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**The characterisation of Plc1:
A phospholipase C enzyme identified in the fission yeast
*Schizosaccharomyces pombe***

A thesis submitted to
The University of Warwick
For the degree of
Doctor of Philosophy

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Dept. Biological Sciences
The University of Warwick
February 2000

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Acknowledgements

I would like to say thank you to:

John Davey for his supervision,

Kevin Davis for all his advice on how to cook lovely food but I still went home to cook marmite on toast or bean feast,

Graham Ladds for all his help and advice even though he supports Manchester United,

Pete Watson for introducing me to the harsh realities of life (ohh why don't you like me Peter??),

Mark (Didbot- every body calls you that) **Didmon** for being a great friend over the past 5 years, ahh just one year out!!,

Cassandra Pateman for all her special cuddles,

Panom Winyayong for teaching me how to say cheeky egg in Thai,

Dale Powner for turning me into a beer monster,

Mark Saxon for being a Chandler like figure when I had my Joey Tribbiani moments and for being a complete sadist in the gym when it came to doing abs sets,

Mark Rapson for introducing me to Jim and Spence, the world goes on standby when they get together!!,

and **Tracey Knott** for her molecular biology help in the first 12 months.

A special thanks to **Clive** and **Carole Brind** for all their help and support over the past 4 years and always believing in my ability.

A special thanks to **Vicky Lamberton** without her I'm not sure that I would have survived the last 18 months.

Thank you to S.G. Rhee who provided the clone of Rat PLC- δ 1

Thank you, to the BBSRC for their funding without it none of this would have been possible.

Declaration

I hereby declare that the work described in this thesis was conducted by myself under the supervision of Dr John Davey with the exception of those instances where the contributions of others has been specifically acknowledged.

None of the information contained herein has been used in any previous application for a degree.

All sources of information have been specifically acknowledged by means of a reference.

Robert Ian Brind

Abstract

The *plc1* gene product (Plc1) of the fission yeast *Schizosaccharomyces pombe* (*Sz. pombe*) encodes a phosphoinositide-specific phospholipase C (PLC) and most closely resembles the δ class of the PLC isozymes. PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂), producing two second messengers, inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG). The work in this thesis is concerned with aspects of PLC signalling in *Sz. pombe* cells.

Sz. pombe cells lacking *plc1* ($\Delta plc1$) display a distinct phenotype. $\Delta plc1$ cells are viable they grow slowly at 29°C (and below) on rich medium, but are unable to grow under stressful conditions such as on minimal medium or at 37°C. Loss of Plc1 function has been shown to cause missegregation of chromosomes and enlarged cells with aberrant morphology (Fankhauser *et al.* 1995). Expression of Plc1 in $\Delta plc1$ cells complemented for the loss of Plc1 and resulted in a wild-type *Sz. pombe* phenotype.

Described in this thesis are the characterisation experiments carried out on Plc1. Extracts prepared from wild-type *Sz. pombe* cells have undetectable Plc1 activity, so an assay was designed, to measure Plc1 activity *in vitro*. The *in vitro* assay and expression of Plc1 in $\Delta plc1$ cells formed the basis of a set of experiments that helped identify a possible regulatory domain and roles for Plc1 in *Sz. pombe* cells. Mutant forms of Plc1 were assayed *in vitro* to determine their level of activity and were then expressed in $\Delta plc1$ cells to see if they were able to complement for the loss of Plc1 activity.

Temperature sensitive mutants of Plc1 were constructed. This illustrated what happened to a *Sz. pombe* cell with an active Plc1 at 23°C but when incubated at 37°C Plc1 activity is switched off. This demonstrated that cells lacking Plc1 activity could no longer divide and defects in cell wall structure began to appear.

Active site mutants of Plc1 were produced to identify whether InsP₃ or DAG are important second messengers in *Sz. pombe* cells. The Plc1 mutants were unable to hydrolyse PIP₂ but could hydrolyse phosphatidylinositol (PI). These mutants hydrolyse PI producing DAG and InsP and were able to rescue the $\Delta plc1$ phenotype. This suggests that DAG production may play an important role in regulating stress response pathways whereas InsP is recycled to produce phosphatidylinositols.

Yeast PLCs have an extended N-terminal domain, which is not seen in any other eukaryotic PLCs. To investigate the role of this N-terminal domain, mutants of Plc1 and PLC- δ were constructed. They demonstrated that without the N-terminus these mutants were active *in vitro* but were unable to complement for the loss of Plc1 activity in $\Delta plc1$ cells. This indicates that the N-terminal domain may play an important regulatory role.

Abbreviations

aa	amino acid
ACh	acetylcholine
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
cpm	counts per minute
dATP	deoxy adenosine triphosphate
ddATP	dideoxy adenosine triphosphate
DMM	defined minimal medium
DMSO	dimethylsulphoxide
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	diaminoethanetetra-acetic acid sodium salt
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(β -aminoethylether) N,N,N',N'-tetraacetic acid
GAP	GTPase-activating protein
GTP	guanosine triphosphate
GDP	guanosine diphosphate
G-protein	guanosine nucleotide-binding protein
h	hour
His ₆	Hexahistidine tag
HPLC	high pressure liquid chromatography
InsP _x	inositol phosphate (x=number of phosphate groups present)
kb	kilo bases
kDa	kilo Dalton
K _m	Michaelis constant
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase

MES	2-[N-morpholino]ethane sulphonic acid
min	minute
mRNA	messenger RNA
NGF	neural growth factor
ORF	open reading frame
PA	phosphatidic acid
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PE	phosphatidylethanolamine
PH	pleckstrin homology region
PI X-K	phosphoinositide kinase (X denotes the position on the inositol ring which is phosphorylated)
PITP	phosphatidylinositol transfer protein
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PtdIns (PI)	phosphatidylinositol
PtdInsP (PIP)	phosphatidylinositol phosphate
PtdInsP ₂ (PIP ₂)	phosphatidylinositol bisphosphate
PtdInsP ₃ (PIP ₃)	phosphatidylinositol trisphosphate
s	second
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>Sz. pombe</i>	<i>Schizosaccharomyces pombe</i>
SDS	sodium dodecyl sulphate
SH	<i>src</i> homology region
td	doubling time
TEMED	NNN'N tetramethyl diamine
Tris	tris (hydroxymethyl) aminomethane
u/v	ultra violet

v/v

volume for volume

w/v

weight for volume

YE

yeast extract

Chapter 1
General Introduction

1.1 Cellular communication

Signal transduction is the process by which cells communicate, regulating their growth, development, and co-ordinating their functions. Extracellular molecules are released by particular cell types and are recognised by membrane-bound receptors. This subsequently results in the activation of an intracellular signal. The large number of stimuli that higher eukaryotic cells are exposed to makes the study of cellular communication *in vivo* difficult. So one alternative is to study signalling in organisms with less elaborate communication pathways. Signalling in the genetically tractable fission yeast *Schizosaccharomyces pombe* (*Sz. pombe*) has been well characterised and provides a simpler model to study cell communication. Phosphatidylinositol-specific phospholipase C (PLC) is an important part of cell signalling as it generates two second messengers which have many functions. *Sz. pombe* contains a PLC and therefore makes this an ideal organism to elucidate the role of PLC in yeast and it may give insights into alternative roles of PLCs in higher eukaryotes.

This general introduction will consider phosphatidylinositols, how they are synthesised, and their role within eukaryotic cells. It will discuss the structure of mammalian PLC isoforms, how they are regulated and will introduce what is known about the structure and function of yeast PLCs.

1.2 phosphoinositide metabolism and its cellular roles

Phosphoinositides and their role within cell membranes was first observed by Hokin and Hokin (1955). This led to the discovery that phosphoinositides are actively metabolised and undergo accelerated turnover when cells are stimulated by various agonists. The metabolism of phosphoinositides and their role in signal transduction has therefore been extensively studied. The current understanding of phosphoinositide-mediated metabolic pathways is summarised in Figure 1. This section will be concerned with discussing the roles of these different phosphoinositides, the different enzymes involved in their synthesis, and finally the regulators of these enzymes.

The major phosphoinositides that are present within resting cells are phosphatidyl inositol (PtdIns), phosphatidylinositol 3-phosphate (PtdIns 3-P), phosphatidylinositol 4-phosphate (PtdIns 4-P) and phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P₂). These phosphoinositides are maintained in equilibrium by a number of kinases and phosphatases.

Perhaps the best characterised signal transduction pathway involving phosphoinositide turnover is the activation of phosphatidylinositol-specific phospholipase C (PLC) (PLC is discussed in detail in section 1.3). Extracellular agonists binding to receptors at the cell surface eventually result in the activation of PLC. Once activated PLC is able to hydrolyse PtdIns, PtdIns 4-P and PtdIns 4,5-P₂ generating two second messengers diacylglycerol (DAG) and soluble inositol phosphates. These include inositol 1-phosphate (Ins1-P), inositol 1,4-bisphosphate (Ins 1,4-P₂) and inositol 1,4,5-tris phosphate (Ins 1,4,5-P₃) respectively. Once these second messengers have performed their function, they are recycled by a series of kinases, phosphatases and phosphatidylinositol synthase to synthesise PtdIns, PtdIns 4-P and PtdIns 4,5-P₂.

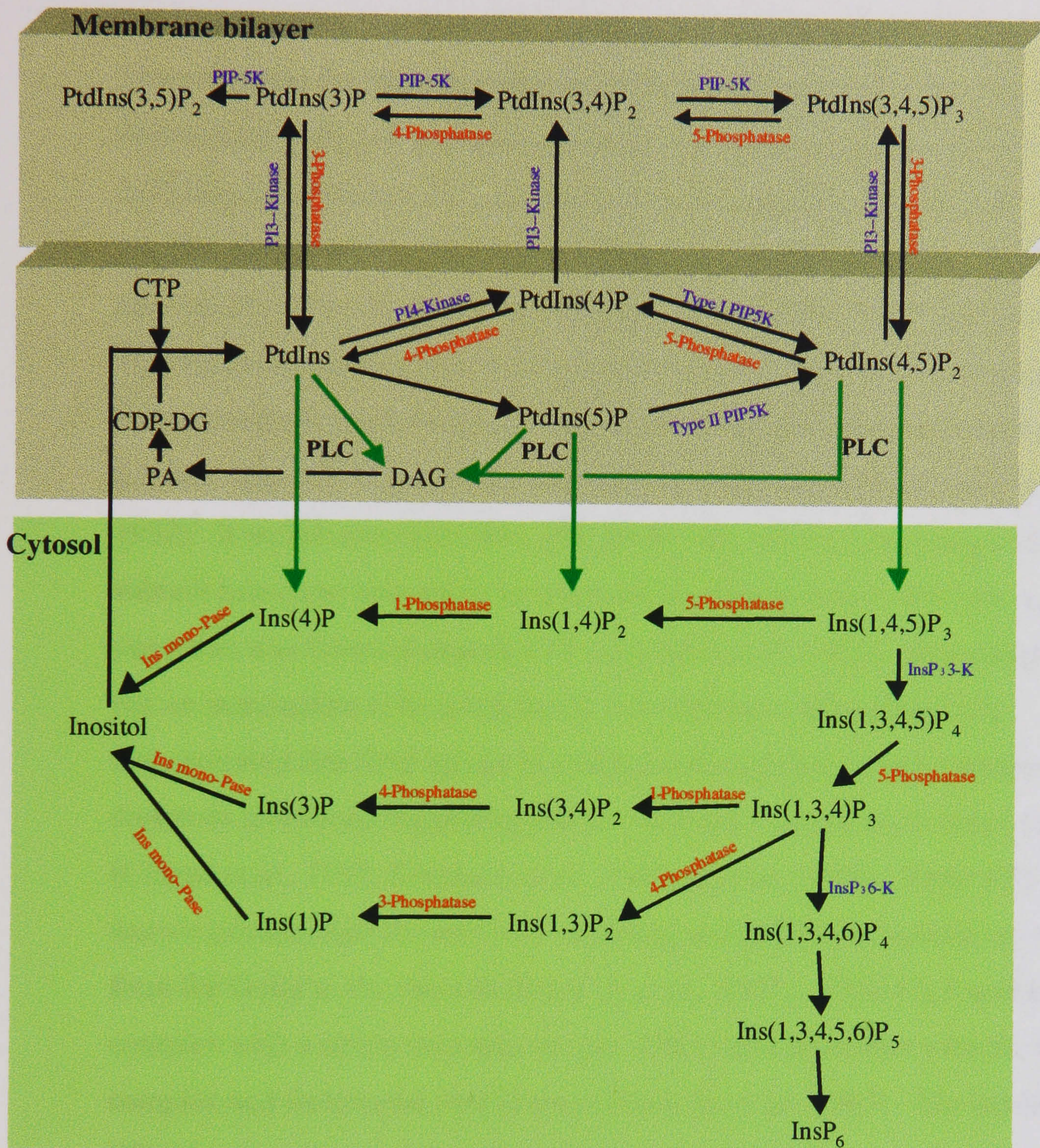


Figure 1 The metabolism of phosphatidylinositols and inositol polyphosphates

A diagram demonstrating that phosphatidylinositol metabolism takes place in the lipid bilayer and inositol polyphosphate metabolism takes place within the cytosol. The link between these two different pathways is phospholipase C whose catalytic action (green arrows) results in the breakdown of phosphatidylinositols into inositol polyphosphates and diacylglycerol (DAG). The blue writing indicates kinase action whereas red writing indicates phosphatase action.

1.2.1 Enzymes involved in the synthesis of phosphoinositides

1.2.1.1 Phosphatidylinositol 3-kinase

The substrates for phosphoinositide 3-kinase (PI 3-K) are PtdIns, PtdIns 4-P and PtdIns 4,5-P₂. PI 3-K phosphorylates the 3-position on the inositol ring resulting in PtdIns 3-P, PtdIns 3,4-P₂ and PtdIns 3,4,5-P₃ respectively. PI 3-K has been purified from a number of sources (Otsu, *et al.*, 1991; Gout, *et al.*, 1992). It consists of two types of subunits with molecular weights of 85kDa and 110kDa. The 85kDa subunit interacts with phosphotyrosine residues in activated growth factor receptors and with tyrosine-phosphorylated substrates of receptor tyrosine kinases. The 110 kDa subunit is thought to be the catalytic subunit (Gout *et al.*, 1992). It would seem therefore, that the 85 kDa subunit brings the 110 kDa subunit into close apposition to activated tyrosine kinases. *VPS34* is a gene in yeast that was found to encode a PI 3-K (Schu *et al.*, 1993). Experiments carried out on mammalian cells using the PI 3-K inhibitor wortmannin have demonstrated that fluid uptake is affected and recycling of transmembrane receptors is reduced suggesting that this enzyme is required for these functions (Clague *et al.*, 1995; Shepherd *et al.*, 1995; Li *et al.*, 1995). Yeast *VPS* mutants encode proteins that are involved in sorting and delivery of soluble hydrolases from the Golgi to the vacuole (Stack (i) *et al.*, 1995). *VPS34* is found in a complex with a serine threonine kinase *VPS15* that associates with the Golgi complex and endosomal membranes (Stack (ii) *et al.*, 1995). The function of *VPS15* is to activate and recruit *VPS34* to the appropriate membrane site (Stack (ii) *et al.*, 1995). One possible function of PtdIns 3-P production at localised membrane areas is that it may promote vesicle coat proteins which might catalyse transport reactions (Stack (i) *et al.*, 1995). This suggests that PI 3-K and its metabolites have the same function as important regulators of vesicular traffic in both mammalian and yeast cells.

1.2.1.2 Phosphatidylinositol-4-phosphate 5-kinase

The enzymes that produce PtdIns 4,5-bisphosphate *in vitro* fall into two related subfamilies, type I and type II phosphatidylinositol-4 phosphate 5-kinase (PIP-5K), based upon their sequence similarity and enzymatic properties (Loijens *et al.*, 1996). It was previously thought that PtdIns 4,5-P₂ was created by first producing PtdIns 4-P from PtdIns using phosphatidylinositol 4-kinase (PI-4K) and then phosphorylating the 5 position of the inositol ring by PIP-5K. As predicted, type I PIP5-K does phosphorylate the 5 position of the inositol ring but surprisingly the type I PIP5-K also phosphorylates the PtdIns at the 4 position of the inositol ring (Rameh *et al.*, 1997). Furthermore reports have indicated that type I and Type II PIP 5-K can phosphorylate PtdIns 3-P at position 4, producing PtdIns 3,4-P₂ (Zhang *et al.*, 1997). PtdIns 3,4-P₂ is not a substrate for type II PIP 5-K but Zhang and Majerus (1998) indicated that PtdIns 3,4,5-P₃ may form directly from PtdIns 3-P without the intermediate (PtdIns 3,4-P₂) dissociating from the enzyme. The type I PIP 5-K can phosphorylate PtdIns 3,4-P₂ to give PtdIns 3,4,5-P₃, but it can also synthesise PtdIns 3,4,5-P₃ directly from PtdIns 3-P in a concerted effort (Zhang and Majerus, 1998). Other reports have demonstrated that type I PIP 5-K converts PtdIns 3-P into PtdIns 3,5-P (Dove *et al.*, 1997). Large quantities of the type II PIP5-K is found in certain tissues including brain, blood cells and platelets (Ling *et al.*, 1989; Borenenkov and Anderson, 1995; Hinchcliffe *et al.*, 1996), suggesting that PtdIns 4,5-P₂ produced by this alternative way may be important in several different cell types. Type II PIP 5-K isolated from platelets, associates with the cytoskeleton and PIP 5-K alters the protein conformation in response to thrombin, indicating that type II PIP 5-K activity may be regulated by various signal transduction pathways (Hinchcliffe *et al.*, 1996)

1.2.1.3 Inositol polyphosphate 5-phosphatase

Inositol polyphosphate 5-phosphatase (5-Pase) hydrolyses the 5-position of the inositol ring. Seven mammalian 5-Pases have been identified and they can be categorised into four groups.

