THE UNIVERSITY OF CALGARY

# Patterns of PKC53E Protein Expression in *Drosophila melanogaster*

by

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A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

CALGARY, ALBERTA

JULY, 1997

# Abstract

Protein kinase C isoenzymes belong to the superfamily of serine/threonine kinases. Many isoenzymes have been isolated in mammals, and homologues have been found in lower eukaryotes. Three PKC genes have been isolated in *Drosophila melanogaster*; *Pkc53E*, *inaC*, and *Pkc98E*. *Pkc53E* is differentially expressed throughout various stages of development. The predicted ORF from the published cDNA, is 75kDa. Anti-PKC53E antibodies were made against a unique peptide from the N-terminus of PKC53E. A band of 75kDa was visualized on Western blots with adult head PKC-rich extracts. A band of similar size was visualized in all three larval stages. In early and mid-pupal stages, a 70kDa band was visualized, however, the 75kDa band was seen again in late pupal stages. Immunohistochemical staining detected protein in neural regions of stage 12 embryos. In late 3rd larval instars, protein was detected in the brain, and in salivary glands.

# Acknowledgements

I would like to thank everyone who helped me throughout my degree. I would like to thank Dr. M.M. Bentley for allowing me the opportunity to learn how to perform research in molecular biology. I would like to thank my committee for their time and effort, in particular, Dr. M. Lohka for his generosity and kindness. This thanks is extended to his lab members for their patience, and help.

I would like to thank the other members of the lab; Renuka, and Kent. Although they all left before me, I thank them for their guidance and support while they were here. I would also like to thank Lauryl for the hours of listening, understanding, and troubleshooting.

I would like to thank my family for their support. They were always here with me in spirit, despite the distance. Thanks especially to my Mom and Bob, for their understanding, and Art, Kirsten, and Elena for their brief interludes of entertainment.

I would also like to thank my acquaintances and friends. They have all made Calgary the wonderful experience it was. In particular I would like to thank Dianne for her support by phone. I would also like to thank Tod and Tanya, for their friendship, and the many 'Friends' nights. Thanks to Heather for the stress relief near the end. I would also like to thank Chris and Catherine, who were my 'family' here in Calgary, and the other part of the 'Friends' nights. I would like to specially thank the host of the 'Friends' nights, Julian, not only for the countless hours of computer help, but for his companionship.

I was supported by the Department of Biological Sciences throughout my studies.

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# Abbreviations

| | |
|---|---|
| ADP | adenosine 5'-diphosphate |
| AP | alkaline phosphatase |
| AMP | ampicillin |
| aPKC | atypical Protein kinase C family |
| APS | ammonium persulfate |
| ATP | adenosine 5'-triphosphate |
| BCIP | 5-bromo-4-chloro-3-indolyl phosphate |
| BLAST | Basic Local Alignment Search Tool |
| *Br-C* | *Broad-complex* genes |
| BSA | bovine serum albumin |
| C# | constant (conserved) domains |
| $Ca^{2+}$ | calcium ion |
| CaM | calmodulin |
| cDNA | DNA derived from RT'd RNA |
| CNS | central nervous system |
| cPKC | classical Protein kinase C family |
| Cys | cysteine |
| DAG | 1,2-*sn*- diacylglycerol |
| DNA | deoxyribonucleic acid |
| DNAse | deoxyribonuclease |
| dNTPs | deoxyribonucleoside triphosphates |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid |
| EMS | ethyl methanesulfonate |
| ER | endoplasmic reticulum |
| FITC | fluorescein isothiocyanate |
| g | grams |
| xg | gravitational force |
| Go/Gq | heterotrimeric G protein (o and/or q family) |
| GAP | GTPase-activating protein |
| GAP-43 | growth associated protein 43 |
| GDP | guanosine 5'-diphosphate |

| | |
|---|---|
| GET | glucose, EDTA, Tris buffer |
| GS | 1,3-β-glucan synthase complex |
| GST | glutathione S-transferase |
| GTP | guanosine 5'-triphosphate |
| *hid* | *head involution defective* gene |
| ICE | interleukin-1β-converting enzyme |
| *inaC* | *inactivation-no-afterpotential C* |
| *inaD* | *inactivation-no-afterpotential D* |
| IPTG | isopropyl β-D-thiogalactoside |
| $IP_3$ | inositol-1,4,5-trisphosphate |
| kb | kilobase pairs |
| kDa | kilodaltons |
| L | litre |
| *Lam* | *Lamin* gene |
| LB | Luria broth |
| M | molar |
| MAPK | mitogen activated protein kinase(s) |
| MARCKS | myristoylated alanine-rich C kinase substrate |
| MCS | multiple cloning site |
| MEK | MAP kinase kinase |
| MEKK | MAP kinase kinase kinase |
| mL | millilitre |
| mM | millimolar |
| MOPS | morpholinopropanesulphonic acid |
| NBT | nitro blue tetrazolium chloride |
| NCBI | National Centre Biotechnology Information |
| NMR | nuclear magnetic resonance imaging |
| nPKC | novel Protein kinase C family |
| *norpA* | *no-receptor potential A* |
| OR | Oregon-R wild type stock |
| ORF | open reading frame |
| PA | phosphatidic acid |
| PBS | phosphate buffered saline |
| PBT | phosphate buffered saline, Triton X-100 |

| | |
|---|---|
| PBTB | phosphate buffered saline, Triton X-100, BSA |
| PC | phosphatidylcholine |
| PCR | polymerase chain reaction |
| PDGF | platelet derived growth factor |
| PDGFR | platelet derived growth factor receptor |
| PEM | PIPES, EDTA, magnesium sulfate |
| PF | paraformaldehyde |
| PI | phosphatidylinositol |
| PI3-kinase | phosphatidylinositol 3-kinase |
| $PIP_2$ | phosphatidylinositol-4,5-bisphosphate |
| PIPES | Piperazine-N,N'-bis[2-ethane-sulfonic acid] |
| PKA | cAMP-dependent protein kinase |
| *Pkc53E* | *protein kinase C 53E* gene |
| *Pkc98E* | *protein kinase C 98E* gene |
| PKC | protein kinase C |
| PKM | catalytically active fragment of PKC |
| $PLA_2$ | phospholipase $A_2$ |
| PLC | phospholipase C |
| PLD | phospholipase D |
| PM | plasma membrane |
| PMA | phorbol 12-myristate 13-acetate |
| PMSF | phenylmethyl-sulfonyl fluoride |
| PNS | peripheral nervous system |
| Pro | proline |
| PS | phosphatidylserine |
| PSD | phosphorylation site domain |
| PTKR | protein tyrosine kinase receptor |
| R | receptor (types; x,i,s,$IP_3$) |
| RACE | rapid amplification of cDNA ends |
| RACKS | receptors for activated C kinases |
| *rdgB* | *retinal degeneration B* gene |
| Rh | rhodopsin |
| RNA | ribonucleic acids |
| RNAse | ribonuclease |
| RTemp | room temperature |

| | |
|---|---|
| *rpr* | *reaper* gene |
| RT | reverse-transcribed |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| Ser | serine |
| SH2 domain | src-homology 2 domain |
| SH3 domain | src-homology 3 domain |
| SOCs | store-operated ion channels |
| SOS | son-of-sevenless |
| TAE | Tris-acetate-EDTA buffer |
| TBS | Tris-buffered saline |
| TBST | Tris-buffered saline, Tween-20 |
| TE | Tris-EDTA buffer |
| TEMED | tetramethylene-diamine |
| Thr | threonine |
| TPA | 12-O-tetradecanoylphorbol-13-acetate |
| TR | Texas red |
| Tris | tris-(hydroxymethyl)-aminomethane |
| *trp* | *transient receptor potential* gene |
| Tyr | tyrosine |
| μg | micrograms |
| μL | microlitre |
| V# | variable domains |
| v/v | volume/volume |
| w/v | weight/volume |

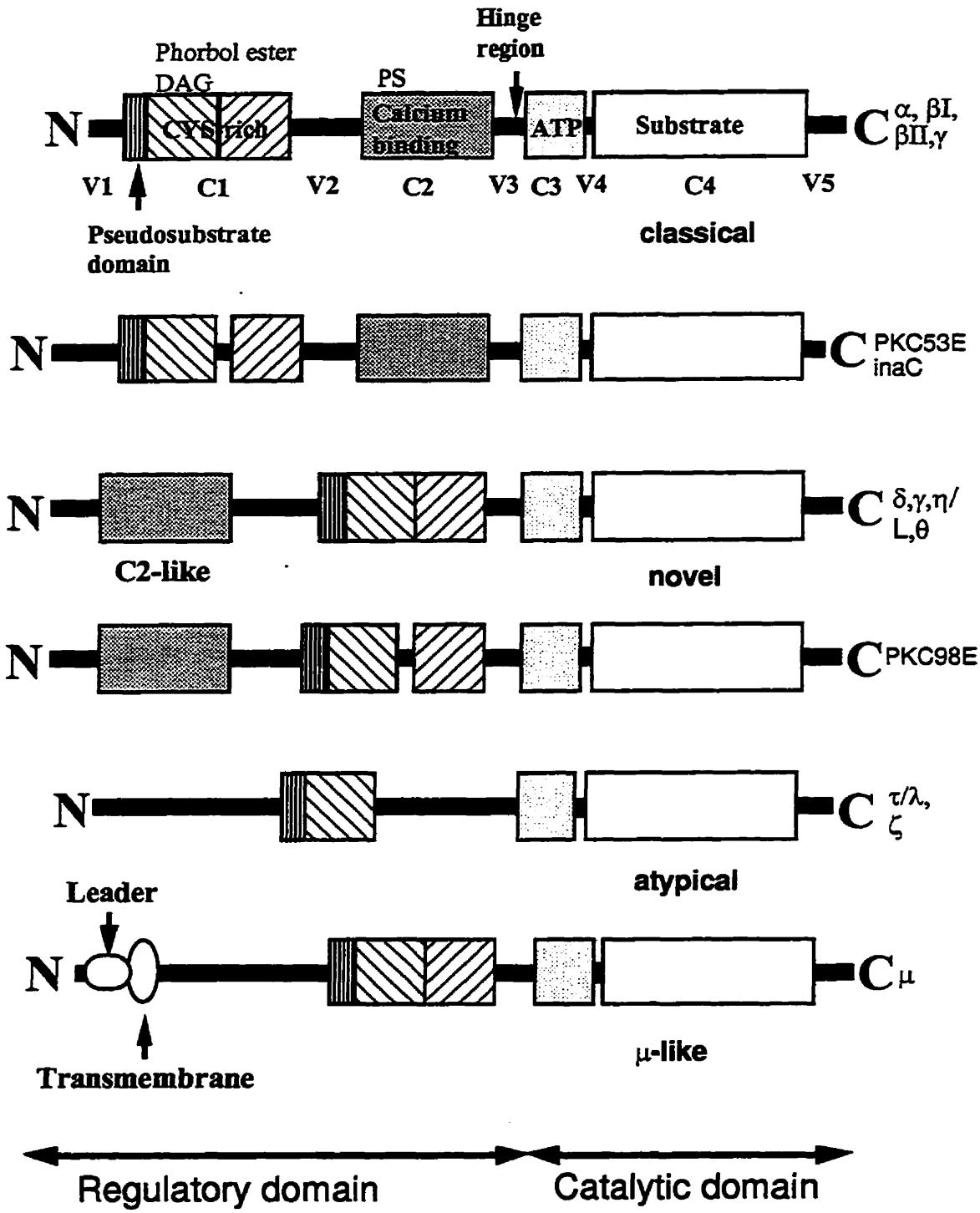
# Chapter 1
# Introduction

Protein kinase C (PKC) comprises a family of kinases, which belong to the superfamily of serine/threonine kinases (Nishizuka, 1986; Nishizuka, 1992). At least 11 mammalian isoenzymes have been identified, and several homologues have been found in lower eukaryotes such as *Drosophila melanogaster* (*D. melanogaster* ), and *Saccharomyces cerevisiae* (*S.cerevisiae*; Newton, 1995; Nishizuka, 1995; Walsh *et al.*, 1996; Schaeffer *et al.*, 1989; Levin *et al.*, 1990). These isoenzymes have been further classified according to their structure as classical, novel, or atypical isoforms. Three *Pkc* genes have been identified in *D. melanogaster*; *Pkc53E*, *inactivation-no-afterpotential C* (*inaC* ), and *Pkc98E* (Rosenthal *et al.*, 1987; Schaeffer *et al.*, 1989). The putative polypeptides encoded by *Pkc53E* and *inaC* have structural similarities with the classical isoenzymes, whereas the putative polypeptide encoded by *Pkc98E* is classified as a novel isoenzyme (Rosenthal *et al.*, 1987; Schaeffer *et al.*, 1989).

## 1.1 PKC Structure

Structurally, the PKC isoenzymes, shown in Figure 1.1, can be divided into two regions; the regulatory region comprising the N-terminal half, and the catalytic region comprising the C-terminal half. The regulatory region interacts with a variety of cofactors required for activation, such as diacylglycerol (DAG), phosphatidylserine (PS), and calcium ($Ca^{2+}$), depending on the isoenzyme. Phorbol esters have been shown to mimic DAG and activate some of the PKC isoenzymes. The catalytic region, which tends to be highly conserved among different isoenzymes within the same species, and between species, contains the adenosine 5'-triphosphate (ATP)-binding site, substrate-binding sites,

Figure 1.1: Structure of the mammalian PKC isoenzymes, and the *D. melanogaster* putative homologues. The cPKCs have four conserved regions, C1, C2, C3 and C4. C1 is composed of two Cys-rich repeats and binds phorbol esters and DAG. C2 binds $Ca^{2+}$ and PS, C3 is the ATP-binding domain, and C4 is the substrate-binding domain. The pseudosubstrate domain is found in the N-terminal portion of C1. Separating the conserved regions are variable domains, V1, V2, V3, V4 and V5, which vary in sequence from one isoenzyme to the next. PKC53E, and inaC are similar to the cPKCs with the exception of a V1' region between the two Cys-rich repeats. The nPKCs are similar to the cPKCs with the exception of a C2-like region, instead of a C2 region. PKC98E is similar to the nPKCs. The aPKCs lack C2, and one of the Cys-rich repeats in C1. *D. melanogaster* isoenzymes with similarity to the aPKCs have not been identified. PKCμ has a transmembrane domain, and leader sequence in the N-terminus of the protein. The rest of the protein is similar to the nPKCs, with two Cys-rich repeats and a C2-like domain. There are no known *D. melanogaster* homologues of this type of isoenzyme (Rosenthal *et al.*, 1987; Schaeffer *et al.*, 1989; Flybase, 1997; Newton, 1995; Nishizuka, 1995; Walsh *et al.*, 1996).


Hinge region
Phorbol ester
DAG
PS
N
CYS rich
Calcium binding
ATP
Substrate
C
α, βI, βII,γ
V1
C1
V2
C2
V3
C3
V4
C4
V5
Pseudosubstrate domain
classical
N
C
PKC53E
inaC
N
C
δ,γ,η/ L,θ
C2-like
novel
N
C
PKC98E
N
C
τ/λ, ζ
atypical
Leader
N
Cμ
Transmembrane
μ-like
Regulatory domain
Catalytic domain

and kinase activity. A hinge region separates the two halves, and is sensitive to proteolytic cleavage, causing the release of a catalytically active fragment (PKM). The protein is rendered inactive when in a folded conformation at this hinge region. PKC isoenzymes have a pseudosubstrate domain in their N-terminus, and the amino acid sequence of this domain varies between classes of isoenzymes and between species. The pseudosubstrate sequence for the mammalian PKCα isoenzyme, RFARKGALRQKNVHEVKN, shares some of the same amino acids (shown in bold) with the *D. melanogaster* putative homologue PKC53E. The pseudosubstrate sequence in all PKC isoenzymes resembles a consensus PKC phosphorylation site (K/RX$_{1/2}$S/TXK/R). A serine/threonine to alanine substitution, however, makes it suitable for interaction with the substrate-binding domain in the catalytic region without phosphorylation. The production of specific cofactors required for activation causes the protein to unfold and to translocate to either the plasma membrane, or to the membrane of an organelle within the cell. Although PKC isoenzymes have been shown to interact with a variety of phospholipids at the membrane, they have also been demonstrated to bind anchoring proteins known as receptors for activated C-kinases (RACKs) (Newton, 1995; Nishizuka, 1995; Walsh *et al.*, 1996; Ron *et al.*, 1995; Ron and Mochly-Rosen, 1995; Mochly-Rosen *et al.*, 1991).

PKC isoenzymes require several post-translational modification events prior to obtaining a mature form. Upon initial translation, they are associated with the detergent-insoluble fraction of cells, and are several kDa lower in molecular weight. The first phosphorylation event occurs in the C-terminus, at Thr-500 in the mammalian βII isoenzyme, by a putative PKC kinase. It is postulated this phosphorylation event is necessary to alter the conformation of the catalytic portion of the protein. This is followed by an autophosphorylation event in the C-terminus, at Thr-641 in the mammalian βII isoenzyme, which stabilizes the catalytic conformation. This is followed by another autophosphorylation event in the C-terminus, at Ser-660 in the mammalian βII isoenzyme,

resulting in the mature detergent-soluble isoenzyme (Newton, 1995; Nishizuka, 1995; Walsh *et al.*, 1996).

The family of PKC isoenzymes is separated into classes, according to differences in their regulatory regions. There are four classes to date; classical (c), novel (n), atypical (a) and μ-like. The three *Pkc* genes in *D. melanogaster*, encode proteins with putative homology to mammalian c and nPKCs. The C-terminus, or catalytic region, is very well conserved among the classes of isoenzymes, and between species (Newton, 1995; Nishizuka, 1995; Walsh *et al.*, 1996; Ron *et al.*, 1995; Ron and Mochly-Rosen, 1995; Mochly-Rosen *et al.*, 1991).

The mammalian classical PKC isoenzymes (cPKCs); α, βI, βII, and γ, have four conserved regions, with five variable regions interspersed between them. Figure 1.1 shows a schematic of the structures of the various groups of PKC isoenzymes and the *D. melanogaster* putative homologues. The structure of these isoenzymes is very well conserved, except C1 has been divided into C1 and C1' in the *D. melanogaster* putative homologues putatively encoded by the genes *Pkc53E* and *inaC*. The first conserved region (C1) consists of two Cys-rich repeats, and each repeat binds two $Zn^{2+}$ ions. This forms a $Zn^{2+}$ butterfly structure, unlike the $Zn^{2+}$ fingers most commonly found in DNA-binding proteins (Newton, 1995). The C1 region has been demonstrated to bind DAG and phorbol esters. The crystal structure and NMR, of the second Cys-rich repeat, from mammalian PKCδ and α respectively, showed that phorbol esters can bind without altering the conformation of this region. Hydrophobic residues and a string of basic amino acids become exposed on the surface, which increase the affinity of the protein for various components of the membrane, such as lipids, and the polar headgroups of phospholipids. At the N-terminus of C1, is the pseudosubstrate domain, which is important for maintaining the protein in an inactive conformation in the absence of substrates. The

presence of activating cofactors causes the protein to unfold and translocate to the membrane (Newton, 1995; Nishizuka, 1995; Walsh *et al.*, 1996).

The second conserved region (C2) is the $Ca^{2+}$-binding domain, and as mentioned above, has also been postulated to interact with PS. The crystal structure of a similar region in synaptotagmin showed a core of five aspartate residues, important in maintaining the folded region postulated to interact with $Ca^{2+}$. Binding $Ca^{2+}$ alters the conformation of the core binding site within C2, shifting the positions of aromatic amino acids for easier interaction with nonpolar components of the membrane. This also alters the position of basic amino acids which can interact more easily with the polar headgroups of phosopholipids such as phosphatidylserine (Newton, 1995; Nishizuka, 1995; Walsh *et al.*, 1996).

The third and fourth conserved regions (C3 and C4, respectively) have been shown to interact with ATP and substrates, respectively. These regions are highly conserved among the different groups of PKC isoenzymes. PKC isoenzymes have cofactor and substrate-dependent ATPase activity, catalyzing a phospho-transfer event from ATP to the bound protein. They have also been demonstrated to have phosphatase activity, in the presence of high ADP concentrations (Newton, 1995; Nishizuka, 1995; Walsh *et al.*, 1996).

Interspersed among the conserved regions, are five variable regions, V1, V2, V3, V4 and V5, which vary considerably in their sequence between isoenzymes and between species. The *D. melanogaster* homologues also contain these regions, except they have an additional region between C1 and C1', referred to as V1'. These regions do not appear to confer any substrate or cofactor specificity. The third variable region, V3, is the hinge region described earlier. It has been demonstrated to be sensitive to proteolytic cleavage, by enzymes such as calpain I and II (Newton, 1995; Nishizuka, 1995; Walsh *et al.*, 1996).

The mammalian novel isoenzymes (nPKCs); δ, ε, η/L, and θ, also have four conserved regions, and the five variable regions as discussed above. This is shown in Figure 1.1, with the schematics of all groups of PKC isoenzymes, and the *D. melanogaster* homologues. PKC98E has highest amino acid sequence homology to mammalian PKCε. The nPKCs are $Ca^{2+}$-independent in their requirements for cofactors of activation. These isoenzymes have a C2-like region, however, it is postulated that two of the Asp amino acid residues are altered, one from Asp to Arg, in the core $Ca^{2+}$-binding site (Newton, 1995; Walsh *et al.*, 1996). This alters the conformation of this region, rendering it in an 'active' state, and mimics the effect of $Ca^{2+}$-binding in the C2 domain in cPKCs. Therefore, this region already has an increased affinity for the polar head groups of phospholipids, and lipid components of the membrane, and no longer requires $Ca^{2+}$. Mammalian PKCδ has a property unique from the other PKC isoenzymes, as the V3 region of PKCδ was found to be sensitive to proteolytic cleavage by ICE-like proteases (Newton, 1995; Nishizuka, 1995; Emoto *et al.*, 1995; Walsh *et al.*, 1996).

The mammalian atypical isoenzymes (aPKCs); ζ and λ/τ, are quite different structurally from the other PKC isoenzymes in their regulatory regions. They do not contain a C2 or C2-like domain, and lack one of the Cys-rich repeats from C1. These are shown schematically in Figure 1.1. There are no known *D. melanogaster Pkc* genes encoding homologues of these isoenzymes. It has been shown that these isoenzymes have reduced or no responsiveness to phorbol esters, and do not require $Ca^{2+}$ for activation (Newton, 1995; Nishizuka, 1995; Walsh *et al.*, 1996).

Another mammalian isoenzyme, PKCμ, is structurally different from the other classes of isoenzymes, and is shown in Figure 1.1. This isoenzyme is not homologous to any known *D. melanogaster* isoenzymes. It has a leader sequence, and transmembrane domain at the N-terminus. The regulatory region has amino acid sequence similarity to the

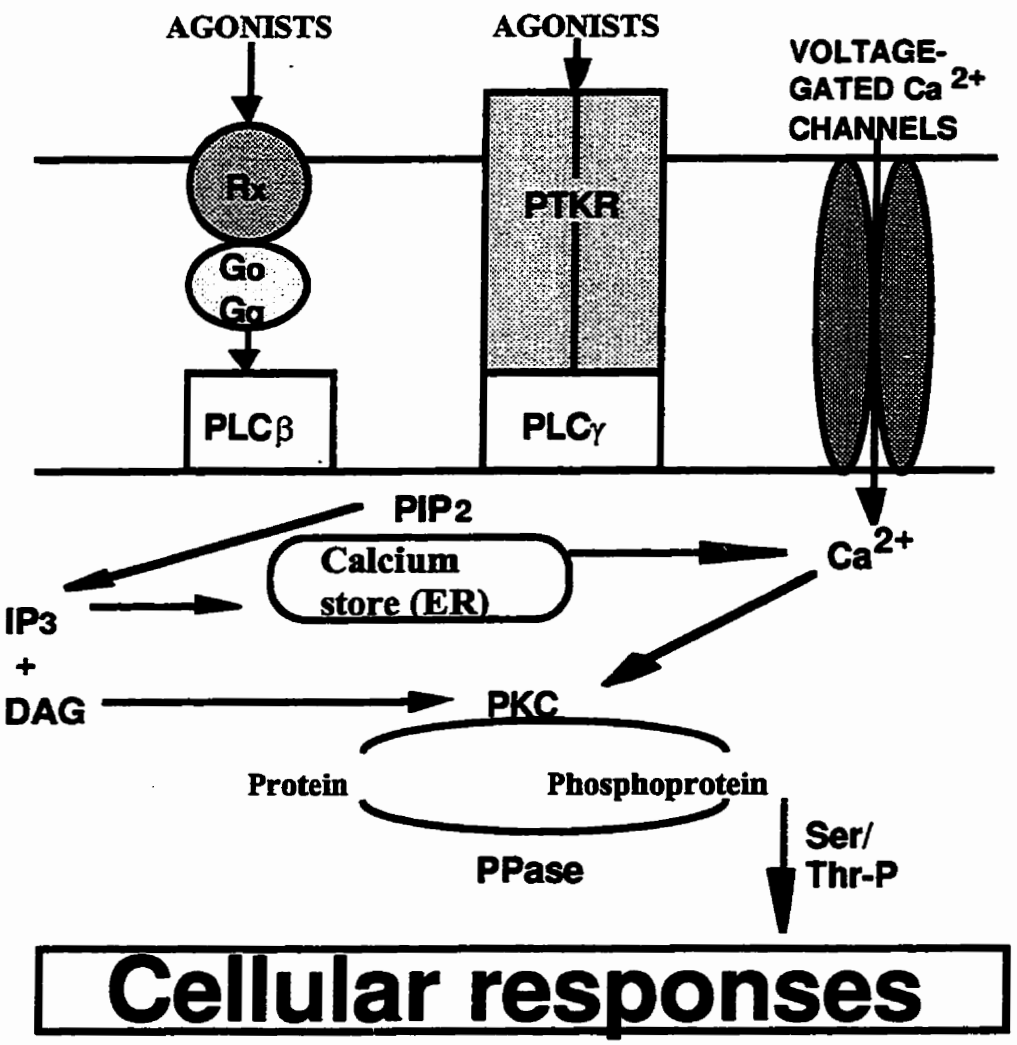
nPKCs, as it has a C1 domain with two Cys-rich repeats, a C2-like domain. The catalytic domain has amino acid sequence similarity to the $Ca^{2+}$-dependent kinases (Newton, 1995; Nishizuka, 1995; Walsh *et al.*, 1996).

## 1.2 PKC in Signal Transduction

PKC isoenzymes are involved in signal transduction pathways leading to a variety of cellular responses such as proliferation, differentiation, learning and light adaptation (Tanaka and Nishizuka, 1994; Buchner, 1995; Zuker, 1996). One of the most characterized pathways mediating classical and novel PKC activation, is through heterotrimeric G protein-coupled seven transmembrane domain receptors, such as muscarinic, serotonergic and adrenergic receptors, shown in Figure 1.2. These receptors typically undergo a conformational change upon ligand binding, resulting in the activation of downstream signaling components. The signal is transduced through heterotrimeric G proteins, composed of three subunits; $\alpha$, $\beta$ and $\gamma$, and the $\alpha$ and $\gamma$ subunits have modified tails which allow them to anchor to the membrane. When the $\alpha$ subunit is in its inactive state, it is bound to guanosine 5'-diphosphate (GDP), and remains associated with the other two subunits. In the presence of guanosine 5'-triphosphate (GTP), the $\alpha$ subunit will exchange GTP for GDP, and dissociate from the $\beta$ and $\gamma$ subunits. The $\alpha$ or $\beta\gamma$ subunits will then interact with downstream effectors, depending on the family of G protein. The $\alpha$ subunit has intrinsic GTPase activity, and after time, will hydrolyze GTP to GDP and inorganic phosphate, causing the subunits to reassociate (Cockcroft and Thomas, 1992; Berridge, 1989; Berridge, 1993; Hug and Sarre, 1993; Neer, 1995).

Phospholipase C$\beta$ (PLC$\beta$) is one of the downstream effectors activated by the $G_q$ or $G_o$ family of G proteins. The *D. melanogaster* homologue, encoded by the *no-receptor-potential* (*norpA* ) gene, will be discussed later in this chapter (Smith *et al.*, 1991; Clapham, 1996; Zuker, 1996). PLC$\beta$ hydrolyzes the substrate phosphatidylinositol-4,5-

Figure 1.2: A schematic of PKC signaling pathways. One of the primary pathways is through heterotrimeric G protein-mediated seven transmembrane receptors. An agonist activates the receptor, which in turn activates the associated heterotrimeric G protein. Upon activation, the Gα subunit dissociates from the βγ subunits, and either α or βγ will activate the downstream effector, PLCβ. PLCβ activation stimulates the hydrolysis of $PIP_2$ to $IP_3$ and DAG. $IP_3$ causes the release of $Ca^{2+}$ ions from internal stores such as the endoplasmic reticulum (ER). DAG activates nPKCs, and $Ca^{2+}$ and DAG activates cPKCs. aPKCs are constitutively active, and do not require cofactors for activation. $Ca^{2+}$ channels in the plasma membrane are stimulated by the depletion of $Ca^{2+}$ ions from internal stores. Upon activation, cPKCs and nPKCs are translocated to the plasma membrane, where they associate with docking proteins, and phosphorylate substrates. PKC can alter gene expression, which may be important for crucial events such as proliferation, or differentiation. PKC activation can also occur through tyrosine kinases (receptor and non-receptor forms). Upon ligand binding, receptor tyrosine kinases interact with SH2 domain-containing proteins, initiating signaling in several possible pathways. PLCγ has an SH2 domain, and can be recruited to these receptors. As described earlier, it hydrolyzes $PIP_2$, to produce DAG and $IP_3$, leading to PKC activation. There are several phospholipids in the plasma membrane which can be hydrolyzed, and other phospholipases may be involved in these pathways (Hug and Sarre, 1993; Fantl *et al.*, 1993; Newton, 1995; Nishizuka, 1995; Walsh *et al.*, 1996). Abbreviations; phosphatase (PPase), seven transmembrane domain (7TM) receptor associated with Go or Gq (Rx), heterotrimeric G proteins (G), phospholipase Cβ (PLCβ), phosphatidylinositol-4,5-bisphosphate ($PIP_2$), diacylglycerol (DAG), inositol-1,4,5-trisphosphate ($IP_3$), protein tyrosine kinase receptor (PTKR), phospholipase Cγ (PLCγ), endoplasmic reticulum (ER), and protein kinase C (PKC).


