of growth during imaginal disc development, the role of the class IA PI3K signalling in the regulation of this process, and the cellular processes that may mediate the ability of this pathway to promote growth (section 1.3, reviewed by Edgar, 1999; Coelho and Leevers, 2000; Day and Lawrence, 2000; Stocker and Hafen, 2000). However, the second part of this discussion will focus on the regulation and execution of apoptosis in Drosophila as this process is particularly pertinent to the results of this thesis (section 1.4). Subsequently, the role of class IA PI3K signalling in promotion of cellular survival will be explored further (section 1.5). Finally, it will be argued that the ability of this pathway to promote growth through the regulation of critical cellular processes such as protein synthesis and metabolism, may in fact also underlie its ability to suppress apoptosis, as suggested by a number of recent studies.
1.3.1 Imaginal disc development

*Drosophila* imaginal discs are epithelial sacs that give rise to the adult epidermal structures or appendages such as eyes, wings and legs (Cohen, 1993). They are derived from precursor cells that are set aside during embryogenesis, proliferate during larval development, and are re-organised during metamorphosis to form the final adult organs. For example, during pupation, the wing imaginal disc undergoes dramatic structural transformation as it is reorganised to form the adult wing blade, hinge and the adjoining body wall of the mesothorax.

The wing disc is generated from approximately 40 primordial cells, arranged as an invaginated cell cluster. These cells begin to proliferate in the first instar of larval development and eventually form an organ of approximately 50,000 cells by the end of the third instar (Madhavan and Scheiderman, 1977). As with other imaginal discs, the development of the wing disc is intrinsically regulated by the sequential organisation of patterning cues that determine the fates of constituent cells within restricted regions. Specifically, the wing disc is divided into anterior and posterior compartments by cell lineage through the restricted posterior expression of the homeotic gene, *engrailed*, and its interaction with compartment-specific signalling cascades. Similarly, dorsal expression of the LIM-homeobox gene *apterous* defines the dorsal and ventral compartments (Serrano and O'Farrell, 1997). A major consequence of the function of these selector genes is the expression of the secreted ligands Decapentaplegic (Dpp) and Wingless (Wg) in a stripe of cells abutting the anterior/posterior boundary and the dorsal/ventral boundary, respectively. In parallel, these two ligands are believed to orchestrate both the growth and patterning of the wing disc and deficiency of either leads to dramatic morphological and/or developmental abnormalities (Nellen et al., 1996; Zecca et al., 1996; Serrano and O'Farrell, 1997). For example, as its name suggests, certain *wingless* mutants do not develop wings (Sharma and Chopra, 1976). Importantly, the vertebrate homologs of Wingless and Dpp (Wnts and bone morphogenetic proteins, respectively) are also involved in the patterning and development of several organs such as the kidneys and dorsal limbs (Roelink, 1996; Sasai and De Robertis, 1997).

The fly eye has a compound structure and is composed of approximately 750 ommatidia. Each ommatidium contains a consistent complement of cells: eight
photoreceptor neurons, four lens-secreting cone cells and two primary pigment cells. There are also six secondary and three tertiary pigment cells which are shared with neighbouring ommatidia. As in the wing disc, the cells of the eye imaginal disc also proliferate during larval development. However, early in the third instar, a dorsal-ventral indentation in the apical surface of the disc, the morphogenetic furrow, sweeps across from the posterior to the anterior of the disc. Ahead of the furrow, the cells continue to proliferate but then arrest in the G1 phase of the cell cycle as they enter the furrow. As the cells emerge from the furrow they are gradually recruited into ‘preclusters’, which then differentiate in a stepwise manner to form the photoreceptors, cone cells and pigment cells that make up each adult ommatidium. Thus, cells in the posterior portion of the disc are differentiating and organising into ommatidial precursors, while cells in the anterior portion of the disc are proliferating. As discussed in section 1.3.1, the correct differentiation of these cells depends upon extracellular signals. These signals include the ligands that activate the receptor tyrosine kinases *Drosophila* epidermal growth factor receptor (DER) and Sevenless, which in turn activate the Ras/MAPK signalling pathway (Hafen et al., 1993a; Hafen et al., 1993b; Freeman, 1996; Dominguez et al., 1998).

In summary, imaginal disc development involves co-ordinated growth, cell proliferation and pattern, and thus resembles the development of many mammalian or vertebrate epithelial organs. Consequently, the *Drosophila* imaginal disc provides a dynamic and powerful model for the study of cell biology, cell signalling and organogenesis *in vivo*.

### 1.3.2 Intrinsic and extrinsic regulation of imaginal disc growth

*Drosophila* imaginal discs have been shown to grow to a consistent final size that is remarkably resistant to perturbation. The growth of these organs is thought to be under the control of extrinsic, systemic factors as well as being responsive to intrinsic cues (Bryant and Simpson, 1984; Milan et al., 1997). For example, *in vitro* culture of imaginal discs with the larval fat body or in fat body-conditioned medium promotes disc growth, suggesting that the fat body may secrete a systemic regulator of growth (Davis and Shearn, 1977). More recently, a family of soluble chitinase-like polypeptides termed imaginal disc growth factors (IDGFs), have been identified and shown to promote the growth *in vitro* of Cl.8+
cells, a clonal imaginal disc cell line (Kawamura et al., 1998). These factors were found to be expressed at high levels in the fat body as well as in variable patterns in the imaginal discs raising the possibility that these molecules may be involved in both long-range and local systemic control of disc growth.

Evidence for the intrinsic control of imaginal disc growth can be found from transplantation studies. Discs from larval donors that have been transplanted into adult hosts grow to a consistent final size that is independent of the developmental stage of the host or transplanted disc (Garcia-Bellido, 1965). Furthermore, ablation or physical disruption of a small region of an imaginal disc results in compensatory growth of the surrounding tissue such that a disc of wild type size still develops (Haynie and Bryant, 1977; Milan et al., 1997). Moreover, although physical damage of a disc during the third larval instar retards pupariation whilst the disc is being repaired, the final size of the remaining discs is not changed indicating that they do not continue to grow past a certain size. Taken together, these experiments imply the existence of one or more intrinsic sensory mechanisms that respond to and compensate for exogenous interference in growth.

Similar transplantation experiments have demonstrated that mammalian organs also possess intrinsic as well as extrinsic growth control mechanisms that regulate their size. For example, if several fetal thymus glands are transplanted into a developing mouse, each grows to its normal adult size, indicating that their growth is mainly controlled by cues intrinsic to the thymus (Metcalf, 1963). In contrast, if the same experiment is repeated with fetal spleens, the total mass attained by the transplanted spleens is equivalent to that of a single normal adult spleen, suggesting that their growth is mainly regulated by extrinsic factors (Metcalf, 1964).

### 1.3.3 Imaginal disc growth in relation to cell division, protein synthesis and patterning

The development of an organ involves the co-ordination of tissue growth (the increase in tissue mass) and patterning. Tissue growth is a function of increases in both cell size and cell number. Cell number, in turn, is the outcome of the balance between cell division and cell death. The sections below will discuss experiments in *Drosophila*
imaginal discs investigating the role of these key cellular processes in relation to tissue growth during disc development.

**1.3.3.1 Relationship between growth and cell division**

Cell division in imaginal discs occurs predominantly in the larval stage of the life cycle and is thus temporally separated from the main period of differentiation that takes place at metamorphosis. The pre-proliferative phase of imaginal disc development is marked by considerable enlargement of both cells and nuclei, possibly to facilitate the subsequent rapid cycles of division that occur soon afterwards (Madhavan and Scheiderman, 1977). These cell divisions initially reduce the size of individual cells dramatically, after which, cell growth and division are roughly co-ordinated so that the average cell size diminishes only slightly as the cells divide and the disc grows. However, near the end of disc development, the rate of cell division is reduced such that cell size increases (Neufeld et al., 1998).

Cell proliferation during disc development has been studied using a variety of methods, including specific labelling of mitotic cells or analysis of marked populations of cells in clones (generated, for example, by X-ray irradiation or by the use of the \textit{flp/FRT} system, see Chapter 3). With respect to the latter technique, the size and morphology of the resulting clones and associated twin spots, identified by specific genetic markers, provides insight into the extent and patterns of proliferating cells during disc development.

Recent experiments have addressed the relationship between cell division and disc growth by manipulation of cell cycle genes in specific compartments or in clones (Weigmann et al., 1997; Neufeld et al., 1998; Su and O'Farrell, 1998). Interestingly, compartment- or clone-specific acceleration of the cell-cycle during wing imaginal disc development by the ectopic expression of the key cell cycle regulators dE2F and DP1 does not affect net growth or final disc size (Weigmann et al., 1997; Neufeld et al., 1998). Rather, more cells of a smaller size are generated. Similarly, no affect on disc growth was observed following inhibition of cell division within one disc compartment using a temperature sensitive allele of Cdc2, or within clones of cells by ectopic expression of the dE2F inhibitor, dRBF (Weigmann et al., 1997; Neufeld et al., 1998). Thus, although there was a reduction in cell number, the cells were larger than normal resulting in a disc of wild
type size. In summary, these results indicate that the cell cycle machinery can be activated independently of the growth machinery, supporting the simple rationale that cell division subdivides mass without increasing it.

1.3.3.2 Relationship between growth and protein synthesis

Minutes are a class of recessive lethal mutations in Drosophila genes that display a genetically dominant phenotype consisting of a retarded rate of development and shorter, finer bristles. Some Minutes have been shown to result from mutations in genes that encode ribosomal components and so are predicted to disrupt protein synthesis. However, despite this, Minute flies are not generally smaller than wild type flies. Instead growth and development are co-ordinately delayed such that an approximately normal body size is attained. Imaginal disc clones of wild type (+/+) cells in a heterozygous Minute (M/+ ) background proliferate at a higher rate than their M/+ neighbours. Consequently, the wild type clones take over large regions of the disc and final organ through a process termed cell competition. However, these mosaic discs develop to the correct final size and shape (Morata and Ripoll, 1975). Thus, these experiments show first, that cells with unimpaired protein synthesis can outgrow protein-synthesis deficient neighbours. Second, this relative increase in the rate of growth is compensated for by a reduction in the absolute growth of other cells in the disc, resulting ultimately in an organ of wild type size.

Recently, Moreno and colleagues proposed a model in which the elimination of slow-proliferating cells through cell competition is the result of a disadvantage in competing for, or transducing, the Dpp signal during disc development (Moreno et al., 2002). The reduction in Dpp signalling in these cells results in the upregulation of the transcriptional repressor gene, brinker (brk), whose expression is normally repressed by Dpp. The excess brk expression is thought to directly or indirectly activate the c-Jun amino-terminal kinase pathway, which in turn induces apoptosis in these cells, thereby eliminating them from a growing population.
1.3.3.3 Relationship between growth and patterning

As discussed previously, patterning mechanisms divide an imaginal disc into compartments during larval development. Early experiments showed that clones of cells can occupy any territory within a disc compartment (Bryant and Schneiderman, 1969). However, clones cannot cross compartment-boundaries. In effect, these boundaries separate the imaginal disc into different 'growth units', as supported by the observation that cell competition between *Minute* heterozygous cells and wild type clones only occurs within a single compartment and not across compartment boundaries (Garcia-Bellido et al., 1973). Thus, mutant growth-impaired cells in one compartment appear to be protected from cell competition from the wild type clone cells in the adjacent compartment. Consistent with this observation, animals with compartment-specific mutations can be generated in which the anterior and posterior compartments are of different sizes (Simpson, 1976).

One clear link between growth and patterning is provided by the morphogens Dpp and Wg. As mentioned above, both Dpp and Wg can affect both growth and cell fate. For example, impaired production of either Dpp or Wg in the wing primordium abrogates wing growth (Sharma and Chopra, 1976; Spencer et al., 1982; Couso and Martinez Arias, 1994; Zecca et al., 1995). Furthermore, clones ectopically expressing Dpp or Wg in the developing wing imaginal disc can generate duplicated structures resulting from additional growth as well as duplicated patterns (Struhl and Basler, 1993; Nellen et al., 1996; Zecca et al., 1996). Interestingly, autonomous activation of Dpp signalling by ectopic expression of an activated Dpp receptor was shown to promote the accumulation of cell mass and cell cycle-progression (Martin-Castellanos and Edgar, 2002). Conversely, a reduction in Dpp signalling by expression of a hypomorphic Dpp receptor or a pathway–specific inhibitor results in a reduction in cell mass accumulation and cell cycle progression (Martin-Castellanos and Edgar, 2002). In both cases, cell mass accumulation and cell cycle progression were modified to the same degree such that cell size was not appreciably altered.

In summary, the compartments of imaginal discs act as growth-control units. The ability of patterning cues to regulate growth is further supported by the observation that modulation of patterning morphogen signals can alter growth, through the co-ordinated regulation of cell mass accumulation and cell-cycle progression. These morphogens may,
therefore, determine an organ’s size and dimensions by instruction of regional and global growth and patterning during disc development (reviewed in Day and Lawrence, 2000).

1.3.4 Molecular mechanisms of growth control

The molecular mechanisms that control organ size are not fully understood. Genetic analysis in Drosophila, however, has identified three classes of genes that affect organ size. Mutations in the first class of genes, termed Drosophila ‘tumour suppressors’, are associated with hyperplastic or neoplastic transformation following loss of heterozygosity (Bryant et al., 1993; Xu et al., 1995). A subset of these genes has been shown to affect imaginal disc growth, resulting in dramatically enlarged discs with altered tissue morphology. Mutations in certain Drosophila tumour suppressors, such as large tumour suppressor (lats, also known as warts) can cause outgrowths of mutant cells in mosaic animals, resembling human tumours (Xu et al., 1995). Although the constituent cells of these neoplasms are often differentiated, the resulting structures do not retain the pattern of the organ from which they originated. Many of the tumour suppressor genes cloned to date encode proteins involved in the formation of gap junctions, indicating that cell-to-cell contacts are important in the control of growth (Bryant et al., 1993). However, the human lats homolog, lats1, was demonstrated to encode a negative regulator of Cdc2/Cyclin A complex, which promotes G2-M progression (Turenchalk et al., 1999; Xia et al., 2002).

Modulation of the second class of genes, such as dpp and wg, cause duplicated outgrowths in mosaic animals and altered organ size by affecting signals that regulate pattern formation (Day and Lawrence, 2000). Mutations in the third class of growth regulatory genes, cause overgrowth or undergrowth of mutant cells in mosaic animals, but do not disrupt normal patterning (Coelho and Leavers, 2000; Stocker and Hafen, 2000). This class includes components of the Drosophila insulin/PI3K pathway (see below). Whereas the first two classes of mutations affect organ size mainly by increasing cell numbers, the third class of mutations affect organ size largely by affecting cell size.
1.3.5 Control of growth by the Drosophila insulin/PI3K pathway

A substantial number of studies have now shown that imaginal disc growth and cell size can be regulated by the *Drosophila* insulin/PI3K pathway growth (reviewed in Edgar, 1999; Coelho and Leevers, 2000; Day and Lawrence, 2000; Stocker and Hafen, 2000). Thus, increased signalling via this pathway results in increased growth and cell size whereas reduced signalling leads to decreased growth and cell size. Both the increase and decrease in growth is sometimes accompanied by parallel changes in cell number. For example, null mutations in Chico and dS6K slow imaginal disc growth and development, eventually giving rise to viable adults that are reduced in size and composed of small cells (Bohni et al., 1999; Montagne et al., 1999). Null mutations of other identified components of the insulin/PI3K pathway are lethal. However, their function in imaginal disc and whole animal development has been investigated by ectopically expressing transgenes, by analysing mutant clones, and by using weak loss-of-function mutations. For example, flies with heteroallelic combinations of weak inr alleles or flies homozygous for hypomorphic dakt alleles are small and have smaller cells (Chen et al., 1996a; Brogiolo et al., 2001; Stocker et al., 2002).

Similar observations on disc and cell size were made following modulation of Dp110 or dAkt activity during eye and wing development. Thus, an increase in cell size is observed following ectopic expression of wild type or membrane-targeted versions of Dp110 and (p66-*) dAkt (Leevers et al., 1996; Verdu et al., 1999; Stocker et al., 2002). Accordingly, cells with reduced or no dAkt, Dp110 or p60 activity show a pronounced reduction in size (Leevers et al., 1996; Verdu et al., 1999; Weinkove et al., 1999). More recently, DPTEN was found to antagonise the effects of this pathway on growth, consistent with the ability of PTEN to dephosphorylate phosphoinositides generated by PI3Ks. Thus, loss-of-function mutations in DPTEN increase cell size whereas overexpression of DPTEN generates small flies with small cells (Goberdhan et al., 1999; Huang et al., 1999; Gao et al., 2000). Interestingly, the increase in cell size associated with loss of DPTEN function is suppressed in a background with reduced dAkt activity (Gao et al., 2000). Furthermore, a mutation in the PH domain of dAkt that reduces its affinity for PtdIns(3,4,5)P3 is sufficient to rescue the lethality of flies devoid of DPTEN activity (Stocker et al., 2002).

* see section 3.1.2

35
Chapter 1: Introduction

The completed Drosophila genome sequence has revealed the presence of at least seven Drosophila insulin-like peptides (DILPs). Ectopic expression of one of these peptides, DILP2, produces bigger flies with bigger cells (Brogiolo et al., 2001). Interestingly, the most prominent region of dilp gene expression during the larval period occurs within bilaterally symmetric clusters of cells within the larval brain, which appear to be functionally analogous to pancreatic islet β cells (Rulifson et al., 2002). These neurosecretory cells express dilp-1, -2, -3 and -5, and release these peptides into the circulatory hemolymph system. Significantly, ablation of these neuronal dilp-expressing cells causes developmental delay and growth retardation, similar to Inr mutants. These defects could be rescued by ubiquitous expression of a DILP2 transgene (Rulifson et al., 2002).

1.3.6 The effects of modulating insulin/PI3K signalling on cell number

Although the effects of modulating the activity of the insulin/PI3K pathway components on growth are obvious, it is not clear whether this pathway has a direct influence on cell number or cell proliferation. Flies that are null for chico or have hypomorphic mutations in inr possess fewer cells (Chen et al., 1996a; Bohni et al., 1999; Brogiolo et al., 2001). Similarly, ectopic expression of DPTEN results in a reduction in cell number (Goberdhan et al., 1999; Huang et al., 1999; Gao et al., 2000). Conversely, ectopic expression of Dp110 in the wing pouch during an extended period of development can result in a mild but significant increase in cell number (Leevers et al., 1996). Furthermore, flies ectopically expressing Inr or DILP2 have more cells (Huang et al., 1999; Brogiolo et al., 2001). Taken together, these experiments suggest that modulation of the insulin/PI3K pathway for extended periods during development alters cell number as well as cell size and growth.

However, these effects on cell number could be an indirect consequence of changes in biosynthesis rather than a direct effect on the cell cycle machinery or its regulators. In this context, impaired biosynthesis will not allow the normal number of cell divisions to occur whereas enhanced biosynthesis may indirectly permit additional cell divisions. Indeed, analysis of clones ectopically expressing Dp110 or p66-dAkt during wing disc development reveals that although the clones are larger in area than wild type clones, they
possess the same number of cells (Verdu et al., 1999; Weinkove et al., 1999). Furthermore, the size reduction observed in flies lacking dS6K function is caused exclusively by a decrease in cell size (Montagne et al., 1999). Thus, growth can be modulated by the insulin/PI3K pathway without a proportional change in cell number.

These observations do not necessarily preclude the possibility that components of the insulin/PI3K pathway also have more direct effects on cell proliferation, perhaps by interacting with other signalling pathways. For example, the dramatic hyperproliferation observed following ectopic expression of Inr during eye development cannot easily be explained by an indirect effect of increased biosynthesis alone. Interestingly, increased MAPK activation was detected in lysates of heads ectopically expressing an activated form of Inr (Sean Oldham and Ernst Hafen, personal communication) indicating that Inr may promote growth and cell division by the activation of two distinct signalling pathways.

Similarly, in contrast to clones ectopically expressing Dp110 or p66-dAkt, Gao and colleagues found that clones of cells possessing the dpten^d189^ loss-of-function mutation are not only larger in area and have bigger cells than control clones, but also have more cells (Gao et al., 2000). Indeed, Goberdhan and colleagues observed a similar increase in cell number in clones of dpten^1^ and dpten^2^, which represent strong null alleles of dpten (Goberdhan et al., 1999). Intriguingly, hypomorphic alleles of the ATP-dependent RNA helicase, eif4A, can partially suppress the hyperproliferation of dpten^d189^ clones but not the increase in cell size. Thus, these authors argue that loss of DPTEN function is not simply equivalent to activation of Dp110 or dAkt, and suggests the existence of an additional PI3K-independent function in controlling cell proliferation. It should be noted that Huang and colleagues identified another dpten allele, dpten^c494^, which affects cell size but not cell number (Huang et al., 1999). However, unlike dpten^d189^, in which the dpten open reading frame is disrupted after amino acid 89, dpten^c494^ carries a single G135E point mutation. Thus, it is possible that the difference in phenotypes between the two studies is because dpten^d189^ represents a null allele whereas the point mutation in dpten^c494^ may not fully inactivate DPTEN.

1.3.7 The insulin/PI3K pathway and cell cycle progression

In contrast to the apparent inability of components of the Drosophila insulin/PI3K pathway to influence cell division directly, the analysis of cell cycle profiles by flow
cytometry reveals that both Dp110 and DPTEN affect the rate at which cells progress through the different phases of the cell cycle. Thus, ectopic expression of Dp110 or loss of DPTEN function reduces the proportion of cells in G1 and increases the proportion of cells in S phase and/or G2-M (Weinkove et al., 1999; Gao et al., 2000). Since clones ectopically expressing Dp110 do not show increased rates of proliferation, this abbreviated G1 may be balanced by a commensurate lengthening of the duration of S and/or G2-M phases. Intriguingly, analysis of cells with loss-of-function mutations in chico, dakt, or ds6k did not reveal differences in the cell cycle phasing (Bohni et al., 1999; Montagne et al., 1999; Scanga et al., 2000). This suggests that manipulating Dp110 or DPTEN activity has a more profound effect on signalling than mutating other components of the insulin/PI3K pathway. Alternatively, Dp110 and DPTEN may have additional functions in cell cycle progression. It should be noted however, that cells ectopically expressing Dp110 are larger than control cells both in G1 and S phase indicating that Dp110 primarily promotes growth rather than S phase entry (Weinkove et al., 1999).

1.3.8 Does insulin/PI3K signalling stimulate growth via effects on protein translation?

The studies discussed above describe a signalling pathway in Drosophila that appears to control the growth of tissues during development. In this paradigm, extracellular insulin-like molecules activate the insulin receptor, triggering the PI3K-dependent generation of phosphoinositide second messengers and the eventual phosphorylation of downstream kinases that regulate growth. It is not clear, however, how this pathway actually promotes biosynthesis in order for growth to occur. Nevertheless, the modulation of protein synthesis and metabolism are likely to be essential components of this process, and the regulation of both these processes has been ascribed to insulin/PI3K signalling in mammals (Katso et al., 2001).

Since the rate of protein synthesis is thought to be limited at the point of translation initiation (Pestova et al., 2001), it is tempting to suggest that an increase or decrease in translation initiation, and hence global protein synthesis, promotes or retards growth, respectively. However, as experiments with Minutes demonstrate, attenuated translational efficiency does not necessarily equate with reduced organ or cell size in Drosophila (see
Chapter 1: Introduction

section 1.3.3.2). The growth phenotype associated with mutations in dS6K has been argued by Thomas and others to provide insight into this apparent inconsistency (Thomas 2000). By selectively modulating the translation of a subset of mRNAs containing 5'-terminal oligopyrimidine (5'TOP) tracts, dS6K alters the pattern of protein synthesis rather than simply affecting global protein synthesis. Since 5'TOP mRNA largely encode components of the translational machinery such as ribosomes (Jeffries et al., 1994), a decrease in dS6K activity would specifically reduce the translation of this subset of mRNAs, such that the translation of non-5'TOP protein mRNAs might be co-ordinately increased. These non-5'TOP mRNAs are proposed to include key molecules that are able to stimulate cell division. Thus, according to this model, when translation of 5'TOP mRNA is suppressed, these key cell-division regulators may achieve a critical level at an earlier stage of the cell cycle allowing the cells to divide at a size that is smaller than wild type. Conversely, increased dS6K activity would initially promote the translation and synthesis of ribosomal protein mRNAs at the expense of the cell cycle regulators such that the cell attains a greater mass than normal before dividing.

The eukaryotic initiation factor 4E (eIF-4E) binds the 7-methylguanosine cap structure of mRNA and, together with 4A and 4γ subunits, contributes to the formation of a functional complex (termed eIF-4F) that mediates the recruitment of mRNA to ribosomes (McKendrick et al., 1999; Pestova et al., 2001). Different mRNAs display different requirements or abilities to compete for eIF-4F (reviewed in Gingras et al., 1999). Thus, as with dS6K, modulation of eIF-4E function may not merely change the global rate of protein synthesis but also alters the specific pattern of protein expression. Indeed, translation initiation can also occur by independent mechanisms that do not require the cap structure or eIF4F. For example, ribosomes can be recruited by binding a region termed the internal ribosome entry segment (IRES, see Jang et al., 1988). Although cap-dependent translation appears to be impaired during mitosis in higher eukaryotes (Scharff and Robbins, 1966), recent studies have shown that IRES-mediated translation is maintained or may become upregulated at mitosis (Pyronnet et al., 2000; Pyronnet and Sonenberg, 2001). This may permit the synthesis of key proteins of the cell division machinery to occur, despite the overall reduction in the global translation rate.
The regulation of eIF-4E is thought to occur both through direct phosphorylation and through its association with inhibitory eIF4E-binding proteins (4E-BPs, see McKendrick et al., 1999). Binding of 4E-BP1 to eIF-4E prevents its association with eIF4G during assembly of eIF-4F. Significantly, this inhibitory effect of 4E-BP is relieved by insulin-induced phosphorylation of 4E-BP (Pause et al., 1994). Similarly, stimulation of Drosophila Schneider S2 cells with bovine insulin was shown to cause phosphorylation of Drosophila 4E-BP (d4E-BP) and its dissociation from Drosophila eIF-4E (deIF-4E, see Miron et al., 2001). Consistent with this observation, Miron and colleagues found that the ectopic expression of mutant 4E-BP1 with an enhanced affinity for eIF-4E (d4E-BP^M) during wing imaginal disc development reduced wing size as a consequence of a decrease in both cell size and number (Miron et al., 2001). Moreover, the growth phenotype elicited by ectopic expression of Dp110 and dAkt could be suppressed when they were co-expressed with d4E-BP^M, suggesting that the Inr pathway may promote growth, at least in part, through the promotion of eIF-4F assembly.

Surprisingly, ectopic expression of wild type d4E-BP or deIF-4E did not affect wing size, in contrast to d4E-BP^M (Miron et al., 2001). Furthermore, wing discs from d4E-BP null mutants are wild type in size (Miron et al., 2001). Nevertheless, flies with phosphorylation-defective mutations in deIF-4E, and therefore unable to become activated by growth factor signalling, show reduced cell, organ, and body size (Lachance et al., 2002). Taken together, these observations suggest that initiation of protein translation is necessary but not sufficient for growth.

The insulin/PI3K signalling pathway may also promote protein translation through the activation of a second initiation factor, eIF2B, as discussed briefly in section 1.5.2.

1.3.9 Control of growth by Drosophila Myc and Ras1

Aberrant activation of the small GTPase, Ras, and overexpression of the Myc transcription factor have both been implicated in tumourigenesis. Both proto-oncogenes are proposed to link extracellular mitogenic signals to intracellular mechanisms that control cell proliferation, most notably by regulating progression through the G1/S transition of the cell cycle. Thus, studies in mammals investigating the oncogenic potential of these genes have mainly focused on how they may promote malignant transformation through the
misregulation of the cell cycle. However, recent studies in *Drosophila* have suggested that the effects of these oncogenes on the cell cycle may be secondary to their ability to promote growth (Johnston *et al.*, 1999; Prober and Edgar, 2000).

The hypomorphic female sterile mutation *diminutive*1, which results in a reduced body size and slender bristles, was identified as an allele of *Drosophila* myc (dMyc, see Schreiber-Agus *et al.*, 1997). More recently, using clonal analysis in imaginal discs, Johnston, Prober and colleagues demonstrated that reducing dMyc or *Drosophila* Ras1 (dRas1) function during *Drosophila* disc development reduces growth and cell size (Johnston *et al.*, 1999; Prober and Edgar, 2000). Conversely, ectopic expression of dMyc or constitutively active dRas1 (dRas1V12) promotes growth and increases cell size independently of the cell-cycle phase. As with Dpl10, although both dMyc and dRas1 induce growth and drive G1-S progression, neither is sufficient to promote G2-M progression and increase the rate of cell division.

Since dMyc appeared to be a stronger growth driver than dRas1V12, Prober and colleagues suggested that dRas1V12 could be acting via dMyc. Interestingly, dMyc-induced growth was not affected by ectopic expression of dominant negative dRas1 (dRas1N17), suggesting that dMyc functions downstream of or parallel to dRas1 in promoting cell growth (Prober and Edgar, 2000). Indeed, it was suggested that dMyc expression was increased in clones of dRas1V12, although this effect appeared to be very slight. A further interesting observation is that the secreted signal wingless is able to repress dMyc expression along the dorsal/ventral boundary of the wing imaginal disc, indicating a possible link between patterning signals and growth control (Johnston and Edgar, 1998).

### 1.3.10 Control of growth by TSC1 and TSC2

Loss-of-function mutations in the *Drosophila* homologs of the Tuberous sclerosis complex genes (*tsc1* and *tsc2*) result in increased cell and imaginal disc size, as well as a reduction in the G1 population, similar to that observed with ectopic expression of PI3K, Myc or Ras1 (Gao and Pan, 2001; Potter *et al.*, 2001; Tapon *et al.*, 2001). Co-immunoprecipitation experiments suggest that TSC1 and TSC2 form a complex and function in a common pathway that regulates cellular growth (Gao and Pan, 2001; Potter *et al.*, 2001). Thus ectopic expression of both proteins but not either alone results in a
reduction in both cell and disc size, as well as cell number. Notably, the cell size phenotypes induced by alteration of TSC1/2 levels is genetically epistatic to the growth phenotype associated with modulating Inr and dAkt activity (Gao and Pan, 2001; Potter et al., 2001). Intriguingly, the enhanced growth induced by loss of TSC1 or TSC2 is contingent upon dS6K (Potter et al., 2001). Conversely, the retardation in growth associated with ectopic expression of both TSC1 and TSC2 is rescued by co-expression of dS6K. However, although Potter and colleagues reported that TSC1 was epistatic to DPTEN, Gao and colleagues, together with Tapon and colleagues, found that TSC1 and DPTEN have additive effects on cell size (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001). Thus, it is unclear from these studies whether the TSC proteins function as components of the insulin/PI3K-signalling pathway or in a parallel pathway that modulates insulin/PI3K-dependent signalling.

1.3.11 Regulation of mammalian growth by the insulin/IGF-1/PI3K pathway

The role of the insulin pathway in the intrinsic control of organ growth and cell size is further supported by the evolutionary conservation of this function in mammals, as demonstrated by a number of transgenic/‘knock-out’ mouse studies. For example, experiments with mice in which signalling through the IGF-1 receptor was reduced revealed that these animals had reduced body weights, although the relative contributions of changes in cell size and cell number were not established (DeChiara et al., 1990; Baker et al., 1993; Liu et al., 1993). Consistent with these observations, mice rendered null for IRS1 or IRS2 also demonstrate defects in growth (Araki et al., 1994; Tamemoto et al., 1994; Withers et al., 1999). Furthermore, S6K1-deficient mice are proportionally smaller in size than wild type mice (Shima et al., 1998). Similarly, Akt1 null mice show retarded fetal and post-natal growth that persists into adulthood (Cho et al., 2001).