Group I only hydrolyse the soluble Ins 1,4,5-P₃ and Ins 1,3,4,5-P₄ and the most likely function of this enzyme is to terminate signals transmitted by these second messengers (Laxminarayan *et al.*, 1993).

Group II 5-Pase hydrolyse the four substrates Ins 1,4,5-P₃, Ins 1,3,4,5-P₄, PtdIns 4,5-P₂ and PtdIns 3,4,5-P₃. A particular group II 5-Pase is deficient in patients suffering from a human disorder known as oculocerebrorenal syndrome of Lowe (OCRL). This affects the eye lens, brain and kidneys (Atree *et al.*, 1992). The OCRL group II 5-Pase prefers the lipid substrate PtdIns 4,5-P₂ (Zhang and Majerus., 1998). Another group II 5-Pase is synaptojanin that functions in synaptic vesicle trafficking (McPherson *et al.*, 1996). Synaptojanin may function with dynamin and amphiphysin. Dynamin is a GTPase that participates in recycling of synaptic vesicles membranes and amphiphysin co-localises with dynamin in nerve terminals (McPherson *et al.*, 1994; McPherson *et al.* 1996). In synaptic vesicle endocytosis is regulated by the amounts of specific PtdIns metabolites (Camilli *et al.*, 1996).

Group III enzymes include two tyrosine-phosphorylated 5-Pases, SIP-110 and SHIP-140. This group only hydrolyses Ins 1,3,4,5-P₄ and PtdIns 3,4,5-P₃ (Damen *et al.*, 1996). SIP-110 and SHIP-140 are encoded by the same gene but are expressed as alternatively spliced gene products (Zhang and Majerus, 1998). They contain phosphotyrosine-binding sites and an SH2 domain enabling them to form complexes with signalling molecules after stimulation of cells by cytokines and growth factors.

A group IV 5-Pase has been identified by three groups (Jackson *et al.*, 1995; Liu *et al.*, 1996; Guiherme *et al.*, 1996) although it has not been isolated or cloned. This 5-Pase hydrolyses one substrate, PtdIns 3,4,5-P₃, and this activity has been shown to be associated with PI 3-K but the function for this is unclear (Jackson *et al.*, 1995).

A 5-Pase has been identified in the budding yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*) and has been designated INP51 (Stoltz *et al.*, 1998). The primary structure closely resembles the type II 5-Pase synaptojanin and exhibits 5-Pase activity towards the substrate PtdIns 4,5-P₂. When *INP51* is deleted it results in a phenotype that grows significantly faster than the parental strain at temperatures below 15°C (Stoltz *et al.*, 1998). Cells lacking *INP51* have almost 4 times more PtdIns 4,5-P₂ and Ins 1,4,5-P₃ (Stoltz *et al.*, 1998) suggesting that 5-Pase activity is required for proper maintenance of PtdIns 4,5-P₂ levels.

1.2.1.4 Inositol 1,4,5-P₃ 3-kinase

Inositol 1,4,5-P₃ 3-kinase (Ins1,4,5-P₃ 3-K) is a widely distributed soluble enzyme that converts Ins 1,4,5-P₃ to Ins 1,3,4,5-P₄ in the presence of Mg²⁺ and ATP (Irvine *et al.*, 1986). In hepatocytes, the enzyme is stimulated by the action of Ca²⁺ and protein kinase A (PKA) but is inhibited by protein kinase C (PKC) (Sim *et al.*, 1990). Ins1,4,5-P₃ 3-K is widespread in the brain but is particularly concentrated in the cerebellar Purkinje cells suggesting that its activity is important within these tissues (Mailleux *et al.*, 1992).

1.2.1.5 Inositol polyphosphate 1-phosphatase

Inositol polyphosphate 1-phosphatase (1-Pase) hydrolyses Ins 1,3,4-P₃ and Ins 1,4-P₂ to Ins 3,4-P₂ and Ins 4-P, respectively (Inhorn *et al.*, 1987). 1-Pase

requires Mg^{2+} for activity but is inhibited by Ca^{2+} at physiological concentrations (Inhorn and Majerus, 1988). Lithium ions inhibit both Ins 1,3,4- P_3 and Ins 1,4- P_2 hydrolysis uncompetitively.

1.2.1.6 Inositol monophosphatase

Inositol monophosphatase (mono-Pase) hydrolyses phosphate groups of all inositol monophosphates with the exception of inositol 2-phosphate (Ackermann *et al.*, 1987; Inhorn *et al.*, 1987). Mono-Pase isolated from bovine and rat brain is a homodimer (Gee *et al.*, 1988). Treatment of bovine mono-Pase with the arginine-specific reagent phenylglyoxal resulted in enzyme inactivation, suggesting that an arginine is involved in catalysis (Jackson *et al.*, 1989).

1.2.1.7 Inositol polyphosphate 3-phosphatase

Inositol polyphosphate 3-phosphatase (3-Pase) has been purified and characterised but not cloned. 3-Pase catalyses the hydrolysis of PtdIns 3-P and soluble Ins 1,3- P_2 to PtdIns and Ins 1-P, respectively (Caldwell *et al.*, 1991). Two isoforms of 3-Pase have been identified in rat brain, types I and II, and both are able to hydrolyse both the lipid and soluble substrate but type II has a twenty times less efficiency to Ins 1,3- P_2 (Majerus, 1992).

1.2.1.8 Phosphatidylinositol transfer protein

Phosphatidylinositol transfer protein (PITP) is a cytosolic protein that has the ability to transfer PtdIns and phosphatidylcholine (PC) between membrane bilayers *in vitro* (Wirtz, 1991). Two isoforms of PITP have been identified in mammalian cells, α and β . They were classified depending upon their lipid

binding properties. For example, PITP α can reversibly bind PtdIns and PC, but has a sixteen times greater affinity for PtdIns than for PC (van Paridon *et al.*, 1987). PITP β can transfer sphingomyelin (a lipid formed from ceramide containing a phosphocholine head group) in addition to PtdIns and PC. PITP is involved in many functions including the delivery of PtdIns to signalling complexes including PIP4-K and PIP5-K resulting in PtdIns 4,5-P₂ production (Cunningham *et al.*, 1995; 1996). PITP may also have a role in exocytosis by promoting PtdIns 4,5-P₂ production and recruiting specific proteins required for exocytosis (Fensome *et al.*, 1996). PITP's involvement in vesicular traffic in yeast has also been identified. The gene *SEC14* in the budding yeast *S. cerevisiae* has been shown to encode a PITP (Novick *et al.*, 1980; Bankaitis *et al.*, 1990). The activity of SEC14 appears to act as a sensor of the Golgi membrane phospholipid content. SEC14 has been shown to regulate the cytidine diphosphate-choline pathways, thus regulating PC biosynthesis and maintaining the appropriate lipid concentration (McGee *et al.*, 1994). SEC14 can also functionally substitute for the mammalian PITP in cell free studies suggesting that there may be a general role for this protein in vesicular transport (Ohashi *et al.*, 1995).

1.2.2 The roles of phosphatidylinositols in cells

This section will be concerned with the roles that the phosphatidylinositols play within the cell. Phosphatidylinositols constitute about 10% of the total membrane phospholipid and they have many roles, including signalling, facilitating vesicle traffic, and even helping to organise cytoskeletal development (see Figure 2).

1.2.2.1 Phosphatidylinositol 3-phosphate

Phosphatidylinositols containing a phosphate on the 3' position of the inositol ring may act as second messengers within mammalian cells, but they are not substrates for phospholipase C. They also do not appear to produce soluble inositol polyphosphates (Hughes and Michell, 1993). The production of PtdIns 3P may regulate Golgi to lysosome traffic and might affect endocytosis in yeast. Production of PtdIns 3P by PI 3-K (VPS34) may either recruit or activate effector molecules, such as vesicle coat proteins that catalyse this transport reaction (DeCamilli *et al.*, 1996). Inactivation of VPS34 results in reduced concentrations of PtdIns 3P which appears to affect the latter stages of the endocytosis (Munn and Riezman, 1994).

1.2.2.2 Phosphatidylinositol 3,5-bisphosphate

Yeast cells are able to synthesise PtdIns 3,5-P₂ using PI 3-K in response to hyperosmotic stress. Mammalian cells are also able to synthesise PtdIns 3,5-P₂ but in response to hypo-osmotic shock (Dove *et al.*, 1997). Yeast cells lacking PI 3K have reduced Golgi to vacuole trafficking. Results indicate that PtdIns 3,5-P₂ is synthesised from PtdIns 3-P in response to hyperosmotic stress. PtdIns 3,5-P₂ may therefore be involved in adaptation response that involves accelerated golgi-to-vacuole vesicle trafficking events (Dove *et al.*, 1997).

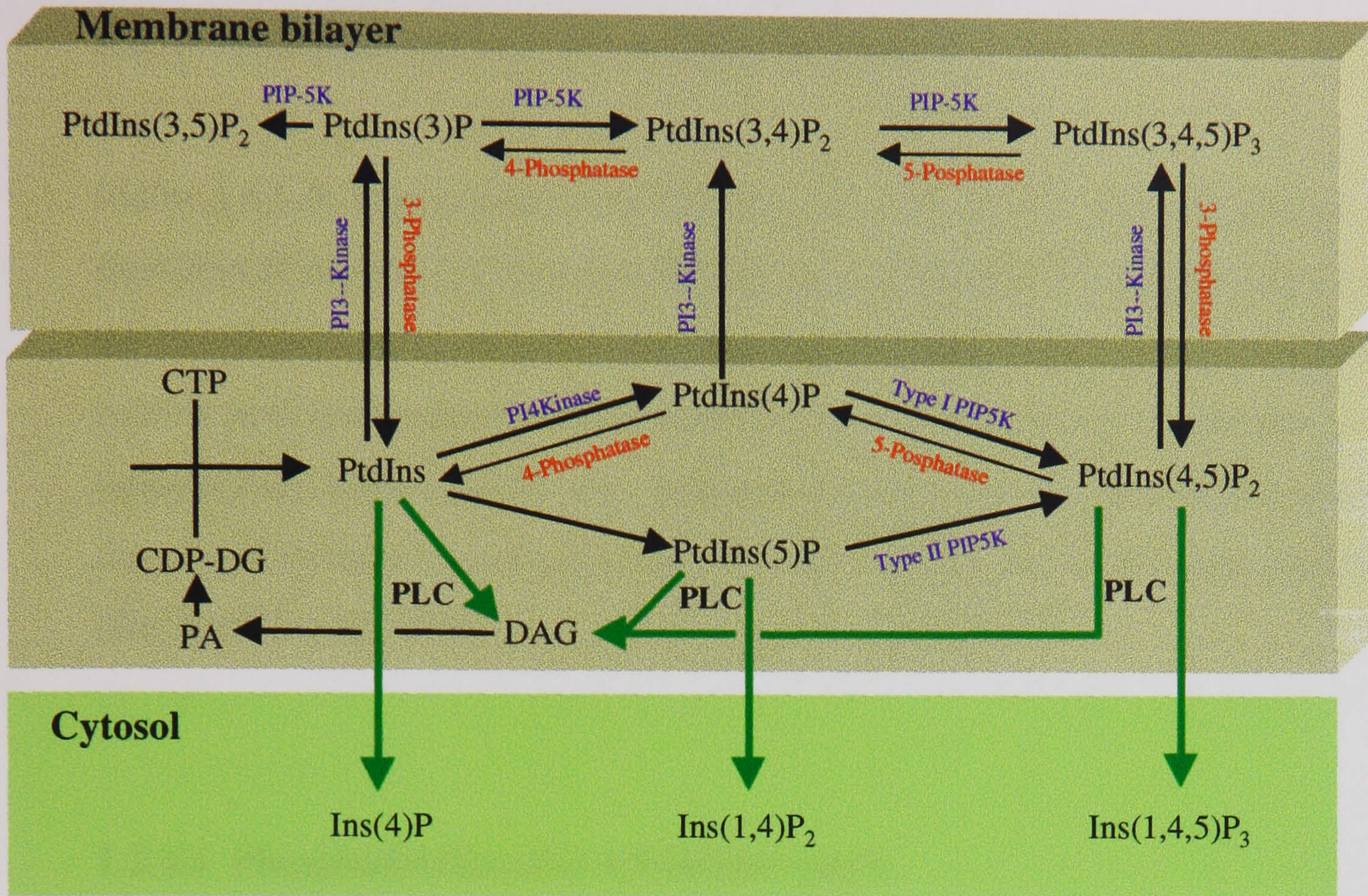


Figure 2 The metabolism of phosphatidylinositols in cells

A schematic diagram of a cell, it demonstrates how phosphatidylinositol metabolism takes place in the lipid membrane bilayer. It suggests how they are synthesised and how there are recycled within the membrane. The roles of these phosphatidylinositols and how their production is regulated is explained in sections 1.2.1.1-1.2.1.8 It demonstrates the role of phospholipase C whose catalytic action (green arrows) results in the break down of phosphatidylinositols into inositol polyphosphate(s) and diacylglycerol (DAG). The blue writing indicates kinase action whereas red writing indicate phosphatase action.

1.2.2.3 Phosphatidylinositol 3,4,5-trisphosphate

PtdIns 3,4,5-P₃ may interact with Rab5 (Li, G. *et al.*, 1995). Rab proteins are members of the Ras GTPase superfamily and are involved in specific stages of the exocytic or endocytic pathways (Simons and Zerial, 1993). Rab proteins are soluble until nucleotide exchange results in them binding to the membrane. It is thought that PtdIns 3,4,5-P₃ may facilitate nucleotide exchange on Rab5 either directly or indirectly resulting in the activation of exocytic or endocytic pathways (DeCamilli *et al.*, 1996).

1.2.2.4 Phosphatidylinositol 4,5-bisphosphate

One of the cellular roles of PtdIns 4,5-P₂ includes regulating vesicle exocytosis. PtdIns 4,5-P₂ has been shown to interact with many proteins involved in the formation and trafficking of vesicles (Terui *et al.*, 1994). PtdIns 4,5-P₂ has been shown to stimulate guanine nucleotide exchange and activate ARF1 (ADP ribosylation factor) (Terui *et al.*, 1994). ARF1 participates in vesicle coat recruitment and is required for the formation of carrier vesicles (Donaldson and Klausner, 1994). GTP-bound ARF1 associates with membranes and activates the attachment of coat proteins, such as COP1 and clathrin (Rothman, 1994). Activated ARF1 also works in co-operation with PtdIns 4,5-P₂, activating an isoform of phospholipase D (PLD) (Figure 3). PLD hydrolyses the phospholipid, phosphatidylcholine (PC) into phosphatidic acid (PA) and choline. PA is then able to activate PIP 5-K generating more PtdIns 4,5-P₂ (Cockcroft *et al.*, 1994). This may be an amplification loop that results in a localised increase in PtdIns 4,5-P₂ and PA in a vesicular carrier (DeCamilli *et al.*, 1996). PtdIns 4,5-P₂ and PA have also been shown to interact with ARF GTPase activating proteins (GAPs) (Liscovitch and Cantley, 1995). GTP hydrolysis results in ARF

returning to its soluble form and this is required for uncoating prior to fusion with the target compartment (Rothman, 1994).