AGONISTS
AGONISTS
VOLTAGE-
GATED Ca 2+
CHANNELS
Rx
Go
Gq
PTKR
PLCβ
PLCγ
PIP2
Calcium
store (ER)
Ca2+
IP3
+
DAG
PKC
Protein
Phosphoprotein
PPase
Ser/
Thr-P
Cellular responses

bisphosphate ($PIP_2$), to produce inositol-1,4,5-trisphosphate ($IP_3$), and diacylglycerol (DAG). This results in a surge of DAG, required for initial cPKC and nPKC activation. aPKCs do not require cofactors for activation, and are most likely constitutively active. $IP_3$ binds to receptors in the membranes of internal $Ca^{2+}$ stores, resulting in an influx of $Ca^{2+}$, and a localized increase in $Ca^{2+}$ ions. $Ca^{2+}$ ions are also required as a cofactor for cPKC activation. Prolonged signaling is enhanced by further $Ca^{2+}$ release through $Ca^{2+}$-induced $Ca^{2+}$ channels in the plasma membrane. A cation ($Ca^{2+}$) channel encoded by the *transient receptor potential* (*trp* ) gene in *D. melanogaster*, is required for $Ca^{2+}$ influx from the extracellular space, and is essential for repeated signaling of inaC (Hardie *et al.*, 1993; Clapham, 1996; Zuker, 1996). *trp* mutants were found to have decreased PKC activity, and a phenotype similar to the *inaC* mutant phenotype (Hardie *et al.*, 1993). There are multiple channels involved in regulating the transient $Ca^{2+}$ concentration, and a mutant in any one of these proteins could disrupt further PKC activation (Berridge, 1989; Berridge, 1993; Hug and Sarre, 1993; Clapham, 1996; Nishizuka, 1992; Nishizuka, 1995; Walsh *et al.*, 1996).

Other phospholipases hydrolyze different phospholipid components of the PM, and produce cofactors for cPKC and nPKC activation, or potentiate activation by DAG. Phospholipase D (PLD) hydrolyzes phosphatidylcholine (PC), resulting in the release of phosphatidic acid (PA) and choline, the former of which is converted to DAG. Phospholipase $A_2$ ($PLA_2$) also hydrolyzes PC, producing *cis* -unsaturated free fatty acids, and lysoPC, which potentiate the DAG response. DAG production by hydrolysis of PC is more sustained, and results in prolonged PKC activation. The initial surge of DAG due to $PIP_2$ hydrolysis is sufficient to initiate PKC activation, however, prolonged activation requires sustained DAG production, due to PC hydrolysis. $IP_3$ is not produced by PC hydrolysis, and is not accompanied by influxes of $Ca^{2+}$. Therefore, nPKCs are most

likely activated by DAG produced through PC hydrolysis (Berridge, 1989; Berridge, 1993; Hug and Sarre, 1993; Nishizuka, 1992; Nishizuka, 1995; Walsh *et al.*, 1996).

Classical and novel PKC isoenzymes may also be activated by some tyrosine kinases, as shown schematically in Figure 1.2. An example of receptor tyrosine kinase-mediated activation of PKC is through the platelet derived growth factor receptor (PDGFR), which is activated in the presence of platelet derived growth factor (PDGF) (Koch *et al.*, 1991). Upon activation, receptor tyrosine kinases homodimerize, and trans-autophosphorylate specific tyrosine residues in the cytoplasmic tails. Proteins such as phospholipase Cγ (PLCγ), Grb2, GAP, and phosphatidylinositol 3-kinase (PI3-kinase), can interact with specific phospho-tyrosine residues via SH2 domains, allowing for the recruitment of complexes and initiated signaling (Fantl *et al.*, 1993). These proteins have been shown to have varying affinities for different phospho-tyrosine residues. As a result, there is cross-talk between some of the different pathways mediated by tyrosine kinases. PLCγ hydrolyzes $PIP_2$ in a similar fashion to PLCβ, producing DAG and $IP_3$, and PKC activation occurs as described earlier. PI3-kinase is one of the key components of a pathway involved in phospholipid turnover, and this is necessary for repeated PKC activation. Grb2 is an adaptor, and has been shown to interact with son-of-sevenless (SOS), a guanine-nucleotide exchange factor essential for Ras-activation. In mammalian cells, Ras-mediated signaling elicits cellular responses, through mitogen-activated protein kinases (MAP kinases), involved in proliferation, and differentiation (Satoh *et al.*, 1992). PKC has been shown to phosphorylate and possibly regulate Raf, the MAP kinase kinase kinase typically activated through Ras (Fantl *et al.*, 1993; Lowy and Willumsen, 1993; Nishizuka, 1995).

Upon activation, the PKC isoenzymes undergo an alteration in conformation, and are translocated to the plasma membrane, or the membrane of an organelle. There is evidence that PKC isoenzymes can interact with docking proteins known as receptors for

activated C-kinases (RACKs) at the membrane (Ron *et al.*, 1994; Ron and Mochly-Rosen, 1995; Mochly-Rosen *et al.*, 1991). PKC isoenzymes have a RACK binding site, which is in a different region of the protein than the substrate-binding domain. Recent work has shown that PKC isoenzymes also have a pseudo-anchoring site, which putatively interacts with the RACK binding site. This keeps the protein folded in an inactive state, in conjunction with the pseudosubstrate-substrate domain interaction. Therefore, the presence of cofactors for activation may not be the only requirement for altering the conformation into an active state. RACKs may dock PKC isoenzymes for closer proximity to substrates, and help to maintain them in an active state (Ron and Mochly-Rosen, 1995).

### 1.3 PKC Substrates

PKC isoenzymes phosphorylate a plethora of substrates *in vitro*, however, most are not likely to be substrates *in vivo*, due to differential temporal and spatial patterns of gene expression. Several mammalian PKC substrates will be discussed, as well as substrates which could interact with the putative isoenzymes encoded by the *D. melanogaster Pkc* genes.

Some of the PKC isoenzymes are directly activated by phorbol esters and tumor promoting factors. This suggests a predominant role for PKC in proliferation, however, the study of mutant cell lines, and cell lines overexpressing PKC, have shown it is not consistently oncogenic (Borner *et al.*, 1991). Despite this finding, PKC is a key component in pathways leading to the regulation and expression of oncogenes. Although $p34^{cdc2}$/cyclin B kinase is postulated to be the primary mitotic lamin kinase, PKC may play a similar role, as one of the mammalian isoenzymes, $PKC\beta_{II}$, has been shown to localize to the nucleus in human leukemic cells (Hocevar *et al.*, 1993; Goss *et al.*, 1994). In many different tissues and cell types, other classical PKC isoenzymes have also been

shown to localize to the nuclear envelope, or nucleoplasm (Olson *et al.*, 1993; Dekker and Parker, 1994; Buchner, 1995).

A putative substrate is Lamin $B_I$, an intermediate filament found in the nuclear lamina, which contains a PKC phosphorylation site in the C-terminus (Hocevar *et al.*, 1993; Goss *et al.*, 1994). Phosphorylation alters it's solubility, and occurs in association with nuclear envelope breakdown (Hocevar *et al.*, 1993; Goss *et al.*, 1994). Experiments have shown that Lamin B may also associate with sites of DNA replication, during S phase, and it's regulation may be important for the organization of chromatin (Buchner, 1995). Lamin, encoded by the *D. melanogaster* gene *Lamin*, is found within the nuclear lamina (Ulizter *et al.*, 1992). This protein is putatively involved in nuclear envelope reassembly, and it would be interesting to postulate phospho-regulation by PKC (Ulizter *et al.*, 1992).

The myristoylated alanine-rich C kinase substrate (MARCKS) family of proteins are another group of PKC substrates (Blackshear, 1993). These proteins are myristoylated at the N-terminus, which is thought to confer higher affinity for membrane association (Blackshear, 1993; Swierczynski and Blackshear, 1996). They also have a positively charged phosphorylation site domain (PSD), which has a PKC phosphorylation site. MARCKS can bind actin, and calmodulin (CaM), in a $Ca^{2+}$-dependent manner (Swierczynski and Blackshear, 1996). Studies have shown that PKC phosphorylation results in membrane dissociation, and decreased affinity for CaM-binding (Swierczynski and Blackshear, 1996). MARCKS bind filamentous actin in a crosslinking manner, causing rearrangements in the cytoskeletal network, and could play an important role in neuronal development (Seki *et al.*, 1995). Mutants in mice have shown that MARCKS, which tend to be ubiquitously expressed, play a role in CNS development, and postnatal viability (Seki *et al.*, 1995). Perhaps through $Ca^{2+}$-dependent mechanisms, PKC and

CaM regulate the ability of MARCKS to interact with actin, altering rates of polymerization and stability in developing neurons, and axonal outgrowth.

Another potential PKC substrate is the microtubule-binding protein, dynamin, which has intrinsic GTPase activity (Sudhof, 1995). This protein plays an important role in synaptic vesicle recycling, possibly in conjunction with synaptotagmin, clathrin, AP2, and AP1880 (Camilli and Takei, 1996). Dynamin forms a ring at the neck of clathrin-coated vesicles, and causes vesicle fission upon GTP-hydrolysis (Camilli and Takei, 1996). However, ATP-hydrolysis is also required for fission, and dynamin could be interacting with, or signaling other proteins, through a GTP-dependent mechanism (Camilli and Takei, 1996). The ability of dynamin to bind and form rings is GTP-independent, as seen by the formation of rings, *in vitro* , around microtubules (Camilli and Takei, 1996). Dynamins have a Pro-rich region in their C-terminus, which has been shown to associate with proteins that contain SH3 domains, such as Grb2, p85, and PLCγ (Camilli and Takei, 1996). This region is dephosphorylated in a $Ca^{2+}$-dependent manner, upon nerve stimulation and depolarization, resulting in decreased GTPase activity (Sudhof, 1995). PKC has been shown to phosphorylate the dynamin protein encoded by the *D. melanogaster* homologue, *Shibire*, which was first isolated as a paralytic mutant with disrupted endocytosis of synaptic vesicles (Kim and Wu, 1987; Robinson *et al.*, 1993). PKC phosphorylation resulted in increased GTPase activity, suggesting that phospho-regulation of dynamin by PKC could be important for synaptic vesicle fission (Robinson *et al.*, 1993).

PKC isoenzymes have been shown to interact with a plethora of neuronal proteins such as the growth associated protein GAP-43 (Tanaka and Nishizuka, 1994). GAP-43 is phosphorylated by PKC in a number of processes, such as exocytosis, axogenesis (in the distal axons and growth cones of growing neurons) and long term potentiation (LTP; Tanaka and Nishizuka, 1994). The GAP-43 homologue in *D. melanogaster* is encoded by

the *Igloo* gene (Neel and Young, 1994). Mutants of this gene had reduced neurite outgrowth. Characterization of the protein showed association with CaM, and phosphorylation by PKC (Neel and Young, 1994). *Igloo* is expressed in the nervous tissue of developing embryos, after 12 hours, and throughout larval, pupal and adult stages, aside from reduced expression during the 3rd larval instar stage (Neel and Young, 1994). Neuroblast differentiation, and initial neurite outgrowth start in embryos after 6 hours, and gene products essential for this process would be expressed at, or prior to, this time (Ashburner, 1989A). The pattern of *Igloo* expression suggests it plays an important role in later stages of axonal outgrowth, in growing neurons (Neel and Young, 1994). Phosphorylated forms of GAP-43 are found only after axogenesis has begun, suggesting phospho-regulation allows for the correct establishment of synaptic connections, but not for initial stages of growth (Mieri *et al.*, 1991; He *et al.*, 1997; Tanaka and Nishizuka, 1994).

Several *D. melanogaster* mutants have shown reduced PKC activity in association with the development, or function and maintenance of nervous tissues. One example is *turnip*, a mutant isolated based on poor learning performance (Choi *et al.*, 1991). Although this mutant had deficient PKC activity, the gene was cytogenetically mapped to a location different from any known *Pkc* genes (Choi *et al.*, 1991). The protein encoded by *turnip* , belongs to the Rho subfamily of GTP-binding proteins, and is involved in the regulation of cytoskeletal elements (Flybase, 1997). Rho proteins have been shown to regulate polymerized actin, an important component of growth cones (Hall, 1992; Hall, 1993). In *S.cerevisiae*, Pkc1 is activated by a RhoI GTPase, and a similar pathway could exist in *D. melanogaster* (Kamada *et al.*, 1996).

A *D. melanogaster* PKC mutant was created through the use of an inhibitor peptide, shown in Figure 1.3 (Broughton *et al.*, 1996; Kane *et al.*, 1997). UAS-PKC inhibitor P-element transgenic flies were made by microinjecting a pUAST-*inaC* vector

Figure 1.3: Amino acid sequence of the *D. melanogaster* inhibitor peptide. This peptide was used as an inhibitor of PKC in transgenic *D. melanogaster*. Also shown is an amino acid sequence comparison between the *D. melanogaster* putative isoenzymes (Broughton *et al.*, 1996).

## *D. melanogaster* inhibitor peptide amino acid comparison

| | | |
|---|---|---|
| PKC53E | 26 | MKSRLRKGALKKKNVFN 43 |
| **InaC** | 52 | MKNRLRKGAMKRKGLEM 67 |
| PKC98E | 52 | AGFNRRRGAMRRRVHQV 67 |

Underline=pseudosubstrate domain

construct, with *inaC* sequence encoding the pseudosubstrate domain (Broughton *et al.*, 1996). Balancer markers were used to create a homozygous stock, which was then crossed with a homozygous hspGAL4 stock (Broughton *et al.*, 1996). This resulted in a stock which was heterozygous for the UAS-PKC inhibitor, and hspGAL4 (Broughton *et al.*, 1996). When these flies were heated to 37°C, GAL4 was produced and bound to UAS, stimulating production of the PKC inhibitor (Broughton *et al.*, 1996). The phenotypic effects of PKC inhibition was examined in both developing neuroblasts dissected from embryos, and learning in adults (Broughton *et al.*, 1996; Kane *et al.*, 1997). The amino acid sequence of the inhibitor peptide was derived from *inaC* and is shown in Figure 1.3, with an amino acid sequence comparison of the other two *D. melanogaster* isoenzymes (Broughton *et al.*, 1996). inaC has a very specific temporal and spatial pattern of expression, and it is likely PKC53E and PKC98E were being inhibited in flies expressing the inhibitor peptide (Schaeffer *et al.*, 1989). PKC inhibition was found to prevent neuroblast differentiation, and neurite outgrowth on dissected neuroblasts *in vitro* (Broughton *et al.*, 1996). In adult flies, PKC inhibition resulted in the dissociation of learning and memory from performance of a task (Kane *et al.*, 1997). This was tested in adult flies by studying courtship performance, where the mated female was the key initiator of several parallel pathways (Kane *et al.*, 1997).

### 1.4 *Pkc1* in *S.cerevisiae*

*Pkc1* was first cloned in *S.cerevisiae* through the use of probes derived from rat cDNA, for PKCγ and PKCβI (Levin *et al.*, 1990). A 2.3kb probe, made from one of the positive clones from the *S.cerevisiae* genomic library, was hybridized with poly(A)$^+$ mRNA and a single band of 4kb was visualized (Levin *et al.*, 1990). The putative ORF spans 3453bp, and encodes a polypeptide of 1151 amino acids, with a molecular weight of 131.5kDa (Levin *et al.*, 1990). Pkc1 is homologous to the classical PKC isoenzymes,

with the exception of an extended N-terminus (Levin *et al.*, 1990). *Pkc1* mutants were phenotypically lethal and arrested in cell cycle after DNA replication with small buds (Levin *et al.*, 1990). Later studies revealed this phenotype was due to altered cell wall integrity, regulated by a RhoI-mediated MAP kinase pathway (Kamada *et al.*, 1996). This pathway, shown schematically in Figure 1.4, transcriptionally regulates *Fks2*, which encodes a component of the 1,3-β-glucan synthase (GS) complex (Kamada *et al.*, 1996). The GS complex produces polymers required for proper formation of the cell wall, and mutants of various components of the pathway result in cell lysis, which is enhanced by stresses such as higher temperatures (Kamada *et al.*, 1996). The *S.cerevisiae RhoI* gene encodes a homologue of the mammalian RhoA protein, and has been implicated as the key activator of Pkc1 (Kamada *et al.*, 1996). RhoI plays a dual role, and also directly regulates the GS complex independently of the Pkc1 pathway (Kamada *et al.*, 1996).

### 1.5 *D. melanogaster* and Cell Death

*D. melanogaster* have several stages of development, and each stage has a unique pattern of gene expression (Andres and Thummel, 1992). Figure 1.5 shows a schematic of the life cycle. A fertilized female lays a diploid zygote, that quickly develops into an embryo (Ashburner, 1989A). The embryo develops into a 1st larval instar, which hatches from the eggshell after a day (Ashburner, 1989A). There are three larval instar stages, and the first two stages each take a day, while the third stage takes about 3 days (Ashburner, 1989A). After the third larval stage, the organism undergoes pupariation for about 4 days, and ecloses as an adult fly (Andres and Thummel, 1992). Throughout these stages, programmed cell death plays an important developmental role (McCall and Steller, 1997). For example, during embryogenesis, neural cells undergo programmed cell death, a process shown to involve gene products from the genes *head involution defective* (*hid* ), and *reaper* (*rpr*; Steller and Grether, 1994; Grether *et al.*, 1995; White *et al.*, 1996;

Figure 1.4: The pathway leading to Pkc1 activation in *S.cerevisiae*. Pkc1 is involved in regulating cell wall integrity, and one of the key activators of this pathway is a Rho1 GTPase. Rho1 interacts with Pkc1 in a GTP-dependent manner, allowing Pkc1 to interact with PS as a cofactor for activation. Pkc1 is translocated to the membrane, where it then activates a MAP kinase cascade; Bck1 (MEKK), Mkk1/2 (MEKs), and Mpk1 (MAPK). This results in altered levels of *Fks2* gene expression, which encodes a key component of the Glucan synthase complex. This complex produces polymers of 1,3-β-glucan in the cell wall, which is important for maintenance of cell wall integrity. Rho1 can also interact with the complex directly, and may have several roles in regulating the pathway (Kamada *et al.*, 1996).

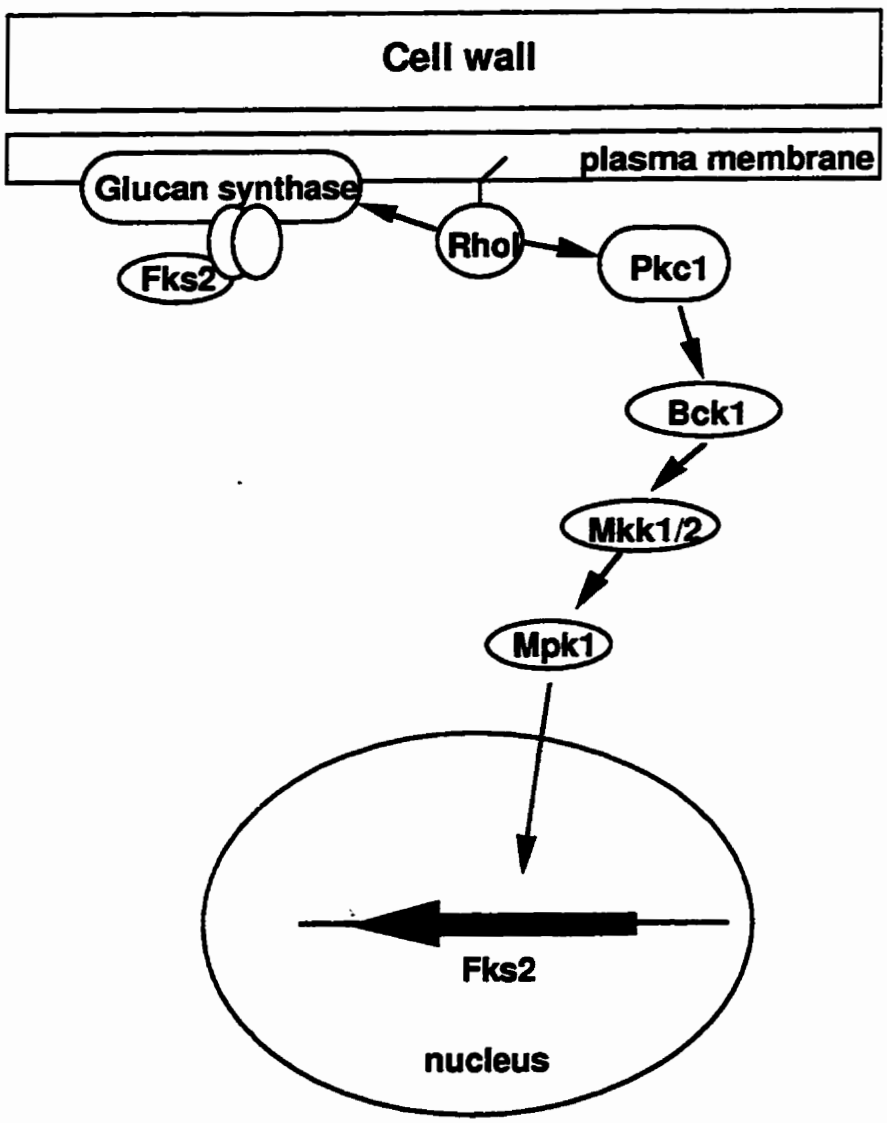
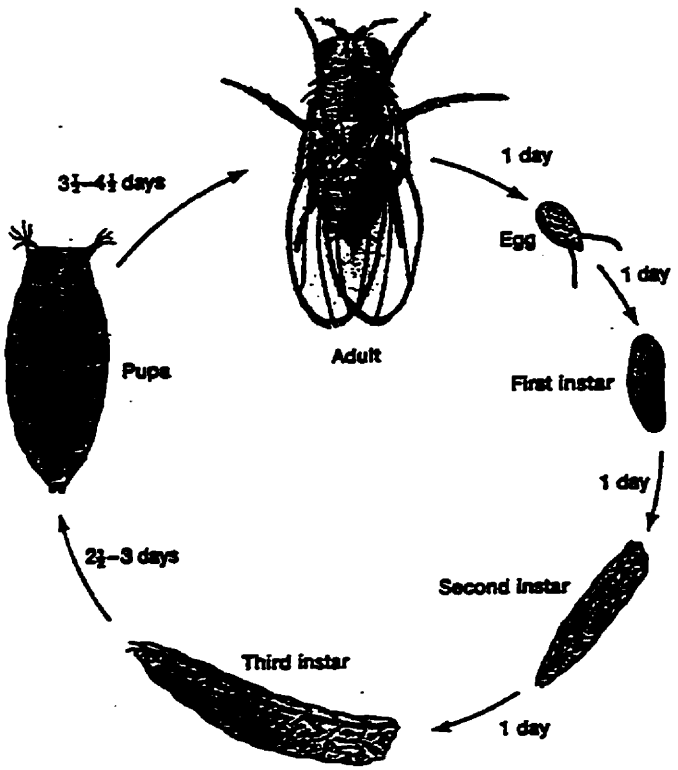

Cell wall
plasma membrane
Glucan synthase
Fks2
Rho1
Pkc1
Bck1
Mkk1/2
Mpk1
Fks2
nucleus

Figure 1.5: A schematic of the life cycle of *D. melanogaster*. The embryos take approximately 21 hours to hatch at 25°C. There are three larval stages; 1st instars, 2nd instars, and 3rd instars. The first two instar stages take about a day each, and the third instar stage takes about 3 days. During pupariation, the larva undergoes metamorphosis, when many adult tissues are formed from the imaginal discs. The pupal stage usually lasts about four days, after which the adult fly ecloses from the pupal casing. The overall cycle takes approximately 9 days at 25°C for wild type flies (Ashburner, 1989A; Griffiths *et al.*, 1996).


1 day
Egg
1 day
First instar
1 day
Second instar
1 day
Third instar
2½–3 days
Pupa
3½–4½ days
Adult

McCall and Steller, 1997). Throughout the larval stages, there are continued periods of moulting, which are signaled through the hormone ecdysone (Andres and Thummel, 1992). Several pulses of ecdysone signal massive programmed cell death during pupariation, causing the organism to undergo a dramatic change during metamorphosis (Andres and Thummel, 1992; Steller and Grether, 1994; McCall and Steller, 1997). Many larval tissues undergo massive programmed cell death, or histoylsis. Some tissues remain until late pupal stages, and the adult structures form largely from the imaginal discs (Andres and Thummel, 1992; Steller and Grether, 1994; McCall and Steller, 1997). Ecdysone stimulates the expression of genes, as shown by the formation of chromosome puffs, involved in metamorphosis and in the development of adult structures (Andres and Thummel, 1992). The salivary glands contain polytene chromosomes in their nuclei, which will puff at loci undergoing transcriptional activity (Andres and Thummel, 1992). Some of the loci of these puffs have been analysed to uncover genes which are actively turned on during ecdysone stimulation (Andres and Thummel, 1992). Many of these genes encode structural pupal proteins, transcription factors, steroid hormone receptors, and other putative signaling proteins (Andres and Thummel, 1992). There are still some genes which have yet to be uncovered, and many that remain to be characterized (Steller and Grether, 1994).

The massive programmed cell death, or histolysis of larval tissues in *D. melanogaster*, is an interesting system to study cell death genes. Mammalian programmed cell death pathways are different in the signals they receive, and the tissues that are programmed to die. However, there may still be some conserved functional homology with components of the pathway in *D. melanogaster*. The *D. melanogaster* gene, *reaper* (*rpr* ), encodes a protein homologous to mammalian proteins with a death domain, and is expressed in all cells programmed for death (Pronk *et al.*, 1996; White *et al.*, 1996; McCall and Steller, 1997). *hid* and *grim* are also involved in *D. melanogaster* cell death

pathway(s), although no mammalian homologues are currently known, and the putative ORFs encode proteins with a similar sequence in the N-terminus to rpr (Grether *et al.*, 1995; Chen *et al.*, 1996; McCall and Steller, 1997). This could be a novel region that is important for signaling in the cell death pathway(s) (Grether *et al.*, 1995; Chen *et al.*, 1996; McCall and Steller, 1997). A large number of cells die during metamorphosis. However, cell death pathway(s) have not been extensively studied during this stage. Preliminary studies show that *rpr* and *hid* are required as essential components of the cell death pathway in tissue histolysis during pupariation (Thummel, pers. comm. 1997). The *Broad-Complex* mutant, *bpr-5*, displays a phenotype where salivary glands fail to histolyze (Thummel, pers. comm. 1997). Analyses of these salivary glands showed reduced expression of *rpr* and *hid*, suggesting the same cell death pathway(s) observed during embryogenesis also occurs during histolysis of salivary glands (Thummel, pers. comm. 1997).

Mammalian PKCδ has been demonstrated to play a role in the apoptosis of B lymphocytes. It contains an interleukin-1β-converting enzyme (ICE) protease cleavage site, and cleavage results in the release of a catalytically active PKM fragment. Cell death is visualized four hours after cleavage (Emoto *et al.*, 1995). Although this may simply be a consequence of cell death, this does provide evidence for the involvement of PKC isoenzymes.

Lamins have been shown to be targeted for proteolytic cleavage by ICE-like proteases during apoptosis (Buchner, 1995). Mammalian Lamins are typically found in association with the cytoskeletal network, and Lamin B$_I$ is found at the nuclear envelope (Buchner, 1995). Previous studies suggested that mammalian Lamin B$_I$ is phosphorylated by PKC, and is associated with nuclear envelope breakdown (Fields *et al.*, 1988; Hocevar *et al.*, 1993). Lamins are conserved in lower eukaryotes, and several homologues have

been identified in *D. melanogaster* (Ulitzer *et al.*, 1992). One, encoded by the *Lam* gene, is found at the nuclear envelope, and has PKC phosphorylation sites (Ulitzer *et al.*, 1992).

### 1.6 *Pkc* genes in *D. melanogaster*

Three *Pkc* genes have been isolated in *D. melanogaster* ; *inaC*, *Pkc53E*, and *Pkc98E*. *Pkc53E*, named according to its cytogenetic location on the 2nd chromosome, was first identified using a bovine probe to *Pkcα* with a *D. melanogaster* cDNA library (Rosenthal *et al.*, 1987). The original published cDNA sequence is 3.2kb, and was derived from two overlapping clones (Rosenthal *et al.*, 1987). The putative ORF encodes a protein of approximately 75kDa, with highest similarity to the mammalian PKCα isoenzyme (Rosenthal *et al.*, 1987). *inaC* was identified using bovine probes to *Pkcα*, and *Pkcβ*, and was originally named *eye-Pkc* according to the pattern of RNA localization by *in situ* hybridizations (Schaeffer *et al.*, 1989). The current name is derived from the mutant phenotype observed (Smith *et al.*, 1991). Cytogenetically, *inaC* maps to a locus within 25kb of *Pkc53E*, and it is likely that one of the genes arose from a duplication event (Smith *et al.*, 1991). The putative ORF encodes a protein of approximately 80kDa, which is homologous to the mammalian classical PKC isoenzymes (Schaeffer *et al.*, 1989; Smith *et al.*, 1991). *Pkc98E*, named according to its cytogenetic location, was also discovered by the same screen used for *inaC* (Schaeffer *et al.*, 1989). The putative ORF encodes a protein of 71kDa with closest similarity to the mammalian PKCε isoenzyme (Schaeffer *et al.*, 1989).

*inaC* expresses one major transcript of 2.4kb, as shown in Table 1.1 (Schaeffer *et al.*, 1989). It was found to be expressed specifically in adult photoreceptor cells, through *in situ* hybridizations of cryostat sections (Schaeffer *et al.*, 1989). Smith *et al.* (1991), created a mutation in the gene using EMS mutagenesis, and found the inaC isoenzyme was essential for light adaptation. Its activity was responsible for light-sensitive retinal

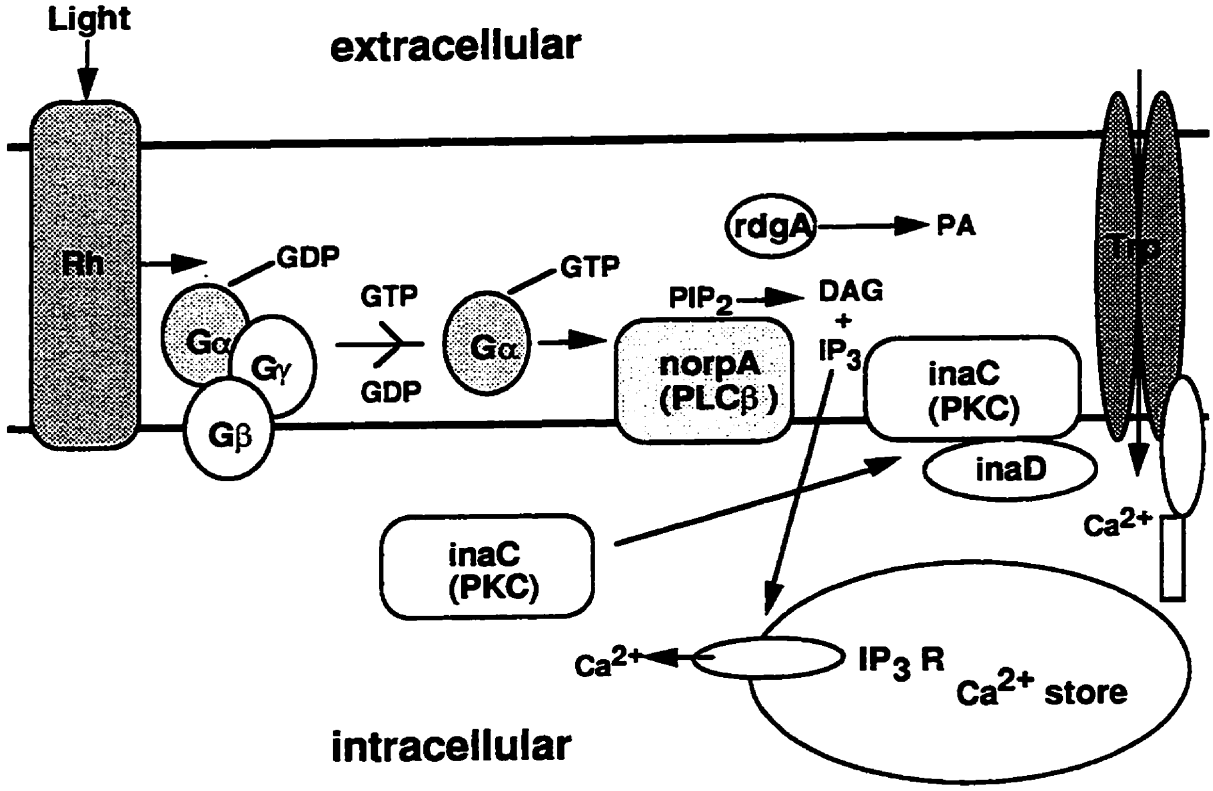
Table 1.1: Transcript sizes and temporal patterns of *Pkc* expression in *D. melanogaster*.

| Gene | Transcript size | Localization |
|---|---|---|
| *inaC* | 2.4kb | adult photoreceptors |
| *Pkc98E* | 4.3/4.5kb | embryos, larvae, pupae |
| | 5.5kb | adult heads |
| *Pkc53E* | 2.4kb | larvae, pupae, adult ovaries, adult testes, adult heads |
| | 3.0/3.4kb | larvae |
| | 4.0/4.3kb | pupae, adult heads |

degeneration in *retinal degeneration B* (*rdgB* ) mutants. Several other mutants were discovered with similar phenotypes, and these genes were found to encode essential components of a heterotrimeric G protein-mediated pathway (Smith *et al.*, 1991; Hardie *et al.*, 1993). The schematic is shown in Figure 1.6, and involves several proteins which are highly conserved in mammalian heterotrimeric G protein-mediated signal transduction pathways (Smith *et al.*, 1991; Clapham, 1996; Zuker, 1996).