Additional mammalian experiments have documented the effects of the insulin/PI3K pathway on cell size, in addition to organ growth. Shioi and colleagues generated transgenic mice expressing constitutively active or kinase dead forms of PI3K (p110α) and Akt1 specifically in the heart (Shioi et al., 2000; Shioi et al., 2002). Cardiac specific expression of PI3K or Akt1 resulted in mice with larger hearts, whereas expression of dominant negative PI3K or Akt1 resulted in smaller hearts. Remarkably, the increase
and decrease in heart size was associated with a comparable increase or decrease in cardiomyocyte size. Furthermore, the proportions of each heart chamber, the myocardial architecture, and the cardiac function were not significantly perturbed by the modulation of PI3K or Akt1 activity, suggesting a specific role in organ size determination. Similarly, transgenic mice in which constitutively active Akt1 is expressed in pancreatic β-cells show a significant increase in both β-cell size and total islet mass (Tuttle et al., 2001).

Other studies have revealed that mice homozygous for neuronal-specific deletions of PTEN show progressive cerebral enlargement and dysplasia, accompanied by the development of seizures and ataxia (Backman et al., 2001; Groszer et al., 2001; Kwon et al., 2001). The PTEN mutant cells from these mice show a cell-autonomous increase in soma size and elevated phosphorylation of Akt.

In summary, observations from experiments in mammals and flies show that the insulin/PI3K pathway is an evolutionarily conserved critical regulator of growth in both Drosophila and mammals.

1.4 Apoptosis in Drosophila

The development of a correctly proportioned imaginal disc requires the coordinated regulation of cell number and cell growth by patterning cues (see section 1.3). Cell number is, in turn, determined by the rate of cell division and cell death. In the developing wing disc, a small number of epithelial cells undergo apoptosis at any given time, except during the larval molts when there is a dramatic increase in cell death (Milan et al., 1997). During inter-molt periods, random patterns of apoptosis are observed, with single cells or small clusters of neighbouring cells undergoing synchronous cell death (Nordstrom et al., 1996; Milan et al., 1997). Later in development, wing discs display a more restricted pattern of cell death. Thus, in third instar wing discs, apoptotic cells are preferentially located at the border between the presumptive wing and notum, whereas during the pupal stage, dead cells are observed at the wing hinge and, eventually, in the wing blade.

Milan and colleagues demonstrated that when the local regulation of apoptosis is experimentally disrupted in the wing disc, cell death in other regions is modified to compensate against potential disproportion in growth (Milan et al., 1997). For example,
Chapter 1: Introduction

ectopic expression of the cytotoxin, ricin, in the posterior compartment of the wing disc initially increased cell death within this compartment and ultimately resulted in compensatory cell death in the anterior compartment (Milan et al., 1997). This localised increase in cell death is also accommodated by changes in cell proliferation. Thus, following expression of ricin and the induction of apoptosis in the posterior compartment of the wing disc, the number of mitotic cells increases significantly in the directly affected area. In contrast, cell proliferation is substantially reduced in the anterior compartment and is followed by an increase in apoptosis prior to the resumption of normal cell division. Thus, if the expression of ricin is temporary, the wing disc eventually attains the correct proportion and size. However, extended periods of ricin expression resulting in chronic cell death does reduce the final size of the wing disc. Nevertheless, the correct proportion and patterning of these smaller organs is maintained (Milan et al., 1997).

In summary, apoptosis and growth are co-ordinately regulated during Drosophila development in order to generate correctly proportioned and patterned imaginal discs. The role of cell death in the development of the eye imaginal disc is discussed in section 1.4.6.1.

1.4.1 Apoptosis: the basics

The core apoptotic machinery was first identified genetically in studies of C. elegans. During development of the hermaphrodite worm, 131 of 1090 somatic cells die by programmed cell death, leaving an adult of 959 cells. Through genetic screens, loss-of-function mutations in egll, ced 3, ced 4, were found to prevent the death of these 131 cells, whereas ced 9 loss-of-function mutants displayed extensive ectopic cell death (reviewed in Yuan, 1995).

Subsequent characterisation of these and similar genes has revealed that there is remarkable conservation in the machinery that controls and executes cell death in C. elegans, Drosophila and mammals. Consequently, the versatility of invertebrate model organisms has been invaluable in identifying many key proteins involved in apoptosis and in establishing the mechanisms of initiation, execution, and resolution of apoptosis, as discussed below. A summary of the basic apoptotic machinery in Drosophila and mammals is shown in figures 1.5A and 1.5B, respectively, and will be discussed in the sections below.
Figure 1.5A The apoptotic system in *Drosophila*

See text for details. Note that, unlike in mammals, cytochrome c is suggested to remain tethered to the outer mitochondrial membrane in *Drosophila*, where it may undergo a conformational change (based on Meier et al, 2000)
Figure 1.5B The apoptotic system in mammals

See text for details. Prototypic molecules are used to represent families of proteins; for example, Bax and Bcl-2 represent pro- and anti-apoptotic members of the Bcl-2 family, respectively. Survival signalling pathways include the Ras/MAPK and PI3K/Akt pathways. Bad is one of several apoptosis regulatory and effector components whose activity is proposed to be modulated by these pathways. Compare with figure 1.5A (based on Meier et al, 2000)
1.4.2 Caspases: cellular executioners

The majority of apoptosis is executed through the action of a continuously expanding class of cysteine-dependent aspartate-specific proteases or ‘caspases’, of which the protein encoded by *ced-3* is a member. These core executioners transduce pro-apoptotic signals, generated in specific subcellular compartments, and cleave key proteins involved in cellular homeostasis, such as lamins, kinases, DNA repair enzymes and proteins involved in mRNA splicing and DNA replication (Thornberry and Lazebnik, 1998). The cleavage events (which usually inactivate, but occasionally activate, the target substrates) gives rise to many of the biochemical and morphological changes associated with apoptosis (reviewed in Thornberry and Lazebnik, 1998). Caspases are named in reflection of their predisposition for a carboxyl-terminal aspartic acid within a specific tetrapeptide sequence at the cleavage site of their substrates, although some caspases can also cleave after a glutamate residue (see below). They are expressed as zymogens with little or no intrinsic catalytic activity that are composed of three precursor domains: an N-terminal pro-domain, a large 20kDa domain containing the catalytic cysteine, and a smaller 10kDa carboxy-terminal domain (Cryns and Yuan, 1998). Proteolysis of the caspases at specific inter-domain sites results in their activation, releasing the large and small subunits from the prodomain, and enables them to oligomerise and form functional heterotetramers. The inter-domain cleavage sites themselves conform to caspase consensus cleavage motifs, conferring the potential for auto-proteolysis.

Caspases can be divided into two groups on the basis of their variable length amino-terminal prodomains: upstream, or initiator, caspases (group 1) and downstream, or effector, caspases (group 2). Initiator caspases, such as CED 3, have extended prodomains, which, in mammals, have specific sequence motifs known as death effector domains (DEDs) or caspase recruitment domains (CARDs). These prodomains are thought to mediate homophilic interactions with specific adapter or scaffolding proteins during the assembly of multiprotein pro-apoptotic complexes (Earnshaw *et al.*, 1999). The formation of these complexes or ‘apoptosomes’ brings the initiator caspases into close proximity, enabling autoprocessing and activation to occur (Salvesen and Dixit, 1999). The processed initiator caspases are then thought to cleave and activate downstream effector caspases,
which are characterised by shorter prodomains. Thus, following initial activation by an
upstream caspase, a cascade of auto- or trans-activation ensues resulting in eventual
cellular demise (Alnemri, 1997). However, as will be discussed in section 1.4.5, apoptosis
can also occur through caspase-independent mechanisms, in which mitochondria appear to
play a central role.

_Drosophila_ has at least 7 caspases: DCP-1 (Song et al., 1997b), DCP-2/DREDD
(Inohara et al., 1997; Chen et al., 1998), drICE (Fraser et al., 1997), DRONC (Dorstyn et
al., 1999a), DECAY (Dorstyn et al., 1999b), STRICA/Dream (Doumanis et al., 2001) and
DAMM/Daydream (Harvey et al., 2001). Three of these caspases, DCP-2, DRONC and
STRICA/Dream, have long prodomains and are probably initiator caspases (Vemooy et al.,
2000). In addition to the different prodomains, other functional distinctions between the
_Drosophila_ caspases have been observed. For example, DRONC has a divergent active site,
which seems to confer unusual substrate specificity. Thus DRONC is able to cleave after
 glutamate in addition to aspartate (Hawkins et al., 2000; Meier et al., 2000b). Furthermore,
in contrast to other _Drosophila_ caspases, DRONC is resistant to inhibition by the
baculovirus protein, p35, a promiscuous caspase inhibitor (Grether et al., 1995; Chen et al.,
1996c; White et al., 1996).

A small number of studies have investigated the function of these caspases during
development. For example, _in vitro_ immunodepletion experiments suggest that drICE is
required for apoptotic activity in embryonically derived _Drosophila_ S2 cell lines (Fraser et
al., 1997). Furthermore, ectopic expression of wild type DRONC or a truncated form
(lacking the prodomain) in the developing eye induces extensive cell death and ablation of
all retinal structures (Meier et al., 2000b). However, with the exception of _dcp-1_, analysis
of the _in vivo_ function of the _Drosophila_ caspases has been hindered by the lack of loss-of-
function mutations. Interestingly, homozygous _dcp-1_ mutants are embryonically viable and
display normal patterns of cell death, suggesting that the perdurance of the maternally
loaded protein or mRNA is sufficient for embryogenesis and that zygotic _dcp-1_ is
unnecessary at this stage (Song et al., 1997b). Alternatively, other caspases may be
responsible for embryonic cell death and, thus, embryonic DCP-1 function is dispensable.
In summary, caspases are key apoptotic executioners involved in the majority of programmed cell death. These proteases are regulated by a number of different mechanisms, which will be discussed in the sections below.

1.4.3 Inhibitors of apoptosis proteins: key caspase inhibitors

Apoptosis in *Drosophila*, as in mammals, is negatively regulated by members of a family of evolutionary conserved proteins termed inhibitors of apoptosis, or IAPs, that were originally identified in baculoviruses (Deveraux and Reed, 1999). The mechanism by which IAPs suppress cell death is still not clear, though it is proposed that these proteins may bind to and inhibit caspases. IAPs contain one to three amino-terminal baculovirus IAP repeats, or BIRs, in which a conserved arrangement of cysteines and histidines bind a zinc ion to form a zinc finger-like motif (Hinds et al., 1999). Several IAPs also possess a RING domain located near the carboxy terminus. Note, however, that both these domains are also found in other proteins that function in different contexts and that not all proteins with these domains are IAPs (Yang and Li, 2000). The *Drosophila* genome encodes four proteins that share homology with the viral IAPs: DIAP1, the product of the thread locus (Hay et al., 1995), DIAP2 (Hay et al., 1995; Duckett et al., 1996; Liston et al., 1996; Uren et al., 1996), Deterin, a homolog of Survivin (Jones et al., 2000), and dBRUCE, a homolog of human BRUCE (Genbank accession number CG6303).

Ectopic expression of either DIAP1 or DIAP2 suppresses normal developmental cell death (Hay et al., 1995) whereas loss-of-function mutations in DIAP1 induce early embryonic lethality accompanied by apoptosis (Wang et al., 1999; Lisi et al., 2000). Similarly, ectopic expression of Deterin in S2 cells inhibits cytotoxin-induced apoptosis, whereas attenuation of Deterin expression by double-stranded RNA-mediated interference (see section 5.3.5) induces significant apoptosis (Jones et al., 2000).

Several cellular IAPs have been shown to bind to and directly inhibit the activity of caspases. Indeed, DIAP1 can inhibit the proteolytic activity of active drICE and DCP-1 in *vitro* (Kaiser et al., 1998; Hawkins et al., 1999; Wang et al., 1999). IAPs may also act indirectly by binding to the extended prodomains of initiator caspases, thereby preventing their sequestration into activating apoptosome complexes. In support of this, Meier and colleagues demonstrated that DIAP1 could rescue the eye ablation phenotype induced by
ectopic expression of DRONC but not truncated DRONC lacking the pro-domain (Meier et al., 2000b).

1.4.4 Mechanisms of caspase activation

Several pathways have been described that lead to caspase activation in mammals. One pathway involves direct delivery of the serine protease, granzyme B, from cytotoxic T cells into the cytoplasm of target cells where it activates executioner caspases (Trapani et al., 2000). Two other pathways, described briefly below in sections 1.4.4.1 and 1.4.4.2, involve cytoplasmic adaptor proteins that link a cell death signal transducer to initiator caspase activation (Meier et al., 2000a). A fourth pathway, also discussed below (section 1.4.4.3), has been identified in Drosophila that involves the transcription and post-translational regulation of pro-apoptotic cytoplasmic proteins. Direct homologs of these molecules have not yet been identified in mammals or C. elegans.

1.4.4.1 Death receptor pathway

A major extrinsic or instructive pro-apoptotic pathway in mammals is initiated by binding of ligands such as Fas to receptors of the tumour necrosis factor/CD95 receptor superfamily, often referred to as ‘death receptors’ (Meier et al., 2000a). Ligand-dependent death receptor multimerisation initiates the recruitment of cytoplasmic adaptors such as Fas-associated death domain (FADD) that mediate apoptosome assembly. Caspase activation is induced via the oligomerisation of DEDs present in the receptor-bound adaptors, and in certain initiator caspases.

Although no death receptors or corresponding ligands have been identified in invertebrates, a Drosophila homolog of the FADD adaptor, dFADD, has been identified and shown to interact with the prodomain of DCP-2 (Hu and Yang, 2000). Furthermore, ectopic expression of the cytoplasmic region of mammalian CD95 receptor in Schneider S2 cells induces apoptosis (Kondo et al., 1997). More recently, a Drosophila TNF homolog termed Eiger was cloned and characterised (Moreno et al., 2002). Eiger is a potent inducer of apoptosis. However, the apoptotic effect of Eiger does not appear to require the activity of the caspase-8 homolog DREDD, but rather completely depends on its ability to activate the JNK pathway. Moreover, Eiger-induced cell death requires DRONC and DARK.
1.4.4.2 Drosophila Apaf

A second, evolutionary conserved apoptotic pathway is initiated by cell-intrinsic developmental cues or cytotoxic agents and involves factors that promote apoptosome formation such as the C. elegans protein, CED4, and its vertebrate homolog, Apaf-1. Apaf-1 interacts with and mediates the activation of caspase 9 through CARD interactions, a process that is regulated by an auto-inhibitory carboxy-terminal WD40 repeat domain (Zou et al., 1997). Inhibition of Apaf-1 by the WD-40 domain is relieved by binding of holocytochrome c, which, when released from the intermembrane space of mitochondria, appears to be an important trigger of apoptosis in vertebrates (Li et al., 1997b; Zou et al., 1997; Green and Reed, 1998).

Drosophila also has a CED4/Apaf-1 homolog, named Dark/HAC-1/Dapaf-1 (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). Loss-of-function mutations in Dark reduce developmental programmed cell death. However, Dark does not appear to be required for viability, as mutants develop into adult flies, albeit with a number of morphological abnormalities including hyperplasia of the central nervous system, ectopic melanotic tumours and male sterility (Rodriguez et al., 1999). Ectopic expression of Dark into Schneider S2 cells induces cell death, which could be completely suppressed by caspase-inhibitory peptides (see section 5.3.4) or substantially attenuated by co-expression of an active site mutant of DCP-2 but not drICE (Rodriguez et al., 1999). This indicates that Dark-induced apoptosis, like that of Apaf-1, is likely to be mediated through the activation of certain initiator caspases. Indeed, ectopic expression studies have shown that Dark can co-immunoprecipitate with DCP-2 and DRONC (Kanuka et al., 1999; Rodriguez et al., 1999). Like Apaf-1, Dark also contains inhibitory WD-40 repeats, which enhances apoptosis when deleted, suggesting that cytochrome c may also modulate Dark activity in Drosophila (Rodriguez et al., 1999). Consistent with this observation, full length Dark but not truncated Dark lacking the WD-40 can be co-immunoprecipitated with cytochrome c. Indeed, during apoptosis in Drosophila, translocation of cytochrome c from the mitochondria to the cytoplasm also occurs, though the protein remains tethered to outer mitochondrial membranes (Varkey et al., 1999).
Chapter 1: Introduction

1.4.4.3 Reaper, Grim and Hid

A screen for *Drosophila* mutations that result in defective cell death identified a 300-kb genomic interval on chromosome 3 (75C) required for almost all programmed cell death during *Drosophila* embryonic development. Embryos carrying overlapping deletions that map to this region (such as *Df(3R)H99*) show essentially no embryonic apoptosis and die towards the end of embryogenesis due to the accumulation of a significant excess of cells, particularly in the CNS (White *et al.*, 1994). Subsequent analysis of this region has identified three novel genes, *reaper (rpr)*, *grim*, and *head involution defective (hid)*, which are involved in the initiation of programmed cell death in *Drosophila* (White *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1996c). Apoptosis induced by extrinsic insults such as ionising radiation and cytotoxin-induced stress was also profoundly defective in *Df(3R)H99* embryos, suggesting that these genes function in both physiological and pathological programmed cell death (Nordstrom *et al.*, 1996). Importantly, the apoptotic machinery appears to be intact in *Df(3R)H99* homozygotes since high doses of ionising radiation can still induce apoptosis (White *et al.*, 1994). In addition, apoptosis in late stage *Drosophila* nurse cells does not require genes within the *Df(3R)H99* region (Foley and Cooley, 1998). Together, these data imply that *rpr*, *grim*, and *hid* are necessary for the initiation of most, but not all, programmed cell death.

Ectopic expression of either *rpr*, *grim* or *hid* during embryogenesis or eye development results in extensive cell death, embryonic lethality, and an eye ablation phenotype, respectively (Grether *et al.*, 1995; Hay *et al.*, 1995; Chen *et al.*, 1996c; White *et al.*, 1996). The endogenous expression of *rpr* and *grim* appears to be confined to and predictive of cells that are destined to die, and transcripts of these genes can be detected approximately 2 hours prior to the onset of the morphological changes associated with apoptosis (White *et al.*, 1994; Chen *et al.*, 1996c; Robinow *et al.*, 1997). Additionally, *rpr* transcription is induced in response to a variety of apoptotic stimuli, such as ionising irradiation, inhibition of cellular differentiation, and steroid hormone-induced cell death (Nordstrom *et al.*, 1996). In contrast, although *hid* is expressed in many cells that undergo cell death, the correlation is not absolute. For example, while there is considerable cell death detected in the ventral nerve cord during late embryogenesis, *hid* expression is only detected in a fraction of these cells (Grether *et al.*, 1995).
Although the exact mechanism by which \textit{rpr}, \textit{grim} and \textit{hid} mediate apoptosis has not yet been determined, a clear link with caspase activation has been demonstrated. Thus, apoptosis induced by ectopic expression of these genes \textit{in vitro} or \textit{in vivo} can be suppressed following pre-treatment with caspase-inhibitory peptides or by co-expression of the baculoviral caspase-inhibitor protein, p35, respectively (Grether \textit{et al.}, 1995; Chen \textit{et al.}, 1996c; Nordstrom \textit{et al.}, 1996; White \textit{et al.}, 1996). Similarly, reducing caspase activity by heterozygous mutation or expression of dominant negative caspases can suppress the 'rough eye' phenotypes induced by various combinations of Reaper, Grim, and Hid ectopic expression (Chen \textit{et al.}, 1998; Meier \textit{et al.}, 2000b; Harvey \textit{et al.}, 2001).

In genetic screens for modifiers of Reaper and Hid-mediated apoptosis in the eye, Hay \textit{et al.} and Goyal \textit{et al.} identified loss-of-function and gain-of-function mutations in DIAPl as dominant enhancers and suppressors, respectively (Hay \textit{et al.}, 1995; Goyal \textit{et al.}, 2000). In addition, co-expression of either DIAPl or DIAP2 suppresses Reaper-, Grim- or Hid-induced apoptosis in the eye (Hay \textit{et al.}, 1995; Liston \textit{et al.}, 1996). Furthermore, Reaper, Grim and Hid can bind to DIAPl and suppress its ability to inhibit DCP-1 and drICE caspase activity in yeast (Wang \textit{et al.}, 1999). Indeed, the gain-of-function mutations in DIAPl identified in the genetic modifier screen by Goyal and colleagues were shown to impair binding to Reaper and Hid in \textit{in vitro} assays (Goyal \textit{et al.}, 2000). Finally, apoptosis resulting from mutations in DIAPl is epistatic to \textit{Df(3R)H99} (Goyal \textit{et al.}, 2000). Consequently, a double inhibition model has been proposed in which induction of apoptosis by Reaper, Grim and Hid is initiated through their binding to DIAPl, thereby blocking the ability of DIAPl to inhibit caspases. Consistent with this model, despite the fact that Reaper, Hid and Grim show very low overall sequence homology, they do possess limited homology with each other at the amino termini through which they interact with IAPs (see below).

Although homologous proteins have not been identified in other organisms, Reaper, Hid and Grim can induce apoptosis in mammalian cells suggesting that the apoptotic machinery with which they interact is conserved in mammals. Recently, a mammalian protein named Smac/DIABLO was shown to be released from mitochondria into the cytosol during apoptosis, and to have functional similarity with Reaper, Hid and Grim, despite little sequence homology (Wu \textit{et al.}, 2000; Verhagen and Vaux, 2002).
Intriguingly, however, homology is observed in the sequence between the four amino-terminal residues of mature Smac (AVPI), and the amino acid residues of Reaper (AVAF), Grim (AIAY) and Hid (AVPF) (Liu et al., 2000; Wu et al., 2000). Like Reaper, Grim and Hid, Smac can bind IAPs and attenuate their ability to inhibit caspase activation (Chai et al., 2000; Srinivasula et al., 2001). As in Drosophila, this association is also mediated through interactions between the amino terminus of Smac with the BIR domain of IAPs (Wu et al., 2000).

Recently, HtrA2, a member of the HtrA family of serine proteases, was identified as another possible functional homolog of Reaper, Grim, and Hid in mammals (Suzuki et al., 2001). HtrA2 can also bind to and inhibit mammalian IAPs, and its first four amino acid residues (AVPS) show homology with Reaper, Grim and Hid. HtrA2 was identified as an XIAP binding protein and was demonstrated to inhibit XIAP in a similar manner to Smac. However, HtrA2 may also promote apoptosis independently of IAP inhibition and caspase activation through a serine protease activity-dependent mechanism (Suzuki et al., 2001).

### 1.4.5 Mitochondrial regulation of cell death through the Bcl-2 protein family

Mitochondrial function is necessary for the normal maintenance of cellular energy production and, consequently, for cell survival. It is perhaps unsurprising, therefore, that in vertebrates, and probably Drosophila, mitochondria are proposed to be important sites of integration for cell death and survival signals. If cell death signals become dominant, several pro-apoptotic factors are released from the mitochondria. Some of these factors, such as cytochrome c and Smac, regulate caspase-dependent mechanisms of apoptosis. However, it is becoming increasingly clear that apoptosis can also occur through caspase-independent mechanisms, also as a consequence of mitochondrial damage and the release of factors such as HtrA2. Another protein that is released from mitochondria upon receipt of a death signal is apoptosis-inducing factor or AIF (Susin et al., 1999). Following release, AIF translocates to the nucleus where it induces extensive DNA fragmentation, chromatin condensation and caspase-independent apoptosis. The Drosophila genome also encodes an AIF homolog, although this has yet to be functionally characterised (Flybase).

One way in which mitochondrial-induced apoptosis is regulated is via the Bcl-2 protein family. These proteins possess either pro- or anti-apoptotic activity (Vander Heiden
and Thompson, 1999). At least 19 distinct vertebrate Bcl-2 family members have been identified, each of which contain up to four Bcl-2 homology domains (BH1-4). An important characteristic of these proteins is that pro- and anti-apoptotic members can heterodimerise and have the potential to titrate out their opposing functions. Thus, the relative abundance of the pro- and anti-apoptotic members, is thought to be important determinant of the propensity of a given cell to convert death signals into an apoptotic response (Vander Heiden and Thompson, 1999).

Exactly how these proteins modulate cell death is still unclear. Some members of the Bcl-2 family are either resident proteins of the mitochondrial membrane or translocate from the cytosol to the mitochondria following an apoptotic signal (Vander Heiden and Thompson, 1999). Consistent with their ability to form channels in lipid bilayers, a model has emerged in which Bcl-2 proteins are proposed to regulate the release of apoptotic factors such as cytochrome c and AIF from the mitochondria by forming and/or regulating pores on mitochondrial membranes (Shimizu et al., 1999). This may be mediated, at least in part, through association with the mitochondrial voltage-dependent anion channel (see section 1.5.1.1).

In addition to their effects on mitochondria, Bcl-2 family proteins have also been suggested to inhibit programmed cell death by binding to and inhibiting Apaf-1. This is supported by ectopic expression studies demonstrating the association of certain Bcl-2 family members with Apaf-1 (Hu et al., 1998; Inohara et al., 1998; Song et al., 1999). In contrast, however, Moriishi and colleagues did not observe binding in co-immunoprecipitation experiments of the endogenous proteins (Moriishi et al., 1999).

At least two Bcl-2 family members have also been identified in Drosophila. The first is known variously as drob-1 (Igaki et al., 2000), dborg-1 (Brachmann et al., 2000), debcl (Colussi et al., 2000), or dbok (Zhang et al., 2000a; Zhang et al., 2000b). The second gene, known as buffy (Colussi et al., 2000) or dborg-2 (Brachmann et al., 2000), has yet to be functionally characterised. Both proteins have BH1, BH2, and BH3 domains as well as, arguably, weak BH4 domain homology. They both display highest overall homology with the mammalian proapoptotic Bcl-2 family member, Bok. The appearance of dborg-1 and dborg-2 mRNA correlates with programmed cell death in the embryo whereas dsRNA-mediated interference of dBorg-1 expression prevents embryonic apoptosis (Brachmann et
Conversely, ectopic expression of dBorg-1 in cultured cells or transgenic flies induces extensive apoptosis. It is not clear, however, whether caspases play a role in dBorg-1-induced cell death (Colussi et al., 2000; Igaki et al., 2000). Binding assays revealed that dBorg-1 can bind to mammalian anti-apoptotic, but not pro-apoptotic, Bcl-2 family members, indicating that it may function by antagonising the anti-apoptotic potential of the Bcl-2 family. Intriguingly, however, there are no clear candidate genes encoding anti-apoptotic Bcl-2 family members in the fly genome. It is possible that these proteins do not exist in Drosophila or that they have low homology rendering their identification difficult. Nevertheless, ectopic expression of anti-apoptotic mammalian Bcl-2 in Drosophila embryos, wing discs or eye discs can inhibit cell death suggesting that Bcl-2 can have an anti-apoptotic activity in flies (Gaumer et al., 2000).

1.4.6 Regulation of apoptosis by survival signal pathways

Soluble survival factors such as cytokines and hormones play key roles in the establishment and maintenance of metazoan tissues. Particular examples in vertebrates include neurotrophic factors such as NGF that are required for the maintenance of cell viability in the central and peripheral nervous system, or more promiscuous survival factors such as the insulin-like growth factors that are active in a variety of tissues (Raff et al., 1993; Gardner and Johnson, 1996; Parrizas et al., 1997). Studies of the mechanisms by which such factors inhibit apoptosis have implicated the Ras/MAPK and PI3K/Akt pathways in promoting cellular survival. Although subject to significant debate, these pathways appear to act through the direct or indirect inhibition of pro-apoptotic mediators (Xia et al., 1995; Gardner and Johnson, 1996; Datta et al., 1997b; Parrizas et al., 1997). However, as discussed below, they may also act more broadly through the promotion of anabolic activities necessary for maintaining cellular homeostasis. There is also substantial evidence that survival factors, the Ras/MAPK pathway, and, to a lesser extent, the PI3K/Akt pathway are also involved in the establishment and maintenance of various tissues in Drosophila.
1.4.6.1 Control of cell death by Drosophila Ras1/MAPK

As previously mentioned, ommatidia in the developing Drosophila eye are specified in a sequential manner by the recruitment of undifferentiated cells through signalling from their differentiated neighbours. This process is mediated through dRas1 activation by the receptor tyrosine kinases, Drosophila epidermal growth factor receptor (DER) and Sevenless, which stimulate the sequential activation of the MAPKKK, Draf, the MAPKK, Dsor and the MAPK, Rolled (Hafen et al., 1993a; Hafen et al., 1993b; Diaz-Benjumea and Hafen, 1994; Freeman, 1996; Scholz et al., 1997; Dominguez et al., 1998). These kinases, in turn, regulate the activities of the mutually antagonistic transcription factors Pointed P2 and Yan (Hafen et al., 1993a; Hafen et al., 1993b; Diaz-Benjumea and Hafen, 1994; Freeman, 1996; Scholz et al., 1997; Dominguez et al., 1998). Several lines of evidence implicate DER in the regulation of cellular survival during eye development. For example, ectopic expression of argos, a diffusible inhibitor of DER, or expression of a dominant-negative form of DER results in extensive apoptosis (Freeman, 1996; Sawamoto et al., 1998). Conversely, activation of the DER pathway by the ectopic expression of constitutively active DER, inhibits cell death in the developing eye (Miller and Cagan, 1998).

Further studies have shown that the inhibition of apoptosis by DER is mediated by the dRas1 signalling cassette. Kurada and White conducted a genetic screen for modifiers of apoptosis induced in the eye by the ectopic expression of rpr using the glass multimer reporter, GMR (Kurada and White, 1998). They identified loss-of-function and gain-of-function mutations in the dRas1 pathway as dominant enhancers and suppressors, respectively. Subsequent analysis revealed that expression of hid, but not rpr or grim, is directly modulated by this signalling pathway. Specifically, ectopic expression of constitutively active dRas1 or dRaf suppresses hid expression, as does the ectopic expression of Pointed P2. Conversely, ectopic expression of dominant negative DER induced accumulation of hid mRNA and promoted apoptosis in a manner that was sensitive to loss of Hid.

Similar observations were made by Bergman and colleagues using a genetic screen for modifiers of GMR-hid-induced apoptosis (Bergmann et al., 1998). In contrast, however, they found that the eye ablation phenotype of GMR-rpr was not significantly affected by
the ectopic expression of constitutively active or inactive dRas1 mutants. Furthermore, they
demonstrated that site-directed mutagenesis of sequences on Hid that conform to MAPK
phosphorylation consensus motifs, enhances lethality and renders such mutants insensitive
to dRas1/MAPK signalling. Thus, in addition to transcriptional regulation, the
dRas1/MAPK pathways may also be able to directly phosphorylate and, so, inactivate Hid protein.