PtdIns 4,5-P₂ may be involved in the regulation of the cytoskeleton by affecting the interaction of a number of cytoskeletal components with actin. These cytoskeletal components which interact with PtdIns 4,5-P₂ include, profilin and gelsolin. Once interacted with PtdIns 4,5-P₂ profilin promotes and stabilises the formation of actin filaments, whereas, gelsolin severs the actin filaments (Homma and Emori, 1995). Profilin is found in close apposition with the inner leaflet of the plasma membrane where it can bind up to 5 molecules of PtdIns 4,5-P₂ (Goldschmidt-Clermont *et al.*, 1990; Yonezawa *et al.*, 1991). Binding of profilin to PtdIns 4,5-P₂ inhibits the interaction of profilin with actin. It also inhibits PtdIns 4,5-P₂ hydrolysis by PLC (except for PLC- γ 1 when phosphorylated and activated by growth factors it is able to hydrolyse protein bound PtdIns 4,5-P₂ (Goldschmidt-Clermont *et al.*, 1991)). Homma and Emori (1995) demonstrated that gelsolin co-purified with PLC- δ , suggesting that PLC- δ may also be involved in the hydrolysis of protein-bound PtdIns 4,5-P₂ affecting actin assembly.

All other phosphatidylinositols shown in Figure 2 have short half-lives, suggesting that they are only produced as intermediates before producing biologically active molecules. These biologically active molecules include PtdIns 3-P, PtdIns 3,5-P₂, PtdIns 3,4,5-P₃ and PtdIns 4,5-P₂.

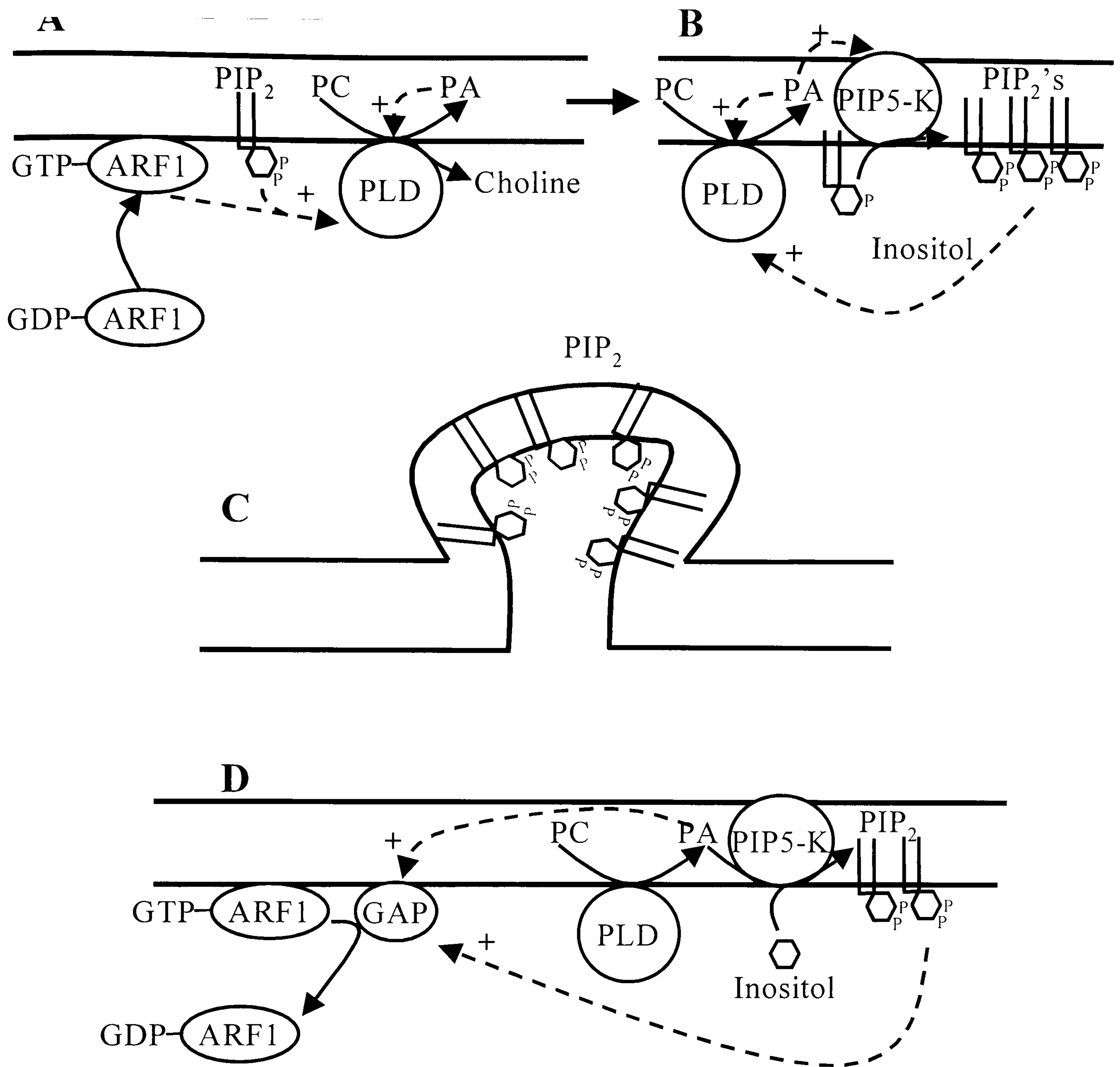


Figure 3 The role of PtdIns 4,5-P₂ in exocytosis

Panel A demonstrates how PtdIns 4,5-P₂ (PIP₂) may stimulate guanine nucleotide exchange on ARF1. Once ARF1 has bound to the membrane it works in co-operation with PtdIns 4,5-P₂ to stimulate PLD. PLD hydrolyses phosphatidylcholine into phosphatidic acid (PA) and choline. Panel B demonstrates that PA activates PIP5-K generating more PtdIns 4,5-P₂. This generation of PtdIns 4,5-P₂ creates an amplification loop as it activates PLD, resulting in a localised increase in PtdIns 4,5-P₂. Panel C shows how this localised increase in PtdIns 4,5-P₂ may result in vesicle production. Panel D demonstrates how this cycle could be switched off; PtdIns 4,5-P₂ and PA are known to interact with GAPs. GAP hydrolyses GTP on ARF1 resulting in its inactivation.

1.2.3 The roles of inositol-polyphosphates in cells

A number of external signals, such as ligands binding to receptors and osmotic stress, generate inositol polyphosphates (InsPPs). InsPPs are soluble molecules that pass through the cytosol to their target binding proteins (Figure 4). The binding proteins include receptors located on the surface of the endoplasmic reticulum and nuclear membranes and many kinases and phosphatases that are involved in InsPPs metabolism (see Sections 1.2.1.3-1.2.1.8).

1.2.3.1 Inositol 1,4,5-trisphosphate

PtdIns 4,5-P₂ hydrolysis by PLC results in the release of second messengers Ins 1,4,5-P₃ and DAG (Figure 5). Ins 1,4,5-P₃ is soluble so it passes through the cytosol to the endoplasmic reticulum (ER) where it binds to its receptor (Furuichi *et al.*, 1989). The Ins 1,4,5-P₃ receptor appears to be a tetramer and each monomer appears to have seven membrane-spanning domains. The majority of the protein is thought to reside in the cytoplasm with the carboxyl terminus residing in the lumen of the ER (Furuichi *et al.*, 1989). The tetramer appears to form the functional Ins 1,4,5-P₃-sensitive Ca²⁺ channel. The Ins 1,4,5-P₃ receptor shows close structural and functional similarity to the ryanodine receptor (RyR) (Takeshima *et al.*, 1989). The RyR however, is almost twice as large as the Ins 1,4,5-P₃ receptor and has an equal number of membrane-spanning domains with both the amino and carboxyl terminus on the cytoplasmic side of the ER membrane (Takeshima *et al.*, 1989). Upon binding of Ins 1,4,5-P₃, the Ins 1,4,5-P₃ receptor undergoes a large conformational change which is perhaps related to the coupling process leading to channel opening (Sudhof, 1990). Both the Ins 1,4,5-P₃ and ryanodine receptors are known to release Ca²⁺; the Ins 1,4,5-P₃ receptor releases Ca²⁺ from the intracellular stores of the ER in response to Ins 1,4,5-P₃ binding to it.

Another protein that binds Ins 1,4,5-P₃, is Ins 1,4,5-P₃ kinase (InsP 3-K). This synthesises Ins 1,3,4,5-P₄ from Ins 1,4,5-P₃. Ins 1,4,5-P₃ kinase is discussed in more detail in section 1.2.1.4. As there is no recognisable Ins 1,4,5-P₃ receptor or Ca²⁺ channel in the *S. cerevisiae* genome, it is unlikely that Ins 1,4,5-P₃ regulates intracellular Ca²⁺ in yeast cells (Ongusaha *et al.*, 1998). Instead, Ins 1,4,5-P₃ production is more likely to be a precursor for inositol phosphate metabolism.

1.2.3.2 Inositol 1,3,4,5-tetrakisphosphate

There is evidence for a protein that binds inositol 1,3,4,5-tetrakisphosphate (Ins 1,3,4,5-P₄) that has been purified from porcine membranes (Donie *et al.*, 1990). The Ins 1,3,4,5-P₄ receptor shows approximately 100 times greater affinity for Ins 1,3,4,5-P₄ than for Ins 1,4,5-P₃ (Majerus, 1992). Ins 1,3,4,5-P₄ receptors have been located in the plasma membrane of endothelial cells which have greater affinity for Ins 1,3,4,5-P₄ than for Ins 1,4,5-P₃ (Ludhov and Clapham, 1992). In olfactory cells there are Ca²⁺ channels that are equally sensitive to the binding of both Ins 1,4,5-P₃ and Ins 1,3,4,5-P₄ (Kalinov *et al.*, 1992).

An alternative target protein for Ins 1,3,4,5-P₄ is 5-phosphatase (Figure 4). Once bound it dephosphorylates the 5' position resulting in Ins 1,3,4-P₃, which is the precursor for inositol hexakisphosphate (Ins-P₆) production in mammalian cells. In yeast cells, only small amounts of Ins 1,3,4,5-P₄ are produced, but this is quickly phosphorylated to Ins 1,3,4,5,6-P₅ and then to Ins-P₆ (Ongusaha *et al.*, 1998).

1.2.3.3 Inositol hexakisphosphate

Inositol hexakisphosphate (Ins-P₆) can be made by receptor-independent pathways or from receptor-generated Ins 1,4,5-P₃ via Ins 1,3,4,5-P₄, Ins 1,3,4-P₃,

Ins 1,3,4,6-P₄ etc. (Figure X). Infusion of Ins-P₆ into the brain stem of rats was found to reduce blood pressure and heart rate (Vallejo *et al* 1987). Subsequent studies have revealed that it may also simulate Ca²⁺ entry into neurones, pituitary cells and adrenal cells (reviewed in Hughes and Michell, 1993). Beck and Keen (1991) have demonstrated that *in vitro* Ins-P₆ inhibits assembly proteins (AP) such as AP-2 and AP-180 which facilitate membrane traffic (Chang *et al.*, 1993; Anderson *et al* 1995). AP-2 is associated with clathrin-coated vesicles at the plasma membrane and is involved in regulating receptor-mediated endocytosis and exocytosis (Chang *et al.*, 1993). AP-180 is a nerve terminal-specific clathrin adaptor (Anderson *et al* 1995). A possible role for Ins-P₆ may be regulating endocytosis and exocytosis of clathrin-coated vesicles (Li, C. *et al* 1995).

Ins-P₆ in pancreatic-β cells inhibits the serine-threonine protein phosphatases, types 1, 2A and 3 (PPases) (Larsson *et al.*, 1997). Protein dephosphorylation by the PPases modulates the activity of the voltage-gated L-type Ca²⁺ channels (Catterall, 1995). Incubation of pancreatic-β cells with glucose resulted in an increase in Ins-P₆ production, this stimulated the activity of L-type Ca²⁺ channels by suppression of the PPases (Larsson *et al.*, 1997).

1.3 Phospholipase C

As discussed in Section 1.2, many cellular signals are derived from the metabolism of phosphoinositides. One enzyme that facilitates the metabolism of phosphoinositides is phospholipase C. Metabolism of phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P₂) by phospholipase C generates the second messengers inositol 1,4,5-trisphosphate (Ins 1,4,5-P₃) and diacylglycerol (DAG) (Figure 5). Ins 1,4,5-P₃ and DAG are known to modulate intracellular responses through the release of Ca²⁺ and activation of PKC, respectively. Once mammalian PLCs had been isolated, conserved domains could be identified. This led to the subdivision of mammalian PLCs into three isozymes, designated β , γ and δ (Rhee *et al.*, 1989). Each isozyme has numerous members. For example, there are at least nine distinct subtypes of PLC isozymes (β 1, β 2, β 3, β 4, γ 1, γ 2, δ 1, δ 2, δ 3 and δ 4) based upon comparisons of their primary amino acid sequences (Rhee and Choi, 1992). Homologues have also been identified in plants, lower eukaryotes and prokaryotes (reviewed in Noh *et al.*, 1995; Heinz *et al.*, 1995). This section will review the PLC isozymes, concentrating on their structure and regulation.

1.3.1 The structure of PLCs

All PLCs contain four conserved domains. These include the X and Y catalytic domain, the pleckstrin homology domain, the EF hand domain, and the C2 domain. The structure and function of these domains are discussed in the following sections.

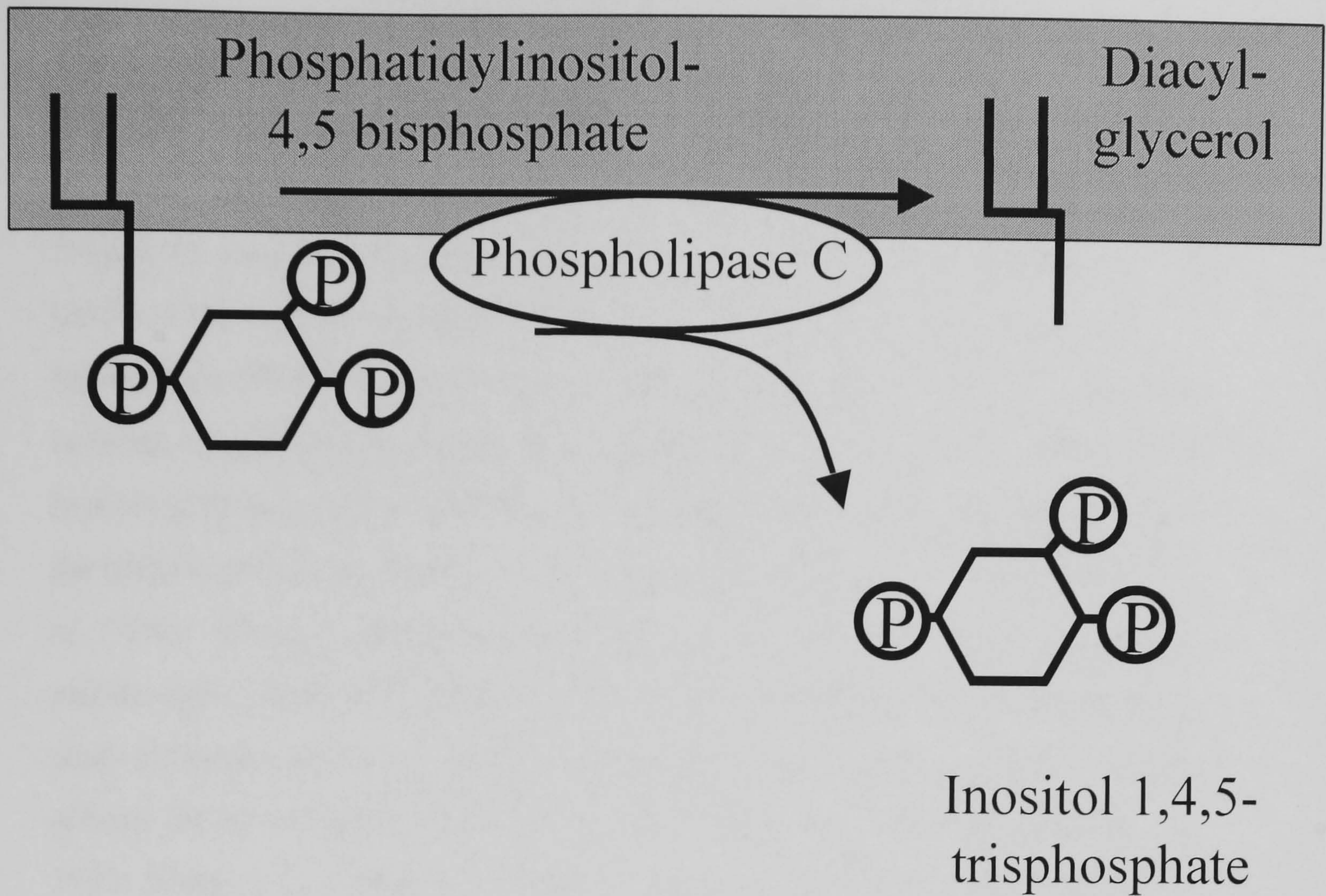


Figure 5 The reaction catalysed by phospholipase C

Phospholipase C hydrolyses PtdIns 4,5- P_2 producing the second messengers soluble Ins 1,4,5- P_3 which passes into the cytosol and DAG which is hydrophobic and remains in the membrane. This bifurcating signalling pathway controls many intracellular responses.