Rhodopsin acts as the receptor for the pathway, which is activated by photons of light (Smith *et al.*, 1991; Clapham, 1996; Zuker, 1996). Rhodopsin is associated with a heterotrimeric G protein, and in the absence of signal, Gα is coupled to GDP (Smith *et al.*, 1991; Clapham, 1996; Zuker, 1996). In the presence of signal, rhodopsin is altered to metarhodopsin, which stimulates Gα to exchange GDP for GTP, and to dissociate from the βγ subunits (Smith *et al.*, 1991; Clapham, 1996; Zuker, 1996). This activates norpA, the downstream effector (Smith *et al.*, 1991; Clapham, 1996; Zuker, 1996). norpA is homologous to the mammalian PLCβ enzyme, and hydrolyzes $PIP_2$ to produce the products DAG and $IP_3$ (Smith *et al.*, 1991; Clapham, 1996; Zuker, 1996). $IP_3$ stimulates the release of $Ca^{2+}$ from internal stores, via the activation of $IP_3$ receptors in the membranes of these stores (Smith *et al.*, 1991; Clapham, 1996; Zuker, 1996). This initial increase in $Ca^{2+}$ is coupled to a second influx of $Ca^{2+}$ ions, through store-operated channels (SOCs) in the plasma membrane (Clapham, 1996; Zuker, 1996). One of the channels identified in this pathway is encoded by the *trp* gene, which putatively senses, and is activated by, the depletion of $Ca^{2+}$ ions from internal stores (Clapham, 1996; Zuker, 1996). There is much debate concerning the mechanism by which SOCs sense the depletion of $Ca^{2+}$ ions from internal stores (Clapham, 1996; Zuker, 1996). The function of the protein encoded by the *trp* gene, was initially identified by its mutant phenotype, which resembled that of mutant *inaC* and *norpA* phenotypes (Hardie *et al.*, 1993). inaC is activated in a manner similar to the classical PKC isoenzymes, in the presence of $Ca^{2+}$,

Figure 1.6: inaC activation in the rhabdomeres of photoreceptor cells in adult *D. melanogaster*. Light activates rhodopsin (Rh) to metarhodopsin, and stimulates Gα to exchange GDP for GTP, and to dissociate from Gβγ. Gα-GTP activates norpA (PLCβ), and produces $IP_3$. $Ca^{2+}$ ions are released from internal stores, through $IP_3$ R activation. $Ca^{2+}$ binds to inaC, causing translocation to the plasma membrane where it interacts with DAG, and is activated. inaD is a putative inaC substrate, and is shown associated with activated inaC. When internal $Ca^{2+}$ stores are depleted, trp, a $Ca^{2+}$ channel in the plasma membrane, results in an influx of $Ca^{2+}$ from the extracellular space. DAG is converted back to PA by rdgA (DAG kinase), and is subsequently recycled by a series of rdg enzymes to restore $PIP_2$ levels (Clapham, 1996; Huber *et al.*, 1996A; Huber *et al.*, 1996B; Zuker, 1996).


Light
extracellular
Rh
GDP
Gα
Gγ
Gβ
GTP
GDP
GTP
Gα
PIP2
norpA
(PLCβ)
rdgA
PA
DAG
+
IP3
inaC
(PKC)
inaD
TRP
Ca2+
inaC
(PKC)
Ca2+
IP3 R
Ca2+ store
intracellular

DAG and phosphatidylserine, whereupon it translocates to the membrane (Schaeffer *et al.*, 1989; Smith *et al.*, 1991; Clapham, 1996; Zuker, 1996). A putative substrate for inaC was identified as inaD, based on the inaD mutant phenotype and on the presence of PKC phosphorylation sites (Huber *et al.*, 1996A; Huber *et al.*, 1996B; Shieh and Zhu, 1996). Immunoprecipitation studies suggest inaD forms a complex with norpA, inaC, and trp, in the presence of cofactors of inaC activation (Huber *et al.*, 1996A; Huber *et al.*, 1996B; Shieh and Zhu, 1996). In the absence of these cofactors, inaC does not associate with the complex, and remains in the soluble fraction (Huber *et al.*, 1996A; Huber *et al.*, 1996B; Shieh and Zhu, 1996). It has been suggested that upon inaC activation, inaD is phosphorylated and signals trp to open its $Ca^{2+}$ channel (Huber *et al.*, 1996A; Huber *et al.*, 1996B; Shieh and Zhu, 1996). Other mutants, such as *rdgB* have been identified based on the requirement for active inaC to elicit the mutant phenotype (Smith *et al.*, 1991). Some of these genes are now known to encode enzymes involved in phospholipid turnover (Clapham, 1996; Zuker, 1996). $PIP_2$ comprises a low proportion of the phospholipids in the plasma membrane, and evidence suggests that a localized $PIP_2$ population is specifically produced when PKC signaling pathways are stimulated (Clapham, 1996; Zuker, 1996). This is most likely functionally conserved in mammalian PKC pathways, and provides an example of the benefit of studying a eukaryote whose genetics are well understood.

Little is known about *Pkc98E*, as the gene has not been characterized beyond the level of the RNA, and mutants have not been discovered. A transcript of 5.5kb is found throughout development, however, two transcripts of 4.3 and 4.5kb are more abundant during earlier stages (Table 1.1). *in situ* hybridizations to cryostat sections of adult heads, show transcripts are found predominantly in adult heads, in particular in the cell bodies of the CNS. The putative protein encoded by this gene is homologous to the mammalian PKCε isoenzyme, and is $Ca^{2+}$-independent (Schaeffer *et al.*, 1989).

*Pkc53E* has been studied extensively at the level of RNA, and at the level of protein. There are several developmental and tissue-specific transcripts (Table 1.1). Initially, Northern blot analyses identified the presence of 4.3, 4.0 and 2.4kb transcripts in adults, and none in 0-3 hour embryos (Rosenthal *et al.*, 1987). Further Northern blot analyses demonstrated the presence of transcripts predominantly in adult heads, and *in situ* hybridizations to cryostat sectioned adult heads showed a ubiquitous pattern of expression (Schaeffer *et al.*, 1989). RNA was found localized in most tissues of the head, including the CNS, and photoreceptors (Schaeffer *et al.*, 1989). Further Northern analyses identified the presence of a 2.4kb transcript in adult gonads, as well as the 4.3, 4.0, and 2.4kb transcripts found in adult heads (Natesan, 1991). The 4.3, 4.0 and 2.4kb transcripts were found throughout pupal stages, however, their proportions varied significantly (Natesan, 1991). Transcripts of 3.4, 3.0 and 2.4kb were also identified in all three larval stages (Natesan, 1991). Although transcript was not evident in embryos of varying stages, transcript abundance increased following 12-O-tetradecanoylphorbol-13-acetate (TPA) treatments. *in situ* hybridizations were performed on stage 12 embryos, and expression was seen in developing neural tissues, possibly in the CNS and PNS (Hughes, 1993). *in situ* hybridizations were also performed in developing oogonia, and transcripts were found, starting around stage 8-9, in the nuclei of nurse cells, and follicle cells (Hughes, 1993). Later stages show evidence of transcript being deposited into the maturing oocyte, from the nurse cells, and alternate probes suggest alternate transcripts are being produced (Hughes, 1993). Transcript was also visualized in testes, in the apical ends of sperm, as well as in the sheath, and in sperm bundles (Hughes, 1993). Cryostat sections of adults showed staining in the CNS regions within the adult heads. Some staining was also visualized in the thorax, most likely in CNS regions, and possibly associated PNS regions (Hughes, 1993).

Further RNA analyses, such as RNAse protection assays, RT-PCR, 3'RACE, and 5'RACE, were performed on transcripts from the adult heads and gonads (Nutter, pers. comm.). Using either primers to the polyA tail, or by selecting poly(A)$^+$ RNA, it became evident the transcript in testes was not polyadenylated. With total RNA extracted from testes, gene-specific primers internal to the polyA tail are able to amplify *Pkc53E* -specific transcripts. The 2.4kb transcript in ovaries is abundant. However, after selecting poly(A)$^+$ RNA, or amplifying using a primer to the polyA tail, it seems a proportion of this message is also not polyadenylated. The 4.3kb and 4.0kb transcripts do not seem to vary significantly in their proportion of polyadenylated RNA. RNAse protection assays imply the presence of alternate messages, with variation occurring primarily in the portion of transcript encoding the N-terminus. 5' RACE analyses revealed alternative splicing in the 5' untranslated region. The 4.3 and 4.0kb transcripts appear to have intron 1 spliced into the message, which increases their length by 600bp from the cloned 3.2kb cDNA (Rosenthal *et al.*, 1987). The difference between the 4.3 and 4.0kb transcripts could be due to the use of an alternative +1 site, as with the two larval transcripts of 3.4 and 3.0kb. The 2.4kb transcripts do not contain intron 1, and experiments have not yet determined whether any exons are spliced out. The cloned cDNA is 3.2kb; however, aside from larvae, transcripts close to this size are not visualized by Northern blot analyses. This clone was derived from two overlapping partial positive cDNA clones, and each was most likely derived from different transcripts (Rosenthal *et al.*, 1987; Nutter, pers. comm.).

The putative *Pkc53E* ORF is 75kDa, and encodes a $Ca^{2+}$ and phospholipid-dependent protein (Rosenthal *et al.*, 1987). During the research leading to this thesis, polyclonal antibodies were made to a small portion of the N-terminus of PKC53E. The antigen is shown in Figure 1.7, with an amino acid sequence comparison between the *D. melanogaster* putative PKC isoenzymes. These antibodies were used to analyze patterns of PKC53E protein expression in *D. melanogaster*, as outlined in the objectives.

Figure 1.7: Amino acid sequence of the antigen. Also shown is a comparison of this region between the *D. melanogaster* PKC isoenzymes (Schaeffer *et al.*, 1989).

## *D. melanogaster* antigen PKC amino acid comparison

| | | | |
|---|---|---|---|
| **PKC53E** | 10 | DPQQQGAEGEAVGENKMKSRLRKGALKKKNVFNVKDHCF | 49 |
| InaC | 36 | PGNLLEITGEANIVNYMKNRLRKGAMKRKGLEMVNGHRF | 85 |
| PKC98E | 36 | EAVVEHTVAVNKEFKERAGFNRRRGAMRRRVHQVNGHKF | 85 |

Underline=pseudosubstrate domain

## 1.7 Objectives

One of the goals of this project was to generate antibodies to PKC53E. These antibodies needed to be unique, to avoid cross reactivity with the other *D. melanogaster* PKC isoenzymes. They also needed to be able to detect PKC53E by Western analyses, and immunohistochemical analyses. The second goal of this project was to use the antibodies to determine patterns of PKC53E protein expression. This was to be accomplished by Western analyses on extracts obtained from various adult tissues, and on extracts obtained from flies of various developmental stages. Another method of determining protein expression was to perform immunohistochemical studies on various adult tissues, and flies from various developmental stages. Discerning temporal and spatial patterns of PKC53E protein expression will lead to a better understanding of its possible function in the overall organism.

# Chapter 2

# Materials and Methods

## 2.1 Materials

### 2.1.1 Chemical and Reagent Suppliers

All chemicals, reagents and supplies were obtained from GibcoBRL, Pharmacia, Boehringer Mannheim, VWR, Fisher, Qiagen, and Amersham. Some reagents and supplies were obtained from various individuals; Dr. S.L. Wong, Department of Biology, The University of Calgary, Dr. M. Lohka, Department of Biology, The University of Calgary, Dr. M. Maloney, Department of Biology, The University of Calgary, Dr. D. Storey, Department of Biology, The University of Calgary, Vanessa Auld, Department of Biology, The University of British Columbia, Dr. M. Walsh, Department of Medical Biochemistry, The University of Calgary, and Dr. S. Lees-Miller, Department of Biology, The University of Calgary. The vector used for cloning, pGEX-4T1, was generously provided by Dr. S.L. Wong. All enzymes, and buffers used for cloning were from GibcoBRL. The primers used for PCR amplification were purchased from Dr. M. Maloney's lab, and the primers used for sequencing were purchased from the University Core DNA Services (University of Calgary). Qiagen columns were used for preparing sequencing-grade DNA (Qiagen). The T7 Sequencing kit from Pharmacia was used for preparing sequencing reactions, and radioactive isotopes were obtained from Amersham. Isopropyl-β-D-thiogalactoside (IPTG) for protein induction was purchased from GibcoBRL, and anti-GST antibodies were generously provided by Dr. D. Storey. A 2mL column was borrowed from Dr. S. Lees-Miller, and the glutathione-coupled Sepharose 4B slurry was purchased from Pharmacia. Reduced glutathione was purchased from GibcoBRL. Coomassie Brilliant Blue R-250, nitrocellulose and phenylmethyl-sulfonyl

fluoride (PMSF) were purchased from GibcoBRL. The rabbits used to raise polyclonal antibodies were purchased and cared for through the University of Calgary, Animal Care Services (Director Dr. D. Morck). All blood samples were taken by, and all injections were carried out by the Animal Care Services. Anti-rabbit alkaline phosphatase (AP) antibodies were purchased from GibcoBRL. Thrombin and Ponceau S stain (Sigma) were kindly provided by Dr. M. Lohka. Protein A-coupled agarose (Sigma) was also kindly provided by Dr. M. Lohka. The PKC assay kit, general anti-PKC antibodies, and substrate peptide were purchased from GibcoBRL. Phosphocellulose units were purchased from Pierce. bPKC$\alpha\beta\gamma$ was kindly provided by Dr. M. Walsh. Anti-rabbit fluorescein isothiocyanate (FITC) antibodies, anti-rabbit biotin-conjugated antibodies, streptavidin-Texas red, and Fluorescent Mounting Media were purchased from GibcoBRL. Goat serum and anti-mouse Texas red antibodies (Jackson Lab.) were kindly provided by Dr. M. Lohka. A monoclonal mouse antibody was kindly given by Vanessa Auld, as a positive control for staining embryos. Compounds used for preparing cryostat sections of *D. melanogaster* tissues were purchased from VWR. All microscope slides used for whole mounts, and cryostat sections were from Fisher.

## 2.2 Methods

### 2.2.1 Molecular Biology Techniques

#### 2.2.1.1 Absorbance Readings

A Beckman DU-640 spectrophotometer was used for DNA and protein quantitation. DNA quantitation using UV light (260/280nm) was followed as outlined (Gallagher, 1994). BioRad Protein Assays for protein quantitation were performed as outlined by the manufacturer, using visible light (595nm), and plotting a standard curve with 1mg/mL bovine serum albumin (BSA).

### 2.2.1.2 Phenol/Chloroform Extractions

This method was used to enhance the quality of DNA used in sequencing and ligation reactions. An equal volume of buffer-saturated phenol was added to the microfuge tubes, vortexed, and centrifuged at 12,000xg for 5 minutes. The aqueous phase was removed, transferred to another microfuge tube, and an equal amount of chloroform was added. Again, the mixture was vortexed, and centrifuged at 12,000xg for 5 minutes and the aqueous phase was drawn off and kept. The DNA was then reprecipitated from solution.

### 2.2.1.3 DNA Precipitations

DNA was precipitated by adding 2.5 times the volume 95 or 100% ethanol v/v, and 0.1 times the volume 3M sodium acetate (or potassium acetate), pH 4.8-5.2. The microfuge tubes were placed at -20°C for at least half an hour, then centrifuged at 12,000xg for 15 minutes at 4°C. The supernatant was removed, and pellets washed with 70% ethanol v/v. They were recentrifuged briefly for 2-5 minutes, then the ethanol was removed, and pellets allowed to air dry for about 15 -30 minutes. The pellets were resuspended in appropriate amounts of TE (pH 7.5-8.0) or dd$H_2O$ (Pharmacia; Moore, 1994).

### 2.2.1.4 Agarose Gel Electrophoresis

Aliquots of 10uL from each PCR reaction were electrophoresed in 2.0% agarose gels, and pGEX-4T1 digested with EcoRI and/or XhoI was electrophoresed in 1.0% gels. These gels were made by dissolving the required amount of agarose in 1 X TAE buffer in the microwave for 2-5 minutes, depending on the final volume used. Agarose was dissolved in 50mL 1 X TAE for the Pharmacia Model GNA-100, and dissolved in 250mL

for the Pharmacia Models GNA-200. Once the agarose solution had cooled to approximately 50°C, 0.5uL 10mg/mL ethidium bromide (GibcoBRL) was added regardless of volume. The solution was then mixed, and poured into a taped tray with the well comb(s) in place. After gel polymerization, the comb(s) and tape were removed, and the tray was placed in a gel box filled with 1 X TAE ensuring the wells were immersed. 400mL 1 X TAE was required for the GNA-100 gel box, and 1-1.5L 1 X TAE for the GNA-200. As per manufacturer's instructions, the 123bp DNA ladder (GibcoBRL) was used as a marker for the 2.0% gels, and the 1kb DNA ladder (GibcoBRL) was used as a marker for the 1.0% gels. The markers were usually set up in a microfuge tube, with $H_2O$ to a final volume of 10uL, and loading dye added in a 1 (dye) : 5 (solution) ratio (Sambrook, 1989; GibcoBRL). DNA loading dye was also added to each sample, and all were loaded into a separate well in the gel, and electrophoresed using a Pharmacia Electrophoresis Power Supply EPS 500/400. The well size varied from 10uL to 40uL, depending on the comb used. The gels were run at 80V, for 1-4 hours, until the dye fronts indicated the gels had run an appropriate amount of time. The gels were then examined under UV light, and a photoimage was taken if necessary (Voytas, 1988).

| | |
|---|---|
| DNA loading dye | 0.25% bromophenol blue w/v, 0.25% xylene cyanol FF w/v, 30% glycerol v/v. Store at 4°C (Sambrook *et al.*, 1989). |
| 50 X TAE | For 1L, 242g Tris base, 57.1mL glacial acetic acid, 37.2g $Na_2EDTA{\cdot}2H_2O$ pH8.5 (Sambrook *et al.*, 1989). |

### 2.2.1.5 Gel Purification

Bands at 80bp were cut out of the gel, and purified. A 100ug/mL silica matrix was used to bind DNA, and 6M guanidine was used as the binding solution. Binding solution was added to the gel slices in microfuge tubes in a 3 : 1 (volume to weight) ratio and were melted at 50°C. 50uL silica was then added to each tube, and the solutions were mixed for 5 minutes at RTemp. The tubes were centrifuged briefly at 12,000xg, then washed with 500uL cold wash buffer. The pellets were resuspended, recentrifuged, and the supernatant was removed. This was repeated twice. A final centrifugation was performed after the last wash buffer had been removed. 25uL TE were added to each tube, and the silica pellets were resuspended and heated at 50°C to elute the DNA. The tubes were centrifuged, and the TE with eluted DNA was collected into new microfuge tubes. Small aliquots were run on agarose gels to check for quantity, and quality of DNA (Boyle *et al.*, 1995).

| | |
|---|---|
| Gel purification wash buffer | 50mM NaCl, 10mM Tris-HCl pH7.5, 2.5mM EDTA pH8.0, 50% ethanol v/v (Boyle *et al.*, 1995). |

### 2.2.1.6 DNA Polymerase I

As per manufacturer's instructions, the amplified 80bp *Pkc53EV1* PCR product was treated with DNA polymerase I to modify any T/A overhangs (GibcoBRL). The modified 80bp fragment was then phenol/chloroform extracted and reprecipitated prior to performing RE digests.

### 2.2.1.7 Restriction Enzyme Digests

As per manufacturer's instructions (GibcoBRL), the modified 80bp *Pkc53EV1* fragment, and the pGEX-4T1 vector were digested with restriction enzymes. The enzymes used were EcoRI, and XhoI, and these were generally left overnight at 37°C to ensure

complete endonuclease cleavage (GibcoBRL). The products were run on agarose gels, and gel purified prior to setting up the ligation reaction.

### 2.2.1.8 Ligation Reactions

As per manufacturer's instructions, the RE digested 80bp *Pkc53EV1* fragment, and the RE digested pGEX-4T1 vector were ligated with T4 DNA ligase (GibcoBRL). The ligations were directional, and due to the small size of the insert, the ratios were altered accordingly. A ratio of 3 parts insert : 1 part vector was most successful; however, due to PCR amplification of the 80bp fragment, the concentration was much higher proportionally than the vector. The concentration of DNA is essential in achieving a successful ligation, and it varies depending on both the size and sequence of the vector and insert. Several ligations of varying amounts of vector and insert were always set up simultaneously. A total reaction volume of 10uL was used, and the ligation reactions were incubated at RTemp overnight. The addition of 1uL 10mM ATP per 10uL volume was found to enhance ligation efficiency. Ligation reactions were diluted five times prior to transformation (GibcoBRL).

### 2.2.1.9 Transformations

Ligation reactions were transformed into *E.coli* DH10B competent cells, made competent with $CaCl_2$. The procedure entailed adding five times diluted ligation reactions to a tube of thawed cells (200uL), and letting them sit on ice for 30 minutes. After this time, they were heat shocked at 42°C for 90 seconds, then 800uL of SOC (GibcoBRL) or LB were added, and they were placed at 37°C for at least 45 minutes. This is the minimum length of time required to achieve growth with cells able to confer ampicillin resistance. It is also advantageous to provide oxygen to the growing cultures by shaking; however, this is not necessary due to the short incubation time. The cells were then plated on LBAmp

plates and placed at 37°C for 12-16 hours. Varying volumes of cells were used for plating. It was beneficial to plate 200uL on one plate, and the remaining 800uL on another (Seidman, 1994).

| | |
|---|---|
| LBAmp plates | For 1L, 10g tryptone, 5g yeast extract, 5g NaCl, 15g select agar and 1mL 1N NaOH. After autoclaving, the medium is cooled to 50°C before adding ampicillin to a concentration of 100ug/mL, then poured into plates (Lech and Brent, 1987). |
| LB plates | For 1L, 10g tryptone, 5g yeast extract, 5g NaCl, 15g select agar and 1mL 1N NaOH. After autoclaving, the medium cooled to 50°C, and poured into plates. Store at 4°C, for a few months (Lech and Brent, 1987). |
| LB broth | For 1L, 10g tryptone, 5g yeast extract, and 10g NaCl and 1mL 1N NaOH. Make fresh before use, or can store at 4°C (GibcoBRL). |
| SOC medium | 2% tryptone w/v, 0.5% yeast extract w/v, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose (GibcoBRL). |

### 2.2.1.10 Inoculating LB

Current protocols emphasizes the use of aseptic techniques in inoculating cultures, and plating. All cultures were handled in the presence of a flame, and all reagents were sterile prior to their addition. A flamed loop was typically used to inoculate LBAmp with

colonies, or to make a streak plate. Vectors and constructs were stored as frozen stocks (Lech and Brent, 1987).

| | |
|---|---|
| LBAmp broth | For 1L, 10g tryptone, 5g yeast extract, and 10g NaCl and 1mL 1N NaOH. After autoclaving, the medium is cooled at least 50°C, and ampicillin is added to a final concentration of 100ug/mL (Pharmacia). |
| Frozen Stock solution | Dilute fresh overnight culture in 65% glycerol v/v, 0.1M $MgSO_4$, 0.025M Tris-HCl pH8.0. Store at -70°C (Lech and Brent, 1987). |

### 2.2.1.11 Minipreps

The procedure used to prepare plasmid DNA from 1.5mL saturated culture of cells was followed as outlined in the GST Gene Fusion System manual (Pharmacia), with some modifications. After the first precipitation, the pelleted DNA was resuspended in 20uL of TE, pH 7.5, and 4uL were used in a digest with 1 unit (U) of RE. RNAseI (Pharmacia) was not added in the first solution, but 1uL of a 10mg/mL stock was added to the 10uL restriction digest. In order to achieve cleaner DNA, the pellet was resuspended in 200uL of TE, and a series of phenol/chloroform extractions were performed. 0.1 times the volume 3-5M sodium acetate (pH 4.8-5.2), or potassium acetate, was used to precipitate the pellet with 2.5 times the volume 95, or 100% ethanol v/v. This procedure can also be upscaled to achieve higher amounts of DNA, by starting with 10mL of cells, and increasing the total volume of TE for resuspension. DNA was quantitated by UV spectrophotometry, and checked for quality on an agarose gel (Pharmacia).

| | |
|---|---|
| Solution I | 100mM Tris-HCl pH7.5, 10mM EDTA pH8.0, 400ug heat-treated RNAse I/mL. Store at 4°C (Pharmacia). |
| Solution II | 0.2M NaOH, 1% SDS w/v. Make fresh before use (Pharmacia). |
| Solution III | For 100mL, 60mL 5M Potassium Acetate, 11.5mL glacial acetic acid, 28.5mL $H_2O$ (Pharmacia). |

### 2.2.1.12 Extraction of Genomic DNA

Genomic DNA was obtained from adult *D. melanogaster* OR flies and utilized as a template for the above PCR reaction. 50 adult flies were collected, 250uL of Buffer A were added, and the flies were homogenized using a sterile homogenizer. Another 250uL of Buffer A were added, and the homogenate was incubated at 65°C for 30 minutes. 75uL of 8M potassium acetate were added, and the solution was mixed, chilled on ice for 20 minutes and centrifuged at RTemp for 5 minutes at 12,000xg. The supernatant was recovered, and recentrifuged. 2.5 times the volume of 95% ethanol v/v was added, kept at RTemp for 5 minutes, then centrifuged at 12,000xg for another five minutes. The pellet was washed with 70% ethanol, and resuspended in 325uL of TE overnight at 4°C. The eluted DNA was centrifuged for 1 minute at 12,000xg at RTemp to remove any excess insoluble material. A series of phenol, phenol/chloroform, and chloroform extractions were performed, then 0.1 times the volume 3M sodium acetate pH 5.2 and 2.5 times the volume 95% ethanol v/v were added. After sitting at RTemp for 5 minutes, the DNA was centrifuged at 12,000xg for 5 minutes, washed with 70% ethanol v/v, and resuspended in 100uL of TE. 5uL aliquots were run on a 0.8% agarose gel to check for purity and yield (Nutter, pers. comm.).

| | |
|---|---|
| Buffer A | For 10mL, 1mL 1M Tris-HCl pH7.6, 2mL 0.5M EDTA pH8.0, 1mL 1M NaCl, 0.5mL 10% SDS w/v (Nutter, pers. comm.). |
| TE | 10mM Tris-HCl pH7.5-8.0, 1mM EDTA pH8.0 (Sambrook *et al.*, 1989). |

### 2.2.1.13 Primer Design

Primers were designed using both the NEB catalogue (with 2 bases preceding an EcoRI site, and 4 bases preceding an XhoI site) and the computer program Amplify 1.2 (Engels, 1993). Test PCR reactions were run using this program to check for possible primer dimers, or other secondary structures which may impede amplification. The primers used for PCR amplification are shown in Figure 2.1, as well as the resulting amplified sequence.

### 2.2.1.14 PCR

An 80bp piece from *Pkc53E* was amplified by PCR, to facilitate cloning, and to ensure that only a region specific to PKC53E was used as an antigen to raise polyclonal antibodies. The primers were designed through the use of the program Amplify 1.2, which checks for primer compatibility, and given the template sequence, whether a theoretical amplification is possible (Engels, 1993). The primers are shown in Figure 2.1. They were designed to introduce an EcoRI and an XhoI cut site into the amplified piece of DNA for directional subcloning into pGEX-4T1, in frame. The PCR reactions were set up with a total reaction volume of 100uL per 0.5mL microfuge tube, and the protocol is listed below. Several controls were used, such as a reaction without primers, another lacking Taq DNA polymerase (GibcoBRL), and a third without template. All reactions were set up on ice,

Figure 2.1: Primers used for PCR amplification. Additional bases were inserted as outlined by the NEB catalogue for RE digests, and genomic DNA extracted from *D. melanogaster* was used as a template. *Pkc53E* sequence is shown in bold, and the EcoRI and XhoI cut sites are underlined.