1.4.6.2 Control of cell death by the Drosophila insulin/PI3K pathway

As discussed in section 1.3.5, recent studies have shown that the insulin/PI3K
pathway plays an essential role in the control of organ size during imaginal disc
development, primarily through increasing cell size. There is also an abundance of data
implicating mammalian PI3K and Akt in the promotion of cell survival (section 1.4.7).
Thus, a number of the Drosophila growth studies addressed the possibility that the effects
of the Drosophila insulin/PI3K pathway on organ growth could, in part, be due to the
suppression of apoptosis. The majority of these studies suggest that insulin/PI3K signalling
is not essential for imaginal disc cell survival. For example, Bohni and colleagues analysed
discs containing either chico null mutant clones or wild type clones by terminal
deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL), which
detects DNA fragmentation, a hallmark of apoptosis (see section 6.4.5; Bohni et al., 1999).
They failed to observe any increase in cell death in chico null clones, nor did they detect
morphological changes characteristic of apoptosis such as pyknosis.

Analysis of DPTEN mutant phenotypes also provides further evidence that the
insulin/PI3K pathway does not play a major role in regulating cell survival in imaginal
discs. First, Gao and colleagues found that clones of DPTEN null cells in eye discs do not
show decreased cell death as assayed by acridine orange staining and flow cytometry (Gao
et al., 2000). Second, the reduction in wing size resulting from ectopic expression of
DPTEN was not suppressed by co-expression of p35. Third, TUNEL analysis did not
reveal significant differences between wild type embryos and dpten-null germ-line clone
embryos that lack both paternal and maternal dpten.

In contrast to the above studies, Huang and colleagues observed that the ectopic
expression of DPTEN behind the morphogenetic furrow of the imaginal eye disc did
increase apoptosis (Huang et al., 1999). This apparent inconsistency could be explained by the fact that different alternative splice variants of DPTEN were used, which may possess distinct functions. The splice variant used by Huang and colleagues (termed DPTEN2) contains a carboxy-terminal sequence PDZ domain binding-motif that may promote apoptosis through physical interaction with other proteins (Smith et al., 1999). Thus, the induction of apoptosis by ectopic DPTEN2 expression may be unique to this protein.

An alternative explanation for the discrepancy could be that the effect of insulin/PI3K signalling on cell survival depends on the cellular context. Indeed, the same group found that ectopic expression of DPTEN2 ahead of the morphogenetic furrow driven by the eyeless enhancer is not associated with an increased incidence of cell death (Huang et al., 1999). Thus, ectopic expression of DPTEN in eye imaginal discs may promote apoptosis in differentiating cells posterior to the morphogenetic furrow but not in anterior proliferating cells (Huang et al., 1999).

Supporting a survival-promoting role of PI3K/Akt signalling, Staveley and colleagues observed that germ-line clone embryos lacking functional dAkt displayed markedly increased apoptosis associated with loss of embryonic cuticle (Staveley et al., 1998). Ectopic expression of p35 completely suppressed the increased cell death in these embryos, indicating a requirement for caspase activity. Furthermore, in a subsequent study, the same group also observed increased apoptosis in embryos ectopically expressing high levels of heat shock inducible catalytically inactive Dpi10 or wild type DPTEN (Scanga et al., 2000). However, these latter findings may be complicated by the increased sensitivity to apoptosis as a consequence of the cellular stress induced by the heat shock regime.

It is possible that the insulin/PI3K pathway does have a moderate effect on cell survival that is secondary to the role played by the MAPK pathway. Indeed, Bergmann and colleagues studied the effects on apoptosis of transgenic flies ectopically expressing effector loop mutants of dRas1V12 which were suggested to specifically activate Raf (dRas1V12S35), Dp110 (dRas1V12C40) or Ral-GDS (dRas1V12G37) (Bergmann et al., 1998). Interestingly, dRas1V12S35 but not dRas1V12G37 strongly inhibited the eye ablation phenotype induced by GMR-hid. dRas1V12C40 had a significant but weak protective effect, supporting the possibility that Dp110 signalling plays a minor role in dRas1-mediated inhibition of apoptosis (Bergmann et al., 1998). However, no evidence was provided for the specificity
of the dRas1<sup>v12</sup> effector loop mutants (see section 4.1.1). Furthermore, this experiment does not address the overall contribution of the insulin/PI3K pathway to the inhibition of apoptosis, independently of dRas1.

Intriguingly, Staveley and colleagues observed that the cuticle phenotype associated with loss-of-function dakt mutant embryos was not rescued in germ line clones also homozygous for the Df(3L)H99 deficiency (Staveley <i>et al.</i>, 1998). Furthermore, there was no significant increase in expression of <i>rpr</i>, <i>grim</i> and <i>hid</i> in dAkt germ line clones. This suggests that loss of dAkt activity induces apoptosis through a mechanism that is distinct from these genes. Thus, the insulin/PI3K pathway may promote cellular survival through a mechanism that does not directly inhibit Reaper, Grim or Hid, for example via the regulation of cytochrome <i>c</i> release or mitochondrial metabolism (see sections 1.4.5 and 1.5).

### 1.4.7 Mammalian PI3K pathway and the regulation of apoptosis

Mammalian PI3K function was first implicated in the suppression of apoptosis by Yao and Cooper when they demonstrated that inhibiting PI3K activity abolished the ability of NGF to promote the survival of the pheochromocytoma cell line PC12 (Yao and Cooper, 1995). Subsequently, it was shown that transfection of cultured cerebellar granule cells with dominant-negative alleles of Akt1 abrogated the ability of IGF-1 and insulin to promote granule cell survival (Dudek <i>et al.</i>, 1997). Since then, there has been a wealth of literature suggesting that the PI3K/Akt pathway is required for growth factor-mediated survival of a range of cell types in culture (reviewed in Datta <i>et al.</i>, 1999; Brazil and Hemmings, 2001). Moreover, PI3K/Akt signalling appears to be sufficient to promote cellular survival in the absence of trophic support and to inhibit cytotoxin-induced apoptosis in vitro.

Transgenic mouse studies have provided evidence for PI3K/Akt-mediated survival <i>in vivo</i>. For example, mouse embryos deficient in PTEN exhibit dramatically increased growth, particularly in the caudal and cephalic regions, that is associated with a reduction in apoptosis (Stambolic <i>et al.</i>, 1998). Furthermore, immortalised embryonic fibroblasts derived from these mice (MEFs) display constitutively elevated levels of Akt activity and phosphorylation and reduced sensitivity to cell death in response to a number of apoptotic
insults. More recently, mice carrying homozygous null alleles of \textit{akt1} were shown to display growth retardation and increased levels of spontaneous apoptosis in testes and thymi (Chen \textit{et al.}, 2001). Additionally, thymocytes and MEFs from these mice are more susceptible to apoptosis induced by a variety of cytotoxic insults such as ultraviolet- or \(\gamma\)-irradiation, and serum withdrawal.

A number of potential targets of the PI3K/Akt signalling pathway that have been identified in mammalian cell culture models have also been proposed to contribute to the ability of this pathway to promote cellular survival. The first such target is the Bcl-2 family member Bad, which promotes apoptosis by binding though its BH3 domain to the anti-apoptotic protein Bcl-\(X_L\) on the mitochondrial membrane (Yang \textit{et al.}, 1995). Phosphorylation of Bad at serine 136 by Akt is proposed to enable its association with 14-3-3 proteins and, consequently, prevent the inhibitory interaction with Bcl-\(X_L\) (Datta \textit{et al.}, 1997b; Blume-Jensen \textit{et al.}, 1998).

A second group of candidate Akt substrates implicated in promoting apoptosis are the Forkhead transcription factors, which may induce the transcription of genes encoding pro-apoptotic proteins such as the cytokine FasL (Brunet \textit{et al.}, 1999). Phosphorylation of FKHRL1 by Akt is proposed to inhibit apoptosis by promoting the sequestration of this FKHRL1 in the cytoplasm, again through association with 14-3-3 proteins. Consistent with this hypothesis, ectopic expression of a FKHRL1 mutant, in which the amino acid residues corresponding to the Akt phosphorylation sites are substituted with an alanine, promotes apoptosis by a FasL-dependent mechanism.

Another transcription factor suggested to be regulated by Akt and implicated in promoting cellular survival is NF-\(\kappa\)B. This ubiquitous, heterodimeric protein is sequestered into the cytoplasm by members of the I\(\kappa\)B family which are in turn regulated by a protein complex that includes two kinases, IKK\(\alpha\) and IKK\(\beta\) (reviewed by Mercurio and Manning, 1999). When phosphorylated and activated, either or both of the IKKs can phosphorylate I\(\kappa\)B, thereby targeting it for ubiquitination and proteosome-mediated degradation. Consequently, NF-\(\kappa\)B is freed and can translocate to the nucleus where it may induce a number of genes that promote survival, such as anti-apoptotic Bcl-2 family members, or IAPs (Mercurio and Manning, 1999). Akt has been shown to enhance the degradation of the I\(\kappa\)Bs (Kane \textit{et al.}, 1999; Ozes \textit{et al.}, 1999).
Interestingly, a number of caspases have Akt phosphorylation consensus motifs (Lawlor and Alessi, 2001). Indeed, recombinant human caspase 9 was shown to be phosphorylated by Akt at serine 196 \textit{in vitro}, which inhibited its protease activity (Cardone \textit{et al.}, 1998). Furthermore, Akt has been shown to be selectively cleaved during the early stages of apoptosis, suggesting that an important feature of the initiation of programmed cell death may be the downregulation of Akt activity (Widmann \textit{et al.}, 1998b); see section 5.3.4).

However, despite the existing evidence, it is still not clear whether the major effect of PI3K signalling is to specifically inhibit activators of the apoptotic machinery or to promote cellular survival through other anabolic effects on cellular homeostasis (see below). Understanding the mechanism of PI3K-mediated cell survival, as well as the specific role played by Akt, is complicated by the multiple outcomes of PI3K activation and by certain caveats with the techniques employed to study Akt function. For example, many of the studies mentioned above are based on the ectopic expression of Akt. However, other AGC kinases share similar substrate specificity to Akt and are also activated by PDK1. It is possible, therefore, that ectopic expression of constitutively active Akt may result in the non-physiological phosphorylation of substrates that are normally regulated by other AGC kinase members (or, indeed, other kinases). Conversely, dominant negative Akt, when ectopically expressed, may also interact with non-physiological substrates thereby preventing them from being phosphorylated by their endogenous upstream kinase. Alternatively, dominant negative forms of Akt may act as 'trapping mutants' or inhibitors of PDK1 and, consequently, may block the activation of other AGC kinases. It is not surprising, therefore, that several putative Akt substrates such as Bad and FKHRL1 are also phosphorylated by other AGC kinases such as S6K and SGK1 \textit{in vitro} with similar stoichiometry (Brunet \textit{et al.}, 2001; Harada \textit{et al.}, 2001).

Aside from potential problems with general methodology, more specific doubts have been raised regarding the validity of the studies mentioned above. For example, the proposed Akt phosphorylation site on human caspase 9 is not conserved in other species. Also, the predicted site of phosphorylation on IKK\(\alpha\) does not correspond to an optimal consensus motif for Akt phosphorylation (Alessi \textit{et al.}, 1996b; Ozes \textit{et al.}, 1999). Additionally, it has yet to be shown that this putative site on IKK\(\alpha\) is actually
Chapter 1: Introduction

Phosphorylated in cells in response to extracellular signals that activate PI3K and Akt. Moreover, regardless of whether or not Bad is a physiological substrate of Akt, it is clear that not all cells express Bad and that cell survival can be regulated independently of both Bad phosphorylation and Akt activation (Scheid and Duronio, 1998; Hinton and Welham, 1999; Kennedy et al., 1999; Pastorino et al., 1999).

Further caution in the interpretation of the above results was indicated by a recent study demonstrating that the FKHRL1 phosphorylation mutants employed by Brunet and colleagues can potently inhibit Akt activity in cells. Thus, some of the observations that have been reported with these mutants may, in fact, be due to the inhibition of the phosphorylation of other Akt substrates (Rena et al., 2001).

1.5 Cell growth and cell survival: two sides of the same coin?

A substantial amount of research has been done to decipher the mechanism through which the PI3K pathway may promote cellular survival. As discussed above, this has largely been biased towards a mechanism involving the direct functional modulation of components of the apoptotic machinery. However, although several promising candidates have been identified, none have been shown conclusively to be physiologically relevant. In an alternative view, given the broad spectrum of cellular effects resulting from PI3K pathway activation, it is possible that the associated reduction in cell death may be a consequence of the promotion of anabolic processes (such as protein synthesis and metabolism) that favour growth and survival. Thus the homeostatic balance is shifted away from cell death towards cell survival, thereby increasing the threshold for the induction of apoptosis. This alternative model is discussed in the sections below.

1.5.1 Regulation of survival through the control of metabolic function

Mitochondrial membrane permeabilisation (MMP) is suggested to be a key event during the early stages of apoptosis that occurs with changes in mitochondrial metabolic function prior to the release of cytochrome c (reviewed in Ravagnan et al., 2002). The mechanism underlying this phenomenon is not clear, but may involve perturbation in the function of the mitochondrial voltage-dependent ion channel (VDAC) and the adenine-
nucleotides translocator (ANT), both of which are components of the permeability transition (PT) pore. A consequence of MMP is the disruption of the electron transfer chain, or the uncoupling of electron transfer from oxidative phosphorylation, resulting in the impairment of ATP synthesis and cellular metabolism. These changes are preceded by the alkalisation of the mitochondrial matrix and ATP synthase-dependent cytosolic acidification, the latter process being necessary for optimum caspase activation (Matsuyama and Reed, 2000). Akt has been shown to maintain mitochondrial integrity, thereby inhibiting cytochrome c release, through an as yet unknown mechanism (Kennedy et al., 1999).

1.5.1.1 Regulation of mitochondrial hexokinases by Akt

Interestingly, a number of studies have raised the possibility that the ability of the PI3K/Akt pathway to promote cellular survival may be directly or indirectly related to effects on cellular metabolism and mitochondrial integrity. Notably, Gottlob and colleagues observed that, in contrast to control cells, stable polyclonal cell lines ectopically expressing constitutively active Akt maintain their intracellular pH and mitochondrial transmembrane potential following combined serum deprivation and UV irradiation (Gottlob et al., 2001). These effects were accompanied by the inhibition of both cytochrome c release and apoptosis. Measurement of cellular phosphocreatine levels, an indicator of VDAC function, suggested that this protective effect was a consequence of the ability of Akt to maintain a normal VDAC configuration thereby preventing its closure, which would otherwise lead to cytoplasmic acidification. Furthermore, in contrast to control cells in which intracellular ATP levels were reduced 10-fold following serum deprivation/UV irradiation, both glycolytic and mitochondrial ATP levels were proportionally elevated in the Akt-expressing cell line.

Intriguingly, the ability of Akt to inhibit cytochrome c release and apoptosis was found to be dependent on the availability of glucose in the culture medium, indicating that Akt-mediated survival requires coupling between glucose metabolism and oxidative phosphorylation. This requirement for glucose is dependent on its conversion to glucose-6-phosphate by the glycolytic enzyme, hexokinase, the first committed step of glycolysis. Consistent with this hypothesis, the non-metabolisable glycolytic inhibitor, 5-thio-glucose,
could suppress Akt-mediated survival. 5-thio-glucose is a linear competitive inhibitor of hexokinase that binds with moderate affinity to the hexokinase glucose-binding site, but is not effectively used as a substrate for the enzyme and is not readily phosphorylated (Wilson and Chung, 1989). In contrast, another glycolytic inhibitor, 2-deoxy-glucose, that can be phosphorylated by hexokinase but is not metabolised further, does not suppress Akt-mediated survival (Chi et al., 1987). Thus, these findings suggested, first, that the initial step of glycolysis was necessary for the promotion of cellular survival by Akt and, second, that this may be due to a requirement for hexokinase activity.

Of the four mammalian hexokinase isoforms, HK-I and HK-II can bind specifically and reversibly to the outer mitochondrial membrane (Wilson, 1995). Significantly, this association may couple glucose metabolism with oxidative phosphorylation since mitochondrial bound hexokinases specifically utilise intramitochondrial ATP to phosphorylate glucose (Cesar Mde and Wilson, 1998). Additionally, hexokinases appear to be integral components of the PT pore and it is suggested that their association and dissociation may mediate transitions between closed and open states of the pore (Beutner et al., 1998). Both the activity and the association of hexokinases with the mitochondrial membrane is inhibited by glucose-6-phosphate, as well as 5-thio-glucose analogues (Wilson and Chung, 1989).

Gottlob and colleagues therefore proposed that Akt, first, increases coupling of glucose metabolism to oxidative phosphorylation and, second, may confer cellular protection from apoptosis by regulating PT pore opening via the promotion of hexokinase-VDAC interactions on the outer mitochondrial membrane. Moreover, ectopic expression of HK-I was demonstrated to significantly inhibit cytochrome c release and apoptosis in a glucose-dependent manner. Consistent with this hypothesis, basal levels of mitochondrial, but not total, hexokinase activity is increased in the Akt-expressing cell lines and maintained following serum withdrawal and UV exposure. This is in contrast to control cells where there is a decline in mitochondrial-bound hexokinase activity.

However, it is not clear how Akt regulates mitochondrial hexokinase activity. Although HK-II contains an Akt consensus phosphorylation motif, its phosphorylation by Akt was not detected (Gottlob et al., 2001). Indeed, so far, there is no evidence that hexokinase activity is regulated by phosphorylation. It is possible that Akt may influence
hexokinase activity indirectly through an, as yet, unidentified co-factor or by modulating glucose-6-phosphate levels. Indeed, Akt has been shown to phosphorylate and activate the downstream glycolytic enzyme, 6-phosphofructo-2-kinase-6 (6-PF2-K), which may affect the steady state level of glucose-6-phosphate and hence HK-II activity (Deprez et al., 1997). However, the observation that 2-deoxy-glucose, which cannot be converted into a substrate for 6-PF2-K (Chi et al., 1987), has no effect on Akt-mediated survival argues against this possibility. Alternatively, Akt may indirectly stimulate hexokinase activity by promoting glucose uptake. Indeed, ectopic expression of Akt in several insulin-responsive cell lines stimulates the uptake of glucose and other nutrients, although the mechanism by which Akt mediates these effects remain unknown (reviewed by Hajduch et al., 2001).

1.5.1.2 Regulation of cell survival by Glycogen Synthase Kinase 3

Further, albeit less direct, evidence for a link between the effect of Akt on metabolism and cellular survival comes from studies on glycogen synthase kinase-3 (GSK-3). Akt can phosphorylate and inactivate GSK-3 which in turn regulates several metabolic proteins, including glycogen synthase (Cross et al., 1995; Shaw et al., 1997; Hajduch et al., 1998; Ueki et al., 1998; van Weeren et al., 1998). Recent studies using neuronal cells in culture have demonstrated that ectopic expression of GSK-3 can induce cell death whereas inhibition of GSK-3 activity, using dominant negative alleles or selective small molecule inhibitors, can suppress apoptosis (Pap and Cooper, 1998; Crowder and Freeman, 2000; Ding et al., 2000; Li et al., 2000; Cross et al., 2001; Culbert et al., 2001; Tong et al., 2001). Significantly, inhibition of GSK-3 activity can block apoptosis induced by the inhibition of PI3K or Akt activity, whereas ectopic expression of GSK-3 can induce cell death even following growth factor treatment (Pap and Cooper, 1998; Crowder and Freeman, 2000; Ding et al., 2000; Cross et al., 2001; King et al., 2001). Notably, caspase-3 activation induced by inhibition of mitochondrial complex I is facilitated by GSK-3 or the PI3K inhibitor, LY294002, and attenuated by IGF-1 or lithium, an inhibitor of GSK-3 (King et al., 2001).

It should be noted that GSK-3 is also regulated by the Wnt signalling pathway and can phosphorylate a number of substrates not involved in glycogen metabolism or PI3K signalling such as β-catenin and Axin (Seeling et al., 1999). Thus, it is not clear what role

66
the Akt-mediated inhibition of GSK-3 may have in the promotion of cellular survival by the PI3K pathway. This point was partially addressed by Culbert and colleagues who examined the protection resulting from the suppression of GSK-3 activity in PC12 cells (Culbert et al., 2001). This was done by using either selective small molecule ATP-competitive GSK-3 inhibitors or FRAT1, a protein proposed to selectively inhibit the activity of GSK-3 towards Axin and β-catenin. Intriguingly, they observed that ectopic expression of FRAT1 is sufficient to confer protection from apoptosis and correlates with inhibition of GSK-3 activity towards β-catenin, but not glycogen synthase, arguing that apoptosis can be suppressed solely through Wnt signalling-mediated inhibition of GSK-3. However, treatment with the small molecule GSK-3 inhibitors provided more potent protection and correlated with the inhibition of GSK-3 activity towards glycogen synthase in addition to β-catenin. This suggests that PI3K signalling-mediated inhibition of GSK-3 is also involved in the suppression of apoptosis.

1.5.2 Protein synthesis and cellular survival

Several recent studies have investigated the regulation of apoptosis through the modulation of translation and protein synthesis (Polunovsky et al., 1996; Henis-Korenblit et al., 2000; Herbert et al., 2000; Polunovsky et al., 2000; Tan et al., 2000). For example, Polunovsky and colleagues observed that ectopic expression of wild type eIF-4E could substitute for serum, PDGF or IGF-1 in maintaining the viability of NIH3T3 fibroblasts and could also inhibit Myc-induced apoptosis (Polunovsky et al., 1996). In a subsequent study, they observed that ectopic expression of 4E-BP1 in Ras-transformed fibroblasts promoted apoptosis and susceptibility to cytotoxic drugs (Polunovsky et al., 2000). Ectopic expression of 4E-BP1 also induced apoptosis of Ras-transformed cells injected into immunodeficient mice and substantially diminished their tumourigenicity. In contrast, cell viability was not altered when 4E-BP1 was ectopically expressed in non-transformed cells. The apoptotic activity of 4E-BP1 was strictly dependent on its ability to sequester eIF4E, preventing the assembly of a functional eIF4F complex. Similarly, ectopic expression of a hypermorphic mutant of Drosophila d4E-BP in the wing imaginal disc was reported to
result in a significant increase in apoptosis, which was suppressed when co-expressed with the caspase inhibitor, p35 (Miron et al., 2001).

Consistent with these studies, ectopic expression of eIF4E has been shown to induce malignant transformation of a variety of cell types, as assayed by growth on soft agar in vitro or the formation in of tumours in nude mice in vivo (reviewed in Gingras et al., 1999). Moreover, a direct correlation between eIF4E mRNA or protein levels and malignancy has been observed in a variety of tumours (reviewed in De Benedetti and Harris, 1999). Indeed, the importance of cap-dependent protein synthesis in promoting cellular survival is further supported by the observation that the initiation factors eIF4G and eIF2α are cleaved by mammalian caspase 3 during early stages of apoptosis, thereby suppressing cap-dependent translation (Clemens et al., 1998; Marissen and Lloyd, 1998; Morley et al., 1998; Marissen et al., 2000).

As discussed in section 1.3.5, the promotion of growth by the PI3K pathway may be mediated, at least in part, by increasing protein synthesis at the level of translation. PI3K signalling can promote translation initiation through the phosphorylation and activation of S6K, or the phosphorylation and inhibition of 4E-BP1 allowing eIF-4E to initiate cap-dependent translation. PI3K signalling may also promote protein translation through the regulation of a second initiation factor, eIF2B, also required for cap-dependent protein translation. eIF2B is inhibited through the phosphorylation of its ε-subunit by GSK-3 (Welsh et al., 1997a; Welsh et al., 1997b; Welsh et al., 1998). Thus, PI3K-mediated inhibition of GSK-3 results in acute activation of eIF2B. It is therefore possible that the PI3K pathway may, at least in part, support cell survival through the promotion of protein synthesis, a key process in the maintenance of cellular homeostasis.

However, inhibition of global translation by approximately 80%-90%, using cycloheximide or pectamycin, does not alter cellular survival or may actually reduce cell death (Herbert et al., 2000; Polunovsky et al., 2000). On the other hand, similar levels of cap-dependent specific translational repression with the mTOR inhibitor rapamycin or ectopic expression of 4E-BP1 has a dramatic apoptotic effect (Herbert et al., 2000; Polunovsky et al., 2000). Thus, there appears to be an important difference between the inhibition of global versus cap-dependent translation and the regulation of apoptosis,
analogous to the relationship between protein synthesis and growth discussed in section 1.3.8.

This dichotomy may be explained by recent reports implicating cap-independent translation in the synthesis of several apoptotic proteins such as Apaf-1 and Myc through the recruitment of ribosomes to internal ribosome entry segments (IRESs) (Coldwell et al., 2000; Henis-Korenblit et al., 2000; Holcik et al., 2000; Stoneley et al., 2000). Although cap-dependent translation is impaired during apoptosis, possibly as a consequence of eIF-4G and eIF-2α proteolytic cleavage, cap-independent translation through IRESs is maintained, enabling the synthesis of key apoptotic proteins and the progression of the cell death programme. Many survival factors and their receptors possess complex 5'-untranslated regions that are also predicted to form stable secondary structures and, consequently, are more dependent on the helicase activity of eIF4F (Coldwell et al., 2000; Henis-Korenblit et al., 2000; Holcik et al., 2000; Stoneley et al., 2000). Thus, one intriguing possibility is that the activation of cap-dependent protein synthesis by, for example the PI3K pathway, results in a profile of cellular proteins that promote cell survival by suppressing apoptosis. Consequently, suppression of PI3K signalling activity attenuates the synthesis of cap-dependent ‘survival’ proteins, whereas cap-independent translation of mRNA encoding apoptotic proteins is maintained, thereby tilting the balance towards apoptosis.

1.6 Overview of thesis

As discussed in section 1.3.9, the Drosophila homologs of the mammalian proto-oncogenes Myc and Ras have both been shown to have similar effects to the Drosophila insulin/PI3K pathway on growth and, in particular, cell size. Moreover, a substantial number of mammalian cell culture studies have implicated the class I PI3K pathway as an effector branch of Ras signalling (as discussed in section 4.1.1). The possibility that the insulin/PI3K, Ras and Myc systems were part of a common growth-regulating signalling network was investigated using an assay that monitors insulin/PI3K pathway activity in vivo. This assay was based on use of phosphorylation state-specific and phosphorylation-independent antibodies to detect the phosphorylation and, hence, activation of a major transducer of this pathway, Drosophila Akt (dAkt), as described in Chapter 3. Thus, using a
combination of the antibody-based assay with immunofluorescence-staining techniques, the relationship between the insulin/PI3K pathway and signalling through dMyc and dRas1 was investigated in vivo during wing disc development, as described in Chapter 4. In particular, the phosphorylation of dAkt was analysed in clones of cells that lacked dRas1 or ectopically expressed constitutively active dRas1 (dRasV12) or dMyc. Significantly, dAkt phosphorylation was increased in dRasV12 clones, but was not altered in dRas1-null clones. This raises the possibility that other Ras-related small GTPases that can also potentially bind to the Ras binding domain of Dp110 may be responsible instead of Ras for endogenous PI3K pathway activation in Drosophila, or indeed, mammals.

The above analysis, however, was complicated by the presence of an intense 'pyknotic' cell staining pattern revealed by immunostaining with one of the phospho-specific dAkt antibodies raised against the phosphorylation site in the carboxyl-terminal hydrophobic motif. This unexpected and intriguing observation was characterised and found to originate from cells undergoing apoptosis.

The identity of this signal was further analysed in Chapter 5. Experiments in imaginal discs or in Drosophila Schneider cells demonstrated that the pyknotic signal and the induction of apoptosis were accompanied by the appearance of an 80kDa signal on phospho-dAkt western blots that co-migrated with the 80kDa isoform of dAkt. Significantly, the appearance of this signal could be blocked by inhibiting caspase activation, suggesting that it was a caspase-dependent process. However, further experiments ruled out the possibility that the 80kDa protein was dAkt.
Chapter 2: Materials and Methods

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Culture of cells and flies</td>
<td>72</td>
</tr>
<tr>
<td>2.1.1 Fly culture</td>
<td>72</td>
</tr>
<tr>
<td>2.1.2 Cell culture</td>
<td>72</td>
</tr>
<tr>
<td>2.1.3 Insulin, CuSO₄ and zVAD-fmk treatment</td>
<td>72</td>
</tr>
<tr>
<td>2.2 Generation and purification of dAkt antisera</td>
<td>72</td>
</tr>
<tr>
<td>2.2.1 Production of rabbit antisera against dAkt peptides</td>
<td>72</td>
</tr>
<tr>
<td>2.2.2 Affinity purification of dAkt antisera</td>
<td>73</td>
</tr>
<tr>
<td>2.3 Biochemical techniques</td>
<td>74</td>
</tr>
<tr>
<td>2.3.1 Preparation of lysates</td>
<td>74</td>
</tr>
<tr>
<td>2.3.2 Protein assays</td>
<td>74</td>
</tr>
<tr>
<td>2.3.3 Immunoprecipitation from cell lysates</td>
<td>74</td>
</tr>
<tr>
<td>2.3.4 Protein kinase assays</td>
<td>75</td>
</tr>
<tr>
<td>2.3.5 SDS polyacrylamide gel electrophoresis</td>
<td>75</td>
</tr>
<tr>
<td>2.3.6 Electroblotting PAGE-resolved proteins</td>
<td>75</td>
</tr>
<tr>
<td>2.3.7 Immunoprobing of western blots</td>
<td>76</td>
</tr>
<tr>
<td>2.3.8 Peptide competition assay</td>
<td>76</td>
</tr>
<tr>
<td>2.3.9 Alkaline phosphatase treatment of immunoblots</td>
<td>76</td>
</tr>
<tr>
<td>2.4 Imaginal disc analysis</td>
<td>77</td>
</tr>
<tr>
<td>2.4.1 <em>Drosophila</em> lines used in experiments</td>
<td>77</td>
</tr>
<tr>
<td>2.4.2 Immunostains of imaginal discs</td>
<td>78</td>
</tr>
<tr>
<td>2.5 Apoptosis techniques</td>
<td>79</td>
</tr>
<tr>
<td>2.5.1 X-ray irradiation of imaginal discs</td>
<td>79</td>
</tr>
<tr>
<td>2.5.2 Acridine orange staining of imaginal discs</td>
<td>79</td>
</tr>
<tr>
<td>2.5.3 Cell death analysis by flow cytometry</td>
<td>79</td>
</tr>
<tr>
<td>2.6 Double-stranded RNA interference of dAkt expression</td>
<td>80</td>
</tr>
<tr>
<td>2.6.1 Design, synthesis and purification of oligonucleotides</td>
<td>80</td>
</tr>
<tr>
<td>2.6.2 DNA amplification by the polymerase chain reaction and purification</td>
<td>80</td>
</tr>
<tr>
<td>2.6.3 In vitro transcription of template DNA</td>
<td>81</td>
</tr>
<tr>
<td>2.6.4 Ethanol precipitation of RNA</td>
<td>81</td>
</tr>
<tr>
<td>2.6.5 dsRNA treatment of Schneider S2 cells</td>
<td>81</td>
</tr>
</tbody>
</table>
Chapter 2: Materials and Methods

2.1 Culture of cells and flies

2.1.1 Fly culture

Flies were raised in tubes and bottles containing standard fly food (0.8% [w/v] agar, 8% [w/v] yeast, 5% [w/v] corn meal, 7% [w/v] sucrose, 0.4% [w/v] nipagin and 0.4% [v/v] propionic acid) at 18°C or 25°C depending on the experimental requirements.