1.3.1.1 The X and Y Catalytic Domain

The X and Y domains are highly conserved and form the catalytic core of the molecule. This was confirmed by the crystal structure of rat PLC- δ 1 (Essen *et al.*, 1996). Although the X and Y domains are separated in the primary structure (Figure 6), once it undergoes tertiary structure formation they form the two halves of the catalytic domain. The X and Y domain create a structure that resembles a triosephosphate isomerase motif (TIM barrel motif). This structure consists of eight interacting β -sheets linked with α -helices (Farber, 1993). PLCs hydrolyse three possible substrates; PtdIns, PtdIns 4-P, and PtdIns 4,5-P₂, with the highest affinity for PtdIns 4,5-P₂ and the lowest affinity for PtdIns (Essen *et al.*, 1996). This is based upon specific interactions created between the substrate and the amino acids that make up the active site. Mutations of specific amino acids within the active site of PLCs results in reduced catalytic activity or altered affinity for the substrate (Cheng *et al.*, 1995; Ellis *et al.*, 1995; Simoes *et al.*, 1995; Wang *et al.*, 1996). See Chapter 7 for more information on active site mutations.

1.3.1.2 The pleckstrin homology domain

PLC isozymes contain a pleckstrin homology (PH) domain located at the N-terminus of the protein (Figure 6). PLCs are not the only proteins that contain a PH domain, they have been identified in more than 100 proteins through sequence and structural similarities (Paterson *et al.*, 1995). Although PH domains display little sequence homology, they show structural similarity. The PH domain consists of seven anti-parallel β pleated sheets with an α helix at the carboxyl-terminal end. The PH domain of PLC- δ 1 binds PtdIns 4,5-P₂ 100 times stronger than the PH domains on pleckstrin, spectrin and dynamin (Macias *et al.*, 1994; Yoon *et al.*, 1994; Ferguson *et al.*, 1995; Hyvonen *et al.*, 1995). Interaction of the PLC- δ 1 PH domain with PtdIns 4,5-P₂ is different from the

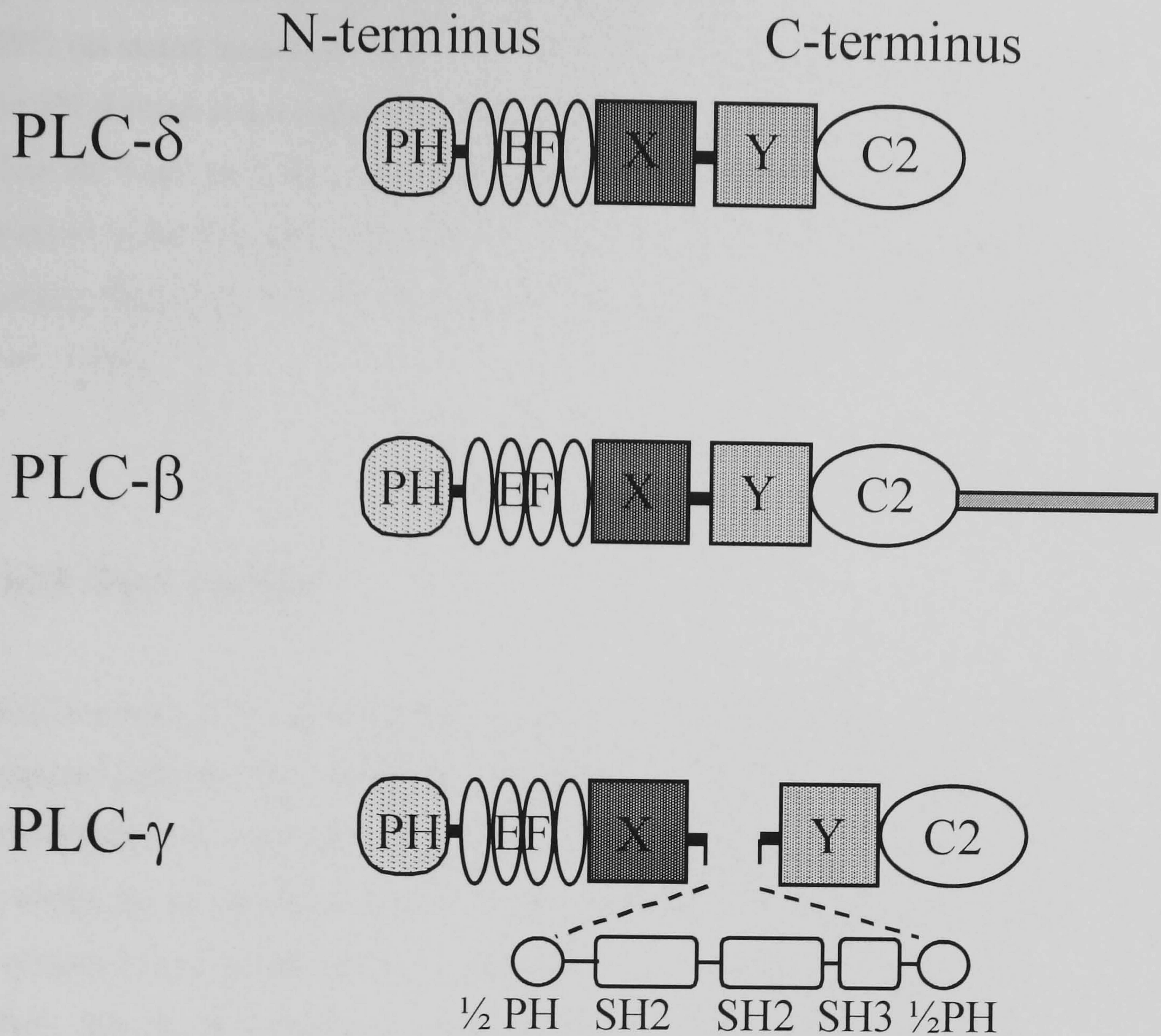


Figure 6 The structure of mammalian PLCs

Mammalian PLCs contain four conserved domains. These include the pleckstrin homology domain (PH), the EF hand domain, the X and Y catalytic domain and the C2 domain. PLC- β has an extended C-terminal domain and PLC- γ has an extra four domains. These extra domains in PLC- β and PLC- γ are involved in their regulation.

catalytic domain, as the PH domain is able to bind PtdIns 3,4,5-P₃ (Garcia *et al.*, 1995) (as stated in section 1.2.2.3, PLCs are unable to hydrolyse PtdIns 3,4,5-P₃). The PH domain is not required for PLC activity but deletion of this domain can affect the function of the enzyme. When any of the basic amino acids were replaced in the N-terminal 60 residues of PLC- δ 1 there was a reduction in ligand binding, PtdIns 4,5-P₂ hydrolysis, and loss of membrane attachment (Yagisawa *et al.*, 1998).

1.3.1.3 The C2 domain

The C2 domain is identified at the C-terminus of all PLCs (Figure 6). The C2 domains were originally identified as homologous regions in Ca²⁺-dependent protein kinase C (Kaibuchi *et al.*, 1989). The structure of the C2 domain in PLC- δ 1 comprises of eight β sheets with four of the sheets creating the core domain, two loops from the ends of the core create a Ca²⁺-binding site (Essen *et al.*, 1996). The function of the C2 domain in PLCs is not fully understood but evidence from other proteins containing C2 domains, such as synaptotagmin and annexin V, suggests that it may be involved in membrane binding. The C2A domain of synaptotagmin binds to acidic phospholipids in a Ca²⁺-dependent manner (Davletov and Sudhof, 1993; Chapman and Jahn, 1994). In the membrane-binding protein annexin V, the recognition site for the head group of phosphatidylserine consists of two adjacent Ca²⁺-binding sites (Essen *et al.*, 1996). A similar mode of phospholipid binding by the C2 domain in PLC- δ may be possible.

Essen *et al.*, (1996) proposed a two step 'tether and fix' membrane binding mechanism for PLC's. The PH domain tethers the enzyme to the membrane by specific binding to PIP₂ and the C2 domain fixes the catalytic domain in a productive orientation relative to the membrane.

1.3.1.4 The EF domain

EF hands were first identified in parvalbumin which, contains two similar Ca^{2+} -binding sites (Herzberg *et al.*, 1986). These Ca^{2+} -binding sites are formed by two α -helices that are separated by a loop. The EF hand is formed by the two α -helices forming a structure that resembles the forefinger and thumb of a right hand; the Ca^{2+} -binding site is the loop between the two helices. Subsequent X-ray crystallographic studies have demonstrated that many proteins that bind Ca^{2+} contain EF hands (Essen *et al.*, 1996). These include PLCs. The EF hands in PLCs consist of four loops (Figure 6) but only loops one and two are able to bind Ca^{2+} (Essen *et al.*, 1996).. The EF hand of PLC- δ 1 is crucial as deletion of about half abolishes PIP_2 hydrolysis activity (Ellis *et al.*, 1993). Mutants where conserved acidic amino acids in the EF motif were replaced by alanine showed nearly the same PLC activity and Ca^{2+} dependency as the wild-type (Nakashima *et al.*, 1995). This suggests that, although the EF hand is required for enzyme activity, it is not the site that regulates the Ca^{2+} -dependence of the reaction (Nakashima *et al.*, 1995; Drayer *et al.*, 1995).

1.3.2 The regulation of the PLC isozymes

From Figure 6 it can be seen that mammalian PLC- β and PLC- γ contain an extended C-terminus and an extra four domains, respectively. This next section will discuss these extra domains identified in PLC- β and PLC- γ and their roles in regulation. PLC- δ consists of the four domains that are common to all PLCs. The regulation of this isozyme is unknown but this next section will identify some of the possible regulators that have been suggested.

1.3.2.1 Regulation of mammalian PLC- β

The PLC- β family are regulated by G-proteins which are coupled to seven-transmembrane (7-TM) spanning receptors (Figure 7) (Blank *et al.*, 1991; Wu *et al.*, 1992). The 7-TM receptors that activate PLC- β via G-proteins include thromboxane, bradykinin, angiotensin, histamine, vasopressin and muscarinic acetylcholine (Gutowski *et al.*, 1991; Shenker *et al.*, 1991; Wange *et al.*, 1991; Bernstein *et al.*, 1992). The domains within the muscarinic acetylcholine receptor that are responsible for interacting with the G-protein are thought to be the second and third intracellular loops of the receptor (Wong *et al.*, 1990). Activation of the receptor results in the G α subunit releasing the bound GDP and binding GTP in its place. This causes dissociation of the G α subunit from the $\beta\gamma$ subunits of the heterotrimeric G-protein and these subunits can then activate PLC- β (for a review of G-proteins see Sprang, 1997).

Stimulatory relationships between the currently known isoforms of PLC- β 1-4 and α subunits of the Gq subfamily (α_q , α_{11} , α_{14-16}) have been firmly established *in vitro*. PLC- β 1 and PLC- β 3 isozymes were shown to be more responsive to G α_q , G α_{11} and G α_{16} (Hepler *et al.*, 1993; Kozasa, *et al.*, 1993; Smrcka *et al.*, 1993) whereas activation of PLC- β 2 is through G α_{16} (Wu *et al.*, 1992). PLC- β 4,

originally identified in mammalian retina (Lee, C-W. *et al.*, 1993), is stimulated by the Gq subfamily (Lee, C-W. *et al.*, 1994).

The $\beta\gamma$ subunits of G-proteins have also proven to be effective regulators of PLC- β activity. This was first reported in liver cells (Blank *et al.*, 1992). Effective regulation of the $\beta\gamma$ subunits requires prenylation of the γ subunit (Dietrich *et al.*, 1994). Comparisons of the purified isoforms of PLC- β suggest that there are different levels of regulation exerted by the $\beta\gamma$ subunits. PLC- β 3 apparently is the most sensitive to $\beta\gamma$ activation with PLC- β 1 being the least sensitive and PLC- β 2 somewhere in-between (Smrcka and Sternweis, 1993; Park *et al.*, 1993). The isoform PLC- β 4 is insensitive to activation by the $\beta\gamma$ subunits (Lee *et al.*, 1994).

From Figure 7 it can be seen that PLC- β isozymes have an extended C-terminal domain. This is thought to be critical for G α activation. Expression of PLC- β 1 lacking amino acids 903 to 1142 (Wu *et al.*, 1993), or PLC- β 2 lacking the C-terminal 350 amino acids (Lee, S.B. *et al.*, 1993), resulted in loss of activation by G α . Mutations of basic amino acid residues in the C-terminus displayed reduced responses to G α (Kim *et al.*, 1996). Expression of PLC- β 2 and PLC- β 3 missing the C-terminus retained full stimulation by $\beta\gamma$ subunits (Lee, S.B. *et al.*, 1993; Blank *et al.*, 1993).

The $\beta\gamma$ subunit may interact with a site on the first half of the Y catalytic domain. Expression of peptides containing the X/Y connector region and first half of the Y catalytic domain could block stimulation of PLC- β 2 by the $\beta\gamma$ subunits (Kuang *et al.*, 1996). A fusion protein containing the first half of the Y catalytic domain was also shown to bind the $\beta\gamma$ subunits (Kuang *et al.*, 1996).

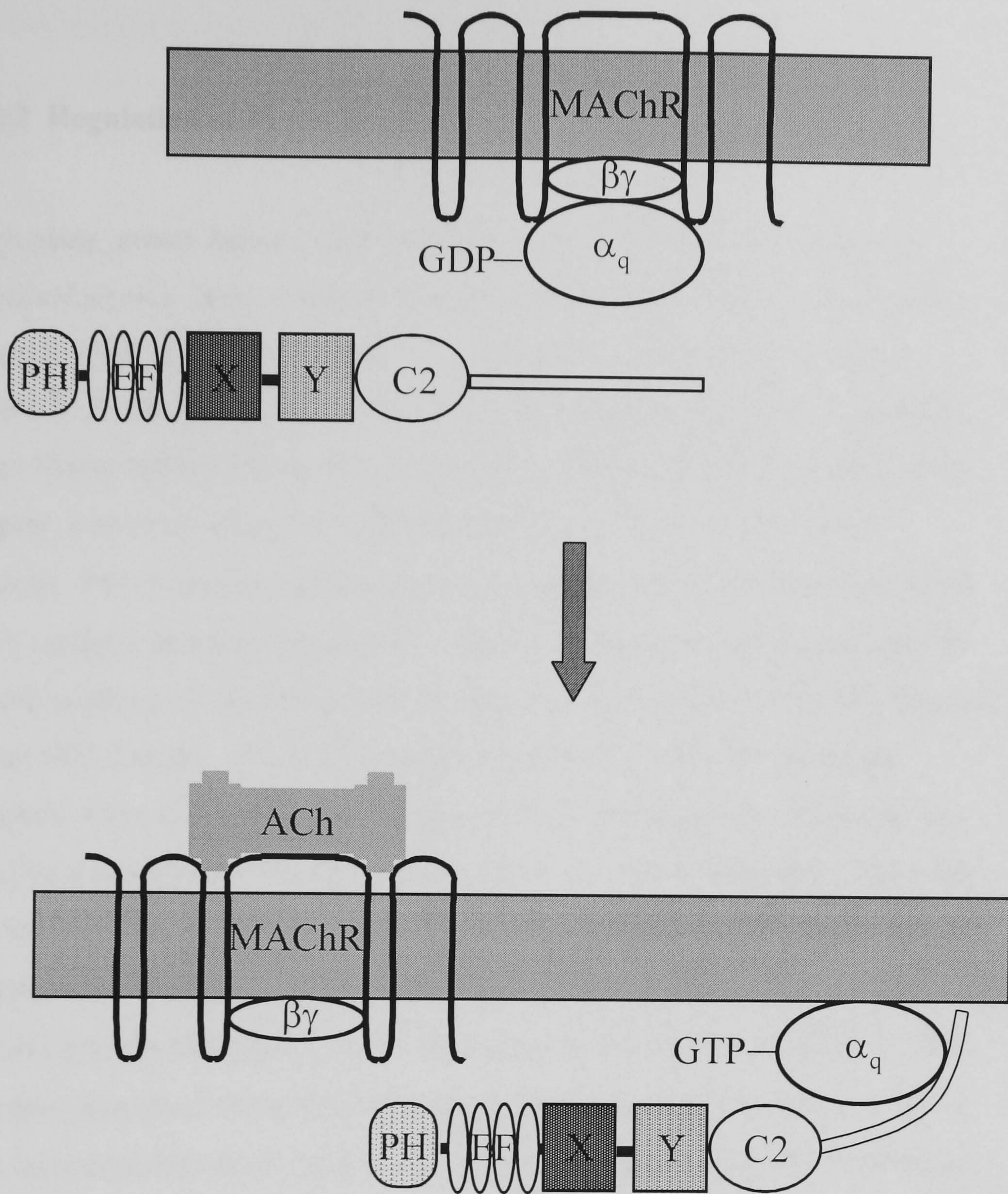


Figure 7 Activation of PLC-β

Activation of PLC-β is illustrated by the M1 muscarinic acetylcholine receptor (MACHR)-dependent activation through the heterotrimeric G-protein, G_q. The ligand acetylcholine (ACh) binds to the MACHR which results in a conformational change in the receptor causing dissociation of the heterotrimeric GDP-bound G_q to yield GTP-bound Gα_q. PLC-β then binds the GTP-bound Gα_q via the carboxyl-terminal region, activating PLC hydrolysis of PtdIns 4,5-P₂.