## PRIMERS

5' strand 5'GTGAATTCGATCCCCAGCAGCAGGGGGCG3'

3' strand 5'GCAGCTCGAGCTTGAGGGCTCCTTTGCGGAG3'

**Bold**=*Pkc53E*

Underlined=RE cut sites

## AMPLIFIED DNA SEQUENCE

5'GTGAATTCGATCCCCAGCAGCAGGGGGCGGAGGGGGAGGCGGTGGGCGAGAACAAAATG

**AAGTCCCGCCTCCGCAAAGGAGCCCTCAAG**CTCGAGCTGC3'

**Bold**=Pkc53E

Underlined=RE cut sites

and the appropriate amount of Taq DNA polymerase was added, according to the manufacturer's instructions, just prior to performing PCR. A Techne Thermal cycler (GENE E) with a heated lid was used for this procedure. The machine was preprogrammed with the desired cycles. The program used was **1**. denaturation at 95°C for 5 minutes **2**. 30 cycles of **a)** denaturation at 95°C for 1minute **b)** annealing at 65°C for 1 minute (Temperature will vary depending on GC-rich content. For amplification of vectors, 55-60°C is acceptable.) **c)** extension at 72°C for 1 minute **3**. polishing at 72°C for 10 minutes. The reactions were applied to the machine after it had been heated to 95°C, as a method of hot-starting the reactions to prevent nonspecific hybridization of primers. 10uL of each reaction were then loaded on a 2% agarose gel with the 123bp DNA ladder (GibcoBRL) as a marker, and electrophoresed (Carpenter, M.S., University of Calgary; Cohen, 1995; GibcoBRL).

| | |
|---|---|
| PCR reactions | 100uL per tube (0.5mL microfuge tubes), 10uL 10 X buffer (GibcoBRL), 10uL 10 X dNTP (2mM each dNTP), 1-5 uL 50mM $Mg^{2+}$ (GibcoBRL) (note that 1.5-2uL gave the best amplification), 5uL each of primers 1 and 2 (each 20uM), 5uL (template should be about 1ng, obtained from genomic DNA isolated from adult OR flies), add $ddH_2O$ to a volume of 99uL, 1uL of diluted Taq DNA polymerase (1U) (GibcoBRL). |

### 2.2.1.15 Plasmid Construction

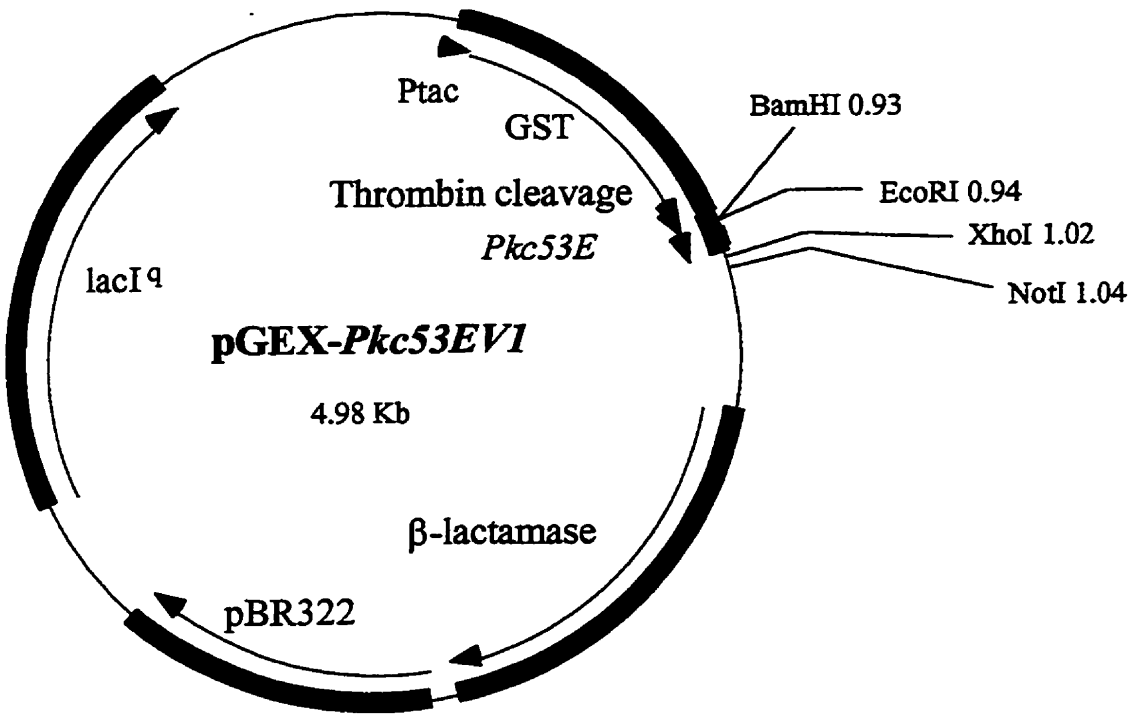
Primers were designed which would amplify DNA sequence encoding V1 and the first part of C1 from PKC53E, while incorporating RE cut sites for directionally

subcloning this piece in frame in the pGEX-4T1 vector. Genomic DNA from *D. melanogaster* was used as a template, and an 80bp piece was amplified by the polymerase chain reaction (PCR). Protocols for genomic extractions and PCR reactions were described in 2.2.1.12 and 2.2.1.14, respectively. This piece was gel purified, and treated by DNA polymerase I (GibcoBRL) to remove any excess bases. Gel purification procedures and DNA modifications with DNA polymerase I were described in 2.2.1.5 and 2.2.1.6, respectively. After phenol/chloroform extractions, the 80bp fragment was cut with EcoRI (GibcoBRL), and XhoI (GibcoBRL). Phenol/chloroform extractions and RE digests were described in 2.2.1.2 and 2.2.1.7, respectively. The fragment was subjected to another phenol/chloroform extraction and reprecipitation. Ligation reactions were then set up with gel purified pGEX-4T1, that had been previously cut with EcoRI and XhoI. DNA precipitations and ligations were described in 2.2.1.3 and 2.2.1.8, respectively. The ligation reactions were transformed into competent *E.coli* DH10B cells, and plated on LBAmp plates. Transformations were described in 2.2.1.9. Colonies were screened by PCR using the same gene-specific primers used for the initial amplification of the 80bp *Pkc53EVI* fragment. A positive colony was selected, midi-prepped using a Qiagen column (Qiagen), and sequenced. Figure 2.2 shows the plasmid map of the pGEX-*Pkc53EVI* construct (Pharmacia).

### 2.2.1.16 PCR Screen

PCR was used to screen transformed *E.coli* DH10B colonies to verify successful subcloning of the 80bp fragment into pGEX-4T1. Colonies were picked, simultaneously plated on LBAmp plates, and inoculated into microfuge tubes set up for PCR reactions. The reactions were prepared as described earlier, using the same gene-specific primers for amplifying the 80bp piece from genomic DNA, as shown in Figure 2.1. A standard

Figure 2.2: A plasmid map of the pGEX-*Pkc53EV1* construct. The vector, pGEX-4T1 was used for subcloning of a region of *Pkc53E* which encodes V1 and part of C1. This 80bp fragment was amplified by PCR, using primers with EcoRI and XhoI cut sites designed for directional subcloning. The fragment was subcloned directly into these sites within the MCS of this vector, in frame with the GST ORF, and sequence encoding the thrombin cleavage site. This results in the expression of a GST-PKC53EV1 fusion protein, when induced with IPTG.


Ptac
GST
Thrombin cleavage
Pkc53E
lacI q
pGEX-Pkc53EV1
4.98 Kb
β-lactamase
pBR322
BamHI 0.93
EcoRI 0.94
XhoI 1.02
NotI 1.04

concentration of 2uL of 50mM $Mg^{2+}$ (GibcoBRL) was used in all of the reactions. The same Techne Thermal cycler, and program was used as described earlier. The products of the reactions were electrophoresed on a 2.0% agarose gel, to verify amplification of the 80bp fragment. Negative controls were performed as described earlier, to ensure all amplification products were specific.

### 2.2.1.17 Dideoxy Sequencing

pGEX-*Pkc53EV1* was prepared for sequencing using a midi-prep Qiagen column purification procedure (Qiagen). All solutions used for this protocol are described below. The procedure was followed as outlined (Qiagen). After the DNA was pelleted, resuspended, and a series of phenol/chloroform extractions were performed, aliquots of DNA were checked for quality and quantity on an agarose gel. The primers used for sequencing were made to the same sequence published in the Pharmacia catalogue, which have been designed for optimal sequencing of the pGEX vectors. In the manual accompanying the T7 Sequencing kit (Pharmacia), part C and part D were followed to denature the template, and prepare the sequencing reactions. Once the sequencing reactions were completed, they were placed at -20°C until needed. Published protocols were used to pour a sequencing gel (Slatko and Albright, 1991). The smaller plates (36cm) were used to obtain about 200-300bp of sequencing data. The short plate was treated on one side with repelSilane (Pharmacia). After the coat dried, it was washed with 70% ethanol v/v as described in the protocol (Slatko and Albright, 1991). Spacers and clamps were used to help keep the plates in place. Once a good seal had been obtained, the bottom and sides were sealed with gel sealing tape, to decrease the amount of leaking. Since urea was difficult to dissolve, 60mL of a 6% acrylamide/bisacrylamide gel solution was made while preparing the plates (Slatko and Albright, 1991). After the solution was degassed, 50uL

tetramethylethylene-diamine (TEMED) and 500uL 10% ammonium persulfate (APS) w/v were added, mixed, and the gel solution was poured (Slatko and Albright, 1991). A syringe was used to pour the gel solution into the plate 'sandwich', tilted such that the solution filled the bottom part of the gel first. The gel polymerized for 45 minutes, then residue was cleaned from the gel plates, and the clamps and tape from the bottom were removed (Slatko and Albright, 1991). The gel sandwich was placed in the Model SA sequencing apparatus (GibcoBRL) and run according to manufacturer's specifications (40W, 1,400V for a 32cm, 0.4mm thick gel), with a Model 4000 power supply (GibcoBRL). The gel was prepared, pre-warmed, and run as outlined (Slatko and Albright, 1991). 3uL samples from the sequencing reactions were aliquoted per lane, and heated at 75-80°C for 2 minutes prior to loading (Slatko and Albright, 1991). The second set of reactions was added when the dye front from the first set had run to the bottom of the gel. The gel was stopped when the dye front from the second set of reactions had run to the bottom, and was treated as outlined, except it was not fixed prior to being placed in the gel drier. The gel was dried on a BioRad Model 583 Gel Dryer, with Gel Blot Paper (Schleicher and Schuell), according to manufacturer's instructions. The second program, which takes 2 hours, was used. After the gel was dried, it was covered with Saran Wrap, removed, and placed in a film cassette with Kodak Scientific Imaging X-OMAT film, for 72 hours (Slatko and Albright, 1991). Meanwhile, the area was cleaned and swiped according to safety regulations for radioactive work, using organic scintillation fluid for swipes, and aqueous scintillation fluid for the buffer. 100uL of buffer were removed to count the radioactivity in radioactive waste. The film was developed according to specifications given for the developer and fixer used in Dr. M. Maloney's dark room. The film was read, and sequence recorded for each reaction.

| | |
|---|---|
| 40% acrylamide/bisacrylamide | 38% acrylamide w/v, 2% bisacrylamide w/v. Do not heat above 55°C. Store at 4°C in dark (Slatko and Albright, 1991). |
| Buffer P1 | 100ug/mL RNAse A, 50mM Tris-HCl pH7.5, 10mM EDTA pH8.0. Store at 4°C (Qiagen). |
| Buffer P2 | 200mM NaOH, 1% SDS w/v. Make fresh before use (Qiagen). |
| Buffer P3 | 3.0M potassium acetate, pH5.5 (Qiagen). |
| Buffer QBT | 750mM NaCl, 50mM MOPS, 15% ethanol v/v, pH7.0, 0.15% Triton X-100 v/v (Qiagen). |
| Buffer QC | 1.0M NaCl, 50mM MOPS, 15% ethanol v/v, pH7.0 (Qiagen). |
| Buffer QF | 1.25M NaCl, 50mM Tris-HCl pH7.5, 15% ethanol v/v, pH8.5 (Qiagen). |
| 6% denaturing acrylamide gel | For 60mL, 25.2g urea, 9.0mL 40% acrylamide/bisacrylamide, 6.0mL 10 X TBE, 24mL $H_2O$. Dissolve, then filter and degas, prior to adding 500uL 10% APS w/v and 50uL TEMED (Slatko and Albright, 1991). |
| 10 X TBE | For 1L, 108g Tris base (890mM), 55g boric acid (890mM), 40mL 0.5M EDTA (20mM) pH8.0. pH8.3-8.9. Will precipitate out of solution over time (Slatko and Albright, 1991). |

### 2.2.2 Biochemical Techniques

#### 2.2.2.1 Protein Assays

Protein assays were carried out using the BioRad Protein Assay system, with bovine serum albumin (BSA) as a control. The concentration of BSA was 1mg/mL, and dilutions of 1ug, 2ug, 4ug, and 8ug were made in a total volume of 1mL, with 200uL of the dye reagent. The solutions were read using visible wavelength (595nm) on a Beckman DU-640 spectrophotometer, and a standard curve was derived. A dilution of the protein sample to be determined was prepared as above for the standard, and read with visible light. The concentration was then determined by extrapolating the value from the standard curve.

#### 2.2.2.2 SDS-PAGE

Proteins obtained through the course of this project were run on SDS-PAGE. Protocols for preparing, running, staining, and destaining SDS gels were followed as outlined (Sambrook *et al.*, 1989). The acrylamide concentration of the gel was adjusted from 8-20% to accommodate the size of the desired proteins. A BioRad Mini 2-D gel apparatus was used for all protein gels, with a Pharmacia Electrophoresis Power Supply EPS 500/400. A continuous Tris-Glycine running buffer was used for this apparatus as per manufacturer's protocol. The separating gel was prepared with a volume of 10mL per gel sandwich (20mL total for both) (Sambrook *et al.*, 1989). The stacking gel was prepared in a volume of 5mL for both gel sandwiches (Sambrook *et al.*, 1989). The apparatus has a gel casting system, and the plates were cleaned with water and 70% ethanol v/v (Sambrook *et al.*, 1989). The separating gel was poured first, and overlaid with 0.1% SDS (Sambrook *et al.*, 1989). After polymerization, the overlay was removed, and the stacking gel was poured on top, with well combs placed accordingly (Sambrook *et al.*, 1989). After polymerization, the wells were rinsed with $H_2O$, and the plates were placed

in the apparatus with running buffer (Sambrook *et al.*, 1989). The BioRad Mini 2-D gels have well sizes of about 30-40uL and prior to loading, 6 X SDS loading buffer was added to the samples in a 1:1 v/v ratio, heated at 65°C for one minute, and placed on ice briefly to cool (Sambrook *et al.*, 1989). 3uL of high molecular weight standard marker (GibcoBRL) were used per gel, and made to a final loading volume of 20uL. The gels were run at 50V until the samples had entered the separating gel, then increased to 120-150V until the dye front had run off. After the gels were stopped, they were either added to Coomassie stain, or placed in transfer buffer, for transfer to nitrocellulose (Sambrook *et al.*, 1989).

| | |
|---|---|
| 30% acrylamide/bisacrylamide | 29% acrylamide w/v, 1% bisacrylamide w/v. Do not heat above 55°C. Store at 4°C in dark (Sambrook *et al.*, 1989). |
| 12% SDS-PAGE (separating) | For 10mL, 4mL 30% acrylamide/bisacrylamide, 3.3mL $H_2O$, 2.5mL 1.5M Tris-HCl pH8.8, 0.1mL 10% SDS w/v 0.1mL 10% APS w/v, 5uL TEMED. Make 10% APS w/v fresh before use. This can be modified for different gel concentrations (Sambrook *et al.*, 1989). |
| SDS-PAGE (stacking) | For 5mL, 3.4mL $H_2O$, 0.83mL 30% acrylamide/bisacrylamide, 0.63mL 1M Tris-HCl pH 6.8, 0.05mL 10%SDS w/v, 0.05mL 10% APS w/v, 5uL TEMED. Make 10% APS w/v fresh before use. Concentration will not change regardless of the concentration of the separating gel (Sambrook *et al.*, 1989). |

| | |
|---|---|
| Coomassie stain | For 100mL, 0.25g Coomassie Brilliant Blue R250, 90mL 1 part methanol : 1 part $H_2O$, 10mL glacial acetic acid (Sambrook *et al.*, 1989). |
| 5 X Tris Glycine buffer | For 1L, 15g Tris base, 72g glycine, and 5g SDS (Sambrook *et al.*, 1989; BioRad). |
| Destain | For 100mL, 90mL 1 part methanol : 1 part $H_2O$, 10 mL glacial acetic acid (Sambrook *et al.*, 1989). |
| 6 X SDS loading dye | For 5mL, 0.35M 1M Tris-HCl pH6.8, 10.28% SDS w/v, 36% glycerol v/v, 5% β-mercaptoethanol v/v, 0.012% bromophenol blue w/v. Store in 0.5mL aliquots at -80°C (Sambrook *et al.*, 1989). |

### 2.2.2.3 Protein Induction

pGEX-4T1 is 4.9kb in size, and contains the open reading frame (ORF) for GST adjacent to sequence encoding a thrombin cleavage site, which is next to the multiple cloning site (MCS). This is under the control of the ptac promoter, which consists of a fusion between the lac and taq promoters. This promoter is inducible by IPTG and repressible by glucose. The vector also contains the *β-lactamase* gene, which confers ampicillin resistance. An 80bp fragment encoding a small portion of the N-terminus of PKC53E was subcloned into the MCS of pGEX-4T1 in frame with the GST ORF. This resulted in the production of a GST-PKC53EV1 fusion protein upon expression. The GST moiety is about 27kDa, and the PKC53E portion encodes a peptide of about 4kDa, resulting in a fusion protein of approximately 31kDa. The protocol and reagents used for induction, are outlined in the GST Gene Fusion System manual (Pharmacia). Cultures were grown in 200-250 mL 2 X YTGAmp with shaking at 37°C until the Absorbance at $OD_{600}$ was 0.6-0.8. IPTG was added to a concentration of 0.1mM, and the cultures were

incubated at 37°C for another 2-4 hours. A higher yield of protein was obtained if the cultures were left for at least four hours after induction. The cells were then centrifuged in Nalgene bottles at 4000xg using the Sorvall RC-5B Refrigerated Superspeed Centrifuge, with the GSA rotor. The supernatant was poured off, and the pelleted cells were stored at -20°C overnight. The cells were maintained at -20°C for a few days if necessary, which was found to enhance cell disruption after sonication. The cells were resuspended in 20mL of GET lysis buffer, and 1mM PMSF was added prior to sonication. The resuspended cells were transferred to 50mL centrifuge tubes, and sonicated using a Kontes Micro Ultrasonic Cell Disruptor on ice for 4-6 pulses at 45 seconds per pulse. Sonication was complete when a clear layer became visible on top of the mixture, indicating the majority of cells had lysed. Oversonication can cause problems with protein denaturation. The sonicate was centrifuged at 9500xg for 30 minutes and the supernatant was then applied to a glutathione-coupled Sepharose 4B column for purification by affinity chromatography (Pharmacia). Upon induction, the fusion construct comprised of a large proportion (20%) of overall protein produced by the cells. The fusion protein was soluble, and quantities of 2-3mg/200mL cells were easily obtained through column purification (Pharmacia).

| | |
|---|---|
| 2 X YTGAmp broth | For 1L, 16g tryptone, 10g yeast extract, 5g NaCl and after autoclaving, and cooling to 50°C, 20% glucose w/v is added. Make fresh before use, or can store at 4°C. Ampicillin is added after the medium is cooled to 50°C to a final concentration of 100ug/mL (Pharmacia). |
| 2 X YTG broth | For 1L, 16g tryptone, 10g yeast extract, 5g NaCl and after autoclaving, and cooling to 50°C, 20% |

glucose w/v is added. Make fresh before use, or can store at 4°C (Pharmacia).

#### 2.2.2.4 Protein Purification

Purification of the GST-PKC53EV1 fusion protein was accomplished by affinity column chromatography with a glutathione-coupled Sepharose 4B column (Pharmacia). The column was set up according to manufacturer's instructions, resulting in a bed volume of 2 mL with a 50% slurry. It was maintained at 4°C, and equilibrated with 1 X PBS when not in use. Due to the small size of the column, the flow rate was slow enough to not require the use of a pump. The fusion protein was expressed, and prepared as discussed in the above protocol, resulting in a sonicate volume of about 20mL. The sonicate was applied to the column, and a series of washes were performed as outlined in the GST Gene Fusion System manual (Pharmacia). The bound GST fusion proteins were eluted with an equal bed volume of glutathione elution buffer, and fractions of purified protein were collected off the column. Although only 2mL of elution buffer was used, three fractions of about 1mL each were collected, due to carry-over from the wash buffer. The third fraction was the most concentrated (1mg/mL), and the first fraction of post wash usually contained some eluted protein as well. The elutions were assayed with the BioRad Protein Assay system to check for quantity. Samples were collected throughout protein purification, and these were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel eletrophoresis (SDS-PAGE), which was stained with Coomassie Blue to check for quantity and for the presence of contaminating proteins. The first purification through the column resulted in an abundance of purified protein, however, contamination with high molecular weight *E.coli* proteins was evident. Therefore, the fractions of purified protein collected in the first run through, were diluted with 1 X PBS, and run through the column again. The eluates collected from this second purification still had a high concentration of purified protein (0.5

mg/mL), however, the proportion of *E. coli* proteins was reduced so they were no longer visible on Coomassie-stained SDS-PAGE (Pharmacia).

| | |
|---|---|
| GET lysis buffer | 150mM NaCl, 50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH8.0 (Pharmacia). |
| Glutathione elution buffer | 10mM reduced-glutathione, 50mM Tris-HCl pH 8.0. Store in 4mL aliquots at -20°C (Pharmacia). |
| 10 X PBS | For 1L, 80g NaCl (137mM), 2g KCl (2.7mM), 11.5g $Na_2HPO_4 \cdot 7H_2O$ (4.3mM), 2g $KH_2PO_4$ (1.4mM) pH7.3 (Sambrook *et al.*, 1989). |

### 2.2.2.5 Thrombin Cleavage

Purified GST-PKC53EV1 fusion protein was incubated in 1 X PBS, with appropriate amounts of thrombin at 37°C for 2-4 hours, and subjected to 20% SDS-PAGE to visualize cleavage. The cleaved antigen was also checked by a Western blot with anti-GST antibodies. The protocol for thrombin cleavage is dependent on the manufacturer. The appropriate units of activity were used for Thrombin (Sigma) accordingly.

### 2.2.2.6 Raising Polyclonal Antibodies

Purified GST-PKC53EV1 protein was used to generate polyclonal antibodies in rabbits. The preparation of antigen for injection was followed as outlined (Dr. M. Lohka, University of Calgary). Two adult female New Zealand white rabbits were purchased and maintained through the University of Calgary Animal Care Services. Prebleeds of 10 mL per rabbit were obtained upon their arrival, for use as preimmune sera. After a period of rest, the initial injections were given. 200ug of protein was adjusted to a volume of 300uL, per rabbit, with sterile water in 1.5mL microfuge tubes, and an equivalent amount of

Freund's complete adjuvant (GibcoBRL) was added per tube. The mixtures were sonicated on ice for about 5 pulses of 45 second duration, with intermittent cooling. Upon completion, the mixtures were white and thick. The mixtures were then drawn into 3cc Becton Dickinson (B-D) LuerLok syringes. This was accomplished using 18G1 1/2 PrecisionGlide syringe needles (B-D) with sawed off bottoms, and by continually recentrifuging the mixtures. After the syringes were filled with a final volume of about 0.5mL per syringe, the needles were replaced with a new needle of the same size for intramuscular injection. Three weeks after the initial injections, each rabbit was given a boost with an emulsion prepared as above, but with 150ug of protein per rabbit mixed with Freund's incomplete adjuvant. Booster injections were given every two weeks using emulsions prepared as for the initial booster, with 150ug of protein per rabbit and Freund's incomplete adjuvant. Test bleeds of 10mL were obtained from each rabbit about 8 days after each injection. Antisera was separated from blood that had coagulated overnight at 4°C, by centrifugation at 5,000xg for 15 minutes. The blood was kept in the vials in which the blood was originally obtained, as they fit in the rotor head of the centrifuge. After centrifugation, the antisera, visible as a clear liquid at the top, was drawn off and aliquoted into microfuge tubes and stored at -20°C for future use. Antisera was used as a primary antibody in Western blots to check for specificity and titer to the antigen. After the third test bleed, a dilution of 1:10000 of the primary antibody was required to detect 0.02ug of antigen, for both rabbits 1 and 2, although the first rabbit gave a stronger signal than the second. Another injection was given, and the first rabbit was bled out. The second rabbit was given a fifth injection and consequently bled out after 7-10 days.

### 2.2.2.7 Western Blots

GST-PKC53EV1 was used as an antigen to raise polyclonal antibodies in rabbits, as described in section 2.2.2.6. These antibodies were used as anti-PKC53E antisera, in a series of Western analyses on different *D. melanogaster* tissues and developmental stages. Table 2.1 lists several different antisera used throughout these experiments. Western blots were also performed with anti-GST antibodies, pre-immune antisera, and anti-PKC53E antisera which had been pre-incubated with either the GST-PKC53EV1 fusion protein, or GST. SDS-PAGE was set up as described above, with proteins from the different extracts in the correct concentrations. Originally, 12% SDS-PAGE was used for the GST fusion protein with 0.02ug per well, and PKC-rich extracts from *D. melanogaster* tissues and developmental stages were subjected to 8-10% SDS-PAGE with 20-40ug per well. However, since both the GST fusion protein and PKC-rich extracts could be separated by 10% SDS-PAGE, this became the standard gel concentration used. After electrophoresing, the gels were placed in transfer buffer for 20 minutes (Sambrook *et al.*, 1989). A piece of nitrocellulose, and six pieces of Whatman 3MM Chromatography paper were cut to size (5.5cm X 8.5cm), per gel. The nitrocellulose was placed in distilled water for 20-30 minutes (Sambrook *et al.*, 1989). The Pharmacia LKB Multiphor II Electrophoresis was used to perform a semi-dry transfer of proteins to nitrocellulose, and was set up according to manufacturer's instructions. Pharmacia Electrophoresis Power Supplies EPS 600 or EPS 500/400 were used as a power source. The typical current, in mAmps, for transferring, is 0.8 times the surface area ($cm^2$) of the blot. Proteins were transferred for 50-60 minutes (Sambrook *et al.*, 1989). The electrodes were wet lightly with transfer buffer, then three pieces of Whatman 3MM Chromatography paper were wet with transfer buffer, and placed on the apparatus, such that no bubbles were present (Sambrook *et al.*, 1989). The nitrocellulose was placed down, then the gel, followed by three more pieces of wet Whatman 3MM Chromatography paper. The bottom plate is attached to the positive

Table 2.1: A list of primary and secondary antibodies used for Western blot analyses, and immunohistochemistry.

| Antibody | Description |
|---|---|
| anti-PKC53E1 | polyclonal primary antibodies against GST-PKC53EV1 antigen from rabbit 1 |
| anti-PKC53E2 | polyclonal primary antibodies against GST-PKC53EV1 antigen from rabbit 2 |
| anti-PKCαβγ | commercially available polyclonal primary antibodies against PKCαβγ (19-36; pseudosubstrate domain) (GibcoBRL) |
| anti-rabbit FITC | commercially available secondary antibodies against rabbit serum, conjugated to FITC (GibcoBRL) |
| anti-rabbit biotin | commercially available secondary antibodies against rabbit serum, conjugated to biotin (GibcoBRL) |
| anti-motor protein | mouse monoclonal antibodies against a motor neuron protein, found in embryos later than stage 12 (Auld) |
| anti-mouse TR | commercially available secondary antibodies against mouse antiserum, conjugated to TR (Jackson Lab.) |
| anti-rabbit TR | commercially available secondary antibodies against rabbit antiserum, conjugated to TR (Jackson Lab.) |

electrode, and the top plate is attached to the negative electrode, so the current transfers the negatively charged proteins down (Sambrook *et al.*, 1989). After the transfer was complete, the blot was checked with Ponceau S stain, and rinsed with water (Sambrook *et al.*, 1989). The blot was marked with a pen where the high molecular weight standards had stained, and cut into strips. The blots were then blocked overnight at 4°C, and incubated with the primary and secondary antibodies as outlined (Sambrook *et al.*, 1989). The blots were incubated with primary and secondary antibodies in blocking buffer. The dilution factors for all commercially obtained antibodies were used according to manufacturer's instructions (GibcoBRL). A 1:5000 dilution of primary antibody was used to achieve consistent results, however, a 1:10000 dilution was also successful with adult head extracts, and resulted in lower background. Antibodies were blocked with antigen and GST by pre-incubating 1:5000 anti-PKC53E antisera with 10-20ug protein at RTemp for 1-2 hours. Affinity-purified antibodies were made as described later in this chapter, and 1:25-1:50 dilution factors were used, depending on the titer obtained. The blots were developed as outlined for Alkaline Phosphatase-conjugated secondary anti-rabbit antibodies (GibcoBRL) according to manufacturer's instructions, and the blot was left to develop at RTemp in the dark. After the reaction was complete, the blots were rinsed with water, and dried on paper towels.