2.1.2 Cell culture

*Drosophila* S2 cells were grown at 23-25°C in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated foetal bovine serum and penicillin-streptomycin (Life Technologies) at a final concentration of 50 units penicillin G and 50 μg streptomycin sulphate per ml of medium. Culture media for *pMT-rpr* cell lines (see section 5.2.2) were also supplemented with 300 μg/ml of hygromycin B (Invitrogen) to maintain selection. For general maintenance, the cells were grown in 75 cm² or 175 cm² tissue culture flasks, and split 1:10 when the cell density was between 10 to 20 x 10⁶ cells/ml, keeping the seeding density greater than 1 x 10⁶ cells/ml. Cells that were to be harvested for lysis or flow cytometry analysis were normally plated onto 90 mm dishes at a seeding density of 1x10⁶ and cultured for 5 days. However, cells that were to be treated with dsRNA prior to lysis were cultured using a modified protocol (see section 2.6.5).

2.1.2.1 Insulin, CuSO₄, and zVAD-fmk treatment

All reagents were from Sigma. Schneider S2 cells were treated with porcine insulin at a concentration of 1μM for 30 min. For induction of the metallothionein responsive promoter in the *pMT-rpr* cell line, cells were exposed to 700 μM CuSO₄ for the durations indicated in the figure legends. To inhibit caspase activity, *pMT-rpr* cells were pre-treated with 20 μM zVAD-fmk (N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) for 90 min prior to CuSO₄-treatment.

2.2 Generation and purification of dAkt antisera

2.2.1 Production of rabbit antisera against dAkt peptides

Peptides pThr423, Thr423, pSer586, Ser586, and CT (section 3.1.1) were synthesised by Graham Bloomberg (Bristol University). Lyophilised pThr423, pSer586 and CT peptides were dissolved in PBS and coupled to Iject maleimide-activated keyhole limpet haemocyanin as directed (Pierce) at a weight to weight ratio of 1:1. The resulting mixtures were dialysed against PBS overnight at 4°C (Slide-A-Lyzer cassettes, Pierce), and divided into aliquots of 200 μg for immunisation. For each peptide, two rabbits were immunised by Eurogentec, Belgium (rabbits SK550 and SK551 to make αpThr423, SK552 and SK553 to make αpSer586, SK554 and SK555 to make αCT). The immunisation and bleeding schedule was as follows. After
taking a preimmune bleed, the rabbits were inoculated three times at two-week intervals, then bled after another week (1st small test bleed). With the exception of SK551 and SK552, the rabbits were inoculated at two further one-month intervals, and bled at the beginning of the 2nd and 4th weeks of each interval. 2nd bleed sera from SK551 and SK552 failed to show significant immunoreactivity towards dAkt and the rabbits were therefore terminated. In contrast, SK553 was inoculated for a further three times and six additional bleeds were obtained. Thus, a total of five bleeds were obtained for SK550, SK554 and SK555, and eleven bleeds for SK553. The crude sera were either stored at -20°C, or at 4°C with 0.03% NaN3.

2.2.2 Affinity purification of dAkt antisera

2nd, 5th and 6th bleed αpSer586 antisera (SK553), as well as 2nd and 5th bleed αpThr423 and αCT antisera (SK550 and SK555, respectively) were affinity purified as follows. Thr423, pThr423, Ser586, pSer586 and CT peptides were first immobilised on to SulfoLink coupling gel as directed (Pierce) using 1-10 mg of lyophilised peptide dissolved in 1 ml of sample preparation buffer (0.1 M sodium phosphate, 5 mM EDTA, pH 7.6). The peptide-coupled SulfoLink columns were then stored in 0.05% NaN3 at 4°C until further processing. Affinity purification was performed as directed (Pierce) with the following modifications. First, the crude sera were filtered to remove debris by allowing the sera to pass through a sterile fibrous sieve placed at the outlet of a plastic funnel. Second, after equilibrating the gel with coupling buffer (50 mM Tris pH 8.5, 5mM EDTA), αpThr423, αpSer586 or αCT crude antisera containing 0.025% NaN3 were cycled through the pThr423-, pSer586- and CT-coupled columns, respectively, overnight at room temperature using a closed-circuit tubing system attached to a peristaltic pump. Third, prior to elution of purified antisera, the columns were washed once with PBS and ten times with 1% (v/v) Triton X100 in PBS, to remove non-specific or low affinity proteins that may have also bound to the immobilised peptides. This was followed by a further two washes with PBS to remove the detergent. The antisera were then eluted using 100mM glycine buffer pH 2.6, and ten to fifteen 1 ml fractions were collected in 1.5 ml eppendorf tubes containing 50μl of 1M Tris, pH 9.5. The elution was monitored by determining the absorbance of the fractions at a wavelength of 280nm to estimate the relative amounts of eluted antibody. The fractions of interest, containing the highest absorbance readings, were then pooled. Fractions with lower readings were also pooled and then concentrated using a centrifugal filter device as directed (Centriprep, Amicon), before being combined with the other fractions. The antibody concentrations of the final pooled fractions were determined as described in section 2.3.2. To reduce the possibility of cross-reaction with unphosphorylated dAkt, the above protocol was repeated with αpSer586 and αpThr423 eluates from the previous purification using the unphosphorylated Ser586 and Thr423 peptide-coupled SulfoLink columns, respectively. The eluates were passed through the columns three times without using a peristaltic pump, and the flow-through containing the final affinity purified antibodies was retained. Affinity purified antibodies were stored at -20°C in 50% (v/v) glycerol.
2.3 Biochemical techniques

2.3.1 Preparation of lysates

All steps were performed at 4°C or on ice, unless otherwise indicated. To prepare S2 cell lysates, the cells were loosened with a sharp tap on the culture dish, poured into 50 ml Falcon tubes containing an equal volume of ice-cold phosphate buffered saline (PBS), and centrifuged at 800 g for 5 min. The supernatant was aspirated and the pellet was washed once with PBS. The centrifugation was repeated and the supernatant was aspirated again. 750 μl of lysis buffer (50 mM tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM NaVO₄, 1 μM pepstatin A, 20 μg/ml aprotinin, 100 μM leupeptin, 100 μM N-α-p-tosyl-L-lysine chloromethyl ketone and 1 mM phenylmethylsulphonyl fluoride) was added per 90 mm culture dish, and incubated for 20-30 min on ice. For protein kinase assays, cells were lysed in lysis buffer as above, supplemented with 20 mM β-glycerophosphate and 15 mM NaPPy. The lysate was clarified by centrifugation at 14 000 g for 10 min, and the supernatant was used for further studies. Detergent lysates of Oregon R third instar imaginal discs were prepared in lysis buffer, supplemented with 0.5% (w/v) sodium deoxycholate and 0.1% SDS, by homogenisation using a small pestle in an eppendorf tube. This lysate was incubated for 2 hours with agitation on a shaking platform, then centrifuged at 14 000 g for 10 min, and the supernatant was removed for analysis.

2.3.2 Protein assays

The approximate protein concentration yielded by lysis of resuspended cells and imaginal discs, or affinity purification of antibodies, was determined by a detergent-compatible protein assay (Bio-Rad DC protein assay). This procedure was based on a modified Lowry protein assay (Lowry et al., 1951). Using known concentrations of protein standards, a standard curve was constructed, and the protein concentration of each sample was determined as directed. The protein standards that were used were bovine serum albumin for cell and disc lysate assays, and bovine gamma globulin for antibody assays. Standards were reconstituted in the same buffer as the samples.

2.3.3 Immunoprecipitation from cell lysates

All incubations were performed at 4°C in lysis buffer containing protease inhibitors, with constant tumbling on a rotating wheel. Each ml of cell lysate was incubated with 3-5 μl of polyclonal antisera for 1.5 hours. 10 μl of a 50:50 slurry of lysis buffer and protein A Sepharose beads (Pharmacia, pre-washed three times in PBS) was then added and the mixture was incubated for a further 30 min. The beads were then washed 5 times in lysis buffer. However, immunoprecipitates that were to be used for protein kinase assays were instead washed three times in lysis buffer, once in salt buffer (100 mM Tris-HCl pH 7.5, 0.5 M NaCl) and once in kinase wash buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol). For
immunoblot analysis, the beads were resuspended in an appropriate volume of 1X sample buffer after the final wash, and heated at 100°C for 5 min.

2.3.4 Protein kinase assays

dAkt kinase activity was measured by the capacity of immunoprecipitates to phosphorylate a peptide substrate (Crosstide) corresponding to the sequence in mammalian glycogen synthase kinase 3β surrounding serine 9 that is phosphorylated by Akt (GRPRTSSFAEG; Cross et al., 1995). Beads from immunoprecipitations were washed as above at 4°C. Kinase assays were performed in 50 μl of 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 50 μM ATP containing 2 μCi [γ³²P] ATP, and 30 μM Crosstide for 30 min at 30°C. Following incubation, the reaction was briefly centrifuged at 16 000 g for 10 sec and transferred to ice. 40 μl of reaction was spotted onto P81 phosphocellulose paper (Whatman) and washed extensively in 75 mM phosphoric acid. The P81 paper was then washed once in acetone and allowed to dry completely. The amount of [γ³²P] incorporation was quantified using a liquid scintillation counter (Beckmann).

2.3.5 SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (PAGE) was performed using a procedure modified from that of Laemmli (1970). 0.75 mm, 1 mm, or 1.5 mm thick mini-gels (7.3 cm x 10.2 cm) or large-gels (16 cm x 20 cm) were run using Bio-Rad or Life technologies apparatus. Resolving gels contained 7.5% (w/v), 8.0% (w/v) or 4-16% (w/v) acrylamide: bisacrylamide in a ratio of 37.5:1 (Amresco), 375 mM Tris-HCl pH 8.8 and 0.1% (w/v) SDS. Stacking gels consisted of 125 mM Tris-HCl pH 6.8, 4% acrylamide: bisacrylamide, and 0.1% (w/v) SDS. Gradient gels were cast using a Model 385 (Bio-Rad) gradient former. Both resolving and stacking gels were polymerised by the addition of ammonium persulphate and TEMED (NNN’N’-Tetramethylethylendiamine). Samples were electrophoresed at 100 V (mini-gels) or 60 V (large-gels) in running buffer (25 mM Tris-base pH 8.3, 192 mM glycine and 0.1% (w/v) SDS).

2.3.6 Electroblotting PAGE-resolved proteins

Resolved proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) by the Trans-Blot system (Bio-Rad) for 60-90 min at 150-200 mA. The Immobilon membranes were pre-wet in methanol prior to use. Electroblotting was performed in transfer buffer (39 mM glycine, 48 mM Tris-base, 0.0375% (w/v) SDS and 20% (v/v) methanol).
2.3.7 Immunoprobing of western blots

Membranes were blocked for 45-60 min at room temperature in Tris-buffered saline (20 mM Tris pH 7.4, 150 mM NaCl) containing 0.1% (v/v) Tween-20 (TBST) and 5% (w/v) powdered milk (Marvel) with agitation on a rotary shaker. Primary antisera were diluted in the same buffer at the appropriate dilution. Standard dilutions for the dAkt antibodies were 1:1000 for 2nd bleed crude antisera, 1 µg/ml for 2nd bleed affinity purified antisera, and 0.25µg/ml for 5th/6th bleed affinity purified antisera. Other standard dilutions were 1:1000 for the mammalian Akt antibody (9272, New England Biolabs), and 1:150 for the β-tubulin antibody (Developmental Studies Hybridoma Bank). The membranes were agitated in the antibody solution overnight at 4°C then rinsed three times in TBST and washed four times for 10 min each at room temperature. The immunoblot was next incubated for 45-60 min with the appropriate secondary antibody conjugated to horse radish peroxidase (DAKO) diluted 1 in 2000 in TBST. Following secondary antibody incubation, the blots were rinsed and washed as above, then gently run over the edge of a plastic container to remove excess buffer and placed in enhanced chemiluminescence reagents for 5 minutes. The standard chemiluminescence kit that was used was Supersignal West Pico (Pierce) with the exception of imaginal disc tissue immunoblots (section 5.2.1) in which case Supersignal West Dura (Pierce) was used. The blots were run over the edge of a plastic container as before to remove excess chemiluminescence buffer and then covered in saran wrap and exposed on X-ray film (Fuji).

2.3.8 Peptide competition assay

Sub-saturating concentrations of αpSer586 and αCT antibodies (2nd bleeds) were pre-incubated at 4°C for 2 hours with three different concentrations of the peptide against which each antibody was raised. Antibody to peptide molar ratios of 1:2, 1:20 and 1:200 were used. Thus, 1 µg/ml of αpSer586 was incubated with 0.159 µg, 0.0159 µg, or 0.00159 µg of pSer586 peptide (molecular weight 1271.28 Da). Similarly, 1 µg/ml of αCT was incubated with 0.154 µg, 0.0154 µg or 0.00154 µg of CT peptide (molecular weight 1229.32 Da). In addition, 1 µg/ml of αpSer586 was incubated with 0.149 µg, 0.0149 µg, or 0.00149 µg of unphosphorylated Ser586 peptide (molecular weight 1191.28 Da). In each case, both antibody and lyophilised peptide were diluted in TBST. Following pre-incubation, the antibody-peptide mixtures were used to probe western blots or immunostains as described in sections 2.3.7 and 2.4.2.

2.3.9 Alkaline phosphatase treatment of immunoblots

Immunoblots were incubated overnight at 4°C in blocking buffer (100 mM boric acid, 25 mM sodium borate, 76 mM NaCl, 3% BSA, 0.1% Tween-20). After blocking, the immunoblots were rinsed three times in borate saline (100 mM boric acid, 25 mM sodium borate, 76 mM NaCl). The blots were then incubated at 37°C for 8 hours in 10 ml of alkaline phosphatase reaction mixture (100 mM Tris-HCl pH 8.6, 0.5 mM MgCl₂, 10 µM ZnCl₂, 50 U calf intestinal alkaline phosphatase [Roche] per ml, 50 µg/ml leupeptin,
25 μg/ml pepstatin A, 100 μM PMSF) or control mixture (reaction mixture without phosphatase). After incubation, the blots were washed twice for 10 min with borate saline containing 100mM sodium pyrophosphate and 1 mM sodium fluoride to inactivate the alkaline phosphatase, followed by two additional 10 min washes with borate saline. The immunoblots were then blocked and probed as described in section 2.3.7.

2.4 Imaginal disc analysis

2.4.1 Drosophila lines used in experiments

Oregon R was used as wild type control line where indicated.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Minute (3)95A lines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yw, hs-flp^{122}; 82B FRT P[arm-lac^{2} w^{+}] P[ry^{+}=A92^2] RpS3^{P^{+}}/TM6B</td>
<td>Bruce Edgar</td>
<td>Prober and Edgar, 2000</td>
</tr>
<tr>
<td>yw, hs-flp^{122}; 82B FRT P[Ubi-GFP w^{+}] P[ry^{+}=A92^2] RpS3^{P^{+}}/TM6B</td>
<td>Ernst Hafen</td>
<td>-</td>
</tr>
<tr>
<td><strong>dakt alleles</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yw, ey-flp glass-lac^{2}; 82B FRT dakt^{844} / TM6B</td>
<td>Ernst Hafen</td>
<td>-</td>
</tr>
<tr>
<td>yw, ey-flp glass-lac^{2}; 82B FRT dakt^{844} / TM6B</td>
<td>Ernst Hafen</td>
<td>-</td>
</tr>
<tr>
<td>yw, ey-flp glass-lac^{2}; 82B FRT dakt^{844} / TM6B</td>
<td>Ernst Hafen</td>
<td>-</td>
</tr>
<tr>
<td>yw, ey-flp glass-lac^{2}; 82B FRT dakt^{184} / TM6B</td>
<td>Ernst Hafen</td>
<td>-</td>
</tr>
<tr>
<td>yw, ey-flp glass-lac^{2}; 82B FRT dakt^{184} / TM6B</td>
<td>Ernst Hafen</td>
<td>-</td>
</tr>
<tr>
<td>yw, ey-flp glass-lac^{2}; 82B FRT dakt^{184} / TM6B</td>
<td>Ernst Hafen</td>
<td>-</td>
</tr>
<tr>
<td>yw, ey-flp glass-lac^{2}; 82B FRT dakt^{184} / TM6B</td>
<td>Ernst Hafen</td>
<td>-</td>
</tr>
<tr>
<td><strong>dp110 null allele</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P[8H] 82B FRT dp110^{+}/TM6B</td>
<td>Sally Leivers</td>
<td>Levers et al., 1996</td>
</tr>
</tbody>
</table>
**2.4.2 Immunostains of imaginal discs**

All incubations and washes were performed at room temperature unless stated otherwise. Where necessary, appropriate genotypes were selected by discriminating against marked balancer chromosomes. Third instar imaginal discs were dissected from larvae and directly fixed in methanol-free 4% (w/v) formaldehyde (Polysciences) for 30-45 min. Following fixation, the discs were washed twice for 15 min each in PBS with 0.1% Tween-20 (PBST) before blocking for 60 min in PBST with 0.1% bovine serum albumin (PBST-BSA). Primary antisera were diluted in PBST-BSA using the appropriate ratio. Standard dilutions were 5 μg/ml for affinity purified 5/6th bleed dAkt antisera, 1/100 for the β-galactosidase antibody, 1/200-1/500 for the anti-Croquemort antibody, and 1/200 for the anti-Myc epitope (9E10; Evan et al., 1985) antibody. The imaginal discs were then incubated with the primary antisera overnight at 4°C. Following primary antibody incubation, the discs were washed five times (2 x 10 min, then 3 x 20 min) in PBS-BSA with 4% (v/v) normal goat serum before incubation in the same buffer with the appropriate fluorophore-conjugated secondary antibodies for 90 min. The standard dilution for fluorescein-, rhodamine- and cyanine-conjugated secondary antibodies (Jackson ImmunoResearch) was 1/200. Following secondary antibody incubation, the discs were washed three times in PBST for 10 min each before being mounted in Fluoro Guard antifade reagent (Bio-Rad).

Immunofluorescent imaginal discs were analysed using a Zeiss Axioplan 2 confocal microscope and Zeiss LSM 2.5 software. Images were processed and assembled in Adobe Photoshop 6.1.

---

<table>
<thead>
<tr>
<th><strong>dral null allele</strong></th>
<th>*<em>yw; 82B FRT <em>dral</em>{46b} P[π-πyc]/TM3, y</em></th>
<th>Bruce Edgar</th>
<th>Prober and Edgar, 2000</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>flp-out clone lines</strong></td>
<td><strong>yw; Actin&gt;CD2&gt; GAL4; UAS-GFP{NLS}</strong></td>
<td>Bruce Edgar</td>
<td>Neufeld et al., 1998</td>
</tr>
<tr>
<td></td>
<td><strong>yw, hs-flp; actin&gt;CD2&gt; GAL4; UAS-GFP{NLS}</strong></td>
<td>Ernst Hafen</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><strong>yw, hs-flp{22}</strong></td>
<td>Bruce Edgar</td>
<td>Neufeld et al., 1998</td>
</tr>
<tr>
<td></td>
<td><strong>yw; P[UAS-dakt]/CyO</strong></td>
<td>Ernst Hafen</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>yw, hs-flp; P[UAS-myc dp110] {SJ9}</strong></td>
<td>Sally Leevers</td>
<td>Leevers et al., 1996</td>
</tr>
<tr>
<td></td>
<td><strong>yw, hs-flp{22}; P[UAS-drasl{1713}]</strong></td>
<td>Bruce Edgar</td>
<td>Prober and Edgar, 2000</td>
</tr>
<tr>
<td></td>
<td><em><em>w</em>;P [ UAS-dras2{1714}]</em>*</td>
<td>Bloomington Stock 2025</td>
<td>Brand and Perrimon, 1993</td>
</tr>
<tr>
<td></td>
<td><strong>yw, hs-flp{22}; UAS-dmyc{42}</strong></td>
<td>Bruce Edgar</td>
<td>Johnston et al., 1999</td>
</tr>
</tbody>
</table>

| **Wing disc GAL4** | **MS1096** | Capdevila and Guerrero, 1994 |
Chapter 2: Materials and Methods

2.5 Apoptosis techniques

2.5.1 X-ray irradiation of imaginal discs

Third instar Oregon R larvae cultured at 25°C were collected and divided into experimental and control samples. The experimental sample were placed in a 60 mm culture dish and exposed to 4000 rads of ionising radiation. Both experimental and control larvae were then transferred to eppendorf tubes containing moist tissue paper and allowed to age at 25°C for 5 hours prior to staining with either acridine orange (section 2.5.2) or the dAkt antisera (section 2.4.2).

2.5.2 Acridine orange staining of imaginal discs

All dissections and incubations were performed in ice-cold PBS supplemented with 1% (v/v) heat-inactivated foetal calf serum (PBS-FC) unless stated otherwise. Wing imaginal discs were dissected from third instar larvae at the desired time point for a period of no longer than 10 min. The dissected discs were then transferred to buffer containing 2 μg/ml acridine orange (3,6-bis [dimethyl-amino] acridine hydrochloride, Sigma) and stained for 10 min with gentle agitation on a rotary shaker. The discs were washed briefly in PBS-FC, mounted in PBS, and viewed immediately under epifluorescence using the fluorescein filter.

2.5.3 Cell death analysis by flow cytometry

CuSO₄-treated pMT-rpr cells, seeded as described in section 2.1.2.1, were detached by gentle tapping of the culture dish and pelleted by centrifugation at 800 g for 5 min. The pellet was washed once in ice-cold PBS and then resuspended and fixed in 70% (v/v) ethanol in PBS for at least 30 minutes at 4°C. The fixed cells were pelleted (1000 g, 3 min) and washed twice in phosphate-citrate buffer (192 mM Na₂HPO₄, 4mM citric acid pH 7.8). To ensure that only DNA and not RNA was stained with propidium iodide (PrI), the cells were treated with 100 μg/ml RNase A (Sigma) in PBS, and PrI was added at 50 μg/ml. PrI fluorescence data from individual cells in each cell population were collected using a Beckton Dickinson FACScan flow cytometer. The data were analysed with the CellQuest programme (Becton Dickinson) and, assuming a linear relationship between PrI fluorescence and the amount of DNA in a cell, the proportion of cells with DNA contents of <2N, 2N, >2N<4N and 4N (N= haploid DNA content) were estimated. Thus the approximate proportion of each cell population in sub-G1, G1, S phase and G2 +M was determined.
2.6 Double-stranded RNA interference of dAkt expression

Double stranded RNA (dsRNA) of 228 bp (dsRNA\textsuperscript{228}), 539 bp (dsRNA\textsuperscript{539}) or 659 bp (dsRNA\textsuperscript{659}) were produced by transcription of both strands of double stranded DNA templates (see section 5.3.5). The templates were amplified by polymerase chain reaction (PCR) from a full length dakt plasmid.

2.6.1 Design, synthesis and purification of oligonucleotides

Each primer used in the PCR contained a 5' T7 RNA polymerase binding site preceded by a 5'GAA overhang (GAATTAATACGACTCACTATAGGGAGA) followed by sense and antisense sequences specific for dakt. The dakt-specific sequences were as follows (corresponding nucleotides in the [p80] dakt gene open reading frame are shown in square brackets):

Sequence 1 (to generate template for dsRNA\textsuperscript{228})

<table>
<thead>
<tr>
<th>sense</th>
<th>TCGATGGTGTTCACGCGCG</th>
<th>[nucleotides 12-32]</th>
</tr>
</thead>
<tbody>
<tr>
<td>antisense</td>
<td>TCCGGAATCGTGTTAGGGGC</td>
<td>[nucleotides 220-240]</td>
</tr>
</tbody>
</table>

Sequence 2 (to generate template for dsRNA\textsuperscript{539})

<table>
<thead>
<tr>
<th>sense</th>
<th>ATGTCAATAAACACAACTTTC</th>
<th>[nucleotides 244-264]</th>
</tr>
</thead>
<tbody>
<tr>
<td>antisense</td>
<td>TCACCTCTTTAACGCGGCTG</td>
<td>[nucleotides 764-784]</td>
</tr>
</tbody>
</table>

Sequence 3 (to generate template for dsRNA\textsuperscript{659})

<table>
<thead>
<tr>
<th>sense</th>
<th>TCCGATGGAAGACTGATGGG</th>
<th>[nucleotides 394-414]</th>
</tr>
</thead>
<tbody>
<tr>
<td>antisense</td>
<td>CGCCACCGTTCACGTACTGC</td>
<td>[nucleotides 1028-1048]</td>
</tr>
</tbody>
</table>

Oligonucleotides were synthesised and quantified using an Applied Biosystems 3948 DNA synthesiser (Ian Goldsmith). The products were purified by high-pressure liquid chromatography (HPLC) and dried down.

2.6.2 DNA amplification by the polymerase chain reaction and purification

PCRs were performed as directed (Qiagen) in 100 μl reaction volumes, containing 1X Qiagen PCR buffer (with 1.5mM MgCl\textsubscript{2}), 0.4 μM of each primer, 200μM dNTPs, and 0.025 U/μl Taq DNA polymerase. Template dakt DNA was provided by 16 ng of pBS-SDE RAC 109 plasmid (Andjelkovic et al., 1995). Due to the length of the primers, two stages of amplification were performed in conjunction using a Peltier thermal cycler. Initially, cycles with a low annealing temperature were used to generate some product. These were followed by cycles at a higher annealing temperature (using two sets of conditions depending on the length of the product required), which minimise the amount of side-products formed by non-specific priming events during the first set of cycles at the lower annealing temperature. Thus, for the first stage of amplification, an initial extended denaturing step was performed (94°C, 3 min), followed by 5 cycles of denaturation (94°C, 30
sec), annealing (50°C, 30 sec) and extension (72°C, 50 sec), completed by a single 5 min extension step at
72°C. The second stage of amplification was performed as follows. After an initial extended denaturing step
(94°C, 3 min), the samples were denatured (94°C, 30 sec), annealed (68°C, 30 sec) and extended (72°C, 50 sec
[dsRNA\(^{53,659}\) or 72°C, 15 sec [dsRNA\(^{238}\)]) for 30 cycles (dsRNA\(^{53,659}\) or 40 cycles (dsRNA\(^{238}\)). A final 5
min extension at 72°C was then performed. The final PCR products were assessed by resolving 0.5 µl of each
on TAE-agarose gels (1% [w/v] agarose, 40 mM Tris.acetate, 1 mM EDTA, 0.5 µg/ml ethidium bromide)
using TAE buffer (40 mM Tris.acetate, 1 mM EDTA). The products were then purified, as directed, using a
QIAquick PCR purification kit (Qiagen), and resuspended in 30 µl of 10 mM Tris-HCl, pH 8.5.

2.6.3 In vitro transcription of template DNA

Purified PCR products containing the T7 RNA polymerase binding site were in vitro transcribed, as
directed, using the MEGAscript transcription kit (Ambion) with an overnight incubation at 37°C. Nuclease-
free water, treated with 0.1% (w/v) diethylpyrocarbonate solution, was used throughout. At the end of the
transcription reaction, the template DNA was degraded by adding 0.1 U/µl DNase 1 and incubating for a
further 15 min at 37°C.

2.6.4 Ethanol precipitation of RNA

RNA that was transcribed using the MEGAscript kit was recovered by ethanol precipitation as
follows. 20 µl of nuclease free water and 5 µl of 3M NaOAc was added to 20 µl of transcription reaction and
thoroughly mixed. 125 µl of absolute ethanol (-20°C) was then added and the mixture was placed under dry
ice for 10 min, before centrifugation at 13 000 g for 15 min at 4°C. The supernatant was then discarded and
the pellet was allowed to air dry for approximately 15 min. The dried pellet was resuspended in 15 µl of
nuclease free water and was incubated at 65°C for 30 min. The RNA was annealed by allowing the mixture to
cool slowly to room temperature, then quantified using a GeneQuant capillary spectrophotometer
(Amersham). The final concentration of the dsRNA was adjusted to 3 µg/µl. A 0.5 µl sample was then run on
a TAE-agarose gel as above to assess the integrity and size of the dsRNA before storage at –20°C.

2.6.5 dsRNA treatment of Schneider S2 cells

S2 cells were plated into 6-well, 35 mm dishes using Drosophila serum-free expression medium
(DES, Invitrogen) with 1 ml per well of 1x10\(^6\) cells/ml. 15 – 30 µg of dsRNA was added immediately and the
plates were gently agitated to mix the RNA and cells before incubating at 23°C for 60 min. 2ml of
Schneider’s Drosophila medium with 10% (v/v) heat-inactivated foetal calf serum was then added and the
plates were incubated for 4, 6, or 9 days before harvesting (section 2.3.1).
Chapter 3: Generation and Characterisation of Phosphorylation State-Specific Antibodies to
Drosophila Akt

3.1 Generation and biochemical characterisation of phosphorylation-specific antibodies to
Drosophila Akt

3.1.1 Strategy for the generation of phosphorylation-specific and phosphorylation state-independent
antisera to dAkt

3.1.2 Immunoreactivity of antisera raised against dAkt peptides

3.1.3 Confirmation that antisera recognise dAkt in vitro in a phosphorylation state-dependent or -
independent manner

3.1.3.1 Immunoprecipitation of p66- and p80-dAkt

3.1.3.2 Peptide competition removes dAkt signal

3.1.4 Affinity purification of dAkt antisera

3.1.5 Correlation between the phosphorylation state and kinase activity of dAkt

3.2 Cell biological characterisation of the phosphorylation-specific antibodies to Drosophila
Akt

3.2.1 dAkt phosphorylation signal is reduced in mitotic clones of dAkt null cells

3.2.2 Dp110 is required for dAkt phosphorylation at Serine 586 in vivo

3.2.3 Ectopic expression of Dp110 induces dAkt phosphorylation in vivo

3.2.4 Identification of a pyknotic signal with apSer586

3.2.5 Ectopic expression of Dp110 in the pouch region of the wing imaginal disc induces increased
dAkt phosphorylation

3.3 Summary
3.1 Generation and biochemical characterisation of phosphorylation-specific antibodies to *Drosophila Akt*

The initial aim of the work described in this thesis was to investigate the activation of the insulin/PI3K pathway *in vivo* during organ growth and development using *Drosophila* imaginal discs as a model system. The second aim was to try to establish the relationship between the insulin/PI3K pathway and other growth-promoting pathways regulated by dRas1 and dMyc to determine whether these systems were part of a common growth-signalling network.

Several approaches have been used to monitor insulin/PI3K pathway activity. Traditionally, these include lipid kinase assays of phosphorylated tyrosine-containing receptor complexes that have been immunoprecipitated using phosphorylated tyrosine-specific antibodies, or extrapolation of insulin/PI3K pathway activity from the activation of downstream effectors. However, many of these techniques have significant limitations. For example, lipid kinase assays of phosphorylated tyrosine immunoprecipitates measure the recruitment of PI3K heterodimers to activated tyrosine-phosphorylated receptor complexes rather than signalling flux through the pathway *per se*. Similarly, direct measurement of intracellular levels of 3'PIs does not necessarily reflect pathway activity. Alternative methods have also been used to infer insulin/PI3K pathway activation such as kinase assays of Akt immunoprecipitates. However, this approach is limited to *in vitro* analysis.

Other techniques that have been used to study insulin/PI3K pathway activation *in vivo* include the use of green fluorescent protein-tagged PH domains. More recently, an alternative technique has been used based on the detection of Akt phosphorylation using phosphorylation-specific antibodies. This method has an additional advantage in that it permits both *in vitro* and *in vivo* analysis of insulin/PI3K signalling, and is also a more specific assay. The major caveats of this technique, however, are that it measures only one branch of insulin/PI3K signalling and that it is possible that Akt may become phosphorylated by PI3K-independent mechanisms. Nevertheless, Akt is a major target of PI3K and thus, its phosphorylation and activation serves as a good indicator of insulin/PI3K pathway activity. Furthermore, although some studies have proposed that PI3K-independent mechanisms of Akt activation occur under certain conditions, these have
been controversial (Konishi et al., 1996; Moule et al., 1997; Sable et al., 1997; Pullen et al., 1998; Shaw et al., 1998; Yano et al., 1998; Filippa et al., 1999).