1.3.2.2 Regulation of PLC- γ isozymes

Polypeptide growth factors, such as platelet derived growth factor (PDGF), epidermal growth factor (EGF) and nerve growth factor (NGF), mediate their responses by binding to their respective receptors on the surface of the cell (Figure 8A). Each of these growth factor receptors have a similar cytoplasmic region that contains a tyrosine kinase domain. The growth factors bind to their receptor, which facilitates the rapid translocation of PLC- γ to particulate fractions. PLC- γ has four additional domains that are positioned between the X and Y catalytic domains (Figure 8A). These four domains comprise of one PH domain made up of two halves (see section 1.3.1.2 for review), two SH2 domains and an SH3 domain. The SH2 domains target PLC- γ to the growth factor receptors whereas targeting to other proteins containing proline rich sequences could be modulated via the SH3 domain (Pawson, 1995). It has been suggested that the SH3 domain might target PLC- γ to the cytoskeleton, and the binding of dynamin (Bar-Sagi *et al.*, 1993; Scaife *et al.*, 1994). Binding to the receptors via the SH2 domain may have a role in regulating the basal activity of PLC- γ . For example, disruption of the SH2/SH3 domain by proteolysis resulted in a PLC- γ with increased activity (Fernald *et al.*, 1994). Co-expression of the N-terminus, incorporating the X catalytic domain of PLC- γ , with the C-terminus including the Y domain, yielded an enzyme with higher activity than the complete enzyme (Hortsman *et al.*, 1996). Finally, when the SH2/SH3 domains were expressed and added to PLC- γ *in vitro*, the activity of PLC- γ was inhibited (Homma and Takenawa, 1992).

The sites where PLC- γ are phosphorylated by the growth factor receptors include Tyr 771, Tyr 783 and Tyr 1254 (Kim *et al.*, 1990; Wahl *et al.*, 1990; Kim *et al.*, 1991). PLC- γ substituted with Phe at Tyr 783 still associated with the PDGF-receptor and was phosphorylated at a serine residue in response to PDGF, similar to wild-type enzyme, but there was no IP₃ produced (Kim *et al.*, 1991). This

suggests that phosphorylation at Tyr 783 is essential for PLC- γ translocation or binding to the receptor but not activation. Unphosphorylated PLC- γ was selectively inhibited in the presence of the actin-binding protein profilin which, like PLC- γ , has a high affinity for PtdIns 4,5-P₂. Only the phosphorylated enzyme catalyses the hydrolysis of profilin-bound PtdIns 4,5-P₂ releasing profilin and affecting actin polymerisation (Goldschmidt-Clermont *et al.*, 1991). This was suggested to be the link between transmembrane signalling and cellular responses such as, cell shape and motility (Goldschmidt-Clermont *et al.*, 1991). It also led to speculation that phosphorylation prevents the interaction of PLC- γ with inhibitory proteins (Noh *et al.*, 1995).

Activation of PLC- γ can also be achieved through the action of non-receptor protein tyrosine kinases (NR-PTK) (Figure 8B), in response to the binding of ligands to cell surface receptors in leukocytes (Todderud *et al.*, 1990). The T-cell antigen receptor complex (TCR) transduces many signals across the plasma membrane, but none of the proteins in the complex are protein kinases. TCR stimulation was shown to result in rapid phosphorylation of PLC- γ on both serine and tyrosine residues (Park *et al.*, 1991; Liao *et al.*, 1992). It is thought that TCR stimulation results in the translocation of a cytosolic kinase, which interacts with the SH2 domains of PLC- γ (Rhee and Choi, 1992)

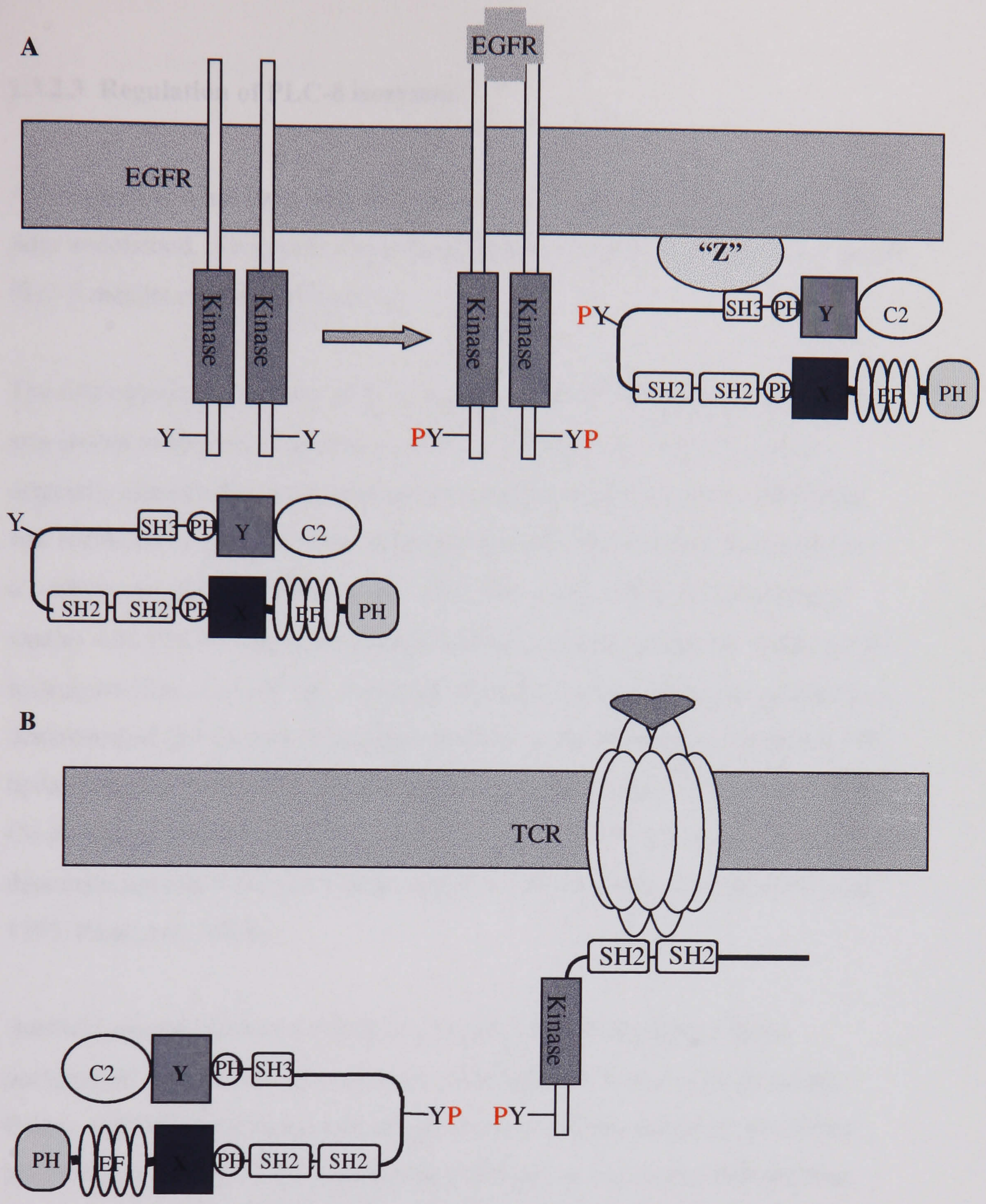


Figure 8 Regulation of PLC- γ

Activation of PLC- γ is illustrated by the epidermal growth factor receptor (EGFR) (A), and the T-cell antigen receptor (TCR) dependent tyrosine phosphorylation of PLC- γ , (B). A demonstrates that EGF binds to its receptor which results in auto-phosphorylation of the receptor and then tyrosine phosphorylation of PLC- γ . This then allows the SH3 domain of PLC- γ to interact with a cytoskeletal protein ("Z"). B demonstrates non-receptor tyrosine kinase activation of PLC- γ . A ligand binds to the TCR activating a cytosolic tyrosine kinase which phosphorylates PLC- γ . Once PLC- γ in both cases has been phosphorylated it binds to cytoskeletal proteins and hydrolyses PtdIns 4,5- P_2 .

1.3.2.3 Regulation of PLC- δ isozymes

Although PLC- δ has been widely characterised, the regulation of PLC- δ is not fully understood. This section will describe three possible mechanisms by which PLC- δ may be regulated (Figure 9).

The first regulatory pathway may be through the GTP-binding protein G_h which was shown to stimulate the activity of PLC- δ (Feng *et al.*, 1996). G_h was originally identified as a transglutaminase enzyme (Nakaoka *et al.*, 1994) that was regulated by guanine nucleotides and was purified in association with the α 1-adrenergic receptor (Baek *et al.*, 1993; Das *et al.*, 1993). Reconstitution studies with PLC- δ , the α 1-adrenergic receptor, and G_h resulted in PtdIns 4,5-P₂ hydrolysis (Das *et al.*, 1993). Research carried out on the oxytocin receptor has demonstrated that G_h may be a signal mediator in this pathway, as PtdIns 4,5-P₂ hydrolysis was increased by oxytocin binding to the receptor (Park *et al.*, 1998). Co-immunoprecipitation of PLC- δ in the oxytocin and α 1-adrenergic systems demonstrated that it only associates with the GTP-bound form of G_h (Das *et al.*, 1993; Park *et al.*, 1998).

Another potential pathway which may regulate PLC- δ involves a novel polypeptide with homology to proteins with RhoGAP activity (Homma and Emori, 1995). The deduced amino acid sequence shows similarity to GTPase activating protein (GAP) and possesses GAP activity for RhoA. Rho proteins have been shown to regulate various cell functions such as morphology, motility and cytokinesis. This novel protein, called RhoGAP (p122), was shown to bind and regulate the activity of PLC- δ but not PLC- β or PLC- γ (Homma and Emori, 1995). This RhoGAP protein may provide a link between actin related cytoskeletal changes and the hydrolysis of PtdIns 4,5-P₂ which binds actin binding proteins including gelsolin and profilin (see section 1.2.2.4 for more information).

Finally, PLC- δ could simply be regulated by Ca^{2+} concentration within the cell. It has been demonstrated that an increase in Ca^{2+} concentration within a physiological range of 0.1-10.0 μM is sufficient to stimulate PLC- δ activity (Allen *et al.*, 1997). An increase in Ca^{2+} concentration on its own was insufficient to stimulate PLC- γ and PLC- β activity (Allen *et al.*, 1997). The activity of PLC- δ can be further enhanced by the presence of phosphatidylinositol transfer protein (PITP) (see section 1.2.1.8 for more information on PITP). It has been suggested that the function of PITP is to supply and present the substrate (PtdIns 4,5- P_2) that would otherwise become limiting (Allen *et al.*, 1997).

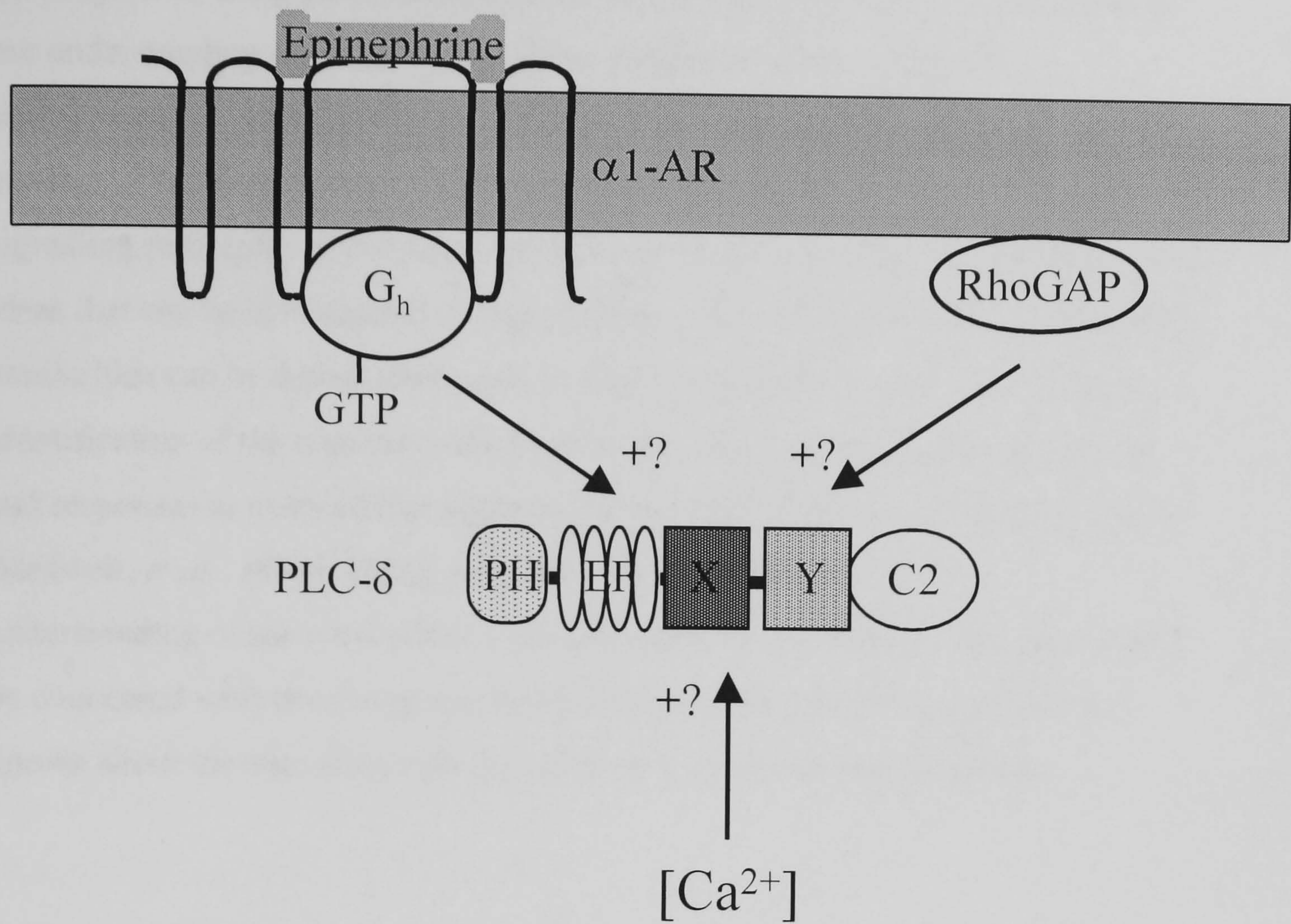


Figure 9 Regulation of PLC- δ

The activation of PLC- δ is not fully understood but several possible regulators of its activity have been identified. PLC- δ could be activated through the G-protein G_h . Epinephrine binds to the 7TM receptor and GTP is substituted for GDP on G_h which is then able to bind PLC- δ , resulting in its activation. PLC- δ may be regulated through a novel protein described as RhoGAP. The binding of RhoGAP to PLC- δ may be sufficient for its activation. Finally, fluctuations in Ca^{2+} concentration within cellular compartments may be sufficient to stimulate PLC- δ .

1.4 Phospholipase C in yeast

Investigations using genetical and biochemical techniques have helped increase our understanding of complex signalling pathways within the yeasts *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Schizosaccharomyces pombe* (*Sz. pombe*). The evolutionary conservation of protein sequences and preservation of signalling principles allows us to use these systems as models, helping to develop ideas that can be investigated in higher eukaryotes. In signalling pathways where similarities can be drawn from yeast to higher eukaryotes, yeast have aided the identification of the regulators involved in the cell cycle progression, secretion and responses to extracellular agonists (Reed, 1992; Bennet and Scheller, 1993; Bardwell, *et al.*, 1994). Using yeast may therefore help increase our understanding of the intricacies of phospholipase C signalling. This section will be concerned with reviewing the identification of the yeast PLCs and what is known about the signalling role they play in *S. cerevisiae* and *Sz. pombe*.