Western blots were scanned in with the Microtek ScanMaker III, and a Power Macintosh 7600/120, using Adobe Photoshop. All blots were modified using the same histogram, and levels of brightness and contrast. They were scanned in at a 300dpi resolution, stored as PICT files, and printed on a black and white laser printer with a 1200dpi.

| | |
|---|---|
| AP substrate buffer | 100mM Tris-HCl pH9.5, 100mM NaCl, and 50mM $MgCl_2$. For 10mL, 44uL NBT, and 33uL BCIP are |

| | |
|---|---|
| | added just prior to incubating membrane. Buffer should be made fresh (GibcoBRL). |
| Blocking buffer | 10% non-fat milk w/v, 0.3% Tween-20 v/v in 1 X PBS. Make fresh before use (Sambrook *et al.*, 1989). |
| TBS | 10mM Tris-HCl pH8.0, 150mM NaCl. Store at 4°C (Sambrook *et al.*, 1989). |
| TBST | TBS + 0.05% Tween-20 v/v (Sambrook *et al.*, 1989; Pharmacia). |
| Transfer buffer | For 1L, 2.92g glycine, 5.81g Tris, 0.375g SDS and 20% methanol v/v (Dr. M. Lohka, University of Calgary; Sambrook *et al.*, 1989). |
| Wash buffer | 0.3% Tween-20 v/v in 1 X PBS (Sambrook *et al.*, 1989; Pharmacia). |

#### 2.2.2.8 Extraction of *D. melanogaster* Proteins

Adult heads were dissected in cold Ringer's solution, and placed in microfuge tubes on ice. The Ringer's solution was removed, and 400 adult fly heads were homogenized in about 600uL of extraction buffer. The homogenate was incubated on ice for 30 minutes, then centrifuged at 10,000xg for 3-5 minutes (Broughton *et al.*, 1996; GibcoBRL). About 300uL of supernatant was collected and quantitated using the BioRad protein assay system. This method was used for the extraction of various *D. melanogaster* proteins. Similar concentrations were achieved with about 50 female ovaries in 100uL buffer, 300 male testes in 50uL buffer, 100 embryos in 50uL extraction buffer, 10-20 adult flies in 200uL extraction buffer, 10-20 pupae and 3rd instar larvae in 200uL extraction buffer, 20-40 2nd instar larvae in 200uL extraction buffer, and 50-70 1st instar larvae in 200uL extraction

buffer. Several reported PKC-rich extraction procedures were performed; however, the best extraction procedure utilized a buffer with 20mM Tris-HCl, 0.5mM EGTA, 0.5mM EDTA, 10mM β-mercaptoethanol, 0.5% Triton X-100, 25ug/mL PMSF and 25ug/mL each leupeptin and aprotinin (Broughton *et al.*, 1996; GibcoBRL). A PKC assay kit was purchased from GibcoBRL, with stock solutions of the above reagents (except PMSF). All dissected tissues used for extraction were collected as described earlier. About 20-40ug of PKC-rich extracts were run per well on SDS-PAGE, which were transferred to nitrocellulose, and analyzed by Western blotting.

| | |
|---|---|
| PKC-rich extraction buffer | 20mM Tris-HCl pH7.5, 0.5mM EGTA, 0.5mM EDTA pH8.0, 10mM β-mercaptoethanol, 0.5% Triton X-100 v/v, 1mM PMSF and 25ug/mL each leupeptin and aprotinin. Make fresh each time before use (GibcoBRL). |

### 2.2.2.9 Immunoprecipitations

As per manufacturer's instructions from a PKC Assay kit (GibcoBRL), immunoprecipitations were performed on PKC-rich extracts from adult heads. Adult heads were dissected in cold Ringer's solution, and placed in microfuge tubes on ice. The Ringer's solution was removed, and they were homogenized in a buffer consisting of 20mM Tris, pH7.5, 0.15M NaCl, 2mM EDTA, 1mM EGTA, 1.0% Triton X-100, 25ug/mL each aprotinin and leupeptin. The homogenate was incubated on ice for 30 minutes, then centrifuged at 10,000xg for 5 minutes and the supernatant was collected. Microfuge tubes with 50-100ug of supernatant were incubated with either 5uL of anti-PKC53E1, or with 10uL of anti-PKCαβγ antibody (GibcoBRL). These antibodies are listed in Table 2.1. They were incubated at 4°C overnight, with rocking. During the

incubation, protein A-coupled agarose (Sigma) was prepared according to manufacturer's instruction. An aliquot (100uL/mL) was transferred to a microfuge tube, centrifuged, and equilibrated in the same buffer used for the above protein extractions. This was repeated several times, then aliquots of protein A-coupled agarose were added to the microfuge tubes containing the antibody-protein samples, for incubation with rocking at RTemp for one hour. The mixture was briefly centrifuged, and washed twice in the buffer used above for extraction. An SDS-PAGE was run at this point, and transferred to nitrocellulose to check for proteins that were specifically bound by the antibodies.

| | |
|---|---|
| Immunoprecipitation buffer | 20mM Tris-HCl pH7.5, 0.15M NaCl, 2mM EDTA pH8.0, 1mM EGTA, 1.0% Triton X-100 v/v, 25ug/mL each aprotinin and leupeptin. Make fresh before use (GibcoBRL). |

### 2.2.2.10 PKC Assays

Immunoprecipitated pellets of PKC53E were collected as described above, and assayed for PKC activity. They were resuspended in an elution buffer of 50mM Tris pH7.5, 0.55M NaCl, 1.0% Triton X-100, 25ug/mL each leupeptin and aprotinin, 2mM EDTA, and 1mM EGTA at a volume of 20uL per reaction. A PKC assay kit from GibcoBRL was utilized for the assays. Two different substrates were used, one was the [Ser 25] 19-36 pseudosubstrate peptide, and the other was an acetylated peptide from myelin basic protein. Negative controls were run with each assay, using the 19-36 pseudosubstrate domain peptide as a specific PKC inhibitor. Essentially, the incorporation of radiolabelled phosphate groups from [$\gamma$-$^{32}$P]ATP into the substrate peptide was an indicator of PKC activity. Phosphocellulose units obtained from Pierce were used to collect the substrate-incorporated radiolabelled phosphates, and placed in scintillation vials

for counting the radioactivity. 1% phosphoric acid v/v was used to wash the phosphocellulose units.

| | |
|---|---|
| PKC assay buffer | 50mM Tris-HCl pH7.5, 0.55M NaCl, 1.0% Triton X-100 v/v, 25ug/mL each leupeptin and aprotinin, 2mM EDTA pH8.0, and 1mM EGTA at a volume of 20uL per reaction. Make fresh before use (GibcoBRL). |

### 2.2.2.11 Blot-affinity Antibody Purification

Anti-PKC53E1 antisera were further purified by a blot-affinity purification method (Tang, 1993). Three SDS gels were run with large wells, each holding 100ug-250ug of antigen, resulting in a total of approximately 300-750ug antigen. The proteins were transferred to nitrocellulose, and detected with Ponceau S stain. The blots were cut to make antigen strips, and placed in blocking buffer overnight at 4°C with gentle rocking. The strips were then incubated with 100uL primary antibody, diluted in 10mL Tris-buffered saline, 0.05% Tween-20 (TBST) with 100uL 10% BSA fraction V, and 100uL 5% sodium azide, 12-16 hours at RTemp with gentle agitation. The strips were then washed 3 X 10 minutes with TBST, and the antibodies were eluted on ice using glycine elution buffer. 1mL glycine elution buffer was incubated with the strips over ice with gentle mixing for 3 minutes, then transferred to a tube with 0.15mL 1M Tris-HCl, pH8.1. This was repeated, then the strips were incubated with 2mL TBST for another 3 minutes, and all eluates were pooled together. The strips were then placed between Whatman 3MM Chromatography paper for storage at -20°C, and were reused several times. Final dilutions of 1:25-1:50 were used successfully in Western blots.

| | |
|---|---|
| Glycine elution buffer | 0.1M glycine, 0.5M NaCl, 0.05% Tween-20 v/v, pH 2.8 (Tang, 1993). |

### 2.2.3 Immunohistochemical Techniques

#### 2.2.3.1 Maintenance of *D. melanogaster* stocks

The Oregon-R wild type strain (OR) was used to obtain protein samples, and organisms from different stages of development were examined for the immunohistochemical studies of PKC53E. This stock has been maintained in Dr. M. Bentley's lab for many years, and was originally derived from flies collected at Roseburg, Oregon. Stocks were maintained on a medium composed of 92.5g agar, 1225g commeal, 440g yeast, 435g sucrose and 850g dextrose, in 16L $H_2O$ ( Add 125mL acid mix (418mL propionic acid, 41.5mL 55% phosphoric acid v/v, in 1L $H_2O$) prior to pouring) (Lewis, 1960). They were kept at room temperature (RTemp), or at 25°C.

#### 2.2.3.2 Collection of Developmental Stages

A schematic of the life cycle is shown in Figure 1.5. Embryos typically hatch after 21 hours at 25°C, pupariation occurs after 5 days, and eclosion after 9 days. Various stages of development were collected for obtaining protein extracts, or for immunohistochemical staining. Various stages of embryos were collected, by placing ~4 day old adult OR flies on Embryo collection plates for 1-2 hours at RTemp. After the adults were removed, the embryos were left for varying periods of time to 'stage' them. For example, a 2-4 hour embryo was left on the plate an additional two hours after the adults were removed, and likewise, a 6-8 hour embryo was left on the plate an additional four hours. Embryos collected for protein extraction were placed directly into harvesting solution in a microfuge tube. The solution was then removed, and the appropriate buffers were added for protein extraction. Embryos collected for immunohistochemical staining

were placed on double-sided scotch tape, and mechanically dechorionated by rolling across the tape. After dechorionation, they were placed directly into microfuge tubes with the appropriate fixing solution. 1st and 2nd instar larvae had been previously collected by Lauryl Nutter, and kept at -80°C. They were simply placed in the appropriate buffer for the extraction of proteins used in Western blots. Immunohistochemistry was not performed on these developmental stages. Early 3rd instar larvae were collected by taking larvae still crawling in the media, and placing them into 20% sucrose w/v. They were then dissected in 1 X phosphate-buffered saline (PBS) for collection of brains, and salivary glands, and placed directly into microfuge tubes with 1 X PBS on ice. Late 3rd instar larvae were collected by taking larvae crawling on the walls, and placing them into 20% sucrose w/v. Brains and salivary glands were then dissected and placed directly into microfuge tubes with 1 X PBS on ice. 3rd instar larvae, used for Western blotting, had been previously collected by Lauryl Nutter and kept at -80°C. Early (white) pupae, eye (tan) pupae, and late (dark) pupae were previously collected by Lauryl Nutter, and kept at -80°C. They were placed in appropriate buffers for protein extraction, and used in Western blots. Immunohistochemistry was not performed with these developmental stages. Four day old male and female adult OR flies were collected, and tissues were dissected in cold Ringer's solution. Tissues were placed in Ringer's solution, in microfuge tubes, on ice. Heads were collected from both males and females, and grouped together, and ovaries and testes were collected from females and males respectively. The Ringer's solution was replaced with the appropriate buffers for protein extraction. Ovaries and testes collected for immunofluorescence were placed in 1 X PBS as described for the larval tissues (Ashburner, 1989A; Ashburner, 1989B; Hughes, 1993).

| | |
|---|---|
| Embryo collection plates | For 400 mL, 200mL fresh grape juice, 192mL $H_2O$ save some for after agar added to help cool), 16g |

| | |
|---|---|
| | agar, 4.2mL 95% ethanol v/v, and 4.0mL glacial acetic acid. Heat grape juice and water to boiling, and stir in agar slowly, then reduce heat and let agar dissolve completely. When dissolved completely, and cooled to 60°C, add ethanol and glacial acetic acid and pour plates. Wrap and store at 4°C (Dr. D. Clark, University of New Brunswick). |
| Harvesting solution | 0.7% NaCl w/v, 0.4% Triton X-100 v/v (Hughes, 1993). |
| Ringer's solution | 182mM KCl, 46mM NaCl, 3mM $CaCl_2$, 10mM Tris-HCl pH7.2, filter sterilized and autoclaved (Ashburner, 1989B). |

#### 2.2.3.3 *D. melanogaster* Tissue Dissections

All dissections of larvae and adults were carried out as demonstrated by Dr. M. Bentley (University of Calgary), and Lauryl Nutter. Schematics for morphology can be found in Drosophila, A Laboratory Handbook (Ashburner, 1989A). Larvae were collected in 20% sucrose w/v, and dissected in cold 1 X PBS. Adults were etherized, and dissected in cold Ringer's solution, or cold 1 X PBS (Hughes, 1993).

#### 2.2.3.4 Immunofluorescence with Whole Mounts

Immunofluorescence was performed with tissues dissected from larvae and adults as described earlier. The tissues were fixed by removing the dissection buffer, replacing it with fresh fixative (4% paraformaldehye (PF)/1 X PBS), and incubating for 20 minutes with gentle agitation at RTemp. After collection, ovaries were treated with collagenase at 37°C for one minute, then washed with 1 X PBS, and incubated with fixative. After

fixing for 20 minutes, the tissues were washed by mixing with 1 X PBTB at RTemp, three times for 10 minutes each. The tissues were then incubated with blocking buffer for 2 -16 hours at 4°C, with gentle mixing. Different types of buffers were used for blocking, and the most effective were 2% skim milk in 1 X PBTB, and 10% normal goat serum in 1 X PBTB. Anti-PKC53E1 antisera were then added to the tissues in appropriate dilutions, and incubated at 4°C overnight with mixing. 1:500-1:1000 were effective dilutions of anti-PKC53E1 antisera. Affinity-purified antibodies were used with a 1:5 dilution, however, this titer may not have been appropriate for some tissues. The tissues were washed again, with 1 X PBTB, three times for 10 minutes at RTemp with gentle mixing. Blocking buffer was added to the tubes after the last wash, and the tissues were incubated at RTemp with mixing for 30 minutes to one hour. Secondary antibodies were added to the tissues at a dilution of 1:2000, for biotin-conjugated, and AP-conjugated anti-rabbit antibodies from GibcoBRL. These antibodies are listed in Table 2.1. However, the Texas red-conjugated secondary anti-rabbit antibodies (Jackson Lab.), required a 1:1000 dilution. If the antibodies were directly conjugated to a fluorochrome, they were kept in the dark by wrapping the tubes in foil. The tissues were incubated with secondary antibodies for 1 - 2 hours at RTemp with mixing, then washed 3 times for 10 minutes each in 1 X PBTB. A Texas red-streptavidin conjugate (GibcoBRL) was used with tissues which had been incubated with biotin-conjugated secondary anti-rabbit antibodies. These tissues were blocked for 30 minutes, then the streptavidin-conjugate was added, and incubated in the dark for one hour at RTemp with mixing. These tissues were washed as before, 3 times 10 minutes with 1 X PBT at RTemp, with mixing. During the washes, the tissues were kept in the dark. After the last wash, Fluorescent Mounting Media (GibcoBRL) was added to the tissues, and they were pipetted onto glass slides, and covered with glass coverslips. They were immediately wrapped in foil, and placed at -20°C until visualized with the microscope. A Leica Aristoplan microscope was used to visualize fluorescence, utilizing

the 40X objective and Texas Red filter. The Leica Wild MP546 camera attached to the microscope was used to take pictures with 400 speed Kodak Ektachrome film, and all exposures were taken in dark field (DF) with a setting of 50.

Slides of whole mounts were scanned in with the Polaroid SprintScan 35, and a Power Macintosh 7600/120, using Adobe Photoshop. All images were modified using the same levels of brightness (-35) and contrast (10). They were scanned in at a 300dpi resolution, stored as PICT files, and printed on a color DyeSublimation printer. Copies were made by color laser photocopying.

| | |
|---|---|
| PBT | 1 X PBS, 0.3% Triton X-100 v/v (Pharmacia; Sambrook *et al.*, 1989). |
| PBTB | 1 X PBS, 0.3% Triton X-100 v/v, 0.2% BSA w/v (Hughes, 1993; Tang, 1993). |
| 4% PF / 1 X PBS | Dissolve 4g PF in 100 mL 1 X PBS by heating over low heat (not past 60$^{o}$C,) add 1N NaOH drops to aid solubility. Make fresh before use (Hughes, 1993). |

### 2.2.3.5 Immunohistochemistry with Embryos

Embryos were stained by a number of different procedures. One is similar to the procedure described above, however, the initial fixation steps were different. Embryos dechorionated by hand were placed in microfuge tubes with 1 part fixative (4% PF/1 X PBS) : 1 part heptane, and incubated for 20 - 45 minutes at RTemp with shaking. The aqueous layer was removed with a pipette, then replaced with methanol. The contents of each microfuge tube were mixed for a minute, then the solution was removed, and replaced with methanol. The methanol was then removed, and replaced with 3 parts methanol : 1 part PF/PBS, and embryos were incubated for 5 minutes at RTemp with mixing. This was

repeated using increasing amounts of PF/PBS (1:1, then 1:3), until the last step when only PF/PBS was added. The embryos were incubated for another 20 minutes with PF/PBS, at RTemp with mixing. Then, they were washed 3 times 10 minutes with PBT, as described above, and placed in blocking buffer 2 hours - overnight at 4°C with gentle mixing. The rest of the staining procedure is described above. Another procedure for fixing embryos used the solution PEM-FA as a fixative. Embryos were collected as described earlier, however, they were placed in 1 part PEM-FA : 1 part heptane, and incubated for 20 minutes at RTemp with mixing. Then the PEM-FA was removed, and replaced with methanol. The embryos were mixed for one minute, then the solution was removed, and replaced with methanol. The embryos were washed with methanol several times, and if desired, the embryos can be kept in methanol for several weeks at 4°C. The methanol was then replaced with 1 X PBTB, and washed three times for 10 - 30 minutes at RTemp with mixing. The embryos were then blocked with 1 X PBTB, and 5% normal goat serum for 30 minutes - 1 hour at RTemp with shaking. Anti-PKC53E1 antisera were then added to the embryos, and they were incubated overnight at 4°C with mixing. Monoclonal antibodies, listed in Table 2.1, were used in a 1:30 dilution. The rest of the procedure is as described above, however 1 X PBTB was used for washing, and 1 X PBTB + 5% normal goat serum was used as the blocking buffer. An anti-mouse secondary antibody conjugated to Texas red (Jackson Lab.), listed in Table 2.1, was used in a 1:1000 dilution.

Immunohistochemical staining in embryos was also performed by colorimetric procedures. The embryos were treated as described above, except they were incubated with 1:2000 dilution of secondary antibody conjugated to AP (GibcoBRL), and after the detergent washes, they were washed twice with TBS. Then, they were incubated in AP substrate with nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in the dark for a half hour. The substrate was washed off with water, and the embryos were placed on a slide for viewing under light microscopy.

Slides of embryos were scanned in with the Polaroid SprintScan 35, and a Power Macintosh 7600/120, using Adobe Photoshop. All images were modified using the same levels of brightness (-35) and contrast (10). They were scanned in at a 300dpi resolution, stored as PICT files, and printed on a color DyeSublimation printer. Copies were made by color laser photocopying.

| | |
|---|---|
| PEM | 0.1M PIPES, 2mM EGTA, 1mM $MgSO_4$, pH6.95. Store at $4^{O}C$ (Auld, University of British Columbia). |
| PEM-FA | 9 parts PEM to 1 part 37% formaldehyde w/v. Make just before use (Auld, University of British Columbia). |

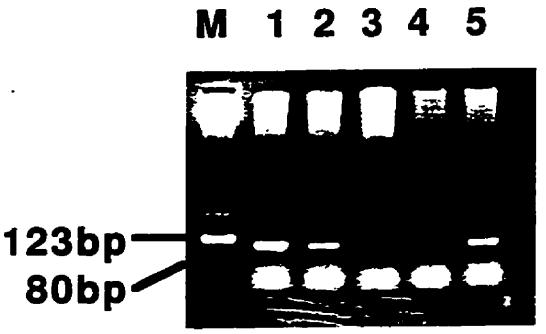
# Chapter 3
# Results

## 3.1 Cloning

The pGEX-*Pkc53EV1* construct was made by subcloning a putative unique portion of *Pkc53E* into pGEX-4T1, as shown in Figure 2.1. This was accomplished by using PCR to amplify an 80bp fragment from genomic DNA, with EcoRI and XhoI cut sites designed in the primers to allow for in frame-directional subcloning. The sequence of the primers used is shown in Figure 2.2, and the 2.0% agarose gel showing the amplified products is shown in Figure 3.1. The first lane (M) shows the 123bp ladder, and all other lanes show the amplified products using varying $Mg^{2+}$ concentrations. The most successful, shown in lanes 1, 2, and 5, resulted in the amplification of an 80bp band. Lanes 3 and 4, had no visible product. Controls were also performed without primers, without template, and without Taq DNA polymerase, and are shown in Figure 3.1. This 80bp fragment encodes the V1 region, and first part of C1, including the pseudosubstrate domain, of the putative ORF for PKC53E, as shown in Figure 2.1. After amplification, the fragment was subcloned into pGEX-4T1, using the unique EcoRI and XhoI cut sites. The transformed DH10B *E.coli* colonies were screened by PCR with the same gene-specific primers used to amplify the original 80bp fragment, as shown in Figure 3.2. The first lane shows the 123bp ladder, the adjacent lane is without template, and all subsequent lanes are amplified products, with different colonies as templates. All colonies shown gave positive results, and to ensure this was not due to contamination, a negative control without template, is shown in Figure 3.2 lane (1). Negative controls without primer, and without Taq DNA polymerase, are shown in Figure 3.1. Products were not visualized, indicating

Figure 3.1: A 2.0% agarose gel with PCR products (A). Also shown is a 2.0% agarose gel with negative controls (B). In gel (A), genomic DNA from *D. melanogaster* was used as template, and *Pkc53E* gene-specific primers were used to amplify an 80bp fragment. Lane (M) shows the 123bp ladder, and all other lanes are the products of PCR reactions, using varying $Mg^{2+}$ volumes from a 50mM stock solution (GibcoBRL). Lanes 1, 2, and 5, are samples from PCR reactions where 1.5uL, 2uL, and 2.5uL 50mM $Mg^{2+}$ were used per 100uL total reaction volume, respectively. An 80bp band was seen as indicated. In Lanes 3 and 4, 3 and 3.5uL 50mM $Mg^{2+}$ were used, respectively, and a band was not visible in these lanes. In gel (B), Lane (M) shows the 123bp ladder. Controls were performed without primers, shown in Lane (1), without template, shown in Lane (2), and without Taq DNA polymerase, shown in Lane (3).

A




B

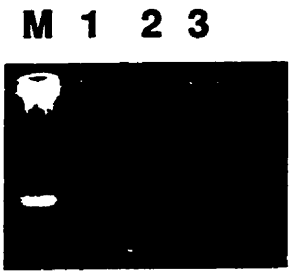
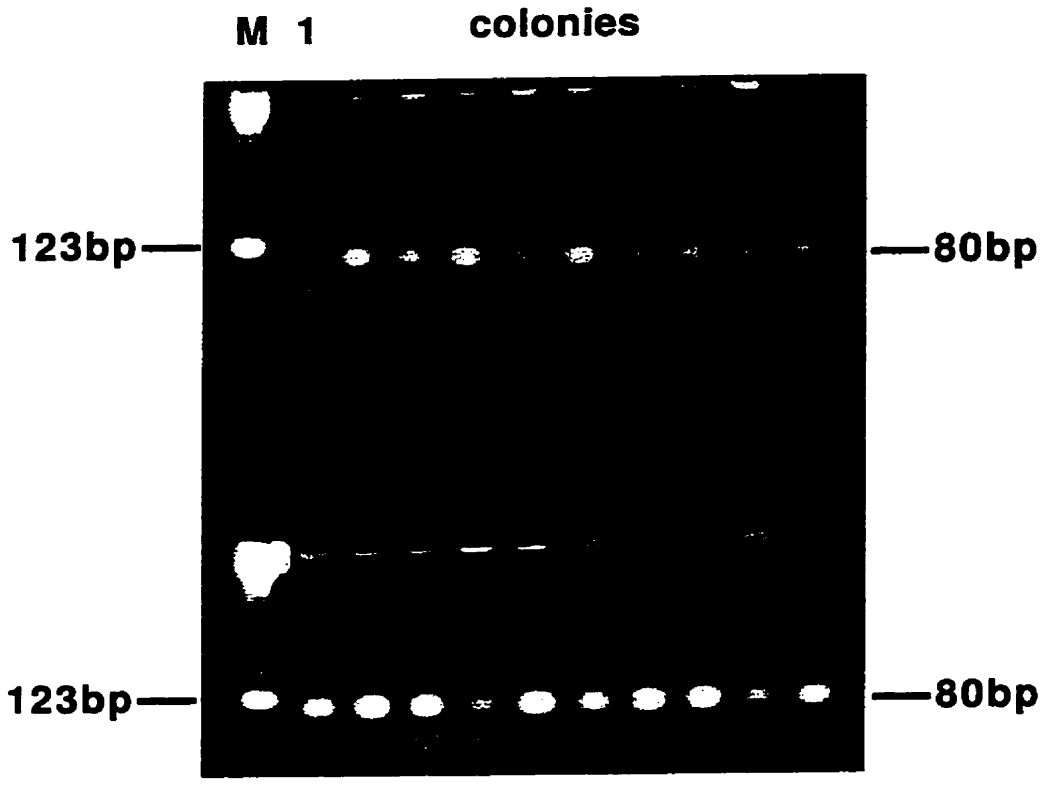


Figure 3.2: A 2.0% agarose gel with PCR products from a colony screen. Transformed DH10B *E.coli* colonies were used as template, with the same *Pkc53E* gene-specific primers as used previously. Lane (M) is the 123bp ladder, Lane (1) is without template, and all subsequent lanes are PCR reactions with different colonies as templates. An 80bp band is visualized as indicated.


M 1
colonies
123bp
80bp
123bp
80bp

the bands in Figure 3.2 were due to amplification of gene-specific sequences. A colony was chosen, midi-prepped, and sequenced by the dideoxy sequencing method. The *Pkc53E* sequence was 100% identical to the published sequence using the National Centre Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) search, and both EcoRI and XhoI cut sites were present (Altschul *et al.*, 1990). The surrounding vector sequence was identical to pGEX-4T1 published sequence, and the position indicated successful subcloning into the MCS.

### 3.2 Expression and Purification

IPTG was added to cells transformed with the pGEX-*Pkc53EV1* construct, resulting in the expression of a GST-PKC53EV1 fusion protein. The GST moiety is approximately 27kDa, and the PKC peptide is about 4kDa, resulting in a fusion protein of about 31kDa on SDS-PAGE. The corresponding amino acid sequence of the putative GST-PKC53EV1 fusion protein encoded by the pGEX-*Pkc53EV1* construct is shown in Figure 3.3. The amino acid sequence in bold corresponds to the PKC53EV1 portion, and the underlined sequence shows the pseudosubstrate domain. Figure 3.4 shows the 15% SDS-PAGE and Western with anti-GST antibodies performed to test bacterial lysates, transformed with pGEX-4T1 and pGEX-*Pkc53EV1*, for the presence of the GST and GST-PKC53EV1 fusion proteins respectively. Lane (1) in both the SDS-PAGE and the Western blot, showed a protein of about 27kDa, the predicted size of GST, and Lane (2) in both the SDS-PAGE and Western blot, showed a protein of about 31kDa, the predicted size of GST-PKC53EV1. There appears to be a doublet, which the anti-GST antibodies recognize. This could be due to partial cleavage of the GST-PKC53EV1 fusion, or due to the usage of alternate translation frames.

Figure 3.3: Amino acid sequence of the GST-PKC53EV1 fusion protein. The N-terminal portion, encoding a protein of approximately 27kDa, is the GST moiety. The C-terminal, in bold face type, encodes a protein of approximately 4kDa, from the V1 region of PKC53E, and the first part of C1. The underlined portion is the putative pseudosubstrate domain, which is less conserved from the mammalian isoenzymes.

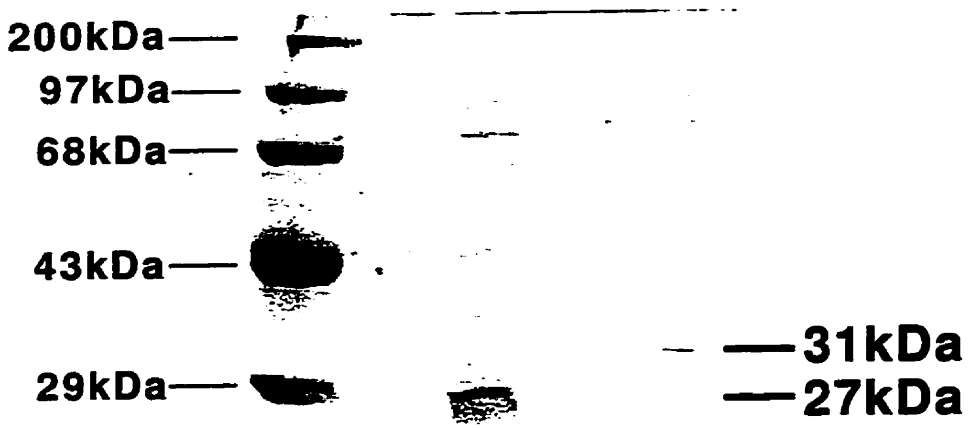
# GST-PKC53EV1

MSPILGYWKIKGLVQPTRLLLEYLEEKYEE
HLYERDEGDKWRNKKFELGLEFPNLPYYID
GDVKLTQSMAIIRYIADKHNMLGGCPKERA
EISMLEGAVLDIRYGVSRIAYSKDFETLKV
DFLSKLPEMLKMFEDRLCHKTYLNGDHVTH
PDFMLYDALDVVLYMDPMCLDAFPKLVCFK
KRIEAIPQIDKYLKSSKYIAWPLQGWQATF
GGGDHPPKSDLVPRGSPEF**DPQQQGAEGE**
**AVGENKMKSRLRKGALKKKNVFNVKDHC**
**F**

**Underlined sequence = pseudosubstrate domain**
**Bold = PKC53EV1/C1**

Figure 3.4: SDS-PAGE and Western blot of transformed *E.coli* lysates. (A) is a Coomassie stained 15% SDS-PAGE with lysates from *E.coli* DH10B transformed with pGEX-4T1 (1), and pGEX-*Pkc53EV1* (2). (B) is the corresponding Western blot for the gel in (A), using 1:5000 dilution of anti-GST antibodies. Lane (1) is with lysates from *E.coli* DH10B transformed with pGEX-4T1, and Lane (2) is with lysates from *E.coli* DH10B transformed with pGEX-*Pkc53EV1*.

A




B

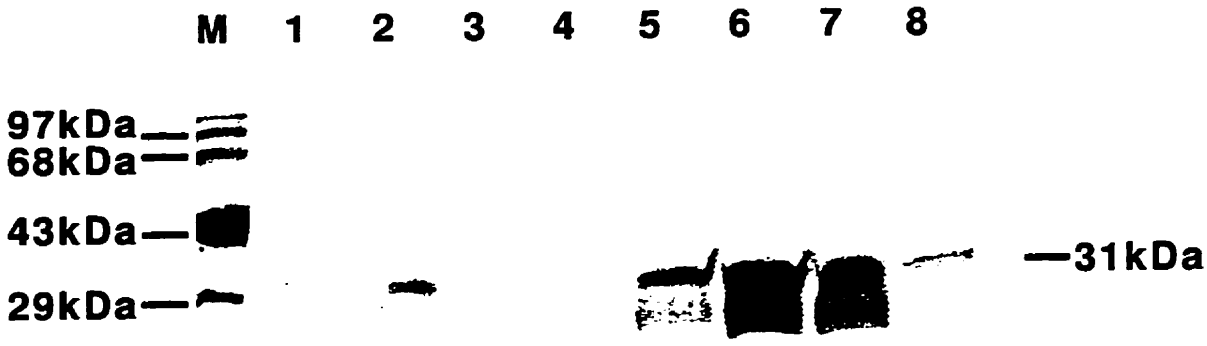
The GST-PKC53EV1 fusion protein was then expressed in larger cultures of the transformed bacterial strain. The protein was purified by affinity-column chromatography, using a glutathione-coupled slurry, followed by elution with reduced glutathione. Figure 3.5 (A) is a 15% SDS-PAGE showing the washes and eluates as they were collected from the second column purification. Lane (M) is the high molecular weight protein marker (GibcoBRL). Lane (1) shows the diluted eluates collected from the first column purification, and Lanes (2-4) are subsequent washes collected at various times from the second column purification. Little protein was visualized in Lane (1), as the eluates collected from the first purification were diluted prior to the second purification. Small amounts of protein were found in subsequent washes. Lanes (5), (6) and (7) show the eluates as collected from the second column purification, following reduced glutathione addition. The eluates obtained were of high purity, as seen by the single band at approximately 31kDa. Lane (8) shows the post-wash, when 1 X PBS had been added to wash the column after collection of the third eluate shown in Lane (7). A faint band is visualized at 31kDa, indicating there was still some protein coming off the column when the wash buffer was added. The eluates were then checked by a Western blot using anti-GST antibodies, and one sample is shown in Figure 3.5 (B).

### 3.3 Thrombin Cleavage

The purified GST-PKC53EV1 fusion protein was also checked by thrombin cleavage to ensure the GST and thrombin cleavage sites had been correctly encoded. Figure 3.6 shows a 20% SDS-PAGE and corresponding Western blot of the fusion protein before and after cleavage. Lane (M) is the marker lane, with the high molecular weight protein marker (GibcoBRL). Lane (1) is the GST-PKC53EV1 fusion protein prior to

Figure 3.5: SDS-PAGE with samples from GST-PKC53EV1 protein purification (A). Also shown is a Western blot to 0.2ug eluate with a 1:2500 dilution of anti-GST antibodies (B). Samples were collected during affinity-column purification, and 10uL of each were run on a 15% SDS-PAGE. Lane (M) is a high molecular weight protein marker (GibcoBRL). The Lane (1) shows the diluted eluate collected from the first column purification, and after applying to the column for a second purification, Lanes (2-4) show fractions from subsequent washes. Lanes (5, 6 and 7) are eluates as collected off of the column from the second purification, immediately after the addition of reduced glutathione. Lane (8) is a post-wash fraction, collected after the third eluate.