Thus, phosphorylation-specific antibodies were raised against dAkt to provide a means of monitoring Drosophila insulin/PI3K pathway activity in vivo and in vitro. A phosphorylation-independent antibody was also generated to measure total dAkt levels. The key phosphorylation sites on dAkt required for maximal kinase activation had not been determined at the time when this research was started. Nevertheless, antibodies were generated against residues in dAkt corresponding to threonine 308 and serine 473 in mammalian Akt1, as these were likely to be functionally conserved and therefore also involved in the activation of dAkt. The corresponding dAkt residues are threonine 423 and serine 586, respectively (see figure 3.1).

3.1.1 Strategy for the generation of phosphorylation-specific and phosphorylation state-independent antisera to dAkt

Rabbits were immunised with various synthetic peptides corresponding to the amino acid sequence of dAkt to generate antibodies against the threonine 423 and serine 586 phosphorylation sites, as well as an antibody that would recognise both phosphorylated and unphosphorylated dAkt. Where possible, sequences were chosen that were most likely to be exposed on the surface of the native dAkt protein. Typically, these are sequences with hydrophilic amino acids. Furthermore, carboxy- and amino-terminal sequences are frequently exposed and often give rise to antisera that are able to recognise the native protein, a key requirement for assays such as immunoprecipitation, as well as many cell staining techniques (Harlow and Lane, 1988). Therefore, to generate an antibody that would recognise total dAkt, a peptide that corresponded to the 10 carboxy-terminal amino acids of dAkt was synthesised: STSTSLASMQ, termed ‘CT’. In addition, two aminoterminal glycine residues were included to provide a spacer for coupling to a carrier protein (see below).

A key concern with the use of phosphorylation specific antibodies is cross-reaction with the unphosphorylated protein. To minimise cross-reactivity with unphosphorylated dAkt and to maximise the specificity of the antibodies, phosphopeptides were used with 4 or 5 amino acids on either side of the phosphorylation site. Thus, to generate antibodies
Figure 3.1 Amino acid sequence comparison of Drosophila Akt with human Akt1

The amino acid sequence of p80- and p66-dAkt aligned with human Akt1. The alignment was generated using the CLUSTALW algorithm (Thompson et al., 1994). Amino acids identical in all three sequences are shaded red. The threonine 308 and serine 473 phosphorylation sites in human Akt1 are marked with an asterisk and the sequences used to generate antigenic peptides are underlined.
against phosphorylated threonine 423 in the activation loop of the kinase domain, the peptide RTTK(pT)FCGTP was used (termed ‘pThr423’). Similarly, for antibodies against phosphorylated serine 586 in the carboxy-terminal hydrophobic motif, the peptide FPQF(pS)YQGD was used (termed ‘pSer586’). Equivalent but unphosphorylated peptides were also synthesised in parallel for later use in affinity purification and peptide competition assays (‘Thr423’ and ‘Ser586’).

Additional amino-terminal cysteines were included in all three peptides to enable coupling to maleimide-activated keyhole limpet haemocyanin (KLH). Each KLH-coupled peptide was used to immunise two rabbits (Eurogentec, Belgium, see section 2.2.1).

3.1.2 Immunoreactivity of antisera raised against dAkt peptides

To assess the antisera raised against the pThr423, pSer586 and CT peptides, their immunoreactivity on western blots of Schneider S2 cell lysates was examined. Strips cut from the same western blot were probed with the pre-immune or immune serum, as described in section 2.3.7. The antisera were tested at different dilutions, with a range of blocking agents and chemiluminescence reagents, in order to determine the optimum conditions for immunoblotting. A commercially available anti-pan mammalian Akt antibody (New England Biolabs) that also recognises dAkt (Javier Verdu, personal communication) was used as a positive control.

Genetic studies suggest that dAkt is a major downstream transducer of the insulin/PI3K signalling pathway in Drosophila, so the immunoreactivity of αpThr423 and αpSer586 on insulin-stimulated S2 cell lysates was investigated. At the time of the experiment, the Drosophila insulin-like peptides (DILPs) had not been identified and, although at least 7 homologs have now been characterised (Brogiolo et al., 2001), none are available purified. Thus, commercially available porcine insulin (SIGMA) was used. Treating S2 cells with 1μM porcine insulin for 30 min has been shown to activate ectopically expressed epitope-tagged (p66-)dAkt in in vitro kinase assays (Javier Verdu, personal communication, Verdu et al., 1999).

Probing western blots with the first and second bleeds revealed that antisera from one of each pair of rabbits immunised with either pThr423 or the pSer586 peptide
(hereafter referred to as αpThr423 and αpSer586, respectively) gave satisfactory immunoreactivity (data not shown). These rabbits were, therefore, boosted to obtain further bleeds. Although antisera from both rabbits immunised with the CT peptide (αCT) gave positive signals, only the rabbit with antisera that gave the strongest immunoreactivity was selected for further boosts and bleeds.

*Drosophila* cells express two isoforms of dAkt from alternative initiation sites within the same mRNA transcript (Andjelkovic *et al.*, 1995; see section 1.2.4 and figures 1.2 and 3.1). Note that the apparent molecular weights of these two isoforms were approximated by Andjelkovic and colleagues to be 66kDa and 85kDa, using western blots of Schneider S2 cell lysates probed with antibodies raised against full-length recombinant dAkt or the amino-terminal extension of the larger dAkt isoform. However, careful determination of the molecular weights by calibration graphs of S2 lysate western blots using the same dAkt antibodies as well as the antisera generated in this study revealed that the larger isoform runs with an apparent molecular weight of 80kDa rather than 85kDa (data not shown). Thus, the isoforms will henceforth be referred to as p66- and p80-dAkt.

Both p66- and p80-dAkt could be detected in the insulin-stimulated and unstimulated cell lysates with αCT antisera (figure 3.2A). In contrast, the αpThr423 and αpSer586 antisera revealed bands corresponding to both dAkt isoforms primarily in the lanes loaded with insulin-stimulated cell lysate, with little immunoreactivity detected in lysate from untreated cells (figures 3.2B and C). This observation is consistent with these antibodies recognising only phosphorylated dAkt in insulin-stimulated S2 cells.

Several cross-reacting bands were also observed. However, with the exception of the strong band revealed by αpThr423 (* in figure 3.2A, lanes 3 and 4), these disappeared or were significantly attenuated in later bleeds and following affinity purification (see section 3.1.4).
Figure 3.2 Immunoreactivity of antisera raised against dAkt peptides

Western blots of serum-starved control or insulin-stimulated Schneider S2 cell lysates (containing approximately 50μg protein per lane) were cut into three strips. Two of each set of strips were individually probed with one of the three dAkt crude antisera (2nd bleeds) or the cognate preimmune sera. The third strip from each blot was also probed with a commercial affinity purified anti-pan mammalian Akt antibody (αPKB). All antibodies were used at a 1/1000 dilution. Order of dAkt immunoblots from top to bottom are αCT (A), αpThr423 (B) and αpSer586 (C). Both the dAkt antisera and mammalian Akt antibody, but not the preimmune sera, detected a 66kDa and 80kDa band corresponding to dAkt. Note the major cross-reacting band (*) in the αpThr423 blot.
A

Preimmune \(\alpha\)CT \(\alpha\)PKB

- + - + - +

160
105
75
50

p80 dAkt
p66 dAkt

Insulin

B

Preimmune \(\alpha\)pThr423 \(\alpha\)PKB

- + - + - +

160
105
75
50

p80 dAkt
p66 dAkt

Insulin

C

Preimmune \(\alpha\)pSer586 \(\alpha\)PKB

- + - + - +

160
105
75
50

p80 dAkt
p66 dAkt

Insulin
3.1.3 Confirmation that antisera recognise dAkt in vitro in a phosphorylation state-dependent or -independent manner

3.1.3.1 Immunoprecipitation of p66- and p80-dAkt

To confirm that the antisera were recognizing dAkt and to determine whether they could recognize the protein in its native confirmation, the ability of each antibody to immunoprecipitate dAkt was examined. Insulin-stimulated and unstimulated S2 cell lysates were incubated with an aliquot of each antiserum and the immune complexes captured with protein A-sepharose beads. These were then resolved by SDS gel electrophoresis, transferred onto a western blot and probed with the commercially available anti-pan mammalian Akt antibody. Figure 3.3 shows that αCT could immunoprecipitate both the p66 and p80 forms of dAkt, whereas αpThr423 and αpSer586 could not.

On the simplest level, the absence of a signal in immunoprecipitates of αpThr423 and αpSer586 indicated that the antibodies are intrinsically unable to detect phosphorylated dAkt. However, given the results of the previous section, it was more likely that they do not recognize native phosphorylated dAkt, or that the avidity of the antibody-antigen complex was relatively weak. Alternatively, it was possible that phosphorylated dAkt was dephosphorylated during the immunoprecipitation procedure. To establish which of these possibilities was true, dAkt was immunoprecipitated from insulin-stimulated and unstimulated S2 cell lysates with αCT. Following immunoprecipitation, the supernatant was retained, and the protein A sepharose-antisera complexes were washed, boiled in SDS sample buffer and serially diluted. These samples were then divided between three SDS PAGE gels, together with the supernatant, and immunoblotted with αpThr423 and αpSer586.

On the western blot probed with αCT, a similar level of immunoreactivity was observed between lanes loaded with insulin-stimulated and unstimulated cell lysate immunoprecipitates, confirming that total dAkt levels were similar (figure 3.4A). Furthermore, dAkt could be detected in the immunoprecipitate supernatants indicating that the immunoprecipitation was partially efficient. Bands corresponding to phosphorylated p66- and p80-dAkt were also detected with αpThr423 and αpSer586 in the
Figure 3.3 Immunoprecipitation of p66- and p80-dAkt

500μl aliquots of serum-starved control or insulin-stimulated Schneider S2 cell lysates (1mg/ml) were individually incubated with 3μl of each dAkt crude antiserum (2nd bleed) or a commercial affinity purified anti-pan mammalian Akt antibody (αPKB). Both the total cell lysates (50μg per lane) and the captured immune complexes were separated by SDS PAGE (7.5%) and immuno blotted with αPKB (1/1000 or 1μg/ml dilution). Both αCT and αPKB, but not αpSer586 nor αThr423, could immunoprecipitate the 66kDa and 80kDa isoforms of dAkt from control or insulin-stimulated cell lysates.
Figure 3.4 Immunoprecipitation of phosphorylated dAkt

αCT was used to immunoprecipitate dAkt from serum-starved control or insulin-stimulated Schneider S2 cell lysates (1mg/ml). The αCT immunoprecipitates were serially diluted and, together with the respective IP supernatants, were resolved on three SDS-PAGE gels (7.5%). The blots were then probed with αCT (A), αpThr423 (B) or αpSer586 (C) at a 1/1000 dilution (2nd bleeds, crude sera). The IP volume refers to the original volume of lysate / supernatant from which the IP was obtained. Note that a greater amount of immunoprecipitate was loaded for αTh423 since it shows weaker immunoreactivity to dAkt in comparison to αCT and αpSer586. Total dAkt, as determined by the αCT immunoblot, was similar in αCT immunoprecipitates and supernatant from both control and insulin-stimulated lanes. Phosphorylated dAkt was detected predominantly in the insulin-stimulated lanes by both αpThr423 and αpSer586.
Supernatant Immunoprecipitate

<table>
<thead>
<tr>
<th></th>
<th>Supernatant</th>
<th>Immunoprecipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50µl</td>
<td>100µl</td>
</tr>
</tbody>
</table>

**A**

IP: αCT

WB: αCT

- + - + +

Insulin

**B**

IP: αCT

WB: αpThr423

- + - + +

Insulin

**C**

IP: αCT

WB: αpSer586

- + - + +

Insulin
immunoprecipitation supernatant from the insulin-stimulated cell lysate, as shown in figures 3.4B and C. This argues against substantial dephosphorylation of dAkt occurring during the immunoprecipitation procedure. Furthermore, both phosphorylation-specific antibodies detected p66- and p80-dAkt immunoprecipitated by αCT, with a three- to four-fold increase in immunoreactivity in immunoprecipitates from insulin-stimulated vs. unstimulated cells. These observations confirm that both αpThr308 and αpSer586 are more able to recognise dAkt when it is immunoprecipitated from insulin-stimulated than unstimulated cells, consistent with them recognising phosphorylated dAkt. Furthermore, the ability of the independently derived phosphorylation specific antisera, which were raised against different regions of dAkt, to detect the two proteins immunoprecipitated by αCT provides further evidence for the ability of all three antisera to recognise p66- and p80-dAkt.

During the course of this research, two additional dAkt antisera were obtained, which were generated by Mirjana Andjelkovic whilst in the lab of Brian Hemmings. These antisera were raised against recombinant dAkt protein or the amino-terminal extension of p80-dAkt (referred to as αdAkt and αNT, respectively). Probing western blots of immunoprecipitates of these antibodies with αpSer586 provided further confirmation that αpSer586 recognised phosphorylated dAkt (see section 5.3.3).

In summary, although αCT, αpThr423 and αpSer586 are each able to recognise denatured dAkt, only αCT appears to be able to immunoprecipitate native dAkt protein.

### 3.1.3.2 Peptide competition removes dAkt signal

Although the ability of αpThr423 and αpSer586 to recognise dAkt was substantially increased when dAkt was immunoprecipitated from insulin-stimulated cells, the immunoreactivity of these antibodies towards dAkt immunoprecipitated from unstimulated cells was still significant, (lanes 3 and 5 in figures 3.4B and C). Thus, although both αpThr423 and αpSer586 appeared to have a preference for insulin-stimulated and therefore phosphorylated dAkt, it is possible that the residual signal was due to some crossreactivity with unphosphorylated dAkt. Alternatively, the signal in the unstimulated cells could simply reflect basal levels of phosphorylated dAkt. To establish
which of these two possibilities was true, a ‘peptide competition assay’ for αpSer586, as well as αCT, was performed on western blots of insulin-stimulated and unstimulated S2 cell lysates, as described in section 2.3.8. Briefly, a sub-saturating concentration of each antibody was preincubated for 2 hours with three different concentrations of the peptide against which each antibody was raised. Final antibody: peptide molar ratios of 1: 2, 1: 20 and 1: 200 were used.

Preincubation of αpSer586 with as little as a 2-fold molar excess of synthetic pSer586 phosphorylated peptide resulted in inhibition of the dAkt signal (figure 3.5A lanes 1 and 2). In contrast, even a 200-fold molar excess of the identical but unphosphorylated Ser586 peptide had little effect on the signal intensity (figure 3.5A lanes 11 and 12). Taken together, these results confirm that the αpSer586 antiserum specifically recognises phosphorylated and not unphosphorylated dAkt. Similarly, incubation of αCT with a 2-fold molar excess of CT peptide abolished detection of the dAkt bands, confirming the specificity of this antiserum for dAkt (figure 3.5B).

As will be discussed later, further confirmation of the specificity of αCT for dAkt was obtained by probing immunoblots of lysates from cells in which dAkt expression was suppressed (section 5.3.5). In addition, the specificity of αpSer586 for phosphorylated dAkt was confirmed by pre-treatment of immunoblots with alkaline phosphatase prior to probing with αpSer586 (section 5.3.6).

3.1.4 Affinity purification of dAkt antisera

A major aim of this research was to use the dAkt antibodies for in situ immunofluorescence in order to investigate dAkt activation in vivo. A key requirement for the confident use of antibodies in cell and tissue staining techniques is the ability to recognise the target with minimal crossreactivity. In addition to the specific antibodies, polyclonal sera also contain relatively high concentrations of antibodies of unknown specificity which often give rise to unacceptable levels of background that may severely complicate the interpretation of any given staining pattern. Part of this problem will arise from antibodies binding non-specifically to the fixed cell or tissue, and part will be from specific interactions arising from spurious activities in the serum. A method used to
Figure 3.5 Peptide competition of dAkt signal

1μg/ml of αpSer586 and αCT (affinity purified 2nd bleeds) were preincubated for 2 hours with their respective phosphorylated or non-phosphorylated synthetic peptides at antibody : peptide molar ratios of 1:2, 1:20 and 1:200. Western blots strips of control or insulin-stimulated Schneider S2 cell lysates (50μg per lane) were then individually probed with control or peptide-incubated αpSer586 (A) and αCT (B). Preincubation of αpSer586 with pSer586 phosphopeptide but not unphosphorylated Ser586 peptide abolished the ability of the antibody to detect phosphorylated dAkt. Similarly, preincubation of αCT with the CT peptide abolished the detection of total dAkt. Note that although cross-reacting bands (*) were detected with the 2nd bleeds of both αpSer586 and αCT, these were absent in the affinity purified final bleeds used in the immunostains described below and in subsequent experiments.
circumvent these problems is to remove the non-specific antibodies by immunoaffinity purification of the antigen-specific antibodies (however, this does guarantee against the possibility of specific cross reaction with a similar epitope on another protein).

Both αpSer586 and αCT were, therefore, purified by affinity chromatography as described in section 2.2.2. Briefly, the crude sera were passed through a column in which their respective target peptides had been coupled to a crosslinked agarose support via an immobilised iodoacetyl group that reacts with the terminal cysteine on each peptide (Sulfolink, PIERCE). Unbound material was washed away and the bound antibody eluted under mildly denaturing conditions. Both αpSer586 and αpThr423 were also passed through non-phosphorylated peptide-coupled columns (see section 2.2.2) and the purified flow-through was retained. Each eluate was initially screened by peptide ELISA (Enzyme-Linked Immunosorbent Assay). The ability to detect serially diluted, immobilised pSer586 or αCT peptide was tested (data not shown). Furthermore, affinity purified αpSer586 was also tested with the unphosphorylated Ser586 peptide, to ensure that cross-reaction did not occur.

A significant reduction in cross-reacting bands and background was observed on western blots of insulin-stimulated and unstimulated S2 cell lysates following affinity purification of both αpSer586 and αCT, as shown in figure 3.6. Although several attempts were made to affinity purify αpThr423, these were unsuccessful as the major cross-reacting band observed with the crude sera (see figure 3.2) persisted following the purification process. Consequently, the use of this antibody was mainly limited to probing αCT immunoprecipitates.

3.1.5 Correlation between the phosphorylation state and kinase activity of dAkt

To investigate whether phosphorylation of dAkt at serine 586 correlates with kinase activation, in vitro kinase assays were performed on αCT immunoprecipitates from insulin-stimulated and unstimulated S2 cell lysates. Kinase assays were also performed on any proteins that were potentially bound non-specifically to protein A-sepharose incubated in the cell lysates in the absence of antibody. Kinase activity was measured by the capacity of
Figure 3.6 Affinity Purification of the dAkt antisera

The 2nd bleed of αCT and the 5th bleed of αpSer586 crude antisera were purified by affinity chromatography using peptide cross-linked columns. A significant reduction in background signal was observed in immunoblot strips of control and insulin-stimulated Schneider S2 cell lysates (50μg per lane) probed with the affinity purified antibodies (1μg/ml) in comparison to strips probed with the crude antisera (1/1000 dilution). Subsequent bleeds with greater signal to noise ratios were also purified (5th bleed αCT, 6th bleed αpSer586).
the immunoprecipitates to phosphorylate a peptide substrate corresponding to the sequence in mammalian GSK-3β surrounding serine 9 (GRPRTSSFAEG) that is phosphorylated by Akt, as described in section 2.3.4 (Cross et al., 1995). Briefly, αCT immunoprecipitates were incubated at 30°C with GSK-3 peptide in the presence of \([γ^{32}P]ATP\) and 50μM cold ATP in kinase reaction buffer. The phosphorylated peptides were separated from unincorporated \([γ^{32}P]ATP\) by spotting onto P81 phosphocellulose paper and washing extensively in 0.75%(v/v) phosphoric acid. The amount of \([γ^{32}P]\) incorporation was quantified using a liquid scintillation counter. The αCT immunoprecipitates were resolved by SDS PAGE and immunoblotted with αpSer586 and αCT.

Insulin stimulation induced a 17-fold increase in dAkt kinase activity, which was accompanied by phosphorylation at serine 586 (figure 3.7A). While phosphorylation of mammalian Akt1 at serine 473 is clearly a prerequisite for maximal kinase activation, these experiments do not address whether phosphorylation of dAkt at the corresponding serine, serine 586 is similarly necessary. Additionally, the phosphorylation of threonine 423 and its role in dAkt kinase activation had not been tested. Nevertheless, given the sequence similarity and evolutionary conservation of function of this protein, it is highly likely that phosphorylation of both threonine 423 and serine 586 is required for maximal dAkt activation.

As discussed in section 1.2.4, the existence of the 81 residue amino-terminal extension on p80-dAkt raises the possibility that this isoform may be functionally different with respect to the smaller p66-dAkt isoform. It is also possible that the proximity of the extension may sterically hinder or modify the conformation of the adjacent pleckstrin homology domain, which has been shown to be important for the translocation of mammalian Akt to the plasma membrane and, hence, its activation (see sections 1.2.3.1 and 1.2.3.5). However, p80-dAkt is clearly phosphorylated at both threonine 423 and serine 586 following insulin stimulation. Furthermore, \textit{in vitro} kinase assays of αNT immunoprecipitates indicated that p80-dAkt is activated 20-fold following insulin stimulation (figure 3.7B). These data, therefore, suggest that translocation and activation of p80-dAkt is not significantly perturbed compared to that of p66-dAkt.

In summary, phosphorylation of dAkt at serine 586 and threonine 423 following insulin stimulation is associated with a significant increase in kinase activity.
Figure 3.7 Correlation between phosphorylation state of dAkt and kinase activity

Kinase assays of αCT and αNT immunoprecipitates from serum-starved control and insulin-stimulated Schneider S2 cells were performed using a synthetic peptide substrate. A fraction of the immunoprecipitates and total cell lysates were also resolved in parallel by SDS-PAGE (7.5%) and immunoblotted with αpSer586 (affinity purified 6th bleed) or αCT (affinity purified 5th bleed) at a concentration of 1μg/ml. Insulin stimulation induced a 17-fold increase in dAkt activity (upper graph) which was accompanied by phosphorylation at serine 586 (A). αNT immunoprecipitates from insulin-stimulated lysates also showed a marked 20-fold increase in activity (lower graph) that was accompanied by the detection of phosphorylated p80-dAkt in the immunoprecipitate (B). Total dAkt in αCT and αNT immunoprecipitates from both control and insulin-stimulated cell lysates were similar (C).
A

 WB: αpSer586

- + - -

p80 dAkt
p66 dAkt
Insulin

K+rase Activity (cpm)

B

 WB: αpSer586

- + - -

p80 dAkt
p66 dAkt
Insulin

K+rase Activity (cpm)

C

 WB: αCT

- + - - -

p80 dAkt
p66 dAkt
Insulin

101
3.2 Cell biological characterisation of the phosphorylation-specific antibodies to Drosophila Akt

As discussed in section 3.1.4, antibodies intended for use in cell and tissue staining techniques should recognise the target antigen with minimal crossreactivity. However, a second more intrinsic requirement is the ability to recognise the target antigen when it is in an approximately native confirmation, as is likely to be found in fixed tissues. Whilst the αpSer586 antibodies were unable to immunoprecipitate dAkt, this did not necessarily preclude their use in immunostaining. For example, antibodies of relatively low affinity often can work well in staining reactions, possibly reflecting bivalent binding to immobilised antigens present in high local concentrations (Mansen 1992). Furthermore, depending on the fixation method employed, different degrees of protein denaturation occur, which may lead to the unmasking of a partially sterically hindered epitope.

To characterise the ability of the antibodies to recognise phosphorylated or total dAkt during imaginal disc development by in situ immunofluorescence, an assay based on the generation of cell clones by the yeast flp/FRT system was employed. Flipase (FLP) recombinase catalyses the site-specific recombination between FLP recombination target (FRT) sites (Xu and Rubin, 1993). This powerful technique can be used in several ways to study the function of any given protein in vivo, as discussed below.

3.2.1 dAkt phosphorylation signal is reduced in dAkt null mitotic clones

One way in which the flp/FRT system can be used is in the induction of mitotic recombination and the generation of clones of cells that are homozygous for a mutation of interest in a heterozygous but essentially wild type background (Golic, 1991; Xu and Rubin, 1993). To achieve this, a chromosome is generated in which an FRT repeat element is placed between the mutation of interest and the base of the chromosome arm near the centromere (figure 3.8). The sister chromosome, which has a wild type copy of the gene of interest, contains an FRT repeat at the same location as the first FRT, as well as a marker gene. Induction of expression of the FLP recombinase, for example by heat shock if under a heat shock promoter, results in recombination in a single cell creating clones of cells that
Figure 3.8 The flp/FRT system

A diagram illustrating how clones of mutant cells are generated with the flp/FRT system. The FLP recombinase induces a crossover of the chromosome arms at the site of an FRT repeat in a random somatic cell. This event gives rise to a clone of homozygous mutant cells and a clone of cells that are wild type at that locus. The cells from this event can be distinguished by a loss or gain of a marker.
carry the mutation. These ‘mutant clones’ can be identified by the absence of the marker gene. A sister clone or ‘twin spot’, that is created from the same recombination event and which contains two wild type copies of the gene of interest, can be identified by the presence of two copies of the marker gene.

To establish whether the αpSer586 and αCT antibodies recognised dAkt in situ in imaginal discs, clones of cells lacking dAkt were generated to determine whether the immunofluorescence signal was reduced in these clones. As the l(3) 89Bq (1998) identified by Staveley et al. was not a protein-null allele of dakt (see section 1.4.6.2), it was of limited use in this case. Additionally, the Df(3R)sbd deficiency, which deletes dakt and other genes, is cell-lethal and so clones could not be generated.

Additional dakt alleles were provided by Hugo Stocker (Ernst Hafen Lab, Zurich University), which were obtained in a genetic screen for randomly induced mutations that inhibit growth. As part of this screen, six strong loss-of-function dakt alleles were identified and, although these were not molecularly characterised, it is likely that at least some are protein-null alleles. Mitotic clones of cells homozygous for these alleles were generated in the wing imaginal disc. However, since abrogation of dAkt activity inhibits growth, it is difficult to generate clones of significant size. Thus, the clones were conferred with a growth advantage by induction in a background of Minute heterozygous cells. Minutes are a class of mutations in Drosophila genes that dominantly slow growth and cell division during imaginal disc development and are cell lethal when homozygous (see section 1.3.3.2). Several Minute genes have been cloned and found to encode components of the ribosomal machinery, and therefore the Minute phenotypes are proposed to result from impaired protein synthesis (Lambertsson, 1998). Consequently, clones of cells that do not carry a Minute mutation have a growth advantage when surrounded by Minute heterozygous cells, enabling the generation of larger clones than would otherwise be possible.

To identify the dakt mutant clones, non-clonal cells were marked by the ectopic expression of β-galactosidase using the lacZ gene under the armadillo promoter (arm-lacZ), which is expressed ubiquitously. Since the optimum conditions for generating dakt mutant clones of sufficient size had not yet been determined, wing imaginal discs from larvae that had been heat shocked using a variety of conditions were pooled prior to
immunostaining. The different conditions included heat shocking at different times after egg laying (between 24 hours to 72 hours) and at different temperatures (30°C to 37°C). The discs were immunostained with either αpSer586 or αCT, in parallel with a mouse monoclonal anti-β-galactosidase antibody. In contrast to the small dakt mutant clones generated in a wild type background (data not shown), relatively large clones were occasionally observed when induced in a Minute background as shown by the anti-β-galactosidase staining in figure 3.9A. The immunofluorescence signal with either αpSer586 or αCT was significantly reduced in the mutant clones indicating that the signal generated by the antibodies is dependent on dAkt levels. The residual staining within the clones may be due to perdurance of dAkt protein or dakt mRNA from the heterozygous precursor cells that gave rise to these clones, or may be due to a background level of cross-reactivity.

3.2.2 Dp110 is required for dAkt phosphorylation at Serine 586 in vivo

Although class IA PI3Ks are clearly major regulators of mammalian Akt activity, a few studies have suggested PI3K-independent mechanisms of Akt activation, as mentioned in section 3.1. To establish whether Dp110 is necessary for dAkt phosphorylation, mitotic clones of dp110 null cells were generated using the flp/FRT system as described above, and stained with αpSer586 and αCT. Figure 3.9B shows that whereas total dAkt levels remain the same in the absence of Dp110, dAkt phosphorylation at Ser586 is substantially reduced in dp110 null clones. Thus, dAkt phosphorylation, and implicitly activation, appears to be dependent on Dp110 activity in vivo.

3.2.3 Ectopic expression of Dp110 induces dAkt phosphorylation in vivo

To further characterise the dAkt antibodies by immunofluorescence and to investigate the ability of Dp110 to induce dAkt phosphorylation in vivo, wing imaginal discs ectopically expressing Dp110 or dAkt in clonal cells were stained with either αpSer586 or αCT. This was achieved by using the ‘flp-out’ technique (Struhl and Basler, 1993), which allows the generation of permanent, heritable ectopic expression of transgenes in random clones of cells induced at precise time-points during development (figure 3.10). A typical ‘flp-out’ construct consists of a constitutive promoter, followed by
Figure 3.9 dAkt phosphorylation is reduced in clones of dAkt null and Dp110 null cells

The *flp/FRT* recombination system was used to generate mitotic clones of dAkt null and Dp110 null cells in a *Minute (3)95A* heterozygous background 60h±12h AEL. Wing imaginal discs were then dissected from wandering late third instar larvae and immunostained with anti-β galactosidase (1/200, mouse) to identify the clones, which lack staining (red). The discs were co-stained with either αCT (affinity purified 5th bleed) or αpSer586 (affinity purified 6th bleed) at a concentration of 2μg/ml (green). Note that twin spots were not observed since *Minute* homozygotes are cell lethal. Both the αCT and αpSer586 immunostain signal was reduced in dAkt null clones (A). In contrast, the αCT signal was unaltered in Dp110 null clones whereas the αpSer586 signal was reduced (B).
Figure 3.10 The ‘flip-out’ system

(A) The expression of GAL4 by the Actin promoter is disrupted by the presence of a stuffer cassette. (B) Heat shock induction of the FLP recombinase gene causes site-directed recombination between the tandem FLP recombination target (FRT) sites in some cells leading to the removal of the stuffer cassette. (C) GAL4 is now heritably and constitutively expressed in those cells that have removed the stuffer cassette. GAL4, in turn, induces the expression of several transgenes whose expression is under the control of the GAL4-inducible promoter, UAS. For example, in the wing imaginal disc, UAS-green fluorescent protein (GFP) can be used to mark cells fluorescently that also ectopically express a gene of interest (based on Potter and Xu, 2000).
an FRT site, a transcriptional terminator or repressor, a second FRT site and the cDNA of a gene of interest as well as a marker gene. Induction of expression of FLP recombinase by a heat-shock inducible promoter results in the excision of the DNA between the FRT sites, thereby permitting the ectopic expression of the gene of interest in random clones.