1.4.1 The identification of PLC1 in *S. cerevisiae*

Three groups have independently cloned an *S. cerevisiae* gene encoding a phospholipase C, termed *PLC1*, with the predicted protein referred to as PLC1 (Flick and Thorner, 1993; Payne and Fitzgerald-Hayes, 1993; Yoko-o *et al.*, 1993). Flick and Thorner (1993) and Yoko-o *et al.* (1993) used a similar PCR approach that utilised the high percentage of sequence similarity found within the X catalytic domain of PLCs. In contrast, Payne and Fitzgerald-Hayes (1993) isolated *PLC1* from a genetic screen designed to identify mutants that exhibit defects in chromosomal segregation. Molecular analysis by all three groups demonstrated that *PLC1* encoded a protein of 101 kDa with approximately 50 and 26% identity to the highly conserved X and Y domains of PLC- δ 1 (Flick and Thorner, 1993; Payne and Fitzgerald-Hayes, 1993). The overall amino acid identity between PLC1 and bovine PLC- δ 2 is 25.1% which is the highest identity

between PLC1 and other PLC isozymes (Yoko-o *et al.*, 1993). PLC1 does not contain the extended C-terminal domain present in the PLC- β family and it does not contain either of the SH2 or SH3 domains, both of which are present within the PLC- γ family. Partially purified PLC1 using nickel chelate affinity chromatography has shown that PLC1 hydrolyses phosphoinositides in a manner that is similar to higher eukaryotic cells (Flick and Thorner, 1993). On the basis of structural criteria, Yoko-o *et al.*, (1993) and Payne and Fitzgerald-Hayes, (1993) suggested that PLC1 be classified as a member of the PLC- δ family of enzymes.

Disruption of the *PLC1* gene ($\Delta PLC1$) in *S. cerevisiae* is not lethal, demonstrating that PLC1 activity is not required for essential cell functions under optimal growth conditions (Flick and Thorner, 1993; Payne and Fitzgerald-Hayes, 1993; Yoko-o *et al.*, 1993). Instead, $\Delta PLC1$ cells are temperature sensitive for growth. They grow slower than wild-type cells at 23°C and this growth defect was intensified at increasing temperatures until a complete termination of growth at 37°C (Flick and Thorner, 1993). The $\Delta PLC1$ mutants are not just sensitive to temperature but are also sensitive to osmotic stress, these cells do not grow on non-fermentable carbon sources and grow very badly on fermentable carbon sources other than glucose (Flick and Thorner, 1993; Payne and Fitzgerald-Hayes, 1993). It has been known for some time that the growth medium, in particular nitrogen concentration, stimulates signalling pathways within *S. cerevisiae* cells (Schomerus and Kuntzel, 1992). Nitrogen concentrations are known to regulate processes such as meiotic division and consequently sporulation (Flick and Thorner, 1993). The addition of nitrogen, but not glucose, to nutritionally-depleted wild-type cells has been shown to stimulate PIP₂ hydrolysis (Hawkins *et al.*, 1993; Schomerus and Kuntzel, 1992). This suggests that there may be a pathway in *S. cerevisiae* that requires the activation of PLC1 in response to external changes in nitrogen levels.

It has been reported that $\Delta PLC1$ cells at restrictive temperatures (37°C) are unable to perform cytokinesis, resulting in large multi-budded cells with each

bud possessing its own inherited DNA (Flick and Thorner, 1993). Payne and Fitzgerald-Hayes, (1993) reported that a mutant strain defective in PLC1 activity had an increased rate of aberrant chromosomal segregation. It has been argued, however, that these are secondary effects brought about by alterations in PLC1 activity and that the primary role of PLC1 is not fully understood.

Although the $\Delta PLC1$ cells display a temperature sensitive phenotype, they show aberrant morphology and grow slowly on rich medium at 23°C but not at all at temperatures which exceed 30°C. Mutant isoforms of PLC1 were therefore isolated which contained amino acid substitutions in the conserved X and Y catalytic domains. These mutations were found to be temperature sensitive (Yoko-o *et al.*, 1995). These mutants had a functional PLC1 at temperatures below 29°C but PLC1 activity ceased at 37°C. These temperature sensitive mutants may be useful when trying to identify other components in the *S. cerevisiae* PLC1 signalling pathway.

Flick and Thorner (1998) identified two genes *SPL1* (*PHO81*) and *SPL2*, that, when overexpressed, bypassed the temperature sensitive growth defect of $\Delta PLC1$ cells. The *PHO81* gene encodes a protein which inhibits the PHO80 (cyclin) /PHO85 (protein kinase) Cdk complex. The second gene identified, *SPL2*, encodes a 17 kDa protein and displays some similarity to the PHO81 and other Cdk inhibitors. In addition to overproduction of PHO81, two other conditions inactivate the PHO80/PHO85 complex, a $\Delta PHO80$ mutation and growth on low phosphate medium (Flick and Thorner, 1998). The substrate of the PHO80/PHO85 (Cdk complex) is PHO4 a transcriptional regulator. When PHO4 is phosphorylated by the PHO80/PHO85 complex it is excluded from the nucleus, but phosphate starvation stimulates PHO81 to inhibit PHO80/PHO85 Cdk activity (O'Neil *et al.*, 1996). This allows unphosphorylated PHO4 to accumulate in the nucleus and activate transcription of genes (Schneider *et al.*, 1994).

Flick and Thorner (1998) proposed a model to explain the interactions between PLC1, SPL2 and the regulators of the PHO80/PHO85 (Figure 10). In the absence of *PLC1* a pathway that controls growth at elevated temperatures or controls nutrient uptake or utilisation can not function because activity of the PHO80/PHO85 is too high. The activity of PLC1 can either stimulate the function of a downstream target “X” via an alternative pathway or stop the inhibitory action of the PHO80/PHO85 on “X”. Reduction of the PHO80/PHO85 activity by overproduction of PHO81 or SPL2, growth on low phosphate medium, or a Δ *PHO81* mutant, will allow Δ *PLC1* cells to grow at the non-permissive temperature. This study suggests that the gene products of *PLC1*, *PHO81*, *SPL2* and *PHO80/PHO85* are involved in overlapping regulatory pathways necessary for adaptation to changing nutrients and temperatures.

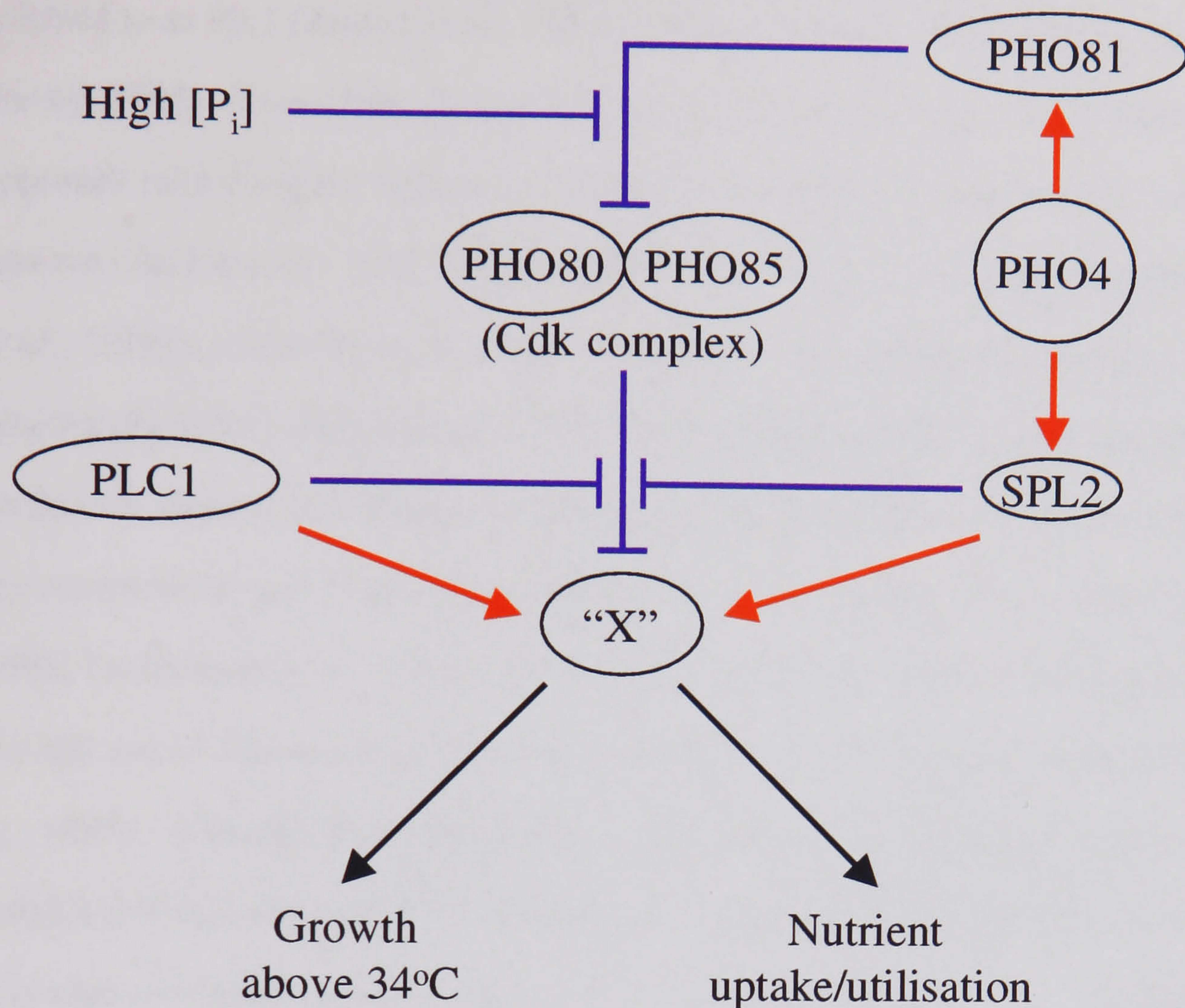


Figure 10 Model suggesting the possible regulatory function of PLC1

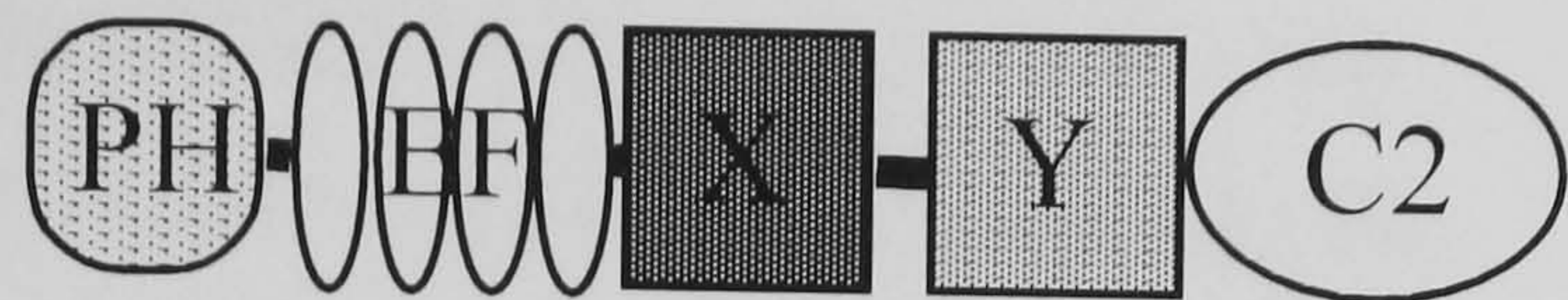
Diagram based on the model suggested by Flick and Thorner, (1998). It demonstrates the regulatory interactions between PLC1, SPL2, PHO81 and the PHO80/PHO85 complex. Inhibitory interactions are indicated by a blue bar and stimulatory interactions are indicated by a red arrow. Black arrows represent the outcome if "X" is stimulated. See text for further explanation.

1.4.2 The identification of Plc1 in *Sz. pombe*

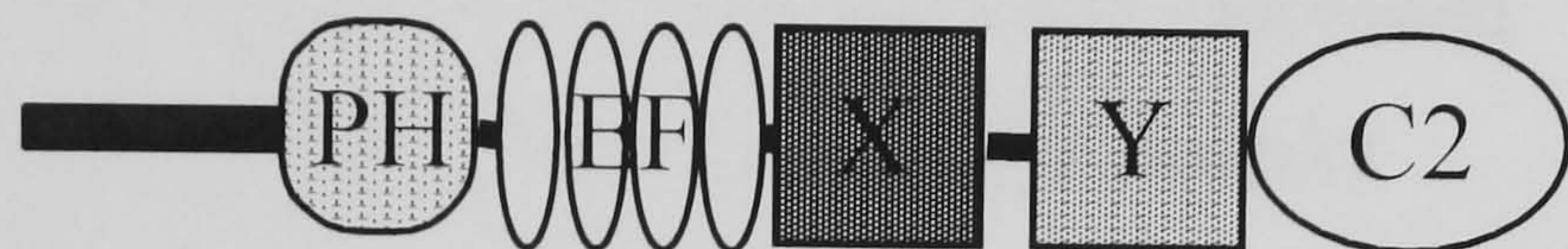
Three groups simultaneously cloned an *Sz. pombe* gene encoding a phospholipase C. This was designated *plc1* with the predicted protein being referred to as Plc1 (Andoh *et al.*, 1995; Fankhauser *et al.*, 1995; Slaaby and Davey, 1995). Two of the groups isolated the *plc1* gene using a PCR-based approach exploiting the high level of sequence similarity found in the X catalytic domain (Andoh *et al.*, 1995; Slaaby and Davey, 1995). Conversely, Frankhauser *et al.*, (1995), isolated the *plc1* gene when screening for mutants defective in sensing phosphate concentrations. The predicted product Plc1 has a calculated molecular mass of 102 kDa and exhibits approximately 55 and 29% identity to the conserved X and Y catalytic domains of the PLC- δ isoform (Andoh *et al.*, 1995; Fankhauser *et al.*, 1995). The *plc1* gene has been physically mapped on the left arm of chromosome II between the genes *rad11* and *mei3* (Fankhauser *et al.*, 1995). Although Plc1 shows closest similarity to PLC- δ , it exhibits an extended N-terminus which is not seen in the mammalian isoform of PLC- δ but it is seen to a lesser extent in the yeast *S. cerevisiae* (see Figure 11). Using HPLC analysis it has been demonstrated that *Sz. pombe* Plc1 hydrolyses PIP₂ into Ins1,4,5-P₃ confirming that *plc1* encodes a phospholipase C enzyme (Slaaby, 1996).

To investigate the role of this *plc1* gene a disruption was carried out resulting in a $\Delta plc1$ cell. Some aspects of the phenotype of the *plc1* disruptants of *Sz. pombe* resemble those of the *PLC1* disruptants of *S. cerevisiae*. Growth of cells was temperature sensitive in rich medium such as yeast extract and cells were unable to grow in minimal medium (Andoh *et al.*, 1995; Fankhauser *et al.*, 1995; Slaaby and Davey, 1995). The $\Delta plc1$ cells of *Sz. pombe* are swollen and are unable to perform cytokinesis (Andoh *et al.*, 1995; Slaaby, 1996). Slaaby (1996) also reported that the $\Delta plc1$ mutant in *Sz. pombe* could not always perform chromosomal segregation similar to the $\Delta PLC1$ mutant identified by Payne and

PLC- δ



S. cerevisiae PLC1



Sz. pombe Plc1

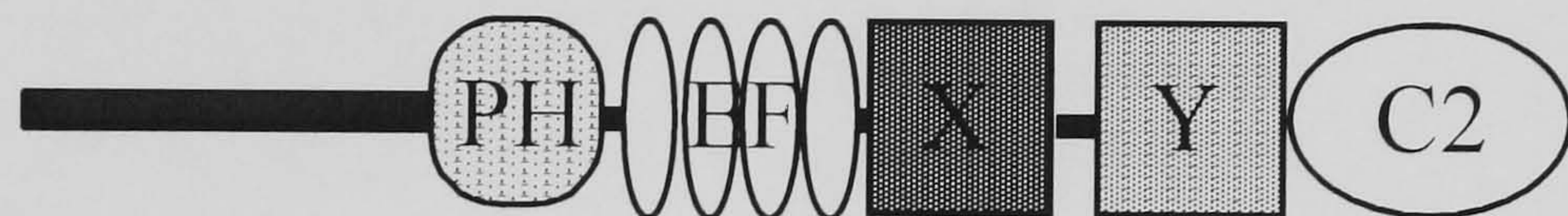


Figure 11 Comparing the structure of PLC- δ to PLC1 and Plc1

PLC- δ contains four conserved domains. These include the Pleckstrin Homology domain (PH), the EF hand domain, the X and Y catalytic domain and the C2 domain. PLC1 from *S. cerevisiae* and Plc1 from *Sz. pombe* contain an extended N-terminus. The function of this N-terminus is not known and there appears to be no sequence similarity between the two yeast PLC N-terminal extensions.