A




B

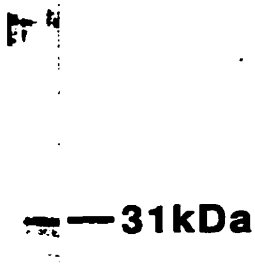


Figure 3.6: SDS-PAGE and Western blot of GST-PKC53EV1 fusion protein cleaved with thrombin. (A) is a 20% SDS-PAGE showing GST-PKC53EV1 fusion before and after cleavage with thrombin. Lane (M) is the high molecular weight protein marker (GibcoBRL). Lane (1) is 0.5ug protein prior to cleavage, and Lane (2) is 5ug protein after cleavage. (B) is the corresponding Western blot with 1:2500 dilution anti-GST antibodies. Lane (1) is the fusion protein prior to incubation with thrombin, and Lane (2) is the fusion protein after incubation with thrombin.


A
M
1
2
31kDa
27kDa
B
1
2
31kDa
27kDa

incubation with thrombin, and Lane (2) is the fusion protein after incubation with thrombin. A band at approximately 31kDa is shown in Lane (1) prior to cleavage, and after cleavage, a band of 27kDa is visualized, shown in Lane (2). As shown in Lane (2), not all of the protein was successfully cleaved. Anti-GST antibodies were utilized in the Western blot, and recognized both the fusion protein before and after cleavage as shown in Lanes (1) and (2) respectively.

### 3.4 Polyclonal Antibodies

The purified GST-PKC53EV1 fusion protein was then used as an antigen to generate polyclonal antibodies in New Zealand female white rabbits. They were bled prior to starting injections, and this pre-immune antisera was used in Western blots with *D. melanogaster* extracts and the antigen to ensure no or little cross reactivity during the experiments performed throughout this project. An immune response was seen after the first booster injection, and the antigen was detected with a 1:1000 dilution of anti-PKC53E1 and anti-PKC53E2 antisera (rabbits 1 and 2, respectively) after only two injections. *D. melanogaster* extracts were also immunoblotted with these antisera, but little protein was detectable at this time. After the fourth injection, 0.02ug antigen was detectable on a Western blot, using a 1:10000 dilution of anti-PKC53E1 antisera. The first rabbit gave a better signal than the second, so it was bled out, and a fifth injection was given to the second rabbit, and subsequently bled out. Anti-PKC53E1 antisera, were used for all Western blots, immunoprecipitations, and immunohistochemical analyses.

### 3.5 Protein Extraction

A variety of extraction procedures was attempted in order to achieve a PKC-rich extract from *D. melanogaster* tissues. Figure 3.7 shows an example of a PKC-rich extract obtained from adult heads on 8% SDS-PAGE (A). Western blots (B) were performed on

Figure 3.7: SDS-PAGE and Western blot with *D. melanogaster* head extracts. (A) is an 8% SDS-PAGE stained with Coomassie, showing the proteins obtained using a PKC-rich extraction procedure with adult heads. Lane (M) is the high molecular weight protein marker (GibcoBRL), and the adjacent lane shows ~20ug of proteins from head extracts. Western blots (B) to 0.02ug antigen (1), with a 1:5000 dilution of anti-PKC53E1 antisera, and to 20ug *D. melanogaster* head extract with a 1:5000 dilution of anti-PKC53E1 antisera preincubated with 10-20ug antigen (2). Western blots to 20ug *D. melanogaster* head extracts with a 1:5000 dilution of preimmune antiserum (3) and with a 1:5000 dilution of anti-PKC53E1 antisera preincubated with 10-20ug GST protein (4). Western blots were also performed to 20ug *D. melanogaster* head extracts with a 1:5000 dilution of anti-PKC53E1 antisera (5).


A
M
97kDa
68kDa
43kDa
29kDa
B
1
2
3
4
5
75kDa
31kDa
antigen block
preimmune
GST block

antigen (1), and *D. melanogaster* head extracts, using either anti-PKC53E1 antisera preincubated with antigen (2), preimmune antisera (3), anti-PKC53E1 antisera preincubated with GST (4) or anti-PKC53E1 antisera (5). The Western blot (5) shown in Figure 3.7 (B), resulted in the detection of a band of approximately 75kDa with a PKC-rich extraction procedure, using EGTA/EDTA, and Triton X-100 in the extraction buffer. Other extraction procedures that were attempted resulted in little or no detection of this 75kDa band. If either Triton X-100, EGTA, or EDTA were not included in the extraction buffer, the 75kDa band would either decrease in intensity, or disappear. The combination of these results suggests the specific detection of a 75kDa protein with the anti-PKC53E1 antisera, due to detection of PKC53E epitopes. Figure 3.8 shows the resultant PKC-rich extracts obtained from a variety of *D. melanogaster* tissues and developmental stages. These extracts were subjected to 10% SDS-PAGE, and were obtained from adult tissues such as ovaries and testes, and developmental stages such as embryos, larvae, and pupae.

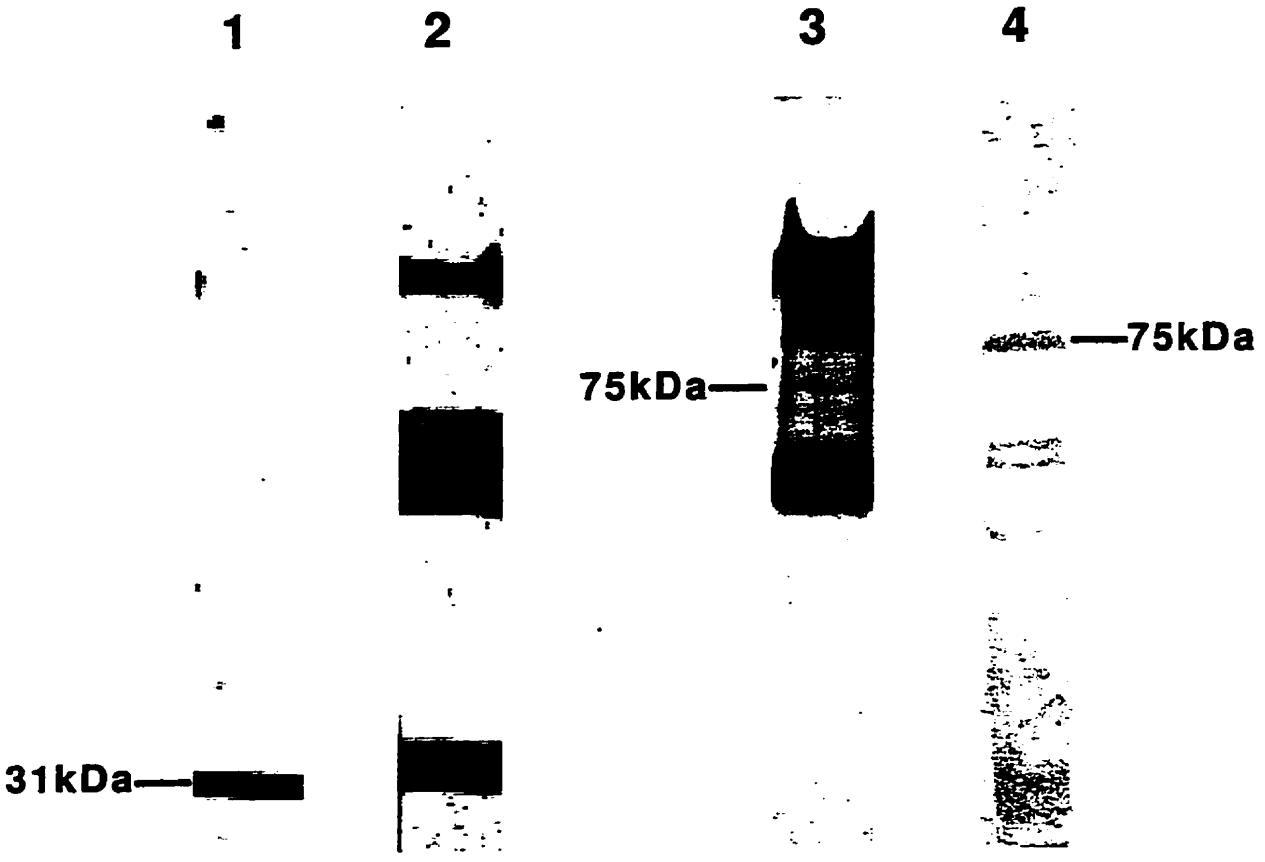
### 3.6 Immunoprecipitations

Immunoprecipitations were performed by incubating *D. melanogaster* adult head extracts with anti-PKC53E1 antisera and protein-A agarose. This was followed by electrophoresis of protein bound to the protein-A beads on a 10% SDS-PAGE, and Western blotting using anti-PKC$\alpha\beta\gamma$ antisera, Figure 3.9. This commercially available polyclonal antiserum was raised against a 19-36 pseudosubstrate peptide which is highly conserved among the classical PKC isoenzymes. Unfortunately, it was derived from sequence for the mammalian isoenzymes, and although there are some similarities in the conserved regions with *D. melanogaster* isoenzymes, there was still some variance in the sequence. A Western blot with anti-PKC53E1 antisera and antigen is shown in Figure 3.9 (1). Also shown is a control (2), where anti-PKC53E1 antisera were used to immunoprecipitate the antigen, and a Western blot was performed using anti-PKC53E2

Figure 3.8: SDS-PAGE with PKC-rich *D. melanogaster* extracts. PKC-rich extracts were obtained from different *D. melanogaster* tissues and developmental stages and subjected to 10% SDS-PAGE and subsequently stained with Coomassie Blue. Shown in Lane (M) is the high molecular weight protein marker (GibcoBRL), and in Lane (1) is 2ug antigen. Lane (2) shows proteins from adult head extracts, and Lane (3) shows proteins from ovaries. Lane (4) is from testes, Lane (5) is from 2-4 hour old embryos, Lane (6) is from 2nd instar larvae, Lane (7) is from 3rd instar larvae, and Lane (8) is from eye (tan) pupae. Concentrations of 20-40ug proteins/well were run on SDS gels for further Western blot analyses although concentrations of proteins from testes were consistently lower at 10ug/well. All gels used for Western blot analyses were transferred to nitrocellulose, and the membranes were checked with Ponceau S stain to ensure successful transfer.


M
1
2
3
4
5
6
7
8
200kDa
97kDa
68kDa
43kDa
29kDa

Figure 3.9: Western blots of antigen and immunoprecipitations. Western blot (1) shows 0.02ug antigen detected with a 1:5000 dilution of anti-PKC53E1 antisera. Western blot (2) shows 5ug antigen immunoprecipitated with anti-PKC53E1 antisera, and detected with a 1:5000 dilution of anti-PKC53E2 antisera. Western blot (3) shows PKC53E immunoprecipitated from 50ug adult head extract with anti-PKC53E1 antisera, and detected with anti-PKCαβγ antibodies (GibcoBRL). Western blot (4) to 20ug adult head extract with anti-PKCαβγ antibodies (GibcoBRL).




immunoprecipitation of antigen with anti-PKC53E1 antisera

immunoprecipitation of head extract with anti-PKC53E1 antisera and detection with anti-PKCαβγ antibodies

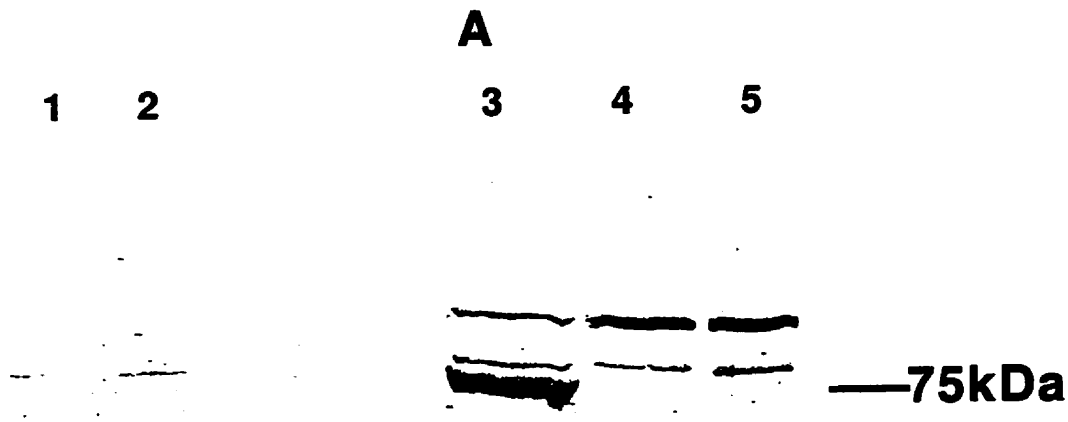
antisera. Some bands are apparent in the 75kDa size region, in the Western blot (3) on head extract immunoprecipitated with anti-PKC53E1 antisera, and detected with anti-PKCαβγ antisera. A Western blot to *D. melanogaster* head extracts with anti-PKCαβγ antisera alone (4) resulted in the detection of faint bands in the 70-80kDa size range. This was expected due to homology in this sequence with the inaC isoenzyme, as shown in Figure 1.7.

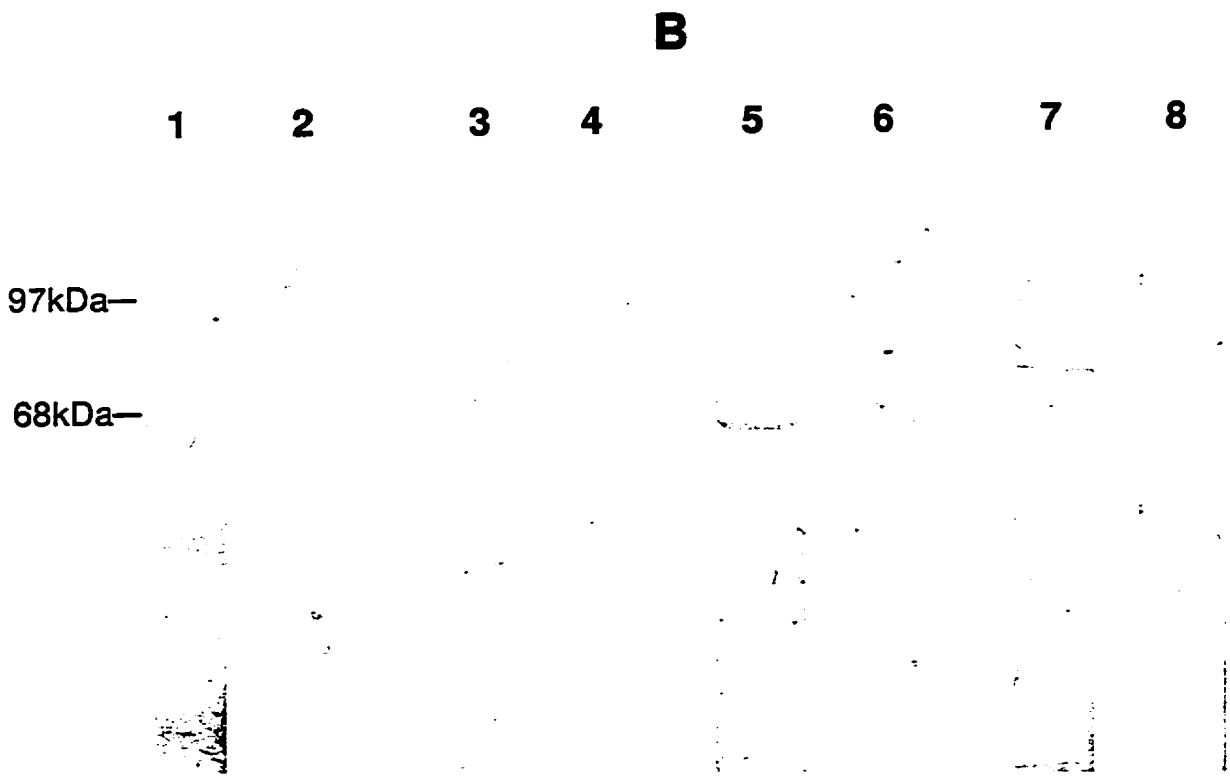
PKC assays were performed on *D. melanogaster* adult head extract proteins immunoprecipitated with the anti-PKC53E1 antisera. The negative controls, using the pseudosubstrate peptide as a competitive inhibitor, failed to result in any significant difference in the incorporated radioactivity (data not shown).

### 3.7 Western Blots with Anti-PKC53E1 Antisera

PKC-rich extracts were obtained from whole adult male and female flies, ovaries and testes. These extracts were electrophoresed on a 10% SDS gel with PKC-rich head extracts, and a Western was performed with anti-PKC53E1 antisera. The results are shown in Figure 3.10 (A). Lane (1) is with proteins extracted from whole adult males, and Lane (2) is with proteins from whole adult females. Faint bands were apparent in the 75kDa region, and these were consistent when subject to controls. Western blots to extracts from whole males and females with preimmune antiserum and anti-PKC53E1 antisera subjected to an antigen block, are shown in (B). (A) Lane (3) is with extracts from adult heads, and the 75kDa band was predominant. Controls shown in Figure 3.7, indicated this band was specific to the anti-PKC53E1 antisera. Two bands of higher molecular weight (80-95kDa) were visible; however, these only appeared after prolonged exposure. These bands were no longer visible after affinity-purifying the anti-PKC53E1 antisera, as described in section 3.8. (A) Lane (4) is with extracts from testes, and no

Figure 3.10: Western blots with various PKC-rich extracts obtained from adult *D. melanogaster* tissues. (A) shows a Western blot with a 1:5000 dilution of anti-PKC53E1 antisera. Lane (1) is with 20-40ug extracts obtained from whole adult males, and Lane (2) is with 20-40ug extracts obtained from whole adult females. Lane (3) is with 20ug extracts from adult heads. Lane (4) is with 10ug extracts from testes, and Lane (5) is with 20-40ug extracts from ovaries. Shown in (B) are controls with Western blots to the same extracts used in (A) with a 1:5000 dilution of preimmune antisera, and anti-PKC53E1 antisera preincubated with 10-20ug antigen, respectively. Western blots to extracts from whole adult males are shown in (1) and (2), to extracts from whole adult females are shown in (3) and (4), to extracts from testes are shown in (5) and (6), and to extracts from ovaries are shown in (7) and (8).


A
1
2
3
4
5
75kDa


B
1
2
3
4
5
6
7
8
97kDa
68kDa

bands in the 75kDa size range were detectable. Two bands of higher molecular weight (80-95kDa) were visible, but controls suggest they were not specific to the anti-PKC53E1 antisera. Western blots to extracts from testes with preimmune antiserum and anti-PKC53E1 antisera subjected to antigen block, are shown in (B). (A) Lane (5) contains extracts from ovaries and a very faint band in the 75kDa size region was visible as well as the two bands of higher molecular weight (80-95kDa). The 75kDa band was not always consistent when subject to controls, and it remains inconclusive as to whether or not it is specifically recognized by the anti-PKC53E1 antisera. Western blots to extracts from ovaries with preimmune antiserum and anti-PKC53E1 antisera subjected to an antigen block, are shown in (B). The two bands of higher molecular weight did not appear to be specific to the antisera recognizing the PKC epitopes.

Western blots were performed to pupal extracts with anti-PKC53E1 antisera. As shown in Figure 3.11 (A), a band at approximately 70kDa was apparent in Lane (1), with proteins from pupal extracts. Lane (2) shows the 75kDa band, with proteins from whole adult female extracts. A smaller band was also visible in Lane (2), and this was most likely due to degradation. Smaller bands were often visualized after an extract had been stored for a prolonged length of time. Figure 3.11 (B) has identical lanes of protein extracts, however the anti-PKC53E1 antisera had been preincubated with antigen prior to incubating with the blot. The bands were no longer visible, supporting specificity to the anti-PKC53E1 antisera.

Western blots were also performed with bovine PKC$\alpha\beta\gamma$ isoenzyme, and a 1:2500 dilution of anti-PKC53E1 antisera. Bands were not visible after prolonged detection (data not shown). As mentioned before, there is some variance in sequence between isoenzymes in the conserved regions, and high variance in sequence between isoenzymes (and species) in the variable regions.

Figure 3.11: Western blots with PKC-rich extracts from *D. melanogaster* pupae and females. Western blots were performed using a 1:5000 dilution of anti-PKC53E1 antisera with PKC-rich extracts obtained from *D. melanogaster* tan (eye) pupae, and whole adult females. Western blot (A) has 20-40ug pupal extracts in Lane (1), and 20-40ug female extracts in Lane (2). Western blot (B) has the same extracts in Lanes (1) and (2), however, the anti-PKC53E1 antisera were preincubated with 10-20ug antigen.


antigen block

### 3.8 Affinity-Purification of Antisera

Anti-PKC53E1 antisera were affinity purified using the blot-affinity purification procedure, in an attempt to reduce some of the cross-reacting bands. Figure 3.12 shows the resulting Western blots using affinity-purified anti-PKC53E1 antisera to detect proteins from adult head extracts. Blot (1) shows the 31kDa antigen recognized by the affinity-purified anti-PKC53E1 antisera. Proteins from adult head extracts were used in the adjacent blots, and no bands were visible in the Blot (2), when the anti-PKC53E1 antisera had been previously preincubated with antigen. The 75kDa band was visible in Western blot (3), when the anti-PKC53E1 antisera had been preincubated with GST protein. The 75kDa band was also visible in Western blot (4), with anti-PKC53E1 antisera.

### 3.9 Developmental Western Blots

Utilizing the same PKC-rich extraction procedure as with adult heads, proteins were extracted from *D. melanogaster* during various stages of development in an attempt to elucidate when protein is first detectable. Figure 3.13 shows Western blots, using a 1:25 dilution of affinity-purified anti-PKC53E1 antisera, with extracts from 1st, Figure 3.13 (A), 2nd, Figure 3.13 (B), and 3rd, Figure 3.13 (C), larval instars . In these Western blots, a band of 75kDa was apparent, although the intensity of the signal varied (Figure 3.13 (A1), (B1), and (C1)). Also shown in Figure 3.13, are Western blots with the same extracts; however, the affinity-purified anti-PKC53E1 antiserum was preincubated with antigen. In these, the band is no longer apparent (Figure 3.13 (A2), (B2), and (C2)). Figure 3.14 show Western blots using a 1:25 dilution of affinity-purified anti-PKC53E1 antisera with extracts from early (white), Figure 3.14 (A), eye (tan), Figure 3.14 (B), and late (dark), Figure 3.14 (C), pupae. In these Western blots, a band of approximately 70kDa was apparent in the early, Figure 3.14 (A1), and eye, Figure 3.14 (B1), pupae, however in the late, Figure 3.14 (C1), pupae, the band seemed to shift, and a larger band

Figure 3.12: Western blots with affinity-purified anti-PKC53E1 antisera on PKC-rich extracts from *D. melanogaster* heads. Blot-affinity purified anti-PKC53E1 were used at a dilution of 1:50. Western blot (1) is with 0.02ug antigen. Western blot (2) is with 20ug PKC-rich extracts obtained from *D. melanogaster* adult heads, with affinity-purified anti-PKC53E1 antisera preincubated with 10-20ug antigen. Western blot (3) is with 20ug of the same head extract; however, the anti-PKC53E1 antiserum was preincubated with 10-20ug GST protein. Western blot (4) is with 20ug of the same head extract.

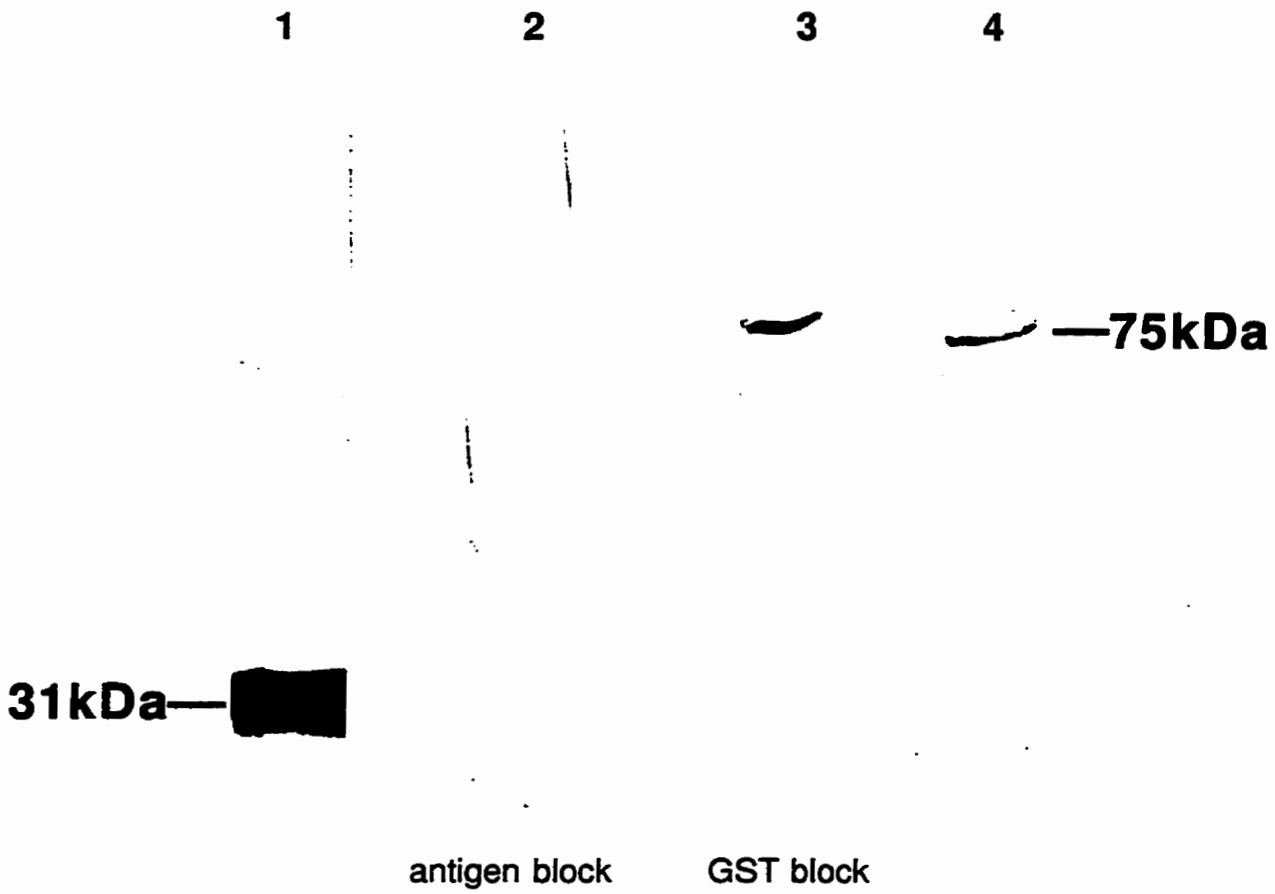

1
2
3
4
—75kDa
31kDa—
antigen block
GST block

Figure 3.13: Western blots with PKC-rich extracts from *D. melanogaster* larvae. Blot-affinity purified anti-PKC53E1 antisera were used at a dilution of 1:25. Western blots (A) to 20-40ug 1st instar larval extracts (1) and to the same extracts with affinity-purified antisera preincubated with 10-20ug antigen (2). Western blots (B) to 20-40ug 2nd instar larval extracts (1) and to the same extracts with affinity-purified anti-PKC53E1 antisera preincubated with 10-20ug antigen (2). Western blots (C) to 20-40ug 3rd instar larval extracts (1), and to the same extracts with affinity-purified anti-PKC53E1 antisera preincubated with 10-20ug antigen (2).