The 'flp-out' system is often used in combination with the yeast UAS/GAL4 system which renders ectopic expression studies significantly more versatile. This latter system involves the construction and integration of a transgene in which the DNA encoding the protein of interest is placed under the control of a promoter containing GAL4-responsive upstream activating sequences (UAS). A fly line carrying the UAS-cDNA construct can then be crossed with any number of existing fly lines that express GAL4 under the control of specific constitutive or inducible promoter, allowing the recombinant protein to be expressed in a temporal- or spatial-specific pattern. Thus, if a 'flp-out' construct of the type described above drives GAL4 expression, this can, in turn, allow the inducible expression in random clones of any number of UAS-cDNA constructs also present in the fly. The 'flp-out' and UAS/GAL4 systems were therefore used to induce clones of cells ectopically expressing either Dp110 or dAkt in wing imaginal discs. This was achieved by removing a CD2 'stuffer cassette' flanked by two FRT sites, inserted between the Actin 5C promoter and the GAL4 coding sequence, using heat shock-inducible FLP. Flies carrying this construct also had a UAS-nuclear green fluorescent protein (nlsGFP) transgene recombined onto the same chromosome. Consequently, clones could be identified by the expression of GFP.

Clones of cells ectopically expressing GFP alone, GFP and dAkt, or GFP and Dp110 were induced 72 hours after egg laying (AEL) following a 30 min heat shock at 37°C. An increase in signal intensity was observed in clones ectopically expressing dAkt when immunostained with either αCT or αpSer586 (figures 3.11A and B, respectively), in comparison to surrounding non-clonal cells, or to control clones expressing GFP alone. Intriguingly, therefore, ectopic expression of dAkt itself appears to result in increased levels of phosphorylation at serine 586. However, this is consistent with the observation that ectopic expression of wild type dAkt in imaginal discs results in increased tissue growth (Verdu et al., 1999). In contrast to the dAkt clones, ectopic expression of Dp110
Figure 3.11 A Ectopic expression of dAkt in cell clones induces increased αCT signal

The ‘flip-out’ system was used to induce clones 72hrs AEL ectopically expressing GFP alone or GFP and dAkt. At 120hrs AEL, wings discs were dissected from wandering late third instar larvae and immunostained with αCT (affinity purified 5th bleed) at a concentration of 5μg/ml. Increased signal was observed with αCT in clones ectopically expressing dAkt but not in control clones.
Figure 3.11B Ectopic expression of dAkt in cell clones induces increased αpSer586 signal

Clones ectopically expressing GFP or GFP and dAkt were induced 72hrs AEL. At 120hrs AEL, wings discs were dissected from wandering late third instar larvae and immunostained with αpSer586 (affinity purified 6th bleed) at a concentration of 5μg/ml. Increased signal was observed with αpSer586 in clones ectopically expressing dAkt but not in control clones.
was associated with an increase in signal within clones in discs immunostained with αpSer586 but not αCT (figure 3.12).

In summary, the data from this section and from the previous section demonstrate, first, that the immunostain signal of both αCT and αpSer586 is dependent on the expression of dAkt, and second, that Dpl110 is both necessary and sufficient to induce phosphorylation of dAkt at serine 586 in vivo.

3.2.4 Identification of a pyknotic signal with αpSer586

Intriguingly, during the course of the experiments described above, it was noticed that αpSer586 immunostains of both wing and eye imaginal discs revealed a random pattern of intensely staining cells (indicated by white arrowheads in figure 3.13). These dense or 'pyknotic' (Greek, pyknos, thick, + -osis, condition) cells were not apparent in discs immunostained with αCT, and appeared to be localised towards the basal membrane of the disc towards which apoptosing cells also migrate. Indeed the pattern of staining in both third instar wing and eye discs strongly suggested an association with apoptosis.

As discussed in sections 1.4 and 1.4.6.1, the correct patterning of the Drosophila wing and eye is achieved in part through the temporal and spatial control of programmed cell death, during which 'excess' cells are removed. The distribution of apoptotic cells in the early stages of wing development appears random, but during the late third instar, clusters of apoptotic cells are preferentially observed in the notum and notum/wing border region along the path of the adjacent tracheal branch. The distribution of the pyknotic cells observed with αpSer586 was very similar to the pattern of cell death in the wing discs, as shown in figure 3.13.

Similar findings were made in third instar eye discs in which cell death is observed in two main regions (Wolff and Ready, 1991). Starting approximately 12 rows posterior to the morphogenetic furrow, apoptotic cells can be seen in an apparently random pattern. A tight band of dying cells is also detected directly ahead of the furrow. Once again, the pyknotic cell staining observed in antennal-eye discs immunostained with αpSer586 displayed a remarkably similar pattern to that of cell death. Detailed characterisation and discussion of this observation will be given in Chapters 4, 5 and 6.
Figure 3.12 Ectopic expression of Dp110 in cell clones induces phosphorylation of dAkt \textit{in vivo}

Clones ectopically expressing GFP with Dp110 were induced 72hrs AEL. At 120hrs AEL, wings discs were dissected from wandering late third instar larvae and immunostained with either $\alpha$CT (affinity purified 5th bleed) or $\alpha$pSer586 (affinity purified 6th bleed) at a concentration of 5µg/ml. Note that these bleeds and concentrations were also used for all subsequent immunostains. Increased signal was observed with $\alpha$pSer586 but not $\alpha$CT in clones ectopically expressing Dp110.
Figure 3.13 Pyknotic signal

Densely staining cells (examples marked by white arrowheads) were observed in immunostains with αpSer586 but not αCT near the basal membrane of both wing discs (upper panel) and antennal-eye discs (middle panel). The distribution of the pyknotic cells was very similar to the characteristic pattern described for apoptosis (see text).
3.2.5 Ectopic expression of Dp110 in the pouch region of the wing imaginal disc induces increased dAkt phosphorylation

During larval stages, the wing imaginal disc is a monolayer epithelium divided by anterior/posterior (A/P) and dorsal/ventral (D/V) compartment boundaries. The MS1096 GAL4 driver allows the expression of UAS-transgenes under the control of GAL4 in the dorsal wing pouch at high levels, and in the ventral wing pouch at lower levels (Capdevila and Guerrero, 1994). As discussed in section 1.3.5, ectopic expression of wild type or membrane-targeted Dp110 under the control of the MS1096 GAL4 driver (MS1096>dpl10 and MS1096> dpl10-CAAX, respectively) results in an enlarged wing blade that has bigger cells. To determine whether the ectopic expression of wild type Dp110, in this situation, results in increased phosphorylation of dAkt, αpSer586 and αCT immunostains were performed on third instar MS1096>dpl10 wing discs. A mouse monoclonal anti-Myc epitope antibody, 9E10 (Evan et al., 1985), was also used to detect the expression of the Myc epitope-tagged Dp110 transgenes. αpSer586 immunostains of MS1096>dpl10 discs showed a relative increase in signal in the region of Dp110 ectopic expression, as shown in figure 3.14A. In contrast, no change in signal intensity was observed with αCT, strongly suggesting that there is increased phosphorylation of dAkt at serine 586 in association with the elevation in Dp110 signalling (figure 3.14B).

Studies in mammalian cell culture have suggested that phosphorylated dAkt is mainly localised at the plasma membrane. However, rather than observing endogenous Akt protein, these studies are largely based on the analysis of ectopically expressed epitope tagged Akt constructs in which the localisation pattern may be artificial rather than physiological. Additional studies have also controversially suggested that Akt translocates to the nucleus following phosphorylation, consistent with the notion that a number of Akt substrates are nuclear localised. Higher magnification of the MS1096>dpl10 wings discs immunostained with αpSer586 revealed that dAkt phosphorylated on serine 586 was predominantly membrane localised (figures 3.15A and 3.16). In contrast, both total dAkt and ectopically expressed Dp110, as indicated by staining with αCT and 9E10 respectively showed a uniform distribution between the membrane and cytoplasm (figures 3.15B and
Figure 3.14 Ectopic expression of Dp110 in the wing disc pouch induces dAkt phosphorylation

The MS1096 GAL4 driver was used to ectopically express myc-epitope tagged Dp110 in the dorsal and ventral compartments of the wing disc pouch region. Discs were subsequently dissected from late third instar larvae and stained with the 9E10 anti-Myc antibody to reveal the region of Dp110 ectopic expression (red). Costaining with αpSer586 (A) revealed a significant increase in endogenous dAkt phosphorylation in the region of Dp110 ectopic expression whereas no change in signal was observed in αCT immunostains (B).
Figure 3.15 Membrane localisation of phosphorylated dAkt

Higher magnification of the MS1096>dp110 wings discs immunostained with αpSer586 revealed that phosphorylated dAkt was predominantly membrane-localised (A). In contrast, both total dAkt and ectopically expressed Dp110, as indicated by staining with αCT and 9E10 respectively, showed uniform distribution between the membrane and cytoplasm (B). All three signals however were excluded from the nucleus (see text).
Figure 3.16 Pyknotic staining cells in MS1096>dp110 wing discs

Higher magnification of the MS1096>dp110 wing discs showed pyknotic staining cells in αpSer586 immunostains (example marked by white arrowhead), which were not observable in 9E10 immunostains (same region indicated by yellow arrowhead).
3.16). However, all three signals were excluded from the nucleus, as confirmed by co-staining with a nuclear dye (data not shown).

As described above, pyknotic staining cells were also observed in αpSer586 immunostains of the MS1096>dpl10 wing discs which were not observable in the 9E10 immunostain (figure 3.16).

3.3 Summary

The experiments in this chapter describe the generation and detailed biochemical and cell biological characterisation of phosphorylation-specific and phosphorylation state-independent dAkt antibodies. These reagents were used to analyse and confirm the phosphorylation and activation of dAkt by insulin in vitro in an embryonically derived Drosophila cell line. In addition, the phosphorylation of dAkt in vivo was examined in cells that lacked or ectopically expressed the Drosophila class IA PI3K homolog, Dp110, during imaginal disc development. Taken together, these data indicate that Dp110 is necessary and sufficient for dAkt activation in vivo.

Intriguingly, immunostaining with αpSer586 also revealed 'pyknotic-staining' cells with significantly elevated signal. These cells were localised near the basal membrane of the disc and showed a characteristically apoptosis-like pattern of tissue distribution. Notably, the pyknotic signal could be competed by pre-incubating αpSer586 with phosphorylated pSer586 peptide but not following pre-incubation with unphosphorylated Ser586 peptide (data not shown).

In the following chapter, the dAkt phosphorylation-specific antibodies are used to investigate the relationship between the insulin/PI3K pathway and other growth-signalling promoters, namely Drosophila Ras1 and Myc. Subsequently, further immunofluorescence-based characterisation of the αpSer586 pyknotic signal is performed.
Chapter 4: Investigation of Growth-Regulation Signalling and Characterisation of Pyknotic Signal

4.1 Investigating the relationship between PI3K, dRas1 and dMyc signalling in vivo ______ 121

4.1.1 Investigating the role of dRas1 in PI3K signalling in vivo ____________________________ 122

4.1.1.1 Ectopic expression of dRas1 induces dAkt phosphorylation in vivo _________________ 124

4.1.1.2 Phosphorylation of dAkt is not reduced in dRas1 null clones _______________________ 126

4.1.2 Ectopic expression of dMyc does not result in increased dAkt phosphorylation in vivo _____ 128

4.2 Characterisation of apSer586 pyknotic signal ___________________________ 130

4.2.1 Pyknotic signal is associated with the induction of apoptosis __________________________ 130

4.2.2 Increased frequency of apSer586 pyknotic signal in irradiated discs _________________ 133

4.3 Summary _________________________________________________________________ 134
4.1 Investigating the relationship between PI3K, dRas1 and dMyc signalling in vivo

dRas1, the *Drosophila* homolog of mammalian N-Ras, K-Ras and H-Ras, has recently been implicated in the control of tissue growth (see section 1.3.9). Ras is believed to function through GTP-dependent interactions with several cellular targets commonly referred to as ‘effectors’, such as the protein kinase Raf and the exchange factorRalGDS. The function of dRaf in dRas1 signalling has been studied extensively in *Drosophila*, particularly in regard to the role of the dRas1/MARK signalling cassette in ommatidial differentiation and cellular survival during eye development (see section 1.4.6.1). Interestingly, however, it was suggested that the effects of dRas1 on growth are at least partially mediated by dMyc (Prober and Edgar, 2000). Specifically, immunostains of wing disc clones ectopically expressing constitutively active dRas1, as well as immunoblots of lysates from these discs, show slightly increased levels of staining with a dMyc-specific antibody. Moreover, FACS analysis from *flp*-out clones coexpressing dMyc and a dominant negative dRas1 mutant (dRas1<sup>ΔN</sup>) revealed cell size and cell cycle profiles similar to those of cells expressing dMyc alone. This suggested that dMyc can rescue the growth defects resulting from inhibition of dRas1. It is not clear, though, how dRas1 increases dMyc levels.

An increasing body of work, based largely on mammalian tissue culture studies, has indicated that the PI3K pathway is also a major effector branch of Ras signalling (reviewed in Katso *et al.*, 2001). All class I PI3K members, including Dp110, have a region that is similar to the Ras binding domain (RBD) found in Raf and Ral-GDS. Consistent with this observation, direct association between class I PI3Ks and Ras has been observed in a GTP-dependent manner using a variety of assay techniques (Rodriguez-Viciana *et al.*, 1996; Rubio *et al.*, 1997; Vanhaesebroeck *et al.*, 1997b; Deora *et al.*, 1998). Indeed, binding of Ras to p110α or p110γ was shown to stimulate lipid kinase activity to a similar or greater extent than phosphotyrosine recruitment of the class IA PI3K regulatory subunits or Gβγ binding to the class IB PI3K, respectively (Rodriguez-Viciana *et al.*, 1996; Pacold *et al.*, 2000). Activation of p110α by Ras is proposed to induce actin rearrangement and inhibit anoikis (Khwaia *et al.*, 1997; Rodriguez-Viciana *et al.*, 1997). Other suggested effects of
Ras-mediated PI3K activation include T cell adhesion and migration (Tanaka et al., 1999), cell transformation (Gire et al., 2000) and inhibition of apoptosis (Kauffmann-Zeh et al., 1997; Khwaja et al., 1997; Xue et al., 2000).

Based largely on mammalian cell culture studies, the transcription factor and proto-oncogene c-Myc has been implicated in the control of cell cycle progression (reviewed in Pelengaris et al., 2000). For example, ectopic expression of Myc in certain quiescent cell types can drive entry into S phase, whereas ectopic expression in cycling cells can accelerate cell division and prevent cell cycle exit (for example Karn et al., 1989; Henriksson and Luscher, 1996). However, more recent in vivo experiments in Drosophila (section 1.3.9) and subsequent experiments in mammals (Iritani and Eisenman, 1999; Schuhmacher et al., 1999) describe a role for Myc in the control of growth. Indeed, the majority of candidate vertebrate Myc gene targets are suggested to be involved in translational control and metabolism, and could function to promote growth (Grandori and Eisenman, 1997; Dang, 1999). Consistent with this observation, the Drosophila Myc target gene, pitchoune, encodes a DEAD box RNA helicase that is potentially involved in translational initiation and ribosomal RNA processing (Zaffran et al., 1998).

The similarity, however, between the cellular phenotypes of dMyc and components of the Drosophila insulin/PI3K pathway also raises the possibility of functional interaction between these two systems. For example, the promotion of growth by components of the insulin/PI3K-pathway may be mediated, in part, through activation of dMyc. Conversely, insulin/PI3K-pathway activity may be epistatic to dMyc function. Alternatively, dMyc may interact synergistically with the insulin/PI3K-pathway such that output from both signalling systems is required for the optimal promotion of growth. For example, although the mRNA cap-binding protein eIF4E, a key regulator of initiation, is regulated post-translationally by PI3K signalling, it also contains two Myc-binding sites in its promoter.

4.1.1 Investigating the role of dRas1 in PI3K signalling in vivo

The recently resolved crystal structure of the Ras·p110γ complex revealed that p110γ interacts with Ras through both the PI3K-RBD and an additional region at the carboxy-terminal of the catalytic domain (Pacold et al., 2000). The exchange of GDP for
GTP causes two regions of Ras, known as switch I and switch II, to change conformation (Pai et al., 1989; Milburn et al., 1990). p110γ appears to interact with many of the same residues in the Ras switch I region that are used by Raf and RalGDS. However, unlike these latter effectors, p110γ also interacts with Ras via the switch II region, a phenomenon which has only previously been observed in complexes of Ras with its upstream regulators (Scheffzek et al., 1997; Boriack-Sjodin et al., 1998). This particular Ras effector interaction may result from the ordering of a loop in the RBD, which prevents Ras from binding to p110γ in the same orientation as Ras in complex with Raf or RalGDS. As a consequence of this unique interaction, Ras establishes novel switch II contacts with both the RBD and catalytic domain of p110γ.

Single amino acid substitutions in the switch I and switch II regions of Ras influence its interaction with target proteins and can confer partial loss-of-function and preferential interaction with specific downstream effectors (Moodie et al., 1995; White et al., 1995). When combined with the constitutively active glycine 12 to valine mutation, these effector domain mutants have been used to dissect the role of different Ras effectors in cellular responses. For example, the tyrosine 40 to cysteine (Y40C) mutant of mammalian Ras was initially documented to preferentially bind p110α isoform with little detectable activity to Raf and RalGDS (Moodie et al., 1995; Rodriguez-Viciana et al., 1997). In contrast, the glutamate 37 to glycine (E37G) mutant of Ras was proposed to preferentially bind RalGDS with no activity to PI3K.

Drosophila Ras1 effector mutants possessing the G12V amino acid substitution in combination with either Y40C (dRas1\textsuperscript{V12,Y40C}) or E37G (dRas1\textsuperscript{V12,E37G}) were employed by two recent studies to investigate the relationship between dRas1 and Dp110/dAkt signalling in vivo. As discussed in section 1.4.6.2, Bergmann and colleagues reported that ectopic expression of dRas1\textsuperscript{V12,Y40C} but not dRas1\textsuperscript{V12,E37G} could partially suppress Hid-induced apoptosis in the eye, and this was suggested to indicate a role for Dp110 in dRas1-mediated cellular survival (Bergmann et al., 1998). However, a recent study by Halfar and colleagues failed to provide evidence for the activation of Dp110 by dRas1\textsuperscript{V12,Y40C} (Halfar et al., 2001). Furthermore, recent studies on p110γ and p110δ revealed a more complex specificity of Ras binding to different PI3K isoforms (Kinashi et al., 2000; Pacold et al., 2000). For example, although the Y40C mutation preserves binding of p110α to Ras, the binding of
p110\(\gamma\) and p110\(\delta\) is substantially reduced or eliminated. In contrast, the E37G mutant, which does not bind p110\(\alpha\), binds p110\(\gamma\) and p110\(\delta\) similarly to wild type Ras. Thus, it is difficult to interpret the results obtained with dRasl effector mutants in the absence of careful characterisation of their binding specificity.

As previously mentioned, a number of studies have investigated the relationship between Ras and class I PI3K signalling \textit{in vitro} and in cell culture, for example following growth factor stimulation of cells transfected with constitutively-active or dominant-negative Ras mutants. The aim of the experiments below were to further investigate the role of Ras in PI3K signalling \textit{in vivo} within a developing organ system using the genetic techniques described in the previous sections in combination with the phosphorylation-dependent and phosphorylation-independent dAkt antibodies.

\subsection*{4.1.1.1 Ectopic expression of dRasl Induces dAkt phosphorylation \textit{in vivo}}

To determine whether dRasl \textit{can} activate the Dp110/dAkt pathway, the ability of constitutively active dRasl\(^{V12}\) to phosphorylate dAkt \textit{in vivo} was investigated. Clones of cells ectopically expressing GFP and dRasl\(^{V12}\) (Prober and Edgar, 2000) were induced 72hrs AEL using a heat shock inducible \textit{flp-ouV} system, as described in section 3.2.3. At 120 hrs AEL, wing discs were dissected from wandering late third instar larvae and immunostained with \(\alpha \text{pSer586}\) and \(\alpha \text{CT}\), as shown in figure 4.1. As observed by Prober and colleagues, clones expressing dRasl\(^{V12}\) were round with relatively smooth borders, suggesting that they mix poorly with neighbouring cells due to altered affinities (Prober and Edgar, 2000). Moreover, several of the dRasl\(^{V12}\) clones formed rosette-like structures, with nuclei in the centre of clones dropping basally. Intriguingly, the dRasl\(^{V12}\) clones displayed increased \(\alpha \text{pSer586}\) staining whereas the \(\alpha \text{CT}\) signal was not significantly altered, indicating that ectopically expressed dRasl\(^{V12}\) can induce phosphorylation of dAkt \textit{in vivo}. Although not easily quantifiable, the intensity of the \(\alpha \text{pSer586}\) signal within dRasl\(^{V12}\) clones relative to surrounding tissue was significantly less than that for dAkt or Dp110 clones. However, this may reflect differences in the levels of expression of the various transgenes rather than a lower intrinsic ability of dRasl\(^{V12}\) to induce dAkt phosphorylation.
Figure 4.1 Ectopic expression of dRas1^{V12} in cell clones induces an increase in αpSer586 signal

Clones ectopically expressing GFP and dRas1^{V12} were induced 72hrs AEL. At 120hrs AEL, wings discs were dissected from wandering late third instar larvae and immunostained with αCT (affinity purified 5th bleed) or αpSer586 (affinity purified 6th bleed) at a concentration of 5μg/ml. Increased signal was observed with αpSer586 but not αCT in clones ectopically expressing dRas1^{V12}. Note that clones expressing dRas1^{V12} appeared round with relatively smooth borders. Several of the dRas1^{V12} clones formed rosette-like structures, with nuclei in the centre of clones dropping basally (red arrowhead).
4.1.1.2 Phosphorylation of dAkt is not reduced in dRas1 null clones

The data presented in the previous section indicates that dRas1 can activate the Dp110 pathway in vivo. However, results obtained with constitutively active Ras may not reflect the physiological situation. Thus, the requirement for endogenous Ras to activate the Dp110/dAkt pathway was further investigated in vivo. Rodriguez-Viciana and colleagues observed that ectopic expression of dominant negative Ras mutant (Ras^{NI7}) into PC12 cells could significantly attenuate PtdIns(3,4)P2 and PtdIns(3,4,5)P3 production after NGF and EGF stimulation (Rodriguez-Viciana et al., 1994). Production of the 3’ phosphorylated phosphoinositides was not completely abolished suggesting that several mechanisms exist whereby growth factors can control PI3K activity, only one of which involves Ras. Indeed, the amount of PI3K activity found in anti-phosphotyrosine immunoprecipitates from NGF- and EGF-stimulated cells was not reduced following Ras^{NI7} expression. This suggested that growth factor control of PI3K through association with phosphotyrosine-containing proteins is preserved despite the attenuation of Ras activity. Consequently, clones of dRas1 null cells would be expected to show reduced, but not completely abrogated, dAkt phosphorylation. To test this possibility, mitotic clones were generated of cells homozygous for the drasI^{40b} allele, in which the entire drasl open reading frame is deleted (Schnorr and Berg, 1996).

dRas1 null clones have impaired growth and poor viability and are eventually eliminated due to competition from the neighbouring, faster growing, wild type cells (Prober and Edgar, 2000). Thus, mitotic recombination was induced in a Minute background to generate relatively large dRas1 null clones (see section 3.2.1), and the discs were stained with either αpSer586 or αCT. To identify the drasI^{40b} clones, non-clonal cells were marked by the expression of GFP. Intriguingly, the dRas1 null clones did not show changes in either αpSer586 or αCT staining, as shown in figure 4.2. One interpretation of this data is that although ectopic expression and constitutive activation of dRas1 is sufficient to induce phosphorylation of dAkt, endogenous dRas1 does not appear to be necessary for dAkt phosphorylation. However, it should be noted that small changes in Dp110/dAkt pathway activity may be outside the range of sensitivity of this assay. Indeed, as mentioned above, activation of the Dp110/dAkt pathway by Ras-independent
Figure 4.2 dAkt phosphorylation is not reduced in mitotic clones of dRas1 null cells

The flp/FRT recombination system was used to generate mitotic clones of dRas1 null cells in a Minute (3)95A heterozygous background 60h±12h AEL. Wing imaginal discs were then dissected from wandering late third instar larvae and stained with either αCT (affinity purified 5th bleed) or αpSer586 (affinity purified 6th bleed) at a concentration of 5μg/ml. Clones were identified by the absence of GFP expression. Immunostaining with either αCT or αpSer586 did not show changes in signal intensity within dRas1 null clones. However, staining with αpSer586 revealed a significant increase in the number of pyknotic cells within clones, especially towards the basal membrane of the disc (right column).
mechanisms would be expected to be maintained. Moreover, abrogation of Ras-mediated PI3K activation may be compensated for through feed-back mechanisms that may exist in vivo within a developing tissue system. Further investigation of these observations is therefore required, as discussed in section 6.3.

As shown in figure 4.2, a substantial increase in αpSer586 pyknotic signal was observed towards the basal membrane of the wing disc within the drasI^40b clone regions. This observation is remarkably similar to the nuclear dye staining of drasl^null clones reported by Prober and Edgar (2000), which also revealed numerous pyknotic nuclei, a morphological characteristic of cell death. Indeed, clones of cells homozygous for another dRas1 null allele (drasI^null) show a significant increase in apoptotic cells as detected by TUNEL staining (Halfar et al., 2001).

4.1.2 Ectopic expression of dMyc does not result in increased dAkt phosphorylation in vivo

To determine whether dMyc promotes growth through the activation of the Dp110/dAkt pathway, the ability of dMyc to promote the phosphorylation of dAkt in vivo was investigated. Clones of cells ectopically expressing GFP and dMyc (UAS-dmyc^42, see Zaffran et al., 1998; Johnston et al., 1999) were induced 72hrs AEL. At 120 hrs AEL, wing discs were dissected from wandering late third instar larvae and immunostained with αpSer586 and αCT, as shown in figure 4.3. In contrast to clones ectopically expressing dAkt or Dp110, no ubiquitous increase in signal was detected with either αpSer586 or αCT within the total dMyc clone region in relation to the surrounding tissue. Intriguingly, however, a dramatic increase in the number of pyknotic staining structures was observed with αpSer586 within clone regions. A three dimensional stack taken through the sagittal plane of the disc revealed that most of the pyknotic structures were located towards the basement membrane of the wing disc (data not shown).

In summary, these data indicate that dMyc does not specifically stimulate dAkt phosphorylation but is associated with increased αpSer586 pyknotic staining (this latter observation will be discussed in greater detail in section 4.2). Although one interpretation of these data is that dMyc and Dp110/dAkt signalling is independent, other possibilities
Figure 4.3 Ectopic expression of dMyc induces increased number of pyknotic staining cells

The 'flip-out' system was used to induce clones 72hrs AEL ectopically expressing GFP and dMyc. At 120hrs AEL, wings discs were dissected from wandering late third instar larvae and immunostained with either αCT (affinity purified 5th bleed) or αpSer586 (affinity purified 6th bleed) at a concentration of 5μg/ml. In contrast to clones ectopically expressing dAkt or Dp110, no increase in αpSer586 signal was observed within the overall clone region. However, there was a dramatic increase in the number of pyknotic staining cells in areas of dMyc ectopic expression.
remain. For example, as previously mentioned, it is possible that dMyc may promote
growth, at least in part, through interaction with components of the insulin/PI3K signalling
network that are independent or downstream of dAkt, such as eIF4E. Indeed, as discussed
in section 1.3.10, it was suggested that the growth- promoting TSC1 and TSC2 proteins
may function in a parallel pathway that may converge on the insulin/PI3K pathway at a
point downstream of dAkt but upstream of dS6K (Gao and Pan, 2001). Lastly, similar to its
suggested role in dRas1-induced growth, dMyc may act downstream of Dp110 and mediate
some of the growth-promoting capacity of the insulin/PI3K pathway, a possibility that has
not been addressed in these experiments.

4.2 Characterisation of αpSer586 pyknotic signal

The observation of the αpSer586 pyknotic signal during the immunofluorescence
experiments described so far was unexpected and potentially very interesting. For
example, the signal appeared to be unique to αpSer586 and was not detectable in αCT
immunostains, suggesting that the pyknotic signal was due to phosphorylation of dAkt.
Furthermore, the morphology and pattern of localisation of the pyknotic staining structures
strongly suggested that they were apoptosing cells, which seemed paradoxical given the
suggested role of Akt as a promoter of cellular survival. Specifically, it was unclear as to
why Akt phosphorylation, and presumably activity, may become increased within dying
cells. Several important questions needed to be addressed initially. First, what was the
identity or source of the signal, second, what was the nature of the signalling event and,
third, what was the functional relevance underlying this signal. The remainder of the results
chapters will therefore focus on the characterisation of this signal in greater detail.

4.2.1 Pyknotic signal is associated with the induction of apoptosis

As discussed in section 4.1.2, ectopic expression of dMyc resulted in a substantial
increase in αpSer586 pyknotic staining structures. Recent evidence indicates that Myc
sensitises cells to a variety of apoptotic triggers (Evan et al., 1992; Juin et al., 1999;
Prendergast, 1999). Experiments in culture suggest that the relative rates of proliferation
and apoptosis induced by ectopic expression of Myc in the presence of growth factors
dictates the overall viability of a cell population (Evan et al., 1992; Harrington et al., 1994). Sensitisation to apoptotic stimuli is an intrinsic activity of Myc which, under normal conditions, is suppressed by a milieu of survival factors (Harrington et al., 1994). Consequently, Myc-induced apoptosis is usually only observed when such survival factors are withdrawn or when cells express very high levels of Myc. To investigate whether ectopic expression of dMyc induced apoptosis, wing discs with clones of cells ectopically expressing dMyc and GFP or GFP alone were stained with the vital dye acridine orange to visualise apoptotic cells (Abrams et al., 1993). Although a few apoptotic cells were observed in control clone cells, the number of dying cells was dramatically increased in clones of cells ectopically expressing dMyc (figure 4.4A).

In contrast to embryos where apoptotic bodies are rapidly engulfed by circulating phagocytes (usually less than 1 hour, see Abrams et al., 1993), dying cells in the wing disc epithelium can remain in situ for up to 6 hours, although are usually engulfed within 2 to 4 hours (Milan et al., 1997). However, it is unlikely that the pyknotic staining structures observed with αpSer586 are phagocytes. Apoptotic cells in the wing disc are usually cleared by a process of translocation towards the basement membrane, where they become enmeshed with the network of fibrous strands that make up the basement membrane structure (Abbott, 1983). Subsequently, it is hypothesised that they are removed by macrophages in the hemolymph that bathes the disc just outside the basement membrane. Indeed, staining dMyc ectopically expressing wing discs with an antibody that recognises the macrophage receptor Croquemort (Franc et al., 1996) did not reveal a staining pattern that resembled the αpSer586 immunostains, and adherent macrophages were rarely observed (data not shown).

A higher magnification of a dMyc clone immunostained with αpSer586 is shown in figure 4.4B. Cells ectopically expressing dMyc are marked by nuclear localised GFP. Nuclei from non-apoptosing cells show homogenous GFP expression. In contrast, condensed nuclei from apoptosing cells contain a prominent non-GFP expressing rounded mass, the identity of which is unknown but may originate from the fine granular material dispersed from the nucleolus that is observable in higher resolution electron microscopy sections (Abbott, 1983). The morphology of nuclei from apoptosing cells varied according to the stage of apoptosis but the nuclei were typically less than one third the size of non-
Figure 4.4 Pyknotic signal in apoptosing cells from clones ectopically expressing dMyc

A) Clones of cells ectopically expressing GFP alone or GFP and dMyc were stained with the vital dye acridine orange to detect apoptosis. Although a small number of apoptosing cells were observed in control GFP clones, ectopic expression of dMyc induced a dramatic increase in apoptosis. B) Higher magnification of a clone ectopically expressing dMyc from a disc immunostained with αpSer586. Apoptosing cells are located mainly to the left in the field of view. The pyknotic staining appears to be perinuclear (white arrowhead), or nuclear localised (yellow arrowhead), perhaps reflecting the stage of apoptosis and the integrity of the nuclear membrane. Generally, no signal is observed in late stage apoptotic bodies (orange arrowhead)
apoptosing cells. A few nuclei were also observed undergoing the process of fragmentation. The intracellular localisation of the pyknotic signal was difficult to ascertain. However, the signal appeared to be either perinuclear (white arrowhead) or nuclear localised (yellow arrowhead), perhaps reflecting the stage of apoptosis and the integrity of the nuclear membrane. Late stage apoptotic bodies (orange arrowhead) showed little or no \( \alpha \text{pSer586} \) signal.