Fitzgerald-Hayes, (1993) in *S. cerevisiae*. The growth defect at 37°C of the $\Delta plc1$ cells are weakly suppressed by a number of supplements which could be added to, or reduced when preparing the growth medium. These include the addition of 0.5M-KCl or 1.2M-sorbitol (Andoh *et al.*, 1995), alternatively the phosphate concentration in the medium could be lowered (Fankhauser *et al.*, 1995). In comparison, a similar level of suppression was seen by an increase in phosphate concentration in *S. cerevisiae* $\Delta PLC1$ cells cultured at 37°C (Flick and Thorner, 1998) but the addition of KCL or sorbitol had no suppressive effect (Andoh *et al.*, 1995). Expression of the *PLC1* gene of *S. cerevisiae* suppressed the growth defect of $\Delta plc1$ cells growing at 37°C further evidence that *plc1* encodes a phospholipase C enzyme (Andoh *et al.*, 1995).

It is clear from these studies that the precise role of Plc1 in *Sz. pombe* is still not fully understood. More research is required to identify the regulator(s) of Plc1 and perhaps the signal transduction pathways that it interacts with.

1.5 Aims of the thesis

The aims of this project were to characterise Plc1 a phospholipase C enzyme identified in the fission yeast *Shizosaccharomyces pombe*.

Chapter 2
Materials and Methods

2.1 Materials

2.1.1 General laboratory reagents

General laboratory reagents were supplied by either BDH Laboratory supplies (Poole, Dorset) or the Sigma Chemical Co. (Poole, Dorset), unless otherwise stated, and were of analytical grade.

2.1.2 Molecular biology reagents

Pharmacia Biotech (St. Albans, Herts.) supplied T7 DNA polymerase, SP6 RNA polymerase II, and all nucleotides. T4 DNA ligase and bacterial alkaline phosphatase were supplied by Gibco BRL (Paisley, Scotland). Boehringer Mannheim (Lewes, East Sussex) supplied PWO polymerase (*Pyrococcus woesei*). Alta Bioscience (University of Birmingham) made all oligonucleotides unless otherwise stated. Sigma Chemical Co. supplied all other molecular biology reagents unless stated otherwise.

2.1.3 Electrophoresis reagents

Agarose (low melting point and Ultrapure type-1) supplied from Gibco BRL. Acrylamide was supplied from Northumbria Biologicals Limited (Cramlington, Northumbria) as a 30% (w/v) solution of 37:5:1 ratio acrylamide: bisacrylamide for SDS PAGE. For DNA sequencing gels, a 40% (w/v) solution 19:1 ratio acrylamide: bisacrylamide solution was used.

2.1.4 Determining Protein concentrations

Protein concentrations were determined colorimetrically using a protein-assay reagent supplied by Bio-Rad (Hemel Hempstead, Herts.) using bovine serum albumin, (BSA) (Sigma A2153) as a standard.

2.1.5 Photographic Supplies

Polaroid 667 land film was obtained from Polaroid UK Ltd. (St. Albans, Herts.). Kodak Biomax MR autoradiographic film was obtained from Sigma Chemical Co. Fuji RX autoradiographic film was obtained from Fuji Photo Film Co., Ltd., Tokyo. Pictures of plates were taken on a UVP GDS 8000 gel documentation system using Grab-it software.

2.1.6 Radioisotopes

[dATP γ ³⁵S (1000 Ci/mmol)], [¹⁴C]-labelled high protein molecular weight markers (Rainbow markers M765), and [dCTP γ ³⁵S (6000 Ci/mmol) were supplied from Amersham Life Sciences (Little Chalfont, Bucks.).

Phosphatidylinositol 4,5 bisphosphate [inositol-2-³H (N)] and phosphatidylinositol [inositol-2-³H (N)] were purchased from DuPont NEN (Boston, MA., USA).

2.1.7 Determining cell volumes

Cell volumes were determined as described in Davey 1991, on non-fixed cells using a C256 Coulter Channelyser supplied by Coulter electronics Ltd. (Luton, Beds.). Isoton II azide-free electrolyte was also supplied by Coulter electronics Ltd.

2.1.8 Scintillation counting

All scintillation counting work was carried out using a Wallac 1450 Microbeta Trilux, Liquid Scintillation and luminescence counter. The effective ³H count for this machine is 50% as informed by the manufacturers instructions.

2.1.9 Growth media

Luria broth, yeast extract and select agar were obtained from Gibco BRL. All components of selective media were obtained from Sigma Chemical Co. Media and plates used for the selective growth of yeast were made and used as described by Moreno *et al.* (1991). Rich medium (YE) is yeast extract, 2% glucose and added amino acids as required (250µg/ml). Plates for the selective growth of yeast were made using DMM or amino acid (a.a.) selective medium plus 1.5% agar. The composition of DMM is described within Davey *et al.* (1995).

2.1.9.1 Selective Medium (a.a.)

Per litre:

Yeast nitrogen base w/o a.a.'s	6.7g
Glucose	20g
Amino acid mix	1.5g
Select aa mix	0.5g

Amino acid mix:

L-alanine	2g
L-asparagine	2g
L-cysteine	2g
L-glutamine	2g
L-glutamic acid	2g
L-glycine	2g
L-isoleucine	2g
L-lysine	2g
L-phenylalanine	2g
L-proline	2g
L-serine	2g
L-threonine	2g
L-tryptophan	2g

L-tyrosine	2g
L-valine	2g
myo-inositol	2g
para-amino benzoic acid	0.4g

Select aa mix (four of the following are selected)

L-histidine	2g
L-methionine	2g
adenine	2g
uracil	2g
L-leucine	4g

2.1.9.2 DMM

Per litre:

NH ₄ Cl	5g
Na ₂ PO ₄	2.2g
Pthalic acid	3g
Glucose	20g
Salts (50x stock)	10ml
Vitamins (1000x stock)	1ml
Minerals (10000x stock)	100µl

Stock solution of salts (50x)

Per litre:

MgCl ₂ .6H ₂ O	52.5g
CaCl ₂ .H ₂ O	735mg
KCl	50g
Na ₂ SO ₄	2g

Stock solution of vitamins (1000x)

Per 100ml:

Nicotinic acid	1g
Inositol	1g

Pantothenic acid	100mg
Biotin	1mg

Stock solution of minerals (10000x)

Per 100ml:

Citric acid	1g
Boric acid	500mg
MnSO ₄ .H ₂ O	500mg
ZnSO ₄ .7H ₂ O	400mg
Molybdic acid	305mg
FeCl ₃ .6H ₂ O	200mg
KI	100mg
CuSO ₄ .5H ₂ O	40mg

2.1.10 Bacterial strains

Escherichia coli (*E. coli*) strain used for general molecular biology:

Strain	Genotype
DH5α	<i>supE44 hsdR17 endA96 thi-1 relA1</i>

2.1.11 Yeast strains

Strain	Phenotype	Genotype
JY291	M	<i>mat1-M Δmat2,3::LEU2</i>
JY292	P	<i>mat1-P Δmat2,3::LEU2</i>
JY330	P leu ⁻	<i>mat1-P Δmat2,3::LEU2⁻, leu1-32⁻</i>
JY383	P leu ⁻ , ura ⁻	<i>mat1-P Δmat2,3::LEU2⁻, ura4-D18</i>
JY402	M leu ⁻ , ura ⁻	<i>mat1-M Δmat2,3::LEU2⁻, ura4-D18</i>
JY681	P leu ⁻ , Δ <i>plc1</i>	<i>mat1-P Δmat2,3::LEU2⁻, Δplc1</i>

JY 291 is a mating type stable *M*-cell, conversely JY 292 is a mating type stable *P*-cell.

JY 330 is a mating type stable *P*-cell but contains the *leu1-32* mutation preventing the cell from producing leucine. This strain as transformed with the expression pREP vectors as these contain the *LEU2* gene as a selectable marker (Maundrell, 1990).

JY 383 is a mating type stable *P*-cell and JY 402 is a mating type stable *M*-cell. These cells also contain the *leu1-32* mutation but they also contain the *ura4-D18* deletion preventing the cell from producing uracil. This will allow genetic disruption to be performed in these strains by re-introducing the *ura4* gene a selectable marker allowing these cells to grow on uracil minus media

JY 681 is a mating type stable *P*-cell, a JY 383 strain which has had the *plc1* gene replaced by *ura4*.

2.2 Methods

2.2.1 General molecular biology techniques

2.2.1.1 Cloning techniques

Standard recombinant DNA technology was achieved as described in Sambrook *et al* (1989), unless otherwise specified. Restriction endonuclease digestions were completed in accordance with the manufacturer's instructions. The digestion products were separated on a low melting point agarose gels. DNA was recovered from the agarose gels using the Gene Clean II Kit supplied by (Anachem, Luton, Beds.) in compliance with the manufacturers instructions. After digestion and purification of the vector DNA, it was treated with bacterial alkaline phosphatase (Amersham Life Science). Before ligation, the vector was subjected to phenol/chloroform extraction.

2.2.1.2 Transformation of *Escherichia coli*

Competent *E. coli* (DH5) were prepared in compliance with Sambrook *et al.* (1989). The transformation of competent *E. coli* was carried out as described by Hanahan (1985).

2.2.1.3 Polymerase Chain Reaction

a) Purified DNA

PCR on purified DNA used a reaction volume of 100 μ l. To make up this volume primers added to a final amount of 1 μ g and approximately 1ng of DNA was added. Generally for products of 1000bp or less, a 1min extension at 72 $^{\circ}$ C and a 30 second 'melt' at 94 $^{\circ}$ C was used. Increased extension times were used on products that were longer; usually 1 min per 1000bp was used. Generally the annealing temperature for primers was 55 $^{\circ}$ C for 1 min, but this could vary

depending on the template. 35 cycles were generally used for each reaction. The reaction concluded with incubation at 72°C for 7 min to allow full extension of the product.

b) Screening plasmid DNA from bacterial cells

The PCR screening of bacterial cells was performed on cells taken from a selective LB plate and placed in 100µl of 5xTE (50mM Tris pH7.5, 5mM EDTA, Sambrook *et al.*, 1989). 1µl of this solution was used in a 100µl PCR reaction. Tween20 was added to the PCR mixture to a final concentration of 0.05% before the PCR reaction was carried out.

c) Yeast Cells

PCR on yeast DNA was performed on cell lysates produced by vortexing the cells with acid-washed glass beads (425-600 micron, Sigma Chemical Co.). After vortexing for 1 min, a 50µl volume of 5xTE was added and the homogenate and glass beads were transferred to a 500µl microfuge tube. The 500µl microfuge tube was pierced and placed in a 1.5ml microfuge tube and centrifuged at 13,000g for 10 s, allowing the homogenate to pass through the pierced holes but not the glass beads. 1µl of this homogenate was used in a PCR reaction.

2.2.1.4 Transformation of Yeast cells

All yeast transformations were carried out using the lithium acetate method (Okazaki *et al.*, 1990). A 50ml culture of yeast was cultured in YE to 1×10^7 cells/ml. These cells were harvested at 2000g for 5 min in a centrifuge prewarmed to 29°C. The cells were then washed in Solution I before resuspension in solution I at 1×10^9 cells/ml. This cell suspension was incubated at 29°C for 60-120 min. A 10µl volume containing 100ng of DNA was added to 290µl of solution II and was prewarmed to 29°C. 100µl of cells were mixed with the DNA and incubated at 29°C for a further 50 min. These cells were heat shocked

at 42°C for 15 min. The cells were collected by centrifugation (1000g for 3 min), resuspended in water and spread onto plates of selective medium.

Solution I: 0.1M lithium acetate, pH 4.9 (with acetic acid)

Solution II: 50% w/v PEG 4000 in solution I

2.2.1.5 Double-stranded DNA sequencing

18µl of DNA (1-2µg of 5kb DNA template) was mixed with 2µl of 10M NaOH and incubated at room temperature for 5 min. 8µl of 5M ammonium acetate was added to the mixture succeeded by 100µl of 100% ethanol, the DNA was subsequently precipitated at -70°C for 20 min. DNA was collected by centrifugation at 14,000g for 10 min at 4°C, this was followed by washing the DNA in 80% ethanol. The DNA was resuspended in 7µl of water. To the DNA 1µl of primer (concentration 6.6ng/µl) and 2µl of sequencing buffer was added. This mixture was incubated at 37°C for 20 min. 5.5µl of labelling mix was added to the mixture and incubated at room temperature for 5 min. Subsequently, 3µl of the labelled mixture was added to each of the four tubes containing 2µl of either ddA, ddC, ddG and ddT termination mixtures and the samples were incubated at 37°C for 5 min. The reactions were stopped by the addition of stop solution. Samples were heated to 80°C for 2 min before loading onto a sequencing gel. The full nucleotide and amino acid sequence of Plc1 can be found in the appendix.

Sequencing buffer: 200mM Tris pH 7.5, 100mM MgCl₂, 250mM NaCl

Labelling mix: 18mM DTT, 0.55µM dCTP, 0.55µM dGTP and 0.55µM dTTP, 0.5U T7 polymerase/µl, 1µCi[dATPγ³⁵SdATP(1000 Ci mmol)]/µl.

Termination mixes: dATP, dGTP, dTTP, and dCTP each at 80µM, 50mM NaCl, 8µM of respective dideoxy nucleotide.

Stop solution: 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 20mM EDTA.

2.2.1.6 Preparation of genomic DNA

Yeast genomic DNA was prepared as described in Guthrie and Fink, (1991).

2.2.1.7 Southern Blot analysis

Southern blot analysis was performed as described in Sambrook *et al.*, 1989. Radioactively labelled probes were created using a Random Primed DNA Labelling Kit supplied from Boehringer Manheim (Lewes, East Sussex) and was used in accordance with the manufacturers instructions.

2.2.2 Assaying Plc1 activity *in vitro*

2.2.2.1 Production of crude yeast extract used for Plc1 assay

400ml of cells were cultured in DMM to 6.5×10^6 cells ml⁻¹ and transferred to 50ml screw-cap tube. The 50ml screw-cap tube were centrifuged at 4000g for 5 min at 4°C; all of the medium was then removed. Each of the yeast cell pellets were washed in 2ml of Solution I and transferred to a 20ml universal. The cells were again centrifuged at 4000g for 5 min at 4°C subsequently the solution I was removed and 5ml of acid washed glass beads (425-600µm. Sigma Chemical Co.) were added, the screw-cap tube were then placed at 4°C. The cells and glass beads were vortexed for 1min, then placed at 4°C for 1 min, this was repeated five times. The 20ml universal bottom was pierced and was placed in a 50ml universal. The beads and homogenate were then washed with 1ml of solution I and centrifuged at 2000g for 1 min. The homogenate passes into the 50ml universal while the glass beads remain in the 20ml universal. The protein concentration was determined using Bio-Rad 'Protein Concentration Kit' (Hemel Hempstead, Herts.) using BSA as a standard. The protein concentration was approximately 10mg/ml. The extracts were stored in 20% glycerol at -20°C.

Solution I: 50mM Tris/HCl pH 7.5
1mM EGTA
20µl/ml Yeast protease inhibitor cocktail (Sigma Chemical Co. P8215)

2.2.2.2 PLC assay

The substrate was prepared in a siliconised glass vial. To the glass vial 2µl of 1.3mg/ml of PIP₂, 2µl of ³H-PIP₂ (0.02µCi) (DuPont NEN, PIP₂ [inositol-2-³H(N)]) and 200µl of chloroform /methanol (2:1). This substrate mixture was dried under a stream of nitrogen. The mixture of radioactively labelled and non-radioactively labelled PIP₂ was resuspended in 25µl of resuspension solution per reaction. This substrate mixture was then sonicated for 30 seconds at setting 5 using the Soniprep 150 (Sanyo); this was repeated three times. For assays where PI hydrolysis was being measured the ³H-PIP₂ was replaced by ³H-PI (0.02µCi) (DuPont NEN, PI [inositol-2-³H(N)]) but all other parameters were kept the same.

Resuspension solution

Per 25µl reaction:

16µl 10% w/v sodium cholate (Sigma C6445)

9µl 50mM Tris/HCl pH 7.5

Sodium cholate was added to the mixture as this is an anionic detergent and at the precise concentration (critical micelle concentration) it creates a micellar structure when sonicated. At lower or higher concentrations of sodium cholate the substrate PIP₂ is not incorporated into a micellar structure which Plc1 can bind to and hydrolyse. 25µl of crude yeast extract was mixed with 25µl of PIP₂ suspension at 30°C. The reaction was stopped after the required length of time by the addition of 250µl of 0.6M HCl, then 1ml of ice cold chloroform/methanol (2:1) was added. The samples were vortexed for 30 s followed by a 4000g centrifugation for 2 min. The aqueous solution containing the ³H-IP₃ was drawn

off and mixed with 400 μ l of scintillation fluid. The radioactivity was determined using a Wallac 1450 Microbeta Trilux, Liquid Scintillation and Luminescence Counter. The effective ^3H count for this machine is 50%.