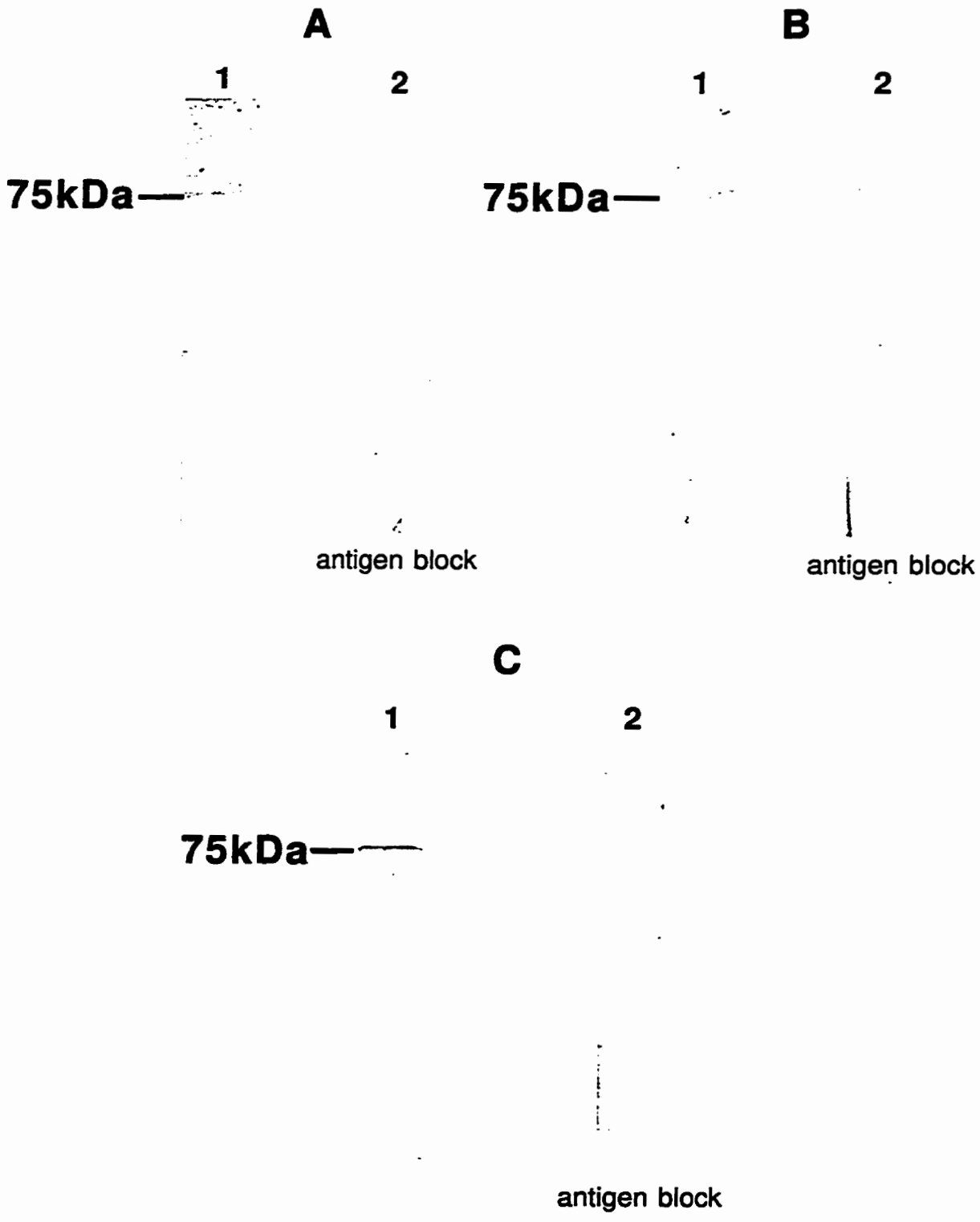
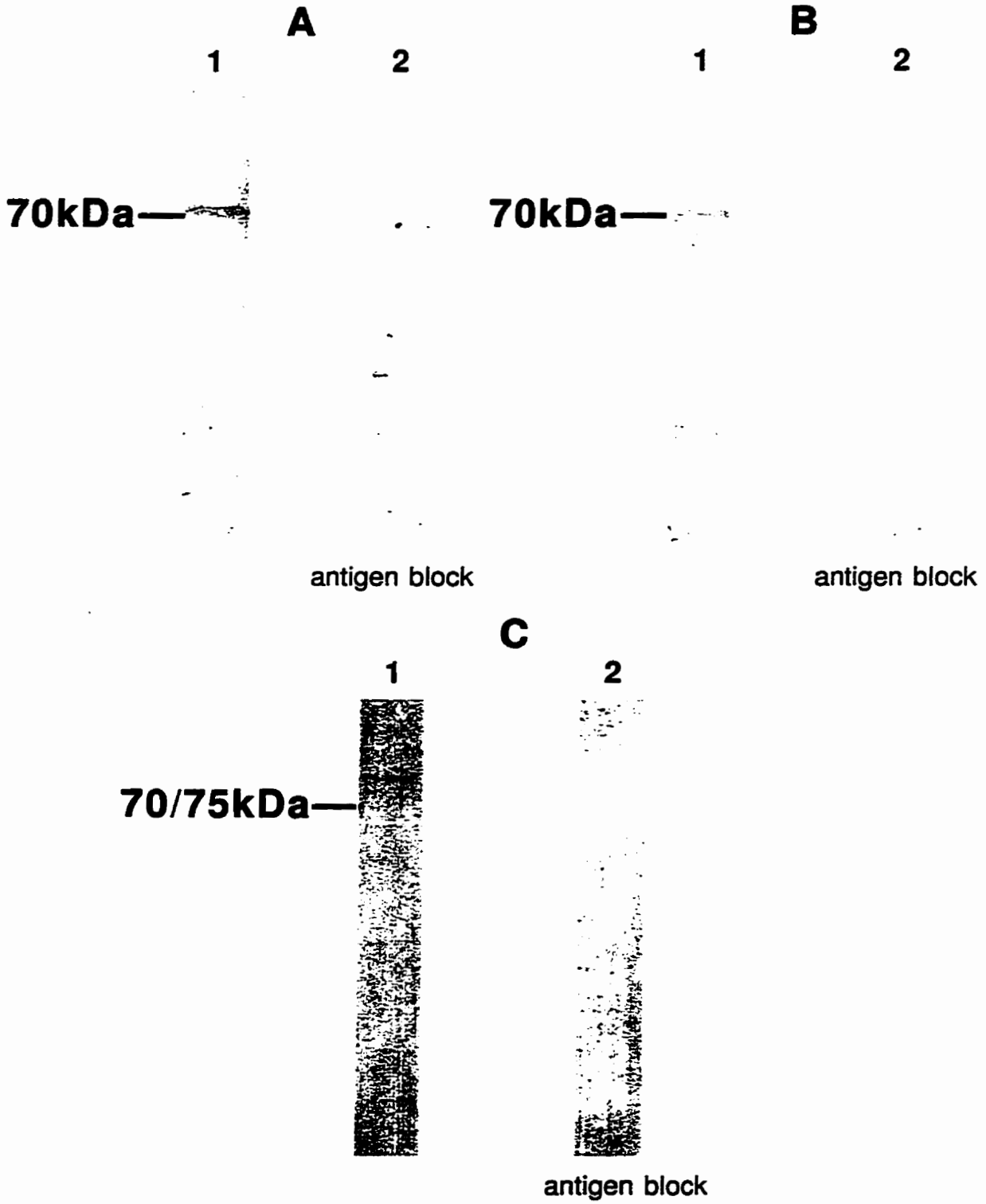

A
1
2
75kDa
antigen block
B
1
2
75kDa
antigen block
C
1
2
75kDa
antigen block

Figure 3.14: Western blots with PKC-rich extracts from *D. melanogaster* pupae. Blot-affinity purified anti-PKC53E1 antisera were used at a dilution of 1:25. Western blots (A) to 20-40ug white (early) pupal extracts (1) and to the same extract with affinity-purified anti-PKC53E1 antisera preincubated with 10-20ug antigen (2). Western blots (B) to 20-40ug tan (eye) pupal extracts (1), and to the same extract with affinity-purified anti-PKC53E1 antisera preincubated with 10-20ug antigen (2). Western blots (C) to 20-40ug dark (late) pupal extract (1), and to the same extract with affinity-purified anti-PKC53E1 antisera preincubated with 10-20ug antigen (2).


A
1
2
70kDa
antigen block
B
1
2
70kDa
antigen block
C
1
2
70/75kDa
antigen block

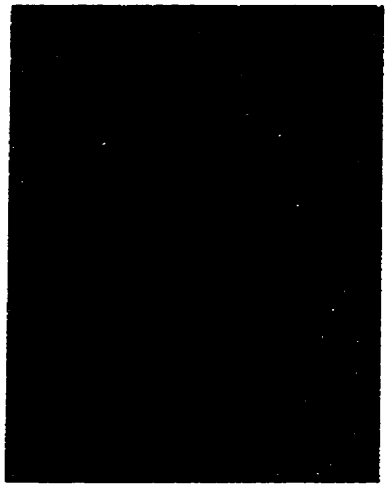
also became apparent. Also shown in Figure 3.14, are Western blots with the same extracts, however the affinity-purified anti-PKC53E1 antisera were preincubated with antigen (Figure 3.14 (A2), (B2), and (C2)). The bands were no longer visible in any of the extracts.

### 3.10 Immunohistochemistry

Immunohistochemical studies were performed on *D. melanogaster* ovaries, testes, numerous stages of embryos, 3rd instar larval brains, 3rd instar salivary glands, cryostat sectioned larvae, and cryostat sectioned adults. The studies on ovaries and testes were inconclusive, as it was difficult to determine whether the signal was specific, due to high cross reactivity with secondary antisera (data not shown). Embryos earlier than stage 12, also failed to result in a specific signal. Figure 3.15 shows the colorimetric immunohistochemical staining visualized in stage 12 embryos. Controls were performed with preimmune antisera (A), and without anti-PKC53E1 antisera (data not shown). Anti-PKC53E1 antiserum was used for colorimetric staining of embryos with anti-rabbit AP secondary antibodies, shown in (B). In these developing embryos, specific signal was seen associated with cell clusters in a symmetrical pattern running the length of the embryo and into the anterior portion. In comparison with an embryo stained for motor neurons, which lie in close association with the CNS, it appeared as though these clusters were most likely in the developing CNS. In the developing head region, small clusters of staining were also observed. This staining was not visualized when negative controls were performed. Embryos were also visualized with immunofluorescence. Controls were performed without anti-PKC53E1 antisera, with anti-rabbit biotin, and with streptavidin-Texas Red (C). The same conditions were used with anti-PKC53E1 antisera (D). Segmentally-specific staining was visualized, which could be due to the formation of early nervous tissues. Stage 14-16 embryos were stained with immunofluorescence using

Figure 3.15: Immunohistochemical staining of stage 12 embryos from *D. melanogaster*. Embryos were visualized with fluorescence microscopy or light microscopy at a magnification of 400X. Embryos were collected and stained after incubation with a 1:500 dilution of anti-PKC53E1 antisera, a 1:30 dilution of anti-motor neuron antisera, without anti-PKC53E1 antisera, or with a 1:500 dilution of preimmune antisera. Earlier stages failed to result in detection of signal. Panel (A) is an embryo stained colorimetrically, after incubation with preimmune antibodies, and a 1:2000 dilution of secondary anti-rabbit AP-conjugated antibodies. Panel (B) is an embryo stained colorimetrically, with anti-PKC53E1 antisera and a 1:2000 dilution of secondary anti-rabbit AP-conjugated antibodies. The arrow points to the staining seen in a very small subset of cells in the developing CNS regions. These cell clusters form a pattern running the length of the embryo and into the anterior portion, or head region. Panel (C) is an embryo without anti-PKC53E1 antisera, and incubated with a 1:2000 dilution of anti-rabbit biotin-conjugated antisera and a 1:1000 dilution of Texas Red-streptavidin. Panel (D) is an embryo stained and visualized after incubation with a 1:500 dilution of anti-PKC53E1 antisera. The arrow points to segmentally specific staining. Panel (E) is a stage 14-16 embryo incubated with monoclonal anti-motor neuron antisera, followed by a 1:1000 dilution of secondary mouse Texas Red-conjugated antisera. The arrow points to the motor neurons found adjacent to the CNS.

A

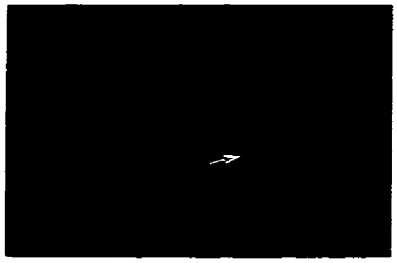


B

C

D

E



mouse monoclonal antibodies to a protein specifically found in motor neurons (E). Secondary anti-mouse Texas Red-conjugated antisera were used, and the embryos were subsequently visualized by fluorescence microscopy. The staining shows the outline of the developing central nervous system, as the motor neurons run adjacent to the CNS cells. Immunofluorescence of 3rd larval instar brains is shown in Figure 3.16. Optic lobes and ventral ganglia were incubated with anti-PKC53E1 antisera (B,D), and controls were performed without anti-PKC53E1 antisera (A,C). The optic lobes displayed staining in concentric circles, with bands of staining in the centre, and not on either side (B). This is indicative of CNS staining. The ventral ganglion also showed CNS staining, as visualized by ubiquitous staining, and the punctate patterns running along the length of the ganglion (D). Unfortunately, when controls were performed, it was difficult to determine whether this staining was specific, due to the high levels of background (A,C). There was a lot of cross reactivity with the secondary antisera, and controls with preimmune antisera gave the same result as with only secondary antisera. Figure 3.17 shows immunofluorescence of 3rd larval instar salivary glands dissected just prior to pupariation. Affinity-purified anti-PKC53E1 antisera were used, with secondary anti-rabbit biotin antisera, and streptavidin-TR (C). Controls were performed without anti-PKC53E1 antisera (A), or with anti-PKC53E1 antisera that had been preincubated with antigen (B). These tissues showed unique staining, as seen by localization to the nuclei of the cells. The pattern seems to be punctate, and it is difficult to discern whether the staining is associated with the nuclear envelope or whether it is internal. Since the staining does not result in the appearance of rims, the staining is more likely internal.

Figure 3.16: Immunofluorescence with *D. melanogaster* 3rd larval instar brains. All whole mounts were visualized with fluorescence microscopy at a magnification of 400X, after incubation with a 1:2000 dilution of secondary anti-rabbit biotin-conjugated antisera and a 1:1000 dilution of streptavidin-Texas Red. Panel (A) shows an optic lobe without the addition of anti-PKC53E1 antisera. Panel (B) shows an optic lobe visualized after preincubation with a 1:500 dilution of anti-PKC53E1 antisera. An arrow points to staining following the pattern of a concentric ring around the mid-portion of the lobe. Panel (C) is a ventral ganglion without prior incubation with anti-PKC53E1 antisera. Panel (D) is the ventral ganglion visualized after incubation with a 1:500 dilution of anti-PKC53E1 antisera. An arrow points to staining in a distinct punctate pattern running parallel to the length of the ganglion. A punctate pattern of staining also runs through the midline portion of the ganglion, although this was also sometimes seen in the controls.

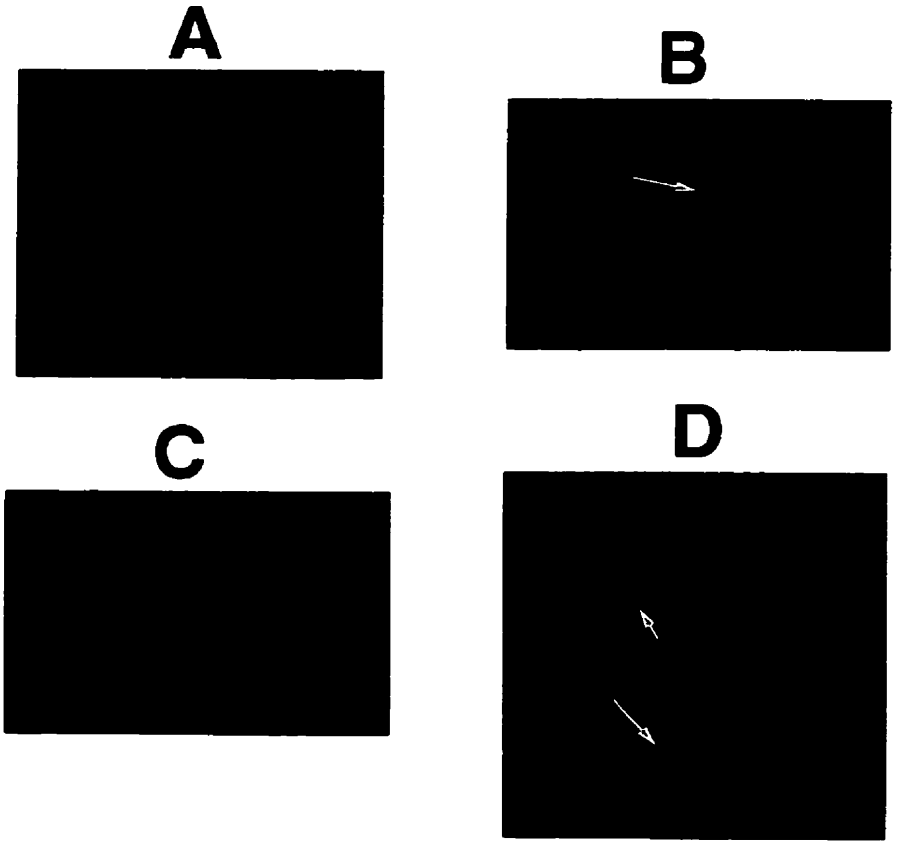
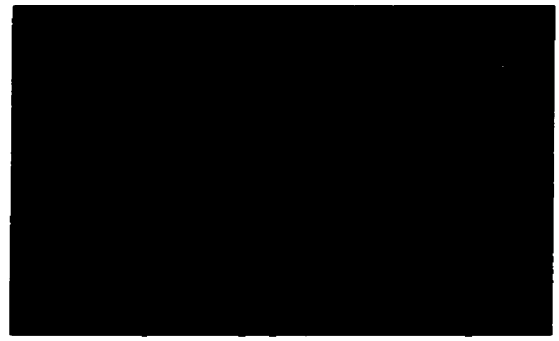

A
B
C
D

Figure 3.17: Immunofluorescence of salivary glands from *D. melanogaster* late 3rd larval instars. Salivary glands were visualized with fluorescence microscopy at a magnification of 400X, after incubation with a 1:2000 dilution of secondary anti-rabbit biotin-conjugated antisera and a 1:1000 dilution of streptavidin-Texas Red. Panel (A) shows salivary glands without affinity-purified anti-PKC53E1 antisera. Panel (B) shows salivary glands visualized after incubation with affinity-purified anti-PKC53E1 antisera preincubated with 50ug antigen. Panel (C) shows the staining of salivary glands after incubation with a 1:5 dilution of affinity-purified anti-PKC53E1 antisera. An arrow points to staining visualized at the nuclei, in a punctate pattern.

A



B

C

# Chapter 4
# Discussion

## 4.1 Cloning

A GST-PKC53EV1 fusion protein was made by subcloning part of *Pkc53E*, which encodes V1 and the first part of C1, into pGEX-4T1. This portion of the protein is unique to PKC53E, as shown in the amino acid sequence comparison between the *D. melanogaster* PKC isoenzymes in Figure 1.7, and contains the pseudosubstrate domain. PCR was used to amplify an 80bp *Pkc53E* fragment from genomic DNA, with cut sites designed for directional subcloning in frame into the pGEX-4T1 vector, as shown in Figure 2.2. Figures 3.1, and 3.2 show the results of PCR, and the PCR screen that was used to check successfully transformed colonies, respectively. Figure 2.1 shows the corresponding plasmid map of the fusion construct, pGEX-*Pkc53EV1*. Figure 3.3 shows the amino acid sequence of the GST-PKC53EV1 fusion protein. The entire fusion protein was used as an antigen for generating polyclonal antibodies as will be discussed later. A protein of at least 20kDa is desirable for eliciting an antigenic response, and due to the small size of the PKC53E peptide, it was more advantageous to retain the GST moiety.

## 4.2 Protein Expression, Purification, and Generation of Polyclonal Antibodies

The GST-PKC53EV1 fusion protein was expressed in *E. coli* DH10B cells, by induction with the lactose analogue IPTG. Figure 3.4 shows the GST-PKC53EV1 fusion protein resulting from induction of the pGEX-*Pkc53EV1* construct, in comparison to the GST protein resulting from induction of pGEX-4T1. The size of GST is 27kDa, and the GST-PKC53EV1 fusion protein is about 31kDa. Anti-GST antibodies were used for the Western blot in Figure 3.4. There appeared to be a doublet in the lane with the GST-

PKC53EV1 fusion protein, which is most likely due to endogenous cleavage (Pharmacia). The GST-PKC53EV1 protein was then purified by affinity-column chromatography using a glutathione-coupled Sepharose 4B column. GST has a binding site for glutathione, which is advantageous for purifying GST fusion proteins (Pharmacia). The GST moiety binds the column via glutathione, and is then eluted with reduced glutathione (Pharmacia). Figure 3.5 shows the SDS-PAGE corresponding to the resulting protein eluates collected from a second column purification. As shown in Figure 3.6, when the GST-PKC53EV1 fusion protein was cleaved by thrombin, the 31kDa protein prior to cleavage, and the resulting 27kDa protein were recognized by anti-GST antibodies. A concentration of approximately 1mg/mL was achieved after protein purification, and this purified protein was used as an antigen for generating polyclonal antibodies in rabbits. A high antibody titre was achieved after the second booster. After four injections, the first rabbit was bled, and anti-PKC53E1 antisera used in various Western and immunohistochemical analyses.

### 4.3 Tissue Extracts, Western Blots and Immunoprecipitations

The predicted size of PKC53E is 75kDa, as postulated by the open reading frame from the 3.2kb cDNA cloned by Rosenthal *et al.* (1987). As shown in Figure 3.7, a predominant band of approximately 75kDa was consistently visualized on Western blots with proteins from *D. melanogaster* head extracts. PKC-rich extracts were obtained using a procedure for the classical PKC isoenzymes, which included the use of $Ca^{2+}$-chelators such as EDTA and EGTA, as well as membrane solubilizing detergents such as Triton X-100, in the buffer (Epand, 1994; GibcoBRL). This provides evidence that the putative open reading frame for PKC53E encodes a PKC with properties similar to the classical PKC isoenzymes, which are both $Ca^{2+}$ and phospholipid-dependent. This procedure was also used to obtain PKC-rich extracts from various tissues and developmental stages from *D. melanogaster*, as shown in Figure 3.8.

Immunoprecipitations were performed using anti-PKC53E1 antisera with PKC-rich adult head extracts. The immunoprecipitations were electrophoresed on SDS gels and transferred to nitrocellulose, for detection with anti-PKCαβγ antisera (GibcoBRL). As shown in Figure 3.9, a band of approximately 75kDa was visualized, however, other bands were also apparent. This was most likely due to non-specific binding of proteins during the immunoprecipitation step, or cross-reactivity with inaC. Western blots performed on adult head extracts using the appropriate dilution of anti-PKCαβγ antisera resulted in the faint detection of several bands in the 70-80kDa size range, shown in Figure 3.9. This was expected, given the amino acid sequence identity in the pseudosubstrate domain between the PKC53E and inaC isoenzymes, as shown in Figure 1.6. The anti-PKCαβγ antisera were raised against a pseudosubstrate peptide (19-36) from the classical mammalian isoenzymes. Although there is some amino acid sequence homology to the *D. melanogaster* PKC53E and inaC isoenzymes, there is some sequence variance, and a strong detection was not expected. Western blots and immunoprecipitations were also performed using bovine classical PKCαβγ isoenzymes, with anti-PKC53E antisera, and anti-PKCαβγ antisera (data not shown). The antigen used to generate anti-PKC53E antisera, shown in Figure 3.3, was unique to PKC53E, and has low amino acid sequence similarity to mammalian PKC isoenzymes. Although part of this antigen includes the pseudosubstrate domain, the diversity in this region, and in the surrounding sequence, would still make it difficult to detect other PKC isoenzymes (Rosenthal *et al.*, 1987; Schaeffer *et al.*, 1989). This actually supports the ability of the antibodies to detect PKC53E specifically.

PKC assays were performed on immunoprecipitated proteins obtained from PKC-rich adult head extracts incubated with anti-PKC53E1 antisera. The pseudosubstrate peptide, which is used as a competitive inhibitor to allow for negative controls in PKC assays, failed to result in a decreased signal (data not shown). One of the reasons this

assay failed to work could be due to the nature of the anti-PKC53E1 antisera, which recognize epitopes in the pseudosubstrate domain. If they bound to this portion of PKC53E, during the immunoprecipitation procedure, it could render the protein constitutively active, preventing it from being susceptible to inhibition. Previous studies have shown similar results, where incubation of PKC isoenzymes with antisera to the pseudosubstrate domain resulted in activation, by causing the pseudosubstrate domain to dissociate from the substrate/kinase domain (Newton, 1995).

### 4.4 Western Blot Analyses

Multiple *Pkc53E* transcripts have been visualized in whole adults, adult heads, adult gonads, and various larval and pupal stages through a variety of RNA analyses (Rosenthal *et al.*, 1987, Schaeffer *et al.*, 1989, Natesan, 1991, Hughes, 1993, Nutter, pers. comm.). At least five different transcripts have been visualized by Northern analyses as shown in Table 1.1. Adult heads have three transcripts of 4.3, 4.0 and 2.4kb, adult gonads have a predominant transcript of 2.4kb, pupae have three transcripts of 4.3, 4.0 and 2.4kb, which vary in proportion according to stage, and larvae have transcripts of 4.3, 3.4 and 3.0kb, which also alter in proportion according to stage. Recent RNAse protection assays, RT-PCR, and 3' and 5' RACE suggest both the use of alternative promoters and alternative splicing in the untranslated region upstream of the ORF (Nutter, pers. comm.). The 4.3 and 4.0kb transcripts appear to have intron 1 spliced in, and are larger than the original cDNA clone of 3.2kb (Rosenthal *et al.*, 1987; Nutter, pers. comm.). This original cDNA was derived from two overlapping clones, using a bovine Pkcα probe with a *D. melanogaster* cDNA library derived from embryos, pupae and adults (Rosenthal *et al.*, 1987). The use of alternative promoters could result in the production of multiple transcripts, and account for size differences. The 3.4 and 3.0kb transcripts are close in size to that predicted by Rosenthal *et al.* (1987), when the cDNA was first characterized. The

2.4kb transcript does not contain the first intron, and it appears that this transcript is not polyadenylated in testes (Nutter, pers. comm.). Given the difference between total RNA, and poly(A)$^+$-selected RNA, some of the 2.4kb transcript found in ovaries is also not polyadenylated (Nutter, pers. comm.).

A series of Western blot analyses was performed with PKC-rich extracts obtained from whole adults, adult heads, and gonads, using the polyclonal antibodies raised against PKC53E, as shown in Figure 3.10. A predominant band of 75kDa was visualized with adult head extracts, and extracts from whole adults. However, a 75kDa band was not visualized with extracts from testes, and a faint 75kDa band was visualized with extracts from ovaries. Two bands of higher molecular weight (80-95kDa), were seen with extracts from heads, ovaries and testes. Controls with preimmune antisera and antigen blocked anti-PKC53E1 antisera, suggest these two bands are not specific to anti-PKC53E1 antisera. The 75kDa band from ovaries was not consistent, suggesting possible contamination from head extracts. Another possibility is that protein levels are low, or vary with age. The intensity of the 75kDa band was very low in whole adults in comparison to adult heads, suggesting the protein is expressed primarily in adult heads. The detection of one specific band at 75kDa, suggests that despite the presence of multiple transcripts, only one PKC53E isoenzyme is present. If other PKC53E isoforms exist, they lack the epitopes recognizable by the anti-PKC53E1 antisera. The 4.3 and 4.0kb transcripts could encode the 75kDa protein visualized predominantly in adult heads, and the 2.4kb transcript may encode another isoenzyme (Rosenthal *et al.*, 1987; Natesan, 1991; Hughes, 1993). This would explain the failure to detect significant amounts of protein in ovaries. The 2.4kb transcript in testes is not polyadenylated, which could explain the lack of detectable levels of protein (Nutter, pers. comm.). It is also possible that the polyadenylated 2.4kb transcript in ovaries is not stable, and is not able to encode significant amounts of protein. The reason for this is not clear; perhaps the 2.4kb transcript is polyadenylated at a different

site, which lowers its stability. There is evidence to suggest there are two polyadenylation sites within 20bp of each other, and although the use of one over the other would not affect size, it could affect stability. The high level of PKC53E expression in adult heads imply it plays an important role in maintenance, or in the general operation of the CNS, and associated tissues. Potential roles for PKC53E in the nervous system will be discussed later in this chapter. *in situ* hybridizations to cryostat sections of adult heads showed the PKC98E isoenzyme is also present in neural tissues, and there could also be some redundancy between the isoenzymes (Schaeffer *et al.*, 1989).

Western blots were performed with extracts from tan (eye) pupae, using anti-PKC53E1 antisera, as shown in Figure 3.11. A smaller band of approximately 70kDa was visualized with tan (eye) pupal extracts. A Western blot was performed with adult whole female extracts for comparison, and the 75kDa band was detectable. Further RNA analyses have not been performed with pupae, larvae or embryos, and these different sized isoforms could result from developmental-specific alternative splicing. The 4.3kb transcript is predominantly expressed during the tan (eye) pupal stage, making it a candidate for the transcript encoding the 70kDa isoenzyme visualized at this time (Natesan, 1991; Hughes, 1993). However, the 4.3kb transcript is also found in other tissues, which do not encode an isoenzyme of this size. This suggests there are post-translational mechanisms which may alter the isoenzyme. PKC isoenzymes have immature forms, prior to phosphorylation of key residues, which can have molecular weights up to 5kDa lower than their mature forms (Newton, 1995). Perhaps the PKC kinase responsible for initial phosphorylation of PKC53E is not expressed, or is not active, during the white (early) and tan (eye) pupal stages, preventing mature forms of the protein from being formed. This would render the protein inactive during these stages, suggesting the protein plays one or more important developmentally-specific roles.

Anti-PKC53E1 antisera were blot-affinity purified, and Figure 3.12 shows the resulting Western blots utilizing a 1:50 dilution of this antisera with PKC-rich adult head extracts. The 75kDa band was detectable, and this band was still visualized when the affinity-purified antibodies were preincubated with GST protein. Western blots using this blot-affinity purified anti-PKC53E1 antisera on proteins from larval and pupal extracts, showed a differential pattern of PKC53E expression. Although consistent signals were not obtained by Western blot analyses of protein extracts from various embryonic stages, PKC53E was detectable in stage 12 embryos by immunohistochemical analyses, and will be discussed later.

A 75kDa protein was detectable throughout larval development, with affinity-purified anti-PKC53E1 antisera, as shown in Figure 3.13. As described earlier, Northern blot analyses showed the presence of 3.0, 3.4, and 4.3kb transcripts in larvae, and the proportions of these transcripts seemed to vary during different larval stages (Natesan, 1991; Hughes, 1993). Since the 75kDa protein was the only detectable protein during these stages, they could all encode the same isoenzyme. It is also possible that alternative splicing results in one or more transcripts encoding a PKC53E isoenzyme which does not possess epitopes recognizable by the anti-PKC53E1 antisera. One or more of these transcripts could be unstable, and the PKC53E isoenzyme is primarily encoded by one of the transcripts. It is also possible the 75kDa isoenzyme in larvae varies from that found in heads, but still contains the same epitopes, and is similar in size. Immunofluorescence studies were performed on several larval tissues, and are discussed below. Evidence from these results suggests there could be multiple isoforms of PKC53E in larvae, and since protein has been found in tissues other than neural regions, these proteins could have alternate functions.

As shown in Figure 3.14, bands of different sizes were detectable throughout the pupal stages examined, using affinity-purified anti-PKC53E1 antisera. A smaller protein

of about 70kDa protein was visualized in white (early) and tan (eye) pupae. However, dark (late) pupae displayed a 'smeary' band, where the protein seemed to shift to a higher size of about 75kDa. As discussed earlier, there are several explanations for the presence of alternate isoforms. It is possible the protein is inactive during early pupal stages, and is required at a later stage during the development of specific adult tissues. The life cycle of *D. melanogaster* , as shown in Figure 1.5, is unique in that the larval stages are very different from the adult (Ashburner, 1989A). There are many genes which are adult-specific, or are larval-specific, and have either completely different roles in the different stages, or are not expressed in these different stages (Ashburner, 1989A). After the 3rd larval instar stage, the organism undergoes pupariation, when the larval tissues are histolyzed, and the imaginal discs develop to form the adult structures (Ashburner, 1989A). If the alternate isoform expressed during pupal stages is active, it could have a specific role in the development of adult structures from the imaginal discs. If this isoform is indeed an immature, inactive form of the protein, then this would indicate that signaling through PKC53E is not required for proper development of adult tissues, until later stages when the higher molecular weight isoform is produced (Newton, 1995). It would be interesting to see if protein localized to any of the imaginal discs during the late 3rd larval instar stages. The protein could also have analogous functions in the developing embryos, as with formation of adult structures. Since protein has been found in adult heads, PKC53E could have a role in the developing CNS structures in both the larva, and in the adults. This will be discussed further with the immunofluorescence studies performed on larval tissues.