In summary, the induction of apoptosis following dMyc ectopic expression was associated with a dramatic increase in the number of pyknotic staining structures detected in \( \alpha \text{pSer586} \) immunostains but not in discs stained with \( \alpha \text{CT} \). Moreover, anti-Croquemort immunostains suggested that this signal did not originate from macrophages. This indicated, first, that the pyknotic staining structures were cells undergoing apoptosis and, second, that one or both of the dAkt isoforms become phosphorylated at serine 586 during apoptosis.

4.2.2 Increased frequency of \( \alpha \text{pSer586} \) pyknotic signal in irradiated discs

The data presented so far indicates that the pyknotic signal is a consequence of apoptosis that occurs during normal developmental or as a result of ectopic expression or loss-of-function of pro-apoptotic or anti-apoptotic genes, respectively. It is not clear, however, whether the signal is also associated with the induction of apoptosis by exogenous non-physiological insults. For example, apoptosis induced by X-ray irradiation appears to have a signalling mechanism that is distinct from developmental cell death, involving the induction of the transcriptional activator and tumour suppressor gene, p53 (White et al., 1994). Consistent with this observation, the \textit{Drosophila} p53 homolog, Dmp53, is required for radiation-induced apoptosis in the wing, but not for the normal levels of cell death that occur in the absence of DNA-damaging agents (Brodsky et al., 2000; Ollmann et al., 2000; Steller, 2000).

Irradiation of larvae with X-rays induces apoptosis by DNA damage and by generation of aneuploids following chromosome segregation in cell division (James and Bryant, 1981). A relatively high dose irradiation (4000 rads) in late third instar wild type wing discs is followed 5 to 8 hours later by a dramatic increase in the number of apoptotic
cells, preferentially localised at the wing margin (Milan et al., 1997). Consequently, wing imaginal discs were dissected from untreated or irradiated late 3rd instar larvae and stained 5-6 hours later with αpSer586 or acridine orange, as described in section 2.5.2. Consistent with previous observations, irradiation induced an abrupt increase in cell death, as determined by acridine orange staining (figure 4.5). Furthermore, this was associated with a substantial elevation in the number of pyknotic structures in wing discs immunostained with αpSer586.

As discussed in section 1.4.4.3, apoptosis in Drosophila is predominantly controlled by three genes that are clustered in a small region of the third chromosome (White et al., 1994). These genes, head involution defective (hid), grim, and reaper (rpr), have been identified as key initiators of the apoptotic machinery (White et al., 1994; Grether et al., 1995; Chen et al., 1996c). Several major observations suggest that ionising radiation-induced apoptosis occurs through a signalling pathway mediated by reaper. First, deletions removing rpr, grim, and hid significantly inhibit radiation-induced apoptosis in embryos (White et al., 1994). Second, expression of Reaper is sufficient to induce apoptosis (Hay et al., 1995; White et al., 1996). Third, reaper transcription anticipates cell death induced by ionising radiation (Nordstrom et al., 1996). More recently, it was shown that the cis-regulatory region of reaper contains a radiation inducible enhancer that includes a consensus p53 binding site, suggesting that reaper is a direct transcriptional target of Dmp53 following DNA damage (Brodsky et al., 2000; Ollmann et al., 2000; Steller, 2000).

The observation that irradiation can induce an increase in the number of pyknotic staining structures suggested, therefore, that the signal was potentially associated with the expression of Reaper. This possibility is investigated further in Chapter 5.

4.3 Summary

The Drosophila homologs of Ras, Myc and components of the insulin/PI3K pathway have been implicated in the promotion of growth during imaginal disc development. This raised the possibility that these systems were part of a common growth-regulating signalling network. Indeed, dRas1 was suggested to promote growth through the post-transcriptional regulation of dMyc. Furthermore, a significant number of studies in
Figure 4.5 Irradiation of wing discs induces an increase in the pyknotic signal

Wing imaginal discs were irradiated at 400 rads and stained 4-5 hours later with acridine orange (AO) or αpSer586. Staining with AO revealed a dramatic increase in the number of apoptosing cells following radiation. This was accompanied by a similar increase in pyknotic staining cells in αpSer586 immunostains, which were predominantly localised within the wing pouch and around the notum-pouch boundary.
mammals have established PI3K as a potential Ras effector. The first section of this chapter described the investigation of the role of dRas1 and dMyc in Dp110/dAkt signalling in vivo within the wing imaginal disc. Interestingly, ectopic expression of a constitutively active dRas1 mutant, dRas1\textsuperscript{V12}, within clones was shown to induce phosphorylation of dAkt. This observation suggested that the activity of the Dp110 pathway is elevated in dRas1\textsuperscript{V12} expressing cells. However, dAkt phosphorylation was not significantly changed in dRas1 null clones, indicating that although enhancing dRas1 activity is sufficient to potentiate signalling through Dp110/dAkt, dRas1 is not necessary for maintaining endogenous levels of Dp110/dAkt signalling in vivo.

The second section of this chapter describes further characterisation of the pyknotic signal detected in αpSer586 immunostains. Cellular pyknosis is a morphological hallmark of apoptosis and, as discussed in Chapter 3, the distribution of the pyknotic signal closely resembled the pattern described for apoptotic cells during wing and eye imaginal disc development. The transcription factor, Myc, has been shown to sensitise cells to apoptosis, and to induce cell death in cultured cells deprived of trophic support. Consistent with this observation, ectopic expression of dMyc in clones during wing imaginal disc development was shown to induce apoptosis within clone regions. This was associated with a substantial increase in the number of αpSer586 pyknotic staining structures, also within clone regions.

As discussed in section 1.4.6.1, dRas1 signalling has been shown to inhibit the activity of the pro-apoptotic mediator Hid, and is an important promoter of cellular survival during imaginal disc development. Consistent with this observation, a significant increase in the number of pyknotic-staining bodies was detected in αpSer586 immunostains of dRas1 null clones. Furthermore, increased αpSer586 pyknotic staining was also observed following irradiation of wing imaginal discs which results in widespread apoptosis via irradiation-induced expression of the pro-apoptotic mediator, Reaper. Taken together, these findings suggest that the increased αpSer586 signal in apoptotic bodies can result from both Hid- and Reaper-mediated mechanisms of apoptosis.

In the following chapter, the αpSer586 apoptosis-associated pyknotic signal is biochemically characterised using cultured cells undergoing apoptosis following ectopic expression of Reaper.
Chapter 5: Characterisation of an 80kDa protein associated with Apoptosis in Drosophila

5.1 Introduction ......................................................... 138

5.2 Characterisation of an apoptosis-associated 80kDa protein .................. 138
  5.2.1 Pyknotic staining is associated with the appearance of an 80kDa protein detected by \( \alpha_{\text{pSer586}} \) 138
  5.2.2 The 80kDa protein is also observed in S2 cells following Reaper expression .......... 139
  5.2.3 Timecourse of appearance of 80kDa protein following ectopic Reaper expression _______ 145
  5.2.4 Appearance of the 80kDa protein is a caspase-dependent event .......................... 145

5.3 The 80kDa protein is not p80-dAkt .................................. 147
  5.3.1 Apoptosis is not associated with phosphorylation of dAkt at threonine 423 ............ 149
  5.3.3 The 80kDa protein cannot be immunoprecipitated using antisera raised against dAkt _______ 151
  5.3.4 dAkt is cleaved following induction of apoptosis .............................................. 151
  5.3.5 Inhibition of dAkt expression does not affect the appearance of the 80kDa band .......... 155
  5.3.6 Detection of the 80kDa protein by \( \alpha_{\text{pSer586}} \) is not phosphorylation-dependent ........ 159

5.4 Summary ........................................................................ 159
5.1 Introduction

The data presented in the previous chapters describe the characterisation of phosphorylation-specific and phosphorylation-state-independent antibodies to Drosophila Akt. These reagents were used to analyse Dp110 pathway signalling in vitro and in vivo during development. Immunostains of imaginal discs with the phosphorylation-specific αpSer586 antibody revealed an elevated, pyknotic signal within dying cells. This observation suggested that dAkt becomes phosphorylated during apoptosis in vivo.

Interestingly, Tang and colleagues recently observed that induction of apoptosis with staurosporine and etopside in NIH3T3 cells was associated with PI3K-mediated Akt phosphorylation at serine 473 and a 6-fold increase in Akt kinase activity (Tang et al., 2001). In this system, ectopic expression of wild type or constitutively active Akt delayed the cleavage of the caspase substrate, poly(ADP-ribose) polymerase (PARP). In contrast, inhibition of Akt activation with the PI3K inhibitor, wortmannin, sensitised the cells to these apoptotic stimuli. Thus, Akt activation was suggested to function as a barrier that proapoptotic signals must overcome, the consequence of which is to delay rather than diminish apoptosis, since the extent of PARP cleavage and the final percentage of apoptosing cells remained the same. A major criticism of this study, however, is that it is not clear whether the Akt phosphorylation and activation occurs in the apoptosing or non-apoptosing population of cells.

Further biochemical characterisation of the αpSer586-pyknotic apoptosis signal is described in this chapter.

5.2 Characterisation of an apoptosis-associated 80kDa protein

5.2.1 Pyknotic staining is associated with the appearance of an 80kDa protein detected by αpSer586

To investigate whether the increased immunostaining with αpSer586 within dying cells was a result of dAkt phosphorylation, the appearance of phosphorylated dAkt in apoptosing imaginal discs cells was investigated biochemically using immunoblots of wing disc lysates. To generate imaginal discs in which a significant number of cells were apoptosing, larvae were irradiated with X-rays as described in section 2.5.1. Lysates were
Chapter 5: Characterisation of an 80kDa Protein associated with Apoptosis in Drosophila

preparing from 50 – 100 wing discs dissected from either irradiated or control non-irradiated late 3rd instar larvae. Samples of the same wing discs were also stained with acridine orange and αpSer586 to confirm cell death and the presence of the pyknotic staining, respectively (see figure 4.5).

The wing disc lysates, as well as lysates of insulin-stimulated and unstimulated S2 cells, were resolved by SDS PAGE and immunoblotted with αpSer586 and αCT, as shown in figure 5.1. As a loading control, the western blots were also probed with an antibody to β-tubulin, which confirmed equal lane loading. Total dAkt levels, as determined with αCT, were similar in both the control and irradiated wing discs lysates, and in the insulin-stimulated and unstimulated S2 cell lysates. Interestingly, in contrast to the untreated S2 cell lysates, significant phosphorylation of p66- and p80-dAkt was detected with αpSer586 in the control wing disc lysates, indicating a basal physiological level of dAkt phosphorylation. Surprisingly, the intensity of the 80kDa signal, thought to correspond to p80-dAkt, was markedly increased in the αpSer586 immunoblot of the irradiated disc lysate. However, no change was observed in the 66kDa signal of p66-dAkt. No other bands were detected with αpSer586 following irradiation (see section 5.2.2).

These data demonstrate that the detection of the pyknotic staining with αpSer586 following the induction of apoptotic cell death is associated with increased signal at 80kDa on αpSer586 immunoblots. This suggested that phosphorylation of p80-dAkt, but not p66-dAkt, was likely to be responsible for the pyknotic signal observed in apoptosing cells.

5.2.2 The 80kDa protein is also observed in S2 cells following Reaper expression

To determine whether the appearance of the 80kDa band was unique to imaginal discs cells or was also present in other Drosophila cells αpSer586 immunoblots of apoptosis-induced Schneider S2 cell lysates were examined. As discussed in section 1.4.4.3, ectopic expression of the Drosophila apoptosis regulators head involution defective (hid), grim, or reaper (rpr) is sufficient to induce cell death in a variety of cell types (White et al., 1994; Grether et al., 1995; Chen et al., 1996b). Consequently, a Schneider S2 cell line that had been stably transfected with a construct containing reaper cDNA under the
Figure 5.1 Detection of an 80kDa protein in irradiated wing imaginal discs

Lysates were prepared from wing discs (30μg per lane, equivalent to 50 discs) dissected from either irradiated or control non-irradiated late 3rd instar larvae. The wing disc lysates, as well as lysates of insulin-stimulated and unstimulated S2 cells (50μg per lane), were resolved by SDS PAGE and immunoblotted with 0.5μg/μl of affinity purified αSer586 (6th bleed) or αCT (5th bleed). As a loading control, the western blot was also probed with an antibody to β-tubulin (1/200 dilution) to confirm equal lane loading. Following irradiation, increased signal was detected at 80kDa with αSer586 but not αCT.
control of a Drosophila metallothionein promoter was obtained from Pascal Meier (Institute for Cancer Research, pMrt-rpr). Treatment of pMrt-rpr cell lines with 0.5-0.7mM CuSO\textsubscript{4} results in a rapid induction of reaper mRNA and Reaper protein, followed by apoptosis several hours later (Pascal Meier, personal communication; see also Nordstrom et al., 1996; Pronk et al., 1996).

To confirm cell death, control and CuSO\textsubscript{4}-treated pMrt-rpr were fixed in 70\% ethanol before staining with the nucleic acid dye, propidium iodide. The samples were then analyzed by flow cytometry, as described in section 2.5.3. This assay is based on the occurrence of endonuclease-mediated DNA fragmentation and the appearance of hypodiploid nuclear fragments with a 'sub-G1' DNA content, a hallmark of programmed cell death (Tounekti et al., 1995). As shown in figure 5.2A, a prominent population in this sub-G1 region was specifically detected in a DNA content frequency histogram 2.5 hours after treatment of the pMrt-rpr cells with CuSO\textsubscript{4}. Treatment of non-transfected or empty vector-transfected S2 cells with CuSO\textsubscript{4} did not induce apoptosis (data not shown).

Lysates from control, CuSO\textsubscript{4}-treated, or insulin-stimulated pMrt-rpr cells were immunoblotted with \textalpha+pSer586 and \textalpha+CT, as shown in figure 5.2B. Probing all three lysates with \textalpha+CT revealed equal levels of dAkt expression. Although no bands were detected in the control pMrt-rpr lysate lane with \textalpha+pSer586, insulin stimulation of pMrt-rpr cells induced phosphorylation of both p66- and p80- dAkt (lanes 1 and 2) in an identical manner to non-transfected S2 cells. As observed with the irradiated disc lysates, an intense signal was detected at 80kDa with \textalpha+pSer586 in the CuSO\textsubscript{4}-treated pMrt-rpr cell lysates (lane 3).

It is possible that CuSO\textsubscript{4} treatment may also result in the appearance of additional proteins that were not within the range of molecular weights resolved in the immunoblot from figure 5.2B. Thus, to determine whether there were other proteins of lower molecular weight that may have been missed in the CuSO\textsubscript{4}-treated cells, the lysates were run on a 4-16\% gradient SDS PAGE gel to increase the range of detection, and immunoblotted with \textalpha+pSer586 as above. As shown in figure 5.3, other than the 80kDa protein, no other proteins with apparent molecular weights between 15kDa to 250 kDa were observed following CuSO\textsubscript{4} treatment.

In summary, the induction of apoptosis in both S2 cells and imaginal discs was associated with an increase in signal at 80kDa in immunoblots probed with \textalpha+pSer586 but
Figure 5.2 Detection of the 80kDa protein during apoptosis induced by Reaper expression

A) To confirm that ectopic expression of Reaper induces cell death, control or CuSO₄-treated pMt-rpr cells were stained with propidium iodide and analysed by flow cytometry. A prominent population in sub-G1 region (black arrowhead) was detected in a DNA content frequency histogram after 2.5 hours treatment of the pMt-rpr cells with CuSO₄. B) Lysates from control, CuSO₄-treated, or insulin-stimulated pMt-rpr cells were immunoblotted with αpSer586 and αCT. As observed in lysates from irradiated wing imaginal discs, induction of apoptosis by Reaper expression resulted in increased signal at 80kDa in immunoblots probed with αpSer586 but not αCT.
**A**

2.5 hrs CuSO₄

**Schneider S2**

- WB: αpSer586
  - p80 dAkt
  - p66 dAkt
  - CuSO₄
  - Insulin

- WB: αCT
  - p80 dAkt
  - p66 dAkt
Figure 5.3 **αpSer586 specifically detects increased signal at 80kDa in lysates from apoptosing cells**

To identify additional apoptosis-associated signals, *pMT-rpr* cell lysates were run on a 4-16% gradient SDS PAGE gel and immunoblotted with **αpSer586** and **αCT**. Other than the 80kDa protein, no other proteins with apparent molecular weights between 15kDa to 250 kDa were observed following CuSO₄ treatment.
Chapter 5: Characterisation of an 80kDa Protein associated with Apoptosis in Drosophila

not αCT. This observation suggested that p80 dAkt becomes phosphorylated during apoptosis. A further implication of this finding is that the amino-terminal extension of p80 dAkt may confer this isoform with a cellular function that is distinct from that of p66 dAkt, for example through differences in binding partners or intracellular localisation.

5.2.3 Timecourse of appearance of 80kDa protein following ectopic Reaper expression

Membrane blebbing, an early morphological marker of apoptosis, is detectable in pMt-rpr cells approximately 2.5-4 hours following CuSO₄ treatment (Nordstrom et al., 1996). The appearance of the 80kDa protein after only 2.5 hours CuSO₄ stimulation suggested that it was an early apoptotic event. To analyse the signal during progression of the cell death programme following Reaper expression, the pMt-rpr cells were treated with CuSO₄ for different durations and lysed. The lysates, in parallel with insulin-stimulated and control cell lysates, were immunoblotted with αpSer586 and αCT, as shown in figure 5.4. Interestingly, the 80kDa protein was detected with αpSer586 during the early stages of apoptosis after 2.5 hours, and persisted for up to 16 hours when the number of cells undergoing apoptosis is maximal (Nordstrom et al., 1996; Pronk et al., 1996). Remarkably, however, no band was observed after 2 hours CuSO₄ treatment, indicating that the signal appears within a 30 min window, a period that also coincides with the initial activation of caspases (Nordstrom et al., 1996).

5.3.4 Appearance of the 80kDa protein is a caspase-dependent event

As discussed in section 1.4.2, the execution of apoptosis is organised by a family of cysteine proteases that exhibit a high degree of primary specificity, usually cleaving after a carboxy-terminal aspartate within a specific tetrapeptide sequence. These cysteine-dependent aspartate-specific proteases (caspases) are expressed as pro-enzymes with little or no intrinsic catalytic activity. Proteolytic cleavage of caspases at specific inter-domain sites results in their activation. These sites of proteolytic activation are themselves caspase consensus cleavage sites. Thus, following initial activation, typically by an ‘initiator’ caspase, a cascade of auto- or trans-activation of caspases ensues (Alnemri, 1997).
Figure 5.4 Timecourse of appearance of the 80kDa protein during apoptosis

The timecourse of appearance of the apoptosis-induced 80kDa signal was analysed during progression of the cell death programme following Reaper expression. \textit{pMel-rpr} cells were treated with CuSO$_4$ for different durations and lysed. The lysates, in parallel with insulin-stimulated and control cell lysates, were immunoblotted with $\alpha$Ser586 and $\alpha$CT. Although the 80kDa protein was not detected following 2 hours treatment, it was observed following only 2.5 hours treatment in $\alpha$Ser586 immunoblots.
To determine whether the appearance of the 80kDa protein was a caspase-related event, since it coincided with initial caspase activation, \( pMt\rightarrow rpr \) cells were pre-treated with a cell-permeant caspase inhibitor-peptide prior to \( \text{CuSO}_4 \) treatment. The synthetic peptide N-benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) is based on the caspase substrate recognition and cleavage motif and is a general vertebrate and invertebrate caspase inhibitor (An and Knox, 1996). Pre-treatment of \( pMt\rightarrow rpr \) cells with zVAD-fmk for 90 min prior to \( \text{CuSO}_4 \) treatment completely inhibited the detection of the 80kDa protein indicating that its appearance is dependent on caspase activity (figure 5.5).

5.3 The 80kDa protein is not p80-dAkt

The observation that the 80kDa signal appears following caspase activation, at a stage that almost invariably commits cells to apoptosis, is paradoxical given the substantial literature implicating mammalian Akt isoforms as a promoter of cell survival (see section 1.4.7). As previously mentioned, a recent study showed that induction of apoptosis with staurosporine and etopside in cultured cells was associated with PI3K-mediated Akt phosphorylation at serine 473 and a several fold increase in Akt kinase activity (Tang et al., 2001). However, in the same study, the phosphorylation of Akt by staurosporine or etopside was not inhibited by pre-treatment with zVAD-fmk, indicating that this was independent of caspase activation. Furthermore, several studies have demonstrated that Akt is cleaved and inactivated during programmed cell death in a caspase-dependent manner. For example, cleavage of Akt1 was observed following induction of apoptosis in Jurkat cells by Fas ligation or in U937 cells by ultraviolet irradiation, and this was inhibited by pre-treatment with cell-permeable caspase inhibitors (Widmann et al., 1998b). Treatment of ovarian epithelial cancer cells with zVAD-fmk and the caspase-3 inhibitor, zDEVD-fmk, also inhibits Akt cleavage following induction of apoptosis by the chemotherapy agent, Cisplatin (Asselin et al., 2001).

To determine whether the 80kDa protein really was dAkt, a number of approaches were taken. First, phosphorylation of the second key site on dAkt required for kinase activity was investigated during apoptosis. Second, the ability to immunoprecipitate the 80kDa protein from lysates of apoptosing cells using various antibodies to dAkt was
Figure 5.5 The appearance of the 80kDa protein is a caspase-dependent event

Pre-treatment of pMt-rpr cells with the pan-caspase inhibitor peptide zVAD-fmk (20μM) for 90 min prior to CuSO₄ treatment completely inhibited the detection of the 80kDa protein by αpSer586.
determined. Third, cleavage of dAkt during apoptosis was studied. Fourth, the detection of the 80kDa protein following suppression of dAkt expression was analysed.

### 5.3.1 Apoptosis is not associated with phosphorylation of dAkt at threonine 423

As discussed in sections 1.2.3.2 and 1.2.3.3, phosphorylation of Akt at threonine 308 is required for kinase activity. To determine whether increased phosphorylation at the equivalent site in dAkt occurred following Reaper-induced apoptosis in S2 cells, αCT immunoprecipitates from lysates of CuSO₄-treated or insulin-stimulated pMt-rpr cells were immunoblotted with αpThr423, as shown in figure 5.6. Although insulin stimulation induced phosphorylation of threonine 423, no increase was observed following 2.5 hours CuSO₄ treatment in either the total cell lysates or in the αCT immunoprecipitates. Furthermore, immunostaining wing imaginal discs with αpThr423 did not reveal the pyknotic cell staining observed with αpSer586 (data not shown).

The absence of increased threonine 423 phosphorylation, however, does not necessarily preclude the possibility that the 80kDa signal detected with αpSer586 was due to phosphorylation of p80-dAkt at serine 586. The phosphorylation of the two equivalent sites on mammalian Akt can, for example, occur independently of each other (Lynch et al., 1999; Persad et al., 2000; Persad et al., 2001). Furthermore, Lynch and colleagues argue that provided there is a basal level of threonine 308 phosphorylation, a substantial increase in serine 473 phosphorylation may result in a significant increase in kinase activity (Lynch et al., 1999). More specifically, they observed enhanced phosphorylation of Akt on serine 473 following ectopic expression of Integrin Linked Kinase in COS cells and this was associated with increased phosphorylation of GSK-3 at serine 21, a downstream target of Akt. However, a major caveat of this study is that Akt kinase activity was not measured directly by in vitro kinase assays, particularly when considering that the increased GSK-3 phosphorylation detected was moderate. Furthermore, it is not clear whether specific phosphorylation of either threonine 308 or serine 473 does actually occur in vivo, in a physiologically relevant context.
Figure 5.6 Phosphorylation of dAkt at threonine 423 is not increased during apoptosis

Lysates of CuSO₄-treated or insulin-stimulated pMT-rpr cells were immunoblotted with αSer586, αThr423 and αCT. Although insulin stimulation induced phosphorylation of threonine 423, no increase in signal at 80kDa was observed with αThr423 in CuSO₄-treated lysates.
5.3.2 The 80kDa protein cannot be immunoprecipitated using antisera raised against dAkt

To further investigate whether the 80kDa protein was p80-dAkt, the ability to detect the signal in dAkt immunoprecipitates from S2 cells following induction of Reaper expression and apoptosis was determined. Lysates of CuSO₄-treated or insulin-stimulated pMt-rpr cells were immunoprecipitated with αCT and, together with the immunoprecipitate supernatant, immunoblotted with αpSer586. Immunoprecipitation was also performed with three other antibodies, two of which were obtained from Brian Hemmings' Lab (Friedrich Miescher Institute, Basel, Switzerland) raised against recombinant dAkt (αdAkt), and against a synthetic peptide corresponding to a sequence in the amino-terminal extension of p80-dAkt, (αNT, see section 1.2.4; Andjelkovic et al., 1995). The third was a commercially available antibody raised against the carboxy-terminal hydrophobic motif of mammalian Akt that also cross-reacted with dAkt (αPKB, see section 3.1.2).

As observed previously, phosphorylated p80-dAkt could be detected in both the supernatant and following immunoprecipitation from the insulin-stimulated pMt-rpr lysate with all four antibodies, as shown in figure 5.7. In contrast, although the 80kDa band could be detected in the supernatants following CuSO₄ treatment, it was not observed following immunoprecipitation with any of the antibodies. Thus, taken together, this data strongly argues against the possibility that the 80kDa band is p80-dAkt.

5.3.3 dAkt is cleaved following induction of apoptosis

Previous studies have shown that Akt is cleaved at three sites to produce 40- and 44-kDa fragments following incubation with active caspases in vitro (Rokudai et al., 2000). Two of these sites are between the PH domain and the kinase domain, the third is in the carboxy-terminal regulatory domain. Interestingly, a sequence alignment reveals that although the first site is not significantly conserved in dAkt, the other two sites are potentially also caspase cleavage sites (figure 5.8A). To determine whether dAkt is also cleaved during apoptosis, lysates of pMt-rpr cells that had been treated with CuSO₄ for different durations were immunoblottedted with αCT to detect potential carboxyl-end fragments, as shown in figure 5.8B. Interestingly, a doublet at approximately 46 and 48
The ability to detect the apoptosis-associated 80kDa protein in dAkt immunoprecipitates was determined. Lysates of CuSO₄-treated or insulin-stimulated pMt-rpr cells were immunoprecipitated with αCT. Immunoprecipitation was also performed with three other antibodies raised against recombinant dAkt (αdAkt), the amino-terminal extension of p80 dAkt, (αNT), and a mammalian Akt antibody that cross-reacts with dAkt (αPKB). Both the immunoprecipitates and the immunoprecipitate supernatants were then immunoblotted with αpSer586. Phosphorylated p80 dAkt could be detected in insulin-stimulated pMt-rpr lysates in both the supernatants and immunoprecipitates. In contrast, the 80kDa signal could be detected in supernatants from CuSO₄-treated pMT-rpr lysates but not following immunoprecipitation with any of the dAkt antibodies.
IP: αPKB
WB: αpSer586

IP: αCT
WB: αpSer586

IP: αdAkt
WB: αpSer586

IP: αNT
WB: αpSer586

IP: αPKB
WB: αCT

IP: αCT
WB: αCT

IP: αdAkt
WB: αCT

IP: αNT
WB: αCT
Figure 5.8 dAkt is cleaved following induction of apoptosis

**A)** A sequence alignment between human Akt1 and *Drosophila* Akt reveals that although the first putative Akt1 caspase cleavage site is not significantly conserved in dAkt (lacks a carboxy-terminal aspartate), the other two sites are also potential cleavage sites in dAkt. **B)** Lysates of pMrt-rpr cells that had been treated with CuSO₄ for different durations were immunoblotted with αCT to detect potential carboxyl-end cleavage fragments. Interestingly, a doublet at approximately 46 and 48 kDa was observed following treatment with CuSO₄ but not in control untreated cells. These may represent protein fragments generated following caspase-induced cleavage of p66- and p80-dAkt after aspartate 150 and aspartate 231, respectively.
kDa was observed following treatment with CuSO₄ but not in control untreated cells, suggesting that one or both of the dAkt isoforms may be cleaved during apoptosis to generate smaller protein fragments. Although not done, this could be confirmed by immunoblotting with the αdAkt antibody, raised against recombinant dAkt, which should detect both amino and carboxyl fragments (see section 5.3.2).

5.3.4 Inhibition of dAkt expression does not affect the appearance of the 80kDa band

If the 80kDa band is p80-dAkt, inhibition of dAkt expression would be expected to significantly reduce the detection of this band with αpSer586 in apoptosing cell lysates. To determine if this was the case, double-stranded RNA-mediated interference (dsRNAi) was employed. This technique permits the potent and specific post-transcriptional silencing of a gene of interest and therefore significantly facilitates reverse genetic studies (reviewed in (Bosher and Labouesse, 2000)). The phenomenon of dsRNAi was first identified following the observation that both sense and anti-sense RNA could efficiently inhibit gene expression. Subsequently, it was discovered that annealing of complementary strands of RNA, producing double stranded RNA, provided an effective method for gene silencing. Delivery of dsRNA into C. elegans or pre-cellularised Drosophila embryos, for example by injection or ingestion, has been shown to phenocopy loss of function mutations (see Fire et al., 1998; Montgomery and Fire, 1998; Montgomery et al., 1998; Fire, 1999).

Double stranded RNAs ranging from 200bp-1000bp in length are able to inhibit gene expression provided the probes are derived from sequences present in the target RNA transcript and not from the promoter or intergenic regions. Furthermore, to induce post-transcriptional gene silencing, the dsRNA should contain mainly exonic rather than intronic sequences, possibly because of the rapid maturation of the precursor messenger RNAs (Fire et al., 1998).

Clemens and colleagues recently demonstrated the potential efficacy of dsRNAi in dissecting signal transduction pathways by inhibiting the expression of specific proteins in different Drosophila cell lines (Clemens et al., 2000). For example, they showed that exposing Schneider S2 cells to dsRNA corresponding to coding sequences from the
**Chapter 5: Characterisation of an 80kDa Protein associated with Apoptosis in Drosophila**

*Drosophila* homolog of the vertebrate insulin receptor substrate family, *chico*, or the *Drosophila* *pten* homolog, *dpten*, resulted in a substantial reduction or increase in dAkt *in vitro* kinase activity, respectively.

To optimise the inhibition of dAkt expression by dsRNAi, different conditions were tested using the parameters determined by Clemens *et al* as a guideline. Since the RNAi process is homology-dependent, the sequences to be targeted for dAkt-specific RNAi were carefully selected to avoid cross-interference between highly homologous sequences. Three different sequences were chosen of varying lengths corresponding to nucleotides 12 – 240 (dsRNA\(^{228}\)), 244 – 783 (dsRNA\(^{539}\)) and 389-1048 (dsRNA\(^{659}\)) of the *dakt* open reading frame. Each dsRNA was produced by transcription from a PCR product that has terminal T7 RNA polymerase binding sites, as described in section 2.6. Following treatment with the dsRNA, *pMT-rpr* cells were incubated for different periods before being lysed. The lysates were then immunoblotted with αCT to determine dAkt expression levels, as well as an antibody raised against β–tubulin to provide a loading control (figure 5.9). Following incubation for 4 days, a substantial reduction in dAkt protein levels was observed with all three dsRNA. Interestingly, the efficacy in inhibition of dAkt expression correlated with the length of the dsRNA, with the shortest being the most potent, although it is not clear if this was coincidental. Although a moderate further reduction in total dAkt levels was observed following incubation for 6 days, no real difference was detected after that. Furthermore, doubling the amount of dsRNA used from 15µg to 30 µg did not significantly enhance the degree of inhibition (data not shown).

Thus, to inhibit dAkt expression prior to induction of apoptosis, *pMT-rpr* cells were treated with dsRNA\(^{228}\) and incubated for 6 days. The cells were then treated with either insulin or CuSO\(_4\), lysed and immunoblotted with αpSer586, αCT and α β tubulin as shown in figure 5.10. The detection of insulin-stimulated phosphorylated dAkt with αpSer586 was abolished following inhibition of dAkt expression. In contrast, the 80kDa protein detected by αpSer586 was still present. Thus, the detection of the caspase-dependent signal is not affected by the removal of dAkt, confirming that this protein is not dAkt.

156
Figure 5.9 Double-stranded RNA-mediated interference of dAkt expression

*pMT-rpr* cells were treated with three different dsRNA fragments corresponding to nucleotides 12–240 (dsRNA228), 244–783 (dsRNA539) and 389-1048 (dsRNA659) of the dakt open reading frame. Following treatment, *pMT-rpr* cells were incubated for different periods before being lysed. The lysates were then immunoblotted with αCT in order to determine dAkt expression levels, as well as an antibody raised against β tubulin to provide a loading control. Incubation of the dsRNA treated cells for 4, 6, or 9 days resulted in a substantial reduction in dAkt protein levels.
Figure 5.10 Inhibition of dAkt expression does not affect the appearance of the 80kDa protein

pMT-rpr cells were treated with dsRNA and incubated for 6 days. The cells were then treated with either insulin or CuSO₄, then lysed and immunoblotted with αSer586, αCT and α-β-tubulin. The detection of insulin-stimulated phosphorylated dAkt with αSer586 was abolished following inhibition of dAkt expression. In contrast, the 80kDa protein was still detected by αSer586.
5.3.5 Detection of the 80kDa protein by αpSer586 is not phosphorylation-dependent

The data presented above show that the 80kDa protein is not p80-dAkt despite having the same apparent molecular weight. This suggests that αpSer586 cross-reacts with another protein that perhaps shares an epitope that is similar to the dAkt sequence against which the antibody was raised against. To determine whether the detection of the 80kDa protein by αpSer586 is also through recognition of a phosphorylation site, immunoblots of lysates from control or CuSO₄-treated pMT-rpr cells were incubated with alkaline phosphatase prior to probing with αpSer586 and reprobing with αCT (see section 2.3.9). The detection of insulin-stimulated phosphorylated dAkt was abolished following preincubation with alkaline phosphatase, as shown in figure 5.11. In contrast, the 80kDa protein in lysates from CuSO₄-treated pMT-rpr cells could still be detected in the phosphatase-treated immunoblot. However, dAkt could be detected in both control and phosphatase-treated immunoblots when reprobed with αCT. This suggested that the recognition of the apoptosis-associated 80kDa protein by αpSer586 was not phosphorylation-dependent.

5.4 Summary

The appearance of the pyknotic signal in apoptosing cells identified in immunostains of imaginal discs with αpSer586 was shown to be associated with the detection of an 80kDa protein on immunoblots of X-ray irradiated wing disc lysates. The protein also appears upon expression of the pro-apoptotic protein, Reaper, in S2 cells, demonstrating that this phenomenon is not unique to imaginal disc cells. These observations strongly suggested that the pyknotic signal identified with αpSer586 in immunostains of imaginal discs was due to phosphorylation of p80-dAkt during apoptosis. Surprisingly, a study of the timecourse of the signal following induction of apoptosis indicated that its appearance coincided with the period of initial caspase activity. Indeed, the detection of the 80kDa band was shown to require caspase activity since it was not observed in lysates from Reaper-induced cells that had been pre-treated with a pan-caspase peptide inhibitor. Since the activation of caspases usually commits a cell to apoptosis,
Figure 5.11 Detection of the 80kDa protein by αpSer586 is not phosphorylation-dependent

Immunoblots of lysates from control or CuSO₄-treated pMT-rpr cells were incubated in buffer with or without alkaline phosphatase prior to probing with αpSer586 and αCT. Although dAkt could be detected with αCT in both control (A) and phosphatase-treated immunoblots (B), the detection of insulin-stimulated phosphorylated dAkt was abolished following preincubation with alkaline phosphatase. In contrast, the 80kDa protein in lysates from CuSO₄-treated pMT-Rpr cells could still be detected on the phosphatase-treated immunoblot. Note that the stronger bands at 80kDa with αCT in the CuSO₄-treated lysates are due to previous probing with αpSer586.
these latter observations seemed inconsistent with the suggested role of Akt in the promotion of cellular survival. Moreover, the findings also contradicted a previous study that had demonstrated that the phosphorylation and activity of Akt is increased in apoptosing cells in a caspase-independent manner as a mechanism to delay the progression of the cell death programme.

Further investigation ruled out the possibility that the 80kDa protein was p80 dAkt. First, the phosphorylation of a second key residue on dAkt that is required for kinase activity is not increased during apoptosis. Second, The 80kDa protein cannot be immunoprecipitated using antisera raised against different regions of dAkt. Third, and most conclusively, inhibition of dAkt expression does not affect the appearance of the 80kDa band.
## Chapter 6: Discussion

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1 Overview</td>
<td>163</td>
</tr>
<tr>
<td>6.2 αpSer586: to use or not to use?</td>
<td>163</td>
</tr>
<tr>
<td>6.3 Does dRas1 regulate the <em>Drosophila</em> PI3K pathway?</td>
<td>164</td>
</tr>
<tr>
<td>6.4 dMyc - a regulator of cell size and cell death</td>
<td>166</td>
</tr>
<tr>
<td><strong>6.5 p80 and the caspase connection</strong></td>
<td>166</td>
</tr>
<tr>
<td>6.5.1 Caspase substrates</td>
<td>168</td>
</tr>
<tr>
<td>6.5.2 Modulating activity through caspase cleavage</td>
<td>168</td>
</tr>
<tr>
<td>6.5.3 p80: how and why?</td>
<td>171</td>
</tr>
<tr>
<td>6.5.4 The biological significance of p80 in apoptosis</td>
<td>172</td>
</tr>
<tr>
<td>6.5.5 Breaking down a cell's defences</td>
<td>172</td>
</tr>
<tr>
<td><strong>6.6 Summary</strong></td>
<td>174</td>
</tr>
</tbody>
</table>
6.1 Overview

Conclusions of the experiments described in this thesis as well as unresolved issues and links to published works have been addressed and summarised as they arose in the main text and at the end of each chapter. The focus of this chapter, therefore, will be confined to three main issues. First, the utility and potential pitfalls of the phosphorylation-specific dAkt antibodies to monitor insulin/PI3K signalling is discussed. Second, the results obtained from the dRas1 and dMyc clones will be expanded upon and suggestions for informative additional experiments will be made. Lastly, the potential biological significance of the αpSer586 apoptosis-associated signal will be discussed further in the context of the current understanding of the role of caspase-regulated proteins in apoptosis.

6.2 αpSer586: to use or not to use?

The finding that αpSer586 not only recognises phosphorylated dAkt but also cross-reacts with a second 80kDa protein (hereafter referred to as p80) raises serious concerns about the utility of this antibody as a specific and useful reagent for the analysis of Drosophila insulin/PI3K signalling in vitro and in vivo. For example, since the identity and mode of regulation of p80 is unknown, it is possible that the cognate signal in immunostains or immunoblots is not restricted to apoptosis. More generally, it is entirely possible that the antibody may also recognise additional proteins under different physiological conditions or during other cellular processes. Consequently, how confident can one be that a given immunostaining pattern or immunoblot signal with αpSer586 represents phosphorylated dAkt and is not due to cross-reaction with p80 or, indeed, any other cross-reacting protein? It should be noted, however, that the problems that have arisen from the use of αpSer586 were compounded by the fact that the cross-reacting protein has the same molecular weight as one of the intended antigen isoforms. Moreover, very little cross-reactivity was observed at other molecular weights, giving the impression that the antibody had high specificity towards the intended antigen.

Nevertheless, the above points raise the importance of having proper experimental controls as well as emphasising the need for conducting parallel experiments that employ
different approaches to address the same question. With respect to this particular reagent, for example, relatively straightforward techniques can be used to validate observations made with αpSer586, such as parallel analysis with αpThr423 and *in vitro* kinase assays using αCT. In addition, and as demonstrated in this thesis, the identity of the signal can be confirmed by dsRNA-mediated interference of dAkt expression. Moreover, since p80 cannot be immunoprecipitated by αCT, levels of phosphorylated dAkt can be specifically analysed by probing αCT immunoprecipitates with αpSer586, where possible. Alternatively, immunoblot analysis with αpSer586 can be restricted to the more dominant p66-dAkt signal, particularly as this dAkt isoform most closely resembles mammalian Akt since it lacks the amino-terminal extension of the larger *Drosophila* isoform which is also absent in the three mammalian homologs.

Finally, analysis of dAkt phosphorylation during imaginal disc development can be performed *in vivo* with reasonable confidence by immunostaining mitotic clones and comparing the staining pattern within a clone with that of the neighbouring tissue. Differences in phosphorylation could then be confirmed by immunoblot analysis of disc lysates with both αpSer586 and αpThr423. Thus, in summary, the unfortunate cross-reactivity of αpSer586 does not necessarily limit the utility of this reagent but rather stresses the importance of having the necessary controls and a well-planned experimental approach.

### 6.3 Does dRas1 regulate the *Drosophila* PI3K pathway?

Keeping the above points in mind, the elevation in immunostain signal with αpSer586 but not αCT in clones of wing disc cells ectopically expressing dRas1<sup>VI2</sup> certainly warrants further investigation. As discussed above, this observation may be confirmed *in vitro* using immunoblots of lysates from these wing discs, which can then be probed with αpSer586 and possibly αpThr423. An increase in signal in both immunostains and immunoblots would strongly suggest that ectopically expressed dRas1<sup>VI2</sup> can activate the Dp110 pathway *in vivo*. On the other hand, given that no decrease in signal was observed in the dRas1 null clones when immunostained with αpSer586, it is not clear whether endogenous dRas1 *does* activate the Dp110 pathway. However, it is possible that
the assay may not be sufficiently sensitive to detect changes in Dp110 pathway activity as a result of reduced input from dRas1, or that the effects of removing dRas1 on the Dp110 signalling are masked by compensatory mechanisms that may exist in vivo. Furthermore, given the diverse roles of dRas1 signalling in a variety of *Drosophila* developmental processes (Wassarman *et al.*, 1995), it is also possible that endogenous dRas1 may activate the Dp110 pathway in certain cases and not others, perhaps in a cell-type or tissue-specific manner, or at a certain developmental stage. An alternative explanation is that in contrast to ectopically expressed dRas1\textsuperscript{V12}, endogenous dRas1 does not activate the Dp110 pathway. Rather, another Ras-related protein that can also bind the Dp110 RBD may be responsible. Indeed, a number of additional proteins that share significant homology with Ras have been identified in *Drosophila*, such as dRas2, which is most similar to R-Ras (Lowe *et al.*, 1987), and dRas3/Roughened, a homolog of the human Rap1 protein (Hariharan *et al.*, 1991). It is noteworthy that unlike Dp110 and dRas1, neither dRas2 nor dRas3 appear to be involved in the regulation of cell size (Hariharan *et al.*, 1991; Brand and Perrimon, 1993) although this observation does not necessarily indicate that these proteins do not interact with Dp110. However, in contrast to dRas1\textsuperscript{V12} clones, clones of cells ectopically expressing a constitutively active mutant of dRas2 (Brand and Perrimon, 1993), do not show increased dAkt phosphorylation (data not shown). Another potential candidate is the *Drosophila* homolog of Rheb (Ras homolog enriched in brain), a member of the Ras superfamily of GTPases. Interestingly, transgenic flies ectopically expressing Rheb, display an overgrowth phenotype in a number of tissues, including the eye, wing and fat body (Saucedo and Edgar, ADRC 2002 abstract). Moreover, as observed with Dp110, cells ectopically expressing *Drosophila* Rheb are significantly larger than controls. It is possible, therefore, that Rheb may function, at least in part, through the activation of Dp110, perhaps following direct interaction via the RBD.

In conclusion, further characterisation of the wing disc observations described in section 4.1.1 is necessary, perhaps *in vitro* by immunoblotting lysates from Schneider S2 cells ectopically expressing dRas1\textsuperscript{V12}, or cells in which dRas1 expression is blocked following dsRNAi treatment. It should also be noted that the requirement for Dp110 pathway activation in the modulation of imaginal disc growth by dRas1 has not been addressed in these experiments. A possible genetic approach is to analyse cell size in *dp110*.
null mitotic clones following transgenic expression of dRas1\textsuperscript{V12} and vice-versa. An observation that the size of dp110 null clone cells is not increased when generated in combination with ectopic expression of dRas1\textsuperscript{V12} would suggest that Dp110 is necessary for the promotion of growth by dRas1. Furthermore, a parallel observation that the reduced size of dras1\textsuperscript{e40b} clone cells is rescued when generated in combination with ectopic expression of Dp110, would further indicate that the Dp110 pathway is also likely to be sufficient for dRas1-mediated growth.

6.4 dMyc - a regulator of cell size and cell death

Analysis of mosaic discs composed of cell clones ectopically expressing dMyc did not reveal differences in dAkt phosphorylation or protein levels when immunostained with \(\alpha_{p\text{Ser596}}\) or \(\alpha_{\text{CT}}\), respectively. This indicates that dMyc signalling is independent of, or possibly acts downstream of Dp110 and dAkt, as discussed in section 4.1.2. Alternatively, dMyc signalling may converge and interact with the Dp110 pathway at a point downstream of dAkt or modulate the activity of common downstream effectors, such as the translation initiation factor, eIF4E (see sections 1.3.8 and 4.1.1).

The detection of the apoptosis-associated pyknotic structures within dMyc clones immunostained with \(\alpha_{p\text{Ser586}}\) prompted further investigation of the role of dMyc in the regulation of cell death. Consistent with observations in mammalian cell culture studies, staining of dMyc clones with the vital dye acridine orange revealed a significant increase in apoptotic cells within clone regions but not the surrounding tissue, confirming that ectopic expression of dMyc can induce cell death \textit{in vivo}. This further supports the 'dual-signal' model in which Myc signalling is proposed to promote both growth/proliferation and apoptosis, depending on the availability of survival factors and the level of Myc expression (Hueber and Evan, 1998).

6.5 p80 and the caspase connection

The detection of p80 raises questions about the identity and functional/biological relevance of this protein. Several key points can be concluded about p80 from the experiments described in this thesis. First, the detection of this protein in \(\alpha_{p\text{Ser586}}\)
immunoblots correlates with the elevated signal within apoptosing cell bodies in αpSer586 immunostains. This suggests that p80, or at least its detection by αpSer586, is associated with apoptosis. This is further supported by the finding that ectopic expression of pro-apoptotic Reaper results in increased p80 signal. Thus, although it cannot be confirmed that p80 is responsible for the pyknotic signal in αpSer586 immunostains, there is significant evidence to support this. Assuming that the pyknotic signal is p80, a second key observation is that the detection of this protein is associated with apoptosis induced by a variety of factors. These include normal developmental apoptosis, genotoxic apoptosis induced by ectopic expression of the pro-apoptotic mediators dMyc and Reaper or following the loss of expression of anti-apoptotic dRas1, and DNA damage-induced apoptosis resulting from an exogenous insult, namely X-ray irradiation. This observation suggests that the appearance of p80 is associated with a common, fundamental apoptotic process. A third point is that p80 runs at an apparent molecular weight of 80kDa, although its actual molecular weight is unknown and may be significantly different. The fourth observation is that the detection of p80 by αpSer586 can be abolished by a cell-permeant caspase inhibitor peptide, suggesting that this protein is directly or indirectly regulated by caspase activation. Lastly, the detection of p80 by αpSer586 does not appear to be due to a phosphorylation event, although this, of course, does not preclude the possibility that this protein becomes phosphorylated at a different site during apoptosis.

Several attempts were made to purify p80 in order to identify this protein. Initial experiments indicated that p80 could be immunoprecipitated with αpSer586 from pMT-rpr cells following 3 hours CuSO₄ treatment. However, the efficiency of these immunoprecipitations was poor, making it unlikely that sufficient amounts of protein could be purified for identification by mass-spectrometry. Nevertheless, during the remainder of this discussion, I will speculate on the possible nature of the relationship between caspase activity and p80, as well as the potential biological and functional significance of this protein during apoptosis.
6.5.1 Caspase Substrates

Many proteases perform general 'house-keeping' tasks or low level activities, such as lysis of nutritional proteins for cellular uptake, remodelling of the extracellular environment, or the destruction and removal of undesired cytosolic proteins. Specificity in proteolytic function is typically achieved indirectly through the action of selective targeting systems that mark key proteins for subsequent degradation (Page and Hieter, 1999). In contrast, caspases are inherently selective proteases due to their requirement for specific tetrapeptide sequences at cleavage sites, as discussed in section 1.4.2. Indeed, analyses by one and two dimensional gel electrophoresis reveal relatively few changes in the overall pattern of cellular proteins in lysates from cells before and during apoptosis (Kaufmann, 1989; Smith et al., 1992; Robaye et al., 1994; Villa et al., 1994; Amess and Tolkovsky, 1995; Gerner et al., 1998). Moreover, not all proteins containing optimal tetrapeptide sequences are cleaved, implying that tertiary structural elements may influence substrate recognition.

Nevertheless, approximately 100 diverse proteins have been reported to be cleaved during programmed cell death following caspase activation. Interestingly, the cleavage of some substrates is cell-type specific. Actin, for example, is cleaved in U937 leukaemia cells, neurons, and thymocytes but not in other cell types undergoing apoptosis (Mashima et al., 1995; Song et al., 1997a; Villa et al., 1998). Furthermore, a few substrates, such as Topoisomerase I, are reported to be cleaved at different sites in different cell types (Samejima et al., 1999). It should also be noted that since other classes of proteases are also activated during apoptosis, not all of the proteins that are suggested to cleaved are necessarily caspase substrates. Calpain, for example, has been implicated in the cleavage of actin during apoptosis, similar to the ability of caspases to cleave actin in vitro (Villa et al., 1998).

6.5.2 Modulating activity through caspase cleavage

In many cases, the biological consequence of a particular substrate cleavage for subsequent apoptotic events is not clear. Furthermore, some of these cleavage events may be inconsequential to the apoptotic process and simply reflect indiscriminate activity of
caspases towards ‘bystander’ proteins that possess caspase cleavage sites. Nevertheless, in many cases, caspase-mediated proteolysis has been shown to result in diverse effects depending on the nature of the substrate and the exact position of the cleavage site in the primary sequence (a schematic representation is shown in figure 6.1). The simplest and probably most common outcome is loss of biological activity (panels a,b). However, limited proteolysis can also result in the generation of cleaved products that antagonise the full length protein (dominant-negative forms) or the production of activated proteins following the removal of inhibitory domains, subunits or binding partners (panels c,d). Notably, a number of kinases that are proteolysed during apoptosis are cleaved within or adjacent to their regulatory domains, releasing the catalytic domains and resulting in constitutive activity. Moreover, where characterised, ectopic expression of these truncated kinases has been shown to promote apoptosis in cells, whereas expression of catalytically-inactive fragments attenuates cell death. This suggests that these cleavage products have functional importance and may mediate many of the effects resulting from caspase activation. Examples include DAP-kinase (Cohen et al., 1997; Ng, 2002), PAK2 (Rudel and Bokoch, 1997; Walter et al., 1998), MST1/Krs and MST2 (Graves et al., 1998; Lee et al., 1998; Ura et al., 2001), MEKK1 (Cardone et al., 1997; Deak et al., 1998; Widmann et al., 1998a), PKCØ (Ghayur et al., 1996; Cross et al., 2000; Basu et al., 2001), PKCØ (Datta et al., 1997a), PKCβ1 (Shao et al., 1997), and PRK2 (Cryns et al., 1997).

Lastly, the cleavage of a particular protein may have secondary effects on the activity of other molecules that may not be caspase substrates themselves. For example, the activation of cyclin-dependent kinases (Cdks) during apoptosis occurs through three indirect mechanisms. First, caspases cleave and inactivate Wee1, a kinase that phosphorylates and inhibits of Cdk1 and Cdk2 during the interphase of the cell cycle (Zhou et al., 1998). Second, they cleave Cdc27, thereby inactivating the anaphase-promoting complex that normally promotes the degradation of mitotic cyclins (Zhou et al., 1998). Third, the Cdk inhibitors p21^{CIP1/Waf1} and p27^{Kip1} are also cleaved and inactivated (Donato and Perez, 1998; Levkau et al., 1998). Thus, the simultaneous inactivation of these proteins results in a significant upregulation of Cdk activity during apoptosis.
Figure 6.1 Caspase substrates

The outcome of caspase-mediated cleavage on protein activity is variable. See text for description of figure.
6.5.3 p80: how and why?

Given the various outcomes of caspase activation, there are multiple mechanisms by which the p80 signal might appear. For example, the apoptosis-associated signal may result from a direct caspase-induced cleavage of a larger precursor molecule. This cleavage may induce a change in protein conformation, which unMASKS a previously sterically hindered epitope or creates a new, perhaps non-contiguous, epitope that is recognised by the αpSer586 antibody. Arguably, the observation that the signal can be detected on immunoblots of lysates from apoptosing cells but not control cells renders these two possibilities less likely, given the harsh denaturing conditions of the western blotting procedure, during which conformation-dependent epitopes should be destroyed and contiguous epitopes unmasked. However, one intriguing possibility comes from the observation that the last four carboxy-terminal residues (YQGD) of the peptide epitope against which αpSer586 was generated (see section 3.1.1) may be a potential caspase-cleavage recognition site. Thus, αpSer586 may be recognising the carboxy-terminal of a protein fragment that was cleaved by caspases at a site containing YQGD or similar residues. This site may have been sterically hindered or in a different molecular conformation in the full-length protein prior to cleavage by caspases.

Alternatively, it is possible that the apoptosis-associated p80 signal is produced in response to caspase-induced cleavage of another protein whose activity in turn may regulate p80 expression, degradation or activity. Thus, the detection of the signal in apoptotic cells but not control cells may be a consequence of caspase-induced transcription, translation or post-translational modification of p80, although, as shown in section 5.3.6, the appearance of the p80 signal does not appear to be due to phosphorylation.

Although it is most likely that the signal results from a caspase-dependent process, the possibility that the signal is due to the activity of another apoptosis-associated protease cannot be ruled out since the fluoromethylketone caspase inhibitor peptide employed in this thesis may be non-specific at the concentration used. This possibility could be addressed by determining whether the signal is present in immunostains or immunoblots of imaginal discs following ectopic expression of p35, which is a specific caspase inhibitor in vivo. However, as noted in section 1.4.2, not all Drosophila caspases are inhibited by p35 and therefore a false negative observation may be made.
6.5.4 The biological significance of p80 in apoptosis

Although the function of p80 is not known, I would like to give a framework for the possible biological significance of this protein to apoptosis in the context of the current understanding of the role of caspase substrates and their downstream targets in cell death.

6.5.5 Breaking down a cell's defences

Raff and colleagues originally proposed a model in which apoptosis is defined as the default state of metazoan cells, counterbalanced though the activity of survival signals that maintain cellular viability (Raff, 1992; Raff et al., 1993). The nature of these signals is diverse, and may include soluble cytokines, hormones, synaptic connections, and direct physical interactions with neighbouring cells or extracellular matrix. Since the specific requirements of each cell type differs, and the availability of these survival signals is limiting, a precise trophic environment is established within the soma to which cell survivability is normally confined. Consequently, displacement of cells from their trophic support through the course of normal or abnormal development, malignancy, or injury, results in their spontaneous destruction and subsequent elimination.

Thus, the cell must monitor and maintain the attachment of its cytoskeleton to these trophic supports, for example through integrin- and cadherin-mediated signalling mechanisms (Raff, 1992). Conversely, in order for apoptosis to progress from a stage of initiation to a phase of execution, caspases and other proteases must target and inactivate the various mechanisms by which cellular survival and homeostasis is promoted. As discussed in section 1.4.6, both the Ras/ERK/MAPK pathway and the PI3K/Akt pathway are proposed to be key mediators of cellular survival. Consistently, both Akt and Raf-1 are cleaved and inactivated during apoptosis (Widmann et al., 1998b; see also section 5.3.4). Similarly, other kinases that are also proposed to promote cell survival, such as focal adhesion kinase and Cbl, are also cleaved and inactivated (Wen et al., 1997; Widmann et al., 1998b). Moreover, caspases can also indirectly downregulate the activity of a number of kinases by activating protein phosphatase 2A through the cleavage of its Aα regulatory subunit (Santoro et al., 1998). In addition, a number of transcription factors that have been
implicated in survival responses, such as NFkB, are also inactivated following caspase-mediated proteolysis (Ravi et al., 1998).

In addition to dismantling protective cellular mechanisms, the activity of proteins that positively regulate pathways that normally promote cell death is potentiated, resulting in positive feedback to the apoptotic program. For example, the cleavage products of three kinases, MEKK1, PAK2, and MST1, may promote apoptosis through the activation of the pro-apoptotic SAPK/JNK (stress-activated protein kinase/Jun N-terminal kinase) pathway (Cardone et al., 1997; Deak et al., 1998; Graves et al., 1998). The sequential activation of c-Jun may contribute to the induction of FasL, which in turn may participate in a positive feedback loop to promote cell death under conditions of cellular stress (Suhara et al., 2002). Similarly, caspase-mediated proteolysis of MDM2 and retinoblastoma protein may result in the enhancement of apoptotic p53 signalling (Pochampally et al., 1999).

Lastly, caspases and other apoptosis-induced proteases can directly mediate cellular disassembly though the proteolysis of a number of cytoskeletal and nuclear molecules. These cleavages may give rise to the characteristic morphological changes that occur during the final stages of apoptosis such as cell contraction and dynamic membrane blebbing, as well as nuclear and cytoplasmic pyknosis. Structural proteins that are cleaved include actin, α-fodrin, paxillin, and cytokeratin 18 (Ku et al., 1997; Wang et al., 1998; Mashima et al., 1999). Cleavage of components of the adherens junctions such as β-catenin and plakoglobin (Herren et al., 1998) may facilitate the detachment of apoptotic cells from their neighbours. Major structural proteins of the nucleus, including the lamins (Rao et al., 1996) and NuMA (Hirata et al., 1998), are also cleaved, which may be necessary for disassembly of the nucleus into membrane-enclosed vesicles.

In addition to direct proteolysis of cytoskeletal proteins, caspases also indirectly promote cellular disassembly through the cleavage and activation of a number of key substrates. For example, ectopic expression of the caspase-mediated cleavage products of gelsolin, and RasGAP results in impairment of the actin cytoskeleton, cell rounding, and detachment from the sub-stratum (Wen et al., 1998; Yang and Widmann, 2001). Similarly, caspase-3 mediated cleavage of Rho-activated serine/threonine kinase, ROCK I, generates a constitutively active truncated kinase that is both necessary and sufficient for the formation of membrane blebs and the translocation of fragmented DNA into blebs and
apoptotic bodies (Coleman et al., 2001; Sebbagh et al., 2001). Interestingly, the cleavage product of PKCδ may also play an important role in the disassembly of the nuclear lamina during apoptosis, perhaps by hyperphosphorylating lamin B (Cross et al., 2000).

Further caspase-mediated proteolytic activity may be generated through the cleavage of ICAD/DFF45 (Liu et al., 1997) and the resulting activation of the CAD/DFF40 nuclease (Enari et al., 1998). This enzyme, in turn, cleaves chromosomal DNA at internucleosomal spacers in vitro giving rise to the condensed nuclear morphology and DNA fragmentation characteristic of apoptosis (although other proteases are also likely to be involved in vivo; see Samejima et al., 1998).

### 6.6 Summary

As outlined above, the activation of caspases does not result in the wholesale degradation of cellular proteins but rather the selected cleavage of a restricted set of targets. Furthermore, these cleavage events have varying effects on protein function, typically resulting in the inactivation or degradation of proteins that are necessary for maintaining cell viability and homeostasis. In certain cases, however, caspase activation may directly or indirectly result in increased biological activity of a protein, serving to disrupt cellular survival mechanisms, to potentiate pro-apoptotic pathways, or to affect cellular disassembly. Although the identity of p80 remains elusive, it is likely that this protein is directly or indirectly involved in the execution phase of apoptosis via one of these processes.
Acknowledgements

I would like to thank Hugo Stocker (Zurich University, Switzerland), Laura Johnston (Columbia University, New York, USA), David Prober (Fred Hutchinson CRC, Seattle, USA) and the Bloomington Drosophila stock centre (Indiana, USA) for providing me with fly stocks. Special thanks to Pascal Meier (Institute for Cancer Research, London, UK) and Kristin White (Harvard Medical School, Massachusetts, USA) for the pMT-rpr cells and for tips and advice on apoptosis. Thanks also to Natalie Franc (LMCB, London, UK) for the Croquemort antibody and Brian Hemmings (Friedrich Miescher Institute, Basel, Switzerland) for the dAkt antibodies. Special thanks to Mary Collins (Winder Institute, London, UK) for allowing me to use her X-ray cabinet and Derek Davies (Cancer Research UK, London) for help with the FACS analysis.

I would also like to thank my friends and colleagues at the Ludwig Institute for Cancer Research (London, UK), and Cancer Research UK (London) including Steven Marygold and Bart Vanhaesebroeck for critically reading my thesis, Richard Foxon and Rob Stein for lending reagents and lab supplies, Alan Entwistle for microscopy guidance, and Heather Mills for preparing fly food.

The following people need to be thanked on a more personal note: Bart Vanhaesebroeck for his critical reading of my thesis, scientific advice and good humour, Rob Stein for his advice and wisdom, and Mike Waterfield for co-supervising me and for his help and guidance.

I would especially like to thank the Growth Regulation Lab (Cancer Research UK), Krishna Vaghela, Ben Kolevski, Steven Marygold, Gemma Bradley and Carmen Coelho, together with Kelly Nikolaidou and Kathy Barrett for their friendship, company and support and for making my research a much more enjoyable experience.

I am especially grateful to my supervisor Sally Levers (Cancer Research UK) for allowing me to work in her lab, for the fantastic scientific training, inspiring enthusiasm, and for her expert advice on the content and format of this thesis. I also thank her for being a good friend.

Finally, I cannot express enough my gratitude for the unending love and support from my mother, father and sister who have contributed in many ways and to great lengths towards my education.


References


References


183
References


References


References


References


References


References


References


Moreno, E., Yan, M., and Basler, K. (2002). Evolution of TNF Signaling Mechanisms; JNK-Dependent Apoptosis Triggered by Eiger, the Drosophila Homolog of the TNF Superfamily. *Current Biology* 12, 1263-1268.


References


References


References


202
References


References


References