### 4.5 Immunohistochemical Studies

Immunohistochemical studies were performed in an attempt to localize the protein *in vivo*. *in situ* hybridizations using a riboprobe to cryostat sections of adult heads

demonstrated that the *Pkc53E* transcripts are expressed ubiquitously (Schaeffer *et al.*, 1989). Further RNA *in situ* hybridizations performed with cryostat sectioned adults, also showed that transcripts were present in the central nervous system (Hughes, 1993). *in situs* hybridizations were also carried out with ovaries, and transcripts were first seen predominantly in the nurse and follicle cells of stage 9 oocytes (Hughes, 1993). This staining increased with stage, until stage 13-14, when the transcript was seen in the mature oocytes (Hughes, 1993). When different riboprobes were used, mature oocytes showed distinct banding patterns, suggesting the presence of alternate transcripts, and a specific role for PKC53E in the development of oocytes, similar to a maternal effect gene (Hughes, 1993). However, a specific signal was difficult to obtain with immunohistochemical staining. Protein appeared to be present in the cytoplasm of stage 9 nurse cells, but the later staged oocytes were impermeable to specific staining, due to the formation of chorion coats (Ashburner, 1989A). Also, the large size of the nurse cells seemed to bind secondary antibody non-specifically, and it was difficult to discern whether the staining visualized was unique to the anti-PKC53E1 antisera recognizing the PKC53E epitopes (data not shown). Further studies also showed staining in the developing oocyte, however, the secondary antibody bound non-specifically (data not shown). It is possible that anti-PKC53E1 antibodies are unable to detect the isoform encoded by the transcripts found in ovaries. Another explanation is that protein is expressed at levels too low to detect, and this is enhanced by non-specific binding of secondary antibodies to other proteins. *in situ* hybridizations were also performed with testes, and transcripts were visualized in the sheath, sperm bundles (Cysts of developing spermatids), and when broken open, the apical ends of sperm (Hughes, 1993). The use of alternate riboprobes, suggested the presence of a singe transcript (Hughes, 1993). Protein was not detectable when immunofluorescence was performed to testes (data not shown). Evidence suggests the transcript in testes is not

polyadenylated, and if this were the only transcript produced, this supports the lack of detectable protein (Nutter, pers. comm.).

Immunohistochemical staining was performed on embryos, and protein was not detectable prior to stage 12. However, by this stage, staining was seen in small clusters of cells in the developing CNS which runs the length of the embryo as shown in Figure 3.15 (B). The motor neurons were visualized by immunofluorescence using anti-motor neuron antisera, also shown in Figure 3.15 (E). This embryo is a later stage, but clearly shows the outline of the CNS as the motor neurons form commisures adjacent to the developing CNS (Ashburner, 1989A; Auld, pers. comm.). Anti-PKC53E1 antisera stained small clusters of cells which seem to be at the centre of the developing CNS. This could explain why it was difficult to detect protein on Western blots, and to detect RNA with *in situ* hybridizations. There was also some segmental-specific staining, however this was difficult to discern since antibodies could be binding non-specifically to the developing folds and denticle belts as shown in Figure 3.15 (D) (Ashburner, 1989A). This segmental-specific staining could be indicative of PNS nerves associated with developing segments. Although transcripts were not detectable in embryos by Northern blot analyses, nor were they were found with *in situ* staining in embryos, transcripts were inducible with phorbol esters (Natesan, 1991; Hughes, 1993). TPA response elements (TPA) may be found in the promoter region of the *Pkc53E* gene (Natesan, 1991). Since the transcripts are inducible in the presence of the right factor, perhaps they are spatially and temporally induced, when required for a developmental process. The pattern of staining visualized upon induction of transcripts was similar but much stronger, and more predominant than that seen with immunohistochemical staining of the protein (Hughes, 1993). There could be very low levels of transcript expression, which are not detectable by *in situ* hybridization, until stimulated. Perhaps transcript and protein levels are kept to a minimum until required, at which point they are induced, then later subside. It is also likely that only

small amounts of protein are required until the embryos reach a very specific stage, at which point protein expression is upregulated. Embryos can not be effectively stained after the formation of cuticle, which occurs around 16 hours, making it difficult to pinpoint when this 'upregulation' could occur (Auld, pers. comm.). Larvae possess a cuticle, which is a protective outer covering they periodically moult as they pass through the different instar stages (Ashburner, 1989A). Since PKC53E is detectable by Western blot analyses in the first larval instar stage, increased protein expression would have to occur just prior to hatching from the egg, which occurs at 21 hours (Ashburner, 1989A). There is evidence to suggest a PKC isoenzyme plays an important role in neuroblast differentiation in stage 6-7 embryos (Broughton *et al.*, 1996). PKC53E is not detectable at this stage, although given the small proportion of cells which are neuroblasts, it would be very difficult to detect any protein from extracts, if it were being expressed (Ashburner, 1989A).

Immunofluorescence studies were also performed on whole mounts of 3rd instar larval brains. The larval brains showed patterns of staining indicative of the CNS. Concentric circles were visualized in the optic lobes, and a symmetrical pattern was visualized along the length of the ventral ganglion, as shown in Figure 3.16 (B,D) (Ashburner, 1989A). High background due to non-specific binding of preimmune, and anti-rabbit biotin antisera, made it difficult to conclude if there was any specific staining. PKC53E could be ubiquitous, as seen with patterns of *Pkc53E* RNA expression in the adult brain (Schaeffer *et al.*, 1989; Hughes, 1993). As discussed earlier, there are several transcripts which are larval-specific, and there may be different isoforms encoded by these alternate transcripts (Rosenthal *et al.*, 1987; Natesan, 1991; Hughes, 1993). However, a single 75kDa band is detected by Western blot analyses. If alternate isoforms exist, they may not contain epitopes recognizable by the anti-PKC53E1 antisera. PKC53E expression in larval brains raises interesting questions regarding its function. In the latter part of this

chapter, potential roles for PKC53E in the development of the CNS and in learning, are discussed.

Immunofluorescence studies were also performed on salivary glands, dissected from 3rd larval instars. These tissues showed protein localization to the nucleus, or perhaps even to scaffold proteins at the nucleus, as shown in Figure 3.17 (C). This staining was visualized with anti-PKC53E1 antisera, before and after affinity-purification, was specifically blocked with antigen, and was not visualized when preimmune antisera were utilized. The presence of PKC53E at the nuclear envelope of cells in salivary glands raises interesting hypotheses as to its function during this stage.

Salivary glands are an important tissue in larvae. They possess polytene chromosomes, which allow for visualization of active gene expression by 'puffs'. They also undergo programmed cell death as larvae enter pupariation to begin metamorphosis, at the end of the 3rd larval instar stage. During metamorphosis, the organism undergoes dramatic changes, and many genes involved during this process are regulated by the hormone ecdysone. Many of these genes have been identified, initially by examining chromosome puffs in response to ecdysone. Some of the genes induced during early ecdysone pulses encode transcription factors that are involved in the regulation of other genes. Ecdysone has also been demonstrated to regulate genes encoding signaling proteins, such as a calcium binding protein homologous to calmodulin. Although *Pkc53E* is not known to be transcriptionally regulated by ecdysone, the production of one or more proteins involved in a calcium-mediated signaling pathway could result in turning on a pathway which utilizes PKC53E. This signaling pathway could be intimately associated with cell death pathways, or could be involved in the development of pupal and adult structures (Andres and Thummel, 1992; Andres and Thummel, 1995).

Several homologues of the cell death pathway have been found in *D. melanogaster*, and evidence suggests this pathway is conserved, at least partially. One recently isolated

gene, *reaper*, encodes a protein with a death-domain. Mammalian proteins with this domain are receptors, which oligomerize and induce apoptosis upon ligand-binding. *reaper* is specifically expressed in cells about to undergo apoptosis, and unpublished results have shown it is upregulated in salivary glands from late 3rd larval instars (Thummel, pers. comm.). *Br-C* mutants, with salivary glands that fail to histolyze, have reduced levels of *reaper* expression (Thummel, pers. comm.). Two other recently identified genes, *grim* and *hid*, also play important roles in eliciting cell death in *D. melanogaster*. The proteins encoded by all three genes have similarities in their N-terminal 14 amino acids, suggesting this portion plays a role in signaling apoptosis. Two or more of these proteins seem to be closely associated in eliciting the cell death response. *hid* was originally identified by its mutant phenotype, with reduced levels of cell death in the head region of developing embryos. Cell death is seen during many stages of *D. melanogaster* development, in particular, in nerve cells of developing embryos. PKC53E is found in developing CNS tissues, and in adult heads, however, a possible function has not yet been discerned. The localization of PKC53E in salivary glands raises a new hypothesis for the function of the protein, which may involve cell death (Steller and Grether, 1994; Steller, 1994; McCall and Steller, 1997).

Recent studies have uncovered putative roles for PKC isoenzymes in programmed cell death. PKCδ has been shown to play a role in apoptosis in B lymphocytes (Emoto *et al.*, 1995). PKCδ is proteolytically cleaved in the V3 region by interleukin-1β-converting enzyme (ICE), resulting in the release of a catalytically active fragment. Cell death is visualized within four hours after this fragment is generated (Emoto *et al.*, 1995). Cleavage of PKCδ could be a consequence of activating the cell death pathway, where ICE and ICE-like proteases specifically target PKC. PKC isoenzymes have been shown to have a plethora of substrates, and are involved in a number of signaling pathways. The release of a catalytically active fragment with no regulation could result in saturating the cell

with signals it can no longer interpret. However, it could also result in the massive phosphorylation of a specific target substrate, such as Lamin B, which plays an important structural role in the nuclear envelope (Fields *et al.*, 1988; Hocevar *et al.*, 1993; Steller, 1995). Lamin phosphorylation and subsequent destabilization has been demonstrated to be an important step in eliciting the apoptotic pathway (Fields *et al.*, 1988; Hocevar *et al.*, 1993; Steller, 1995). It has also been demonstrated that prolonged PKC activation will lead to its degradation (Newton, 1995; Nishizuka, 1995). The release of a catalytically active fragment could result in a quick burst of signaling, followed by downregulation, and this downregulation of signaling could be involved in apoptosis. Another possible explanation for PKC activation, is that it plays a role in a signaling pathway which directly regulates genes involved in programmed cell death. If the protein were proteolytically cleaved, then the regulatory domain, which is recognized by the anti-PKC53E1 antisera, could be translocated to the nuclear envelope where it could play another role. This 'secondary' role could involve interacting with another protein at the nuclear envelope, altering its regulation. This could cause a signal to be transduced into the nucleus, and in turn alter gene expression (Steller and Grether, 1994; Steller, 1995; McCall and Steller, 1997).

Lamins are targeted for proteolysis by ICE-like proteases, and are thought to play a role in apoptosis (Steller, 1995). Some classical mammalian PKC isoenzymes have been shown to interact with Lamin $B_I$ at the nuclear envelope (Fields *et al.*, 1988). Phosphorylation leads to the time-dependent solubilization of Lamin $B_I$, synonymous with mitotic nuclear envelope breakdown *in vitro* (Hocevar *et al.*, 1993). Another study compared PKC$\beta_{II}$ with $p34^{cdc2}$/cyclin B kinase as a putative mitotic lamin kinase. However, PKC$\beta_{II}$ showed higher phosphorylation rates during interphase, in comparison to $p34^{cdc2}$/cyclin B kinase (Goss *et al.*, 1994). Three *lamin* genes have been isolated in *D. melanogaster*, *LamC*, *G-IF*, and *Lam* (Bossie and Sanders, 1993; Riemer and Weber,

1994; Ulitzer *et al.*, 1992). One of the isoforms encoded by the *Lam* gene is found predominantly in the nuclear envelope (Ulitzer *et al.*, 1992). Studies performed by Ulitzer *et al.* (1992) utilized anti-Lamin antibodies, and found a putative role in the assembly of the nuclear envelope after cell division. The *D. melanogaster* PKC53E isoenzyme is homologous to the mammalian classical PKC isoenzymes, and could play a role in the phospho-regulation of Lamin. As shown in this thesis, studies demonstrated PKC53E protein localization to the nuclear envelope of salivary gland cells in late 3rd larval instars. This supports a potential role for PKC53E in phospho-regulating Lamin, however, these cells are about to undergo apoptosis, not proliferation. Phosphorylation of Lamin could lead to nuclear envelope breakdown as a step in the apoptosis of cells, as opposed to nuclear envelope reassembly.

Detectable levels of PKC53E are first seen in late-stage embryos, and it would be exciting to postulate a role for PKC53E in memory or learning. Upon hatching from the egg, the 1st larval instars are the first stage to rely on cues from the environment for survival, and if PKC53E is involved in modulating these signals, it would be required at or just prior to this time (Ashburner, 1989A). Learning is a process that is dynamic, and relies on the constant formation and reformation of synapses (Tanaka and Nishizuka, 1994). If a protein were to be important for such a process, it would be required throughout the organism's life. Homologous PKC isoenzymes have been demonstrated to be important for learning and memory in higher eukaryotes (Tanaka and Nishizuka, 1994). The mechanism by which PKC governs this remains to be elucidated, and it seems that different isoenzymes are expressed in different tissues, and different isoenzymes are activated in response to different signals. Mammalian PKC isoenzymes have been shown to play roles in learning and memory by regulating ion channels, receptors, and neurotransmitter release (Tanaka and Nishizuka, 1994). Mammalian PKCα, which is the closest homologue to the *D. melanogaster* PKC53E isoenzyme, has ubiquitous patterns of

protein expression in the brain, and is found in several different neuronal cell types (Rosenthal *et al.*, 1987; Tanaka and Nishizuka, 1994). PKC isoforms have been found to modulate ion channels and receptors on post-synaptic membranes, as well as affecting other processes such as neurotransmitter release (Tanaka and Nishizuka, 1994). These are all processes which in turn can regulate the storage of information, and have an effect on learning (Tanaka and Nishizuka, 1994). Although GAP-43 will be discussed later on with regard to its role in axonal outgrowth, it has also been shown to play important roles in regulating presynaptic neurotransmitter release, and long term potentiation (Tanaka and Nishizuka, 1994). A *D. melanogaster* mutant, *turnip*, was isolated based on poor learning performance. These mutants had deficient PKC activity, and a 76kDa PKC-substrate protein was found to be important for the learning process (Choi *et al.*, 1991). This gene was cytogenetically mapped to a different location from any of the PKC isoenzymes, suggesting it encodes a protein involved in regulating a PKC-mediated signaling pathway. This protein belongs to the Rho subfamily of GTP-binding proteins, which have been shown to be involved in PKC-mediated signaling pathways (Flybase, 1997). Rho proteins are typically involved in the regulation of cytoskeletal elements, and have been shown to control polymerized actin, an important component of growth cones (Hall, 1992; Hall, 1993).

PKC53E could also be involved in the formation of neuronal processes during differentiation of the nervous system, since protein has been localized to the developing CNS regions in embryos. Broughton *et al.* (1996), have shown the involvement of at least one PKC in the formation of neuronal processes. This was accomplished through the creation of transgenic flies with inducible expression of an inhibitor peptide derived from inaC, a PKC expressed specifically in photoreceptor cells (Broughton *et al.*, 1996). This peptide includes the pseudosubstrate domain, which is highly conserved amongst the *D. melanogaster* PKC isoenzymes, although varies more significantly for PKC98E (Figure

1.3). Inhibition of PKC by this peptide resulted in the inhibition of neurite outgrowth in *D. melanogaster* neuroblasts cultured *in vitro* (Broughton *et al.*, 1996). Previous studies suggest this could occur through regulation of various substrates, such as cytoskeletal elements, important in the formation of lamellipodia and axonal outgrowths (Williams *et al.*, 1994).

The mammalian dynamin proteins are microtubule-binding proteins with intrinsic GTPase activity. They function in clathrin-mediated endocytosis, and have been shown to be PKC substrates (Sudhof, 1995; Camilli and Takei, 1996). Dynamin I is dephosphorylated in a $Ca^{2+}$-dependent manner upon depolarization, resulting in inhibition of GTPase activity (Sudhof, 1995; Camilli and Takei, 1996). The *D. melanogaster* homologue, *Shibire*, was first isolated as a paralytic mutant with disrupted endocytosis of synaptic vesicles (Kim and Wu, 1987). Although the protein encoded by this gene is important for regulating endocytosis in synaptic transmission, *Shibire* mutants also had reduced neurite outgrowth (Robinson, *et al.*, 1993). This suggests a role for dynamin, possibly through PKC phospho-regulation, in axonal outgrowth (Kim and Wu, 1987; Robinson *et al.*, 1993). The *D. melanogaster* GAP-43 homologue, *Igloo*, was recently characterized, and the protein is a putative PKC substrate (Neel and Young, 1994). *Igloo* has a similar developmental pattern of expression to *Pkc53E* (Neel and Young, 1994). It is expressed from late embryonic stages, throughout larval and pupal stages, and in adults, however, 3rd larval instars have lower levels of expression (Neel and Young, 1994). Staining was restricted to the central and peripheral nervous system, predominantly in neurons (Neel and Young, 1994). The mammalian homologue of this protein, GAP-43 (neuromodulin), has been shown to interact with both calmodulin and PKC, most likely in a $Ca^{2+}$-dependent manner (Tanaka and Nishizuka, 1994). It plays an important role in axonal outgrowth, and was found to regulate polymerization of actin in growth cones

(Tanaka and Nishizuka, 1994). Interestingly, phosphorylated forms of GAP-43 were found in growth cones associated with other cells, and calmodulin-associated GAP-43 was found in the retracting edges (He *et al.*, 1997). The pattern of transcript expression suggests that perhaps Igloo is not involved in early neuroblast differentiation, which occurs around stage 6-7, but could be important in the formation of synaptic contacts. The nervous system is dynamic, and a complex organization of proteins is most likely regulating the continual extension of axons and reformation of synapses, especially during the process of learning in response to changes in environmental or developmental cues (Tanaka and Nishizuka, 1994). Another important point is that only non-phosphorylated forms of the mammalian homologue, GAP-43, are found in early neurite outgrowth, and phosphorylated form appears only in the distal portions of the axon and growth cone, when axonogenesis has started (Meiri *et al.*, 1991). This supports a role for PKC and Igloo in later stages of neurite outgrowth, possibly in helping growth cones reach their destination, and in the formation of synaptic contacts (Neel and Young, 1994).

It is interesting that all three PKC isoenzymes found in *D. melanogaster* are expressed in adult heads, and little or no transcripts or protein have been identified in adult bodies. The cDNA for *inaC* was originally cloned by Schaeffer *et al.* (1989) using bovine probes for Pkcα and β. Cytogenetically, *inaC* maps to a position within 25kb of *Pkc53E*, at 53E4-7, and one is thought to have arisen from the other due to a duplication event (Schaeffer *et al.*, 1989; Smith *et al.*, 1991). As shown in Table 1.1, one transcript of 2.4kb has been identified, localized in the photoreceptor cells of the compound eyes and ocelli, starting in late pupal stages (Schaeffer *et al.*, 1989). The protein encoded by this gene is approximately 80kDa, and is homologous to the mammalian classical PKC isoenzymes. inaC has been demonstrated to be involved in light adaptation as shown earlier in Figure 1.4 (Schaeffer *et al.*, 1989; Smith *et al.*, 1991; Hardie *et al.*, 1993). This

protein is activated through a calcium and phospholipid-dependent pathway, and some of the other components of this pathway have been identified through mutational analyses, and immunoprecipitation studies. One of the proteins in this pathway is encoded by the *norpA* (*no-receptor-potential A* ) gene. It is homologous to the mammalian PLCβ enzyme, and upon activation is an effector which hydrolyzes $PIP_2$ (Smith *et al.*, 1991). Another protein involved in the inaC pathway is encoded by the *trp* (*transient receptor potential* ) gene (Hardie *et al.*, 1993). It encodes a light-activated SOC $Ca^{2+}$-ion channel, originally thought to be required to refill calcium stores depleted by $IP_3$ receptors (Hardie *et al.*, 1993). In the absence of functional TRP channels, the calcium stores are not refilled, and inaC is consequently not activated due to the absence of calcium. Recent studies show that TRP is a $Ca^{2+}$ channel that spans the PM, and is activated by the depletion of $Ca^{2+}$ ions from internal stores, causing the influx of $Ca^{2+}$ ions from the extracellular space (Smith *et al.*, 1991; Clapham, 1996; Huber *et al.*, 1996A; Huber *et al.*, 1996B; Shieh and Zhu, 1996). Failure of this channel to open would cause inaC to become inactive, as the $Ca^{2+}$ stores would not replenish, and the $Ca^{2+}$ levels would remain too low (Smith *et al.*, 1991; Clapham, 1996; Huber *et al.*, 1996A; Huber *et al.*, 1996B; Shieh and Zhu, 1996). Both *trp* and *inaC* mutant phenotypes are similar in that they fail to adapt to light (Hardie *et al.*, 1993). Another component of the inaC pathway is encoded by the *rdgB* (*retinal degeneration B* ) gene (Smith *et al.*, 1991). The *rdg* mutant was identified according to the light-sensitive retinal degeneration phenotype, demonstrated to be dependent on inaC activity (Smith *et al.*, 1991). When both *norpA* and *rdgB* were mutated, the eyes were resistant to the light-sensitive degeneration phenotype (Smith *et al.*, 1991). When *rdgB* mutant flies kept in the darkness were treated with phorbol esters, the eyes degenerated (Smith *et al.*, 1991). When both *rdgB* and *inaC* were mutated, the eyes were resistant to light-sensitive degeneration (Smith *et al.*, 1991). This suggests that inaC is a key mediator of the biochemical pathway resulting in RdgB regulation (Smith *et al.*, 1991).

Broughton *et al.* (1996) utilized an inhibitory peptide (Figure 1.3), derived from the pseudosubstrate domain of inaC. A protein of approximately 84kDa, with PKC characteristics, was inhibited from autophosphorylation (Broughton *et al.*, 1996). This inhibition altered neuroblast differentiation and neurite outgrowth *in vitro* (Broughton *et al.*, 1996). The size of this protein and the inhibitor peptide used points to inaC as the PKC involved in this process, however, the specific temporal and spatial pattern of inaC expression and function contradict this. Multiple bands were observed when a rabbit polyclonal antibody recognizing a 7 amino acid consensus sequence for PKC was used, making it difficult to discern whether this was the actual PKC being affected by the inhibitor peptide (Broughton *et al.*, 1996). The pseudosubstrate domain is at least partially conserved among the *D. melanogaster* PKC isoenzymes (Figure 1.7), and the peptide used as an inhibitor (Figure 1.3) could have inhibited one or more of these isoenzymes (Schaeffer *et al.*, 1989).

*Pkc98E* has only been characterized at the level of RNA, and is expressed as a 5.5kb transcript throughout development (Table 1.1). It is found predominantly in the cell bodies of adult heads, with 4.3 and 4.5kb transcripts also being expressed in early stages of development (Schaeffer *et al.*, 1989). The putative ORF for the protein encoded by one or more of these transcripts is 71kDa, with homology to the mammalian novel PKCε isoenzyme (Schaeffer *et al.*, 1989). This protein lacks a $Ca^{2+}$-domain, and is most likely activated through $Ca^{2+}$-independent pathways (Schaeffer *et al.*, 1989). Aside from the C1 and C1' regions, most of the N-terminus varies considerably from the other *D. melanogaster* PKC isoenzymes (Schaeffer *et al.*, 1989). The inhibitory peptide used by Broughton *et al.* (1996) was less likely to inhibit PKC98E than PKC53E, although there is some conservation in the pseudosubstrate domain between all three isoenzymes. PKC98E is significantly smaller than the protein putatively shown to be inhibited by Broughton *et al.*

(1996). Given the developmental pattern of *Pkc98E* expression, however, it could also be a candidate for the PKC involved in neurite outgrowth (Broughton *et al.*, 1996).

The presence of two CNS-specific PKC isoenzymes, PKC53E and PKC98E, can be placed into perspective if there is partial redundancy and partial specificity between them. PKC98E could be specifically activated through $Ca^{2+}$-independent pathways at the onset of development of neural tissue, and is responsible for the primary stages of neuroblast differentiation and neurite outgrowth. This would support PKC98E as the primary target for the inhibitory peptide in the experiments performed by Broughton *et al.* (1996). It is also likely that both PKC53E and PKC98E are targeted for inhibition, and produce the phenotypes seen by Broughton *et al.* (1996). Coordination of the two isoenzymes through $Ca^{2+}$-dependent and independent pathways could confer neuroblast differentiation, subsequent neurite outgrowth, and learning. PKC98E, which is expressed earlier, could interact with specific substrates to regulate neuroblast differentiation in a $Ca^{2+}$-independent manner. PKC53E, which is expressed at a later stage, could then direct latter stages of axonal outgrowth in a $Ca^{2+}$-dependent manner.

Learning processes, governed by the regulation of synaptic transmission, are complex (Tanaka and Nishizuka, 1994; Sudhof, 1995). They most likely require several $Ca^{2+}$-dependent and independent mechanisms, and there could be some redundancy in the substrates required for these processes. For example, PKC98E could potentially interact with turnip, the Rho GTPase discussed earlier (Choi *et al.*, 1991). In mammals, Ras-like proteins are typically involved in tyrosine kinase pathways, which are $Ca^{2+}$-independent (Hall, 1992; Hall, 1993; Lowy and Willumsen, 1993; Fantl *et al.*, 1993). $Ca^{2+}$-independent PKC isoenzymes have also been shown to be activated through these tyrosine kinases, and evidence has shown these enzymes are involved in cross talk with Ras/Raf pathways (Fantl *et al.*, 1993). However, the only PKC isoenzyme found in yeast, Pkc1, has been shown to be directly activated by Rho1, as shown earlier in Figure 1.4 (Kamada

*et al.*, 1996). This isoenzyme is homologous to the mammalian classical PKC isoenzymes and is $Ca^{2+}$-dependent (Levin *et al.*, 1990). Therefore, PKC98E or PKC53E, or both, could be involved in the turnip pathway. If PKC98E is responsible for mediating early neuroblast differentiation and growth, by $Ca^{2+}$-independent mechanisms, this most likely does not include substrates such as Shibire and Igloo, which are phospho-regulated in a $Ca^{2+}$-dependent manner (Robinson *et al.*, 1993; Neel and Young, 1994). These two substrates are more likely to be regulated by PKC53E, supporting a role for PKC53E at later stages of axonal outgrowth, establishing synapses, and even mediating events such as synaptic vesicle endocytosis and other processes important for learning (Robinson *et al.*, 1993; Neel and Young, 1994). It is likely there are many other substrates involved in these processes, which will be uncovered by future work which isolates other components of the PKC53E pathway.

## 4.6 Conclusions

A portion of *Pkc53E* was successfully subcloned, in frame, into the pGEX-4T1 vector. The fusion construct was transformed into *E.coli* DH10B cells, and the GST-PKC53EV1 fusion protein was expressed, and purified by affinity-column chromatography. The GST-PKC53EV1 fusion protein was used to generate polyclonal antisera in New Zealand rabbits. The anti-PKC53E1 antisera recognized a protein of approximately 75kDa in a PKC-rich extract obtained from *D. melanogaster* adult heads. Western blots revealed a protein of similar size in whole adult extracts, although in a much lower proportion. Anti-PKC53E1 antisera failed to recognize a protein in extract from testes, and a faint band of about 75kDa was visualized in extract from ovaries. Anti-PKC53E1 antisera were blot-affinity purified, and the 75kDa band from PKC-rich head extracts, was visualized with a 1:50 dilution. A protein of similar size was visualized throughout all three larval stages, using a dilution of 1:25 affinity-purified anti-PKC53E1

antisera. A protein of approximately 70kDa protein was visualized in early (white) and mid (eye) pupal extracts, and a smeary band of 70-75kDa was visualized in late (dark) pupal extracts, using a dilution of 1:25 affinity-purified anti-PKC53E1 antisera. Immunohistochemical studies revealed the presence of protein in small subsets of neuronal cells in stage 12 embryos. Segmental-specific staining was also visualized. 3rd instar larval brains showed patterns of staining, indicative of CNS, in the optic lobes and ventral ganglia. Protein was also localized at the nuclear envelope of salivary glands dissected from 3rd larval instars.

Patterns of *Pkc53E* RNA and protein expression suggest it plays an important role in the CNS. Although it may not be involved in initial differentiation of the CNS, it may be required for establishing early connections, and/or for maintenance and functioning. The presence of protein at the nuclear envelope in salivary gland cells from late 3rd instar larvae raises additional hypotheses as to its function. PKC53E could play a key role in cell death signaling pathways, initiated by the hormone ecdysone. This function may be transferable to the CNS, as many of these cells are targeted for death throughout development.

### 4.7 Future Directions

The most important step in further characterizing the protein is to determine its function. This could be accomplished through the creation of a mutation in the *Pkc53E* gene, by P-element mutagenesis, or EMS mutagenesis. Another option is to create transgenic flies, using a construct with DNA encoding part of the mRNA in antisense orientation, or a portion of the DNA encoding the pseudosubstrate domain, under the control of a heat shock promoter. Once a mutant is obtained in *Pkc53E*, mutants in other genes potentially involved in the pathway can help discern its function.

Anti-PKC53E1 antisera could be used to perform *in vitro* analyses. PKC53E could be affinity-purified, and subsequently tested for further characterization, and to

determine potential substrates. Anti-PKC53E antisera could be used for immunoprecipitations, which could be electrophoresed on native gels, and tested with antisera to identify interacting components of the pathway(s).

In an attempt to localize PKC53E in adult heads, further immunofluorescence studies could be performed on cryostat sections. Troubleshooting would need to be performed to lower background autofluorescence, and cross-reactivity with secondary antisera. Double-labeling experiments could also be performed to co-localize any other proteins which may be interacting with PKC53E.

The salivary glands from 3rd larval instars could be studied in more depth to delineate the signaling pathway PKC53E may be involved in. There are several mutants in which the salivary glands fail to histolyze, and immunofluorescence studies could be performed on salivary glands dissected from these mutants to discern whether there is any altered pattern of PKC53E protein localization. It would be interesting to determine how the protein is interacting with the nuclear envelope, and this could be accomplished by performing double-labeling experiments, and *in vitro* assays. Studies could also be pursued to discern if there is any correlation between increased PKC53E expression and it's localization to the nucleus, and altered levels of ecdysone. This could potentially be an interesting tissue to study signaling, and the role of signaling in programmed cell death in *D. melanogaster*.

# Chapter 5

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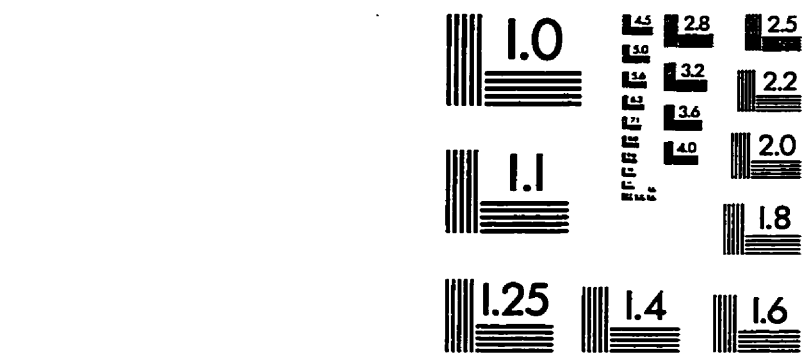


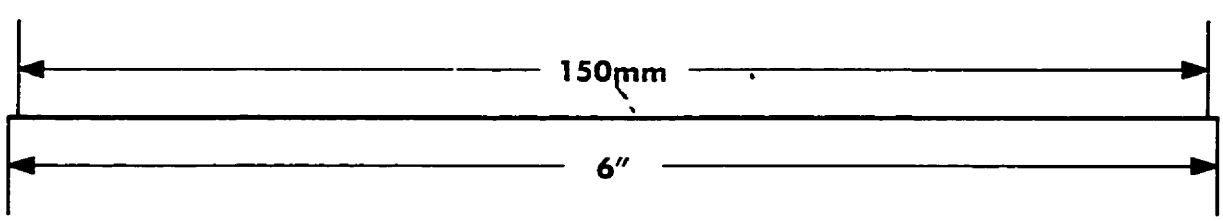


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