2.2.3 Growing cells on microscope slides

The cells were cultured in the required medium at 23°C over night and then diluted to approximately 1×10^3 cells/ml. The cells were placed on a microscope slide containing a small amount of medium containing 1% (w/v) LMP agarose. A cover slip was placed over the top and a small amount of parafin wax, which has been melted, is used to seal the cover slip to the slide. The slide is sealed so that the medium does not dry out affecting cell growth. The cells are incubated at the required temperature for 24 hours and are then viewed using the Nomarski digital camera.

2.2.4 Expression of Plc1 in $\Delta plc1$ strains

The $\Delta plc1$ *Sz. pombe* strain was cultured up overnight in YE medium (see Section 2.1.9) at 23°C. Cells were transformed using the lithium acetate method (Okazaki *et al.*, 1990) (see Section 2.2.1.4) except that, the cell suspension in Solution I was incubated at 23°C for 60-120 min and the cells were heat shocked at 29°C. The transformants were incubated at 23°C on selective medium (aa). Colonies were autotrophically selected for. Colonies were selected and inoculated into 10ml of aa medium and were incubated for 48hrs. The cultures were then inoculated at 5×10^5 cells/ml and incubated at 23°C for 24hrs. The cell volumes were calculated using a C256 Coulter Channelyser (see Section 2.1.7). After the 24hr period the cell volumes were calculated (see Section 2.1.7) and were collected by centrifugation (1000g for 3 min). The cells were resuspended in DMM (see Section 2.1.9.1) at a concentration of 1×10^6 cells/ml and then 5000 cells were spread onto DMM. The plates were incubated at either 23°C or at

37°C and colony formation was recorded by photographing the plates on the gel documentation system.

2.2.5 Statistical treatment of data

All PLC assays were completed in triplicate. The mean value for the assays were calculated and bars on the data indicate the standard range of the mean. This demonstrates the full range of the data from the lowest to the highest value.

Chapter 3

Constructs

3.1 Constructs containing modified versions of the *plc1* gene

3.1.1 General information applying to all modifications of the *plc1* gene introduced by PCR

Due to the size of the pREP vectors, the PCR products of the modified *plc1* genes in figures were first sub-cloned into the plasmid pBluescript KS (Stratagene, Cambridge, UK). Once cloned into the pKS vector, the sequences were confirmed by performing double-stranded DNA sequencing (see Methods for details). On the constructs where inverse PCR was carried out, double stranded DNA sequencing was performed once the construct had been ligated and was circularised. Once the constructs had been sequenced they were subsequently cloned into the yeast expression vectors pREP3x and pREP81x.

3.1.2 Expression vectors

Expression of the *plc1* gene in *Sz. pombe* was achieved by using a set of expression vectors designed by Maundrell, (1990). The pREP vectors are based upon the thiamine-repressible *nmt1* promoter. Two pREP vectors were used in this study; pREP3x and pREP81x. The vectors only differ by six nucleotides within the *nmt1* promoter region. This difference of six nucleotides results in different levels of expression (Maundrell, 1990). The vector pREP3x has the higher level of expression whereas pREP81x has the lower level of expression (Maundrell, 1990; 1993).

3.1.3 Constructs

3.1.3.1 Fusing the His₆ tag to *plc1*

Construction of the N-terminal and C-terminal regions of Plc1 fused to a series of six histidine residues is described in Figures 12 and 13, respectively. These clones have six histidine residues introduced at either the N- or C- terminus of the Plc1 protein

3.1.3.2 Cloning a truncated form of *plc1* to improve PCR efficiency

Due to problems associated with being unable to PCR the full-length *plc1* gene, a truncated form of the *plc1* gene was created. The complete *plc1* gene was digested with *NcoI* and cloned into the *NcoI* site of the vector pGEM-5Zf⁺ to produce the clone JD709 (Figure 14). The clone JD709 was used as a template for the next four constructs (Figures 15-18).

3.1.3.3 The mutagenesis of lysine 582

A modified version of the *plc1* gene was produced which replaced the lysine residue at position 582 with an arginine residue. This construct was produced as described in Figure 15. Another modification was made at position 582 but this time lysine at position 582 was replaced with alanine and this construct was produced as described in Figure 16. These versions of the *plc1* gene were then cloned into both pREP3x and pREP81x.

3.1.3.4 The mutagenesis of arginine 693

A modified version of the *plc1* gene was produced which replaced the arginine residue at position 693 with a lysine residue. This construct was produced as described in Figure 17. Another modification was made at position 693. This time, the arginine was replaced with a leucine and this construct was produced as described in Figure 18. These versions of the *plc1* gene were then cloned into both pREP3x and pREP81x.

3.1.3.5 The use of overlapping PCR to mutagenise arginine 873 and glycine 481

Figure 19 shows a construction of a mutated version of the *plc1* gene where the arginine residue at position 873 has been replaced by a cysteine residue. This procedure involved a process of overlapping PCR where two fragments of the *plc1* gene were produced which had complementary overhangs. The two

fragments were annealed together and PCR was performed to amplify the annealed fragment. After being cloned into pKS it was then cloned into the yeast expression vectors pREP3x and pREP81x. Figure 20 demonstrates that this procedure was repeated to create another mutated form of the *plc1* gene but this time a serine residue replaced the glycine residue at position 481.

3.1.3.6 Cloning of Rat PLC- δ

Figure 21 demonstrates how Rat PLC- δ was cloned from a pMT-2 plasmid containing the gene sent from S.G.Rhee (National Institute of Health, Bethesda, Maryland, USA). The sequence was confirmed in pKS and it was subsequently cloned into the yeast expression vectors pREP3x and pREP81x. The pREP vectors do not contain an *EcoRV* site directly down stream of the promoter, so an end-filled *Bam*HI site was therefore used. The *EcoRV* sites introduced by the primers when cloning the Rat PLC- δ were also end-filled so the PCR product was cloned with blunt ends into the pREP vectors.

3.1.3.7 Producing a truncated form of *plc1*

Figure 22 describes the production of a truncated form of the *plc1* gene. A PCR primer was designed which introduced an initiator codon at the leucine residue position 144. This fuses the A of the Atg (Met1) to the TG of cTG (Leu144), it therefore removes residues 2 to 144 inclusive. The truncated form of *plc1* was then cloned into the pREP3x and pREP81x after the sequence was confirmed in pKS.

3.1.3.8 Fusing the N-terminus of *plc1* to Rat PLC- δ

Figure 23a demonstrates how the *plc1* gene and the Rat PLC- δ gene were cloned into the same pKS vector. The clone JD900 is the gene *plc1* that has had the internal *EcoRV* sites removed by PCR. Although the *EcoRV* restriction sites have been removed the actual protein sequence remains the same. Digestion of

JD900 and partial digestion of JD673 (Rat PLC- δ in pKS) with *EcoRV* allowed ligation of the two genes together. Figure 23b demonstrates how two primers were used in an inverse PCR reaction creating a hybrid PLC. The N-terminus of the *plc1* gene was ligated to the full length Rat PLC- δ gene thus circularising the plasmid. The sequence was confirmed and the subsequent hybrid PLC was cloned into pREP3xr and pREP81xr (pREP vectors that now contain an *EcoRV* site downstream of the promoter) constructed by K. Davis (University of Warwick, UK).

3.1.3.9 Expressing the N-terminal domain of Plc1

Figure 24 demonstrates the amplification of the N-terminus. The primers M13universal and JO622 were used to amplify the first 147 amino acids of *Sz .pombe* Plc1 using the template JD645 (pKS*plc1*). The primer JO622 was designed to introduce a stop codon directly after valine 147 and also introduce a *Bam*HI restriction site. Once the sequence of the PCR product had been confirmed in pKS it was digested with *Bam*HI and ligated into the *Bam*HI digested pREP3x and pREP81x vectors.

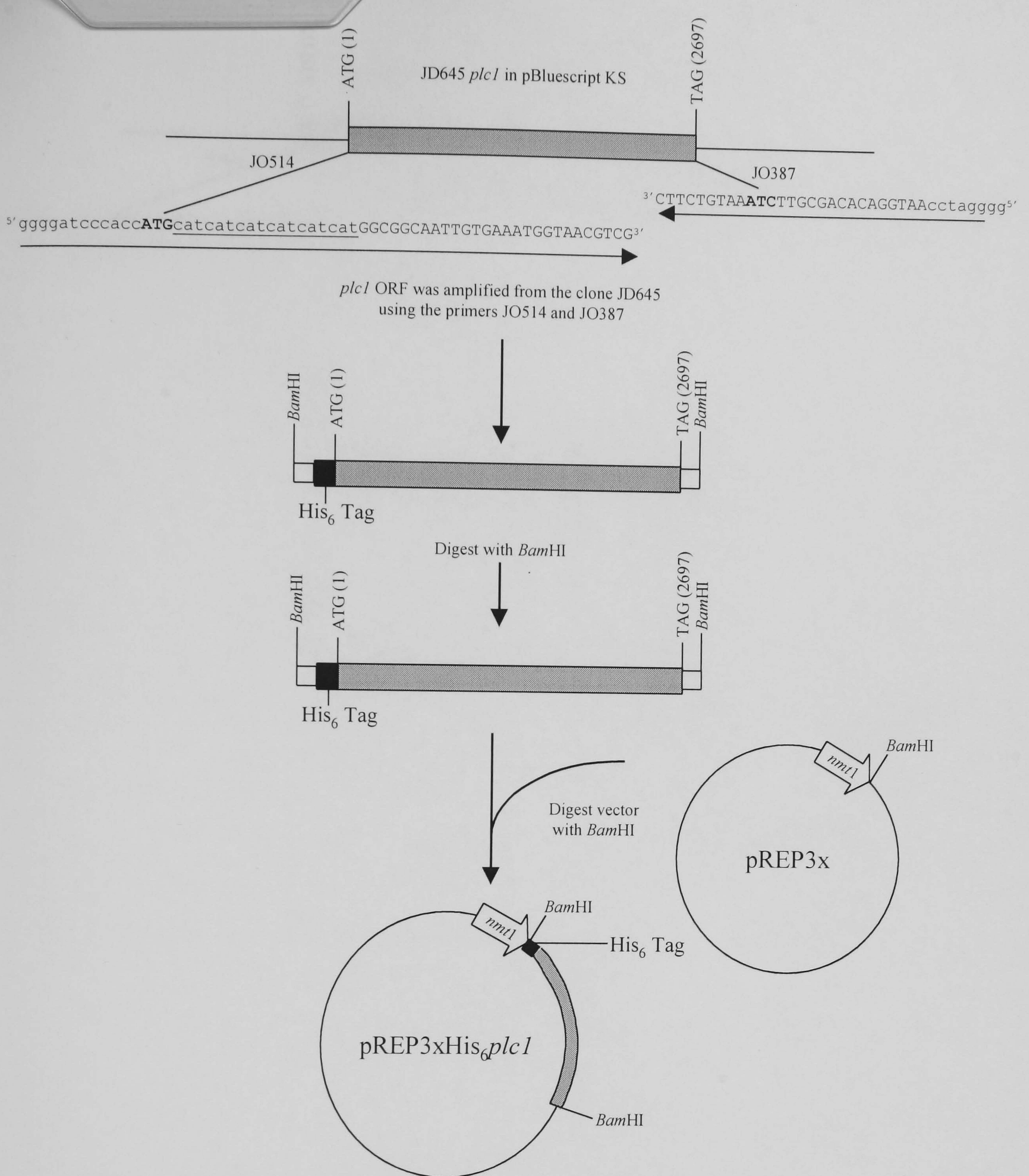


Figure 12 Introducing a His₆ tag into the N-terminus of Plc1

The oligonucleotides JO387 and JO514 were used to PCR amplify *plc1* from the clone JD645. Both primers contain *Bam*HI sites to assist the cloning of the PCR product. Primer JO514 contains a sequence *CCACC* immediately upstream of the initiator codon (shown in bold type) to increase the efficiency of translation (Kozac, 1994; 1986; Yun *et al.*, 1996). The sequence underlined represents the region that will encode the hexahistidine tag. The stop anticodon is shown on primer JO387 in bold type. Lower case letters represent sequence that is used in the primer but is not found in the *plc1* gene.

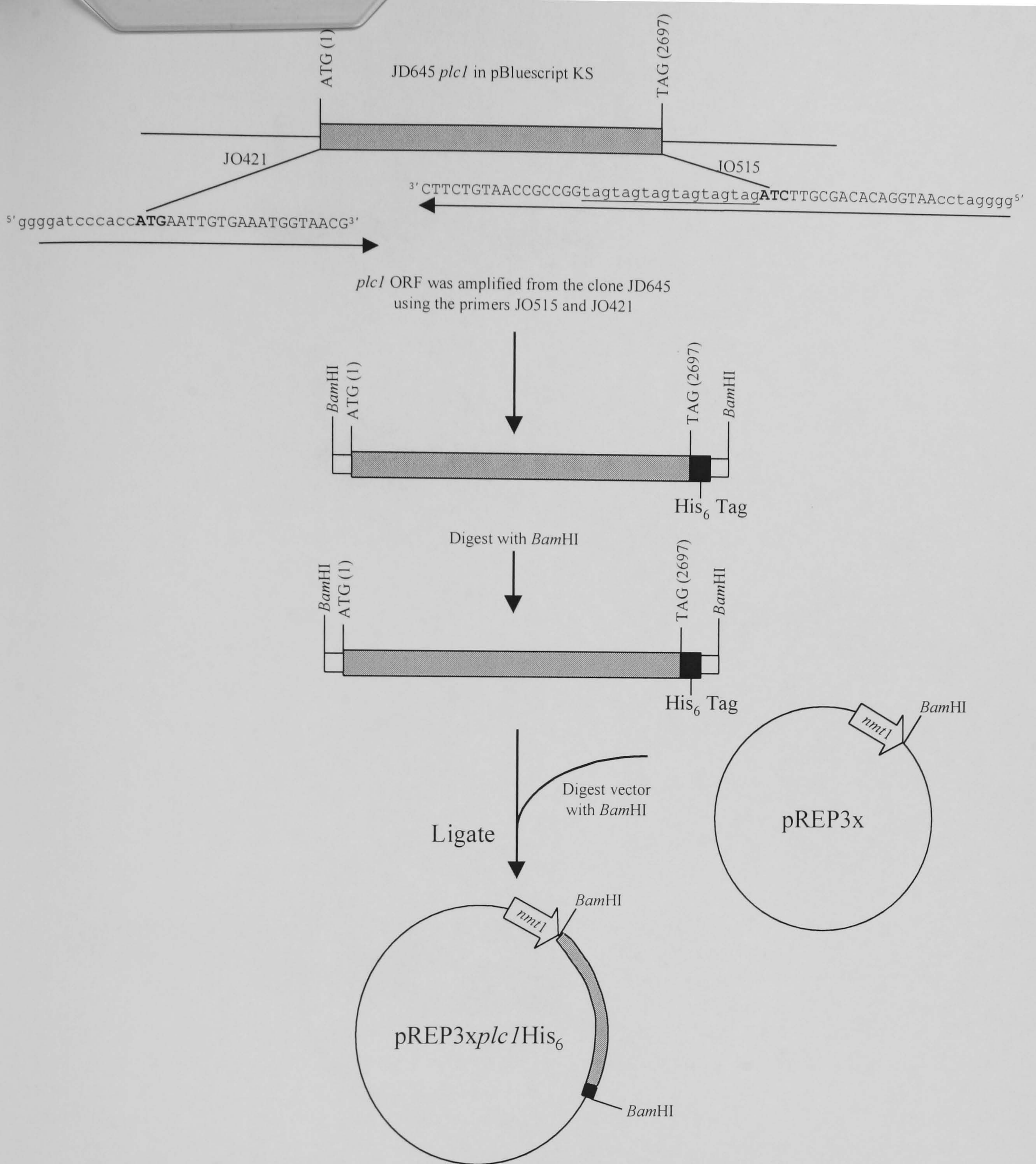


Figure 13 Introducing a His₆ tag into Plc1

The oligonucleotides JO421 and JO515 were used to PCR amplify *plc1* from the clone JD645. Both primers contain *Bam*HI sites to assist the cloning of the PCR product. Primer JO421 contains a sequence *CCACC* immediately upstream of the initiator codon to increase the efficiency of translation (Kozac, 1994;1986; Yun *et al.*, 1996). The stop anticodon is shown on primer JO515 in bold type and the sequence underlined represents the region that will encode the hexahistidine tag. Lower case letters represent sequence that is used in the primer but is not found in the *plc1* gene.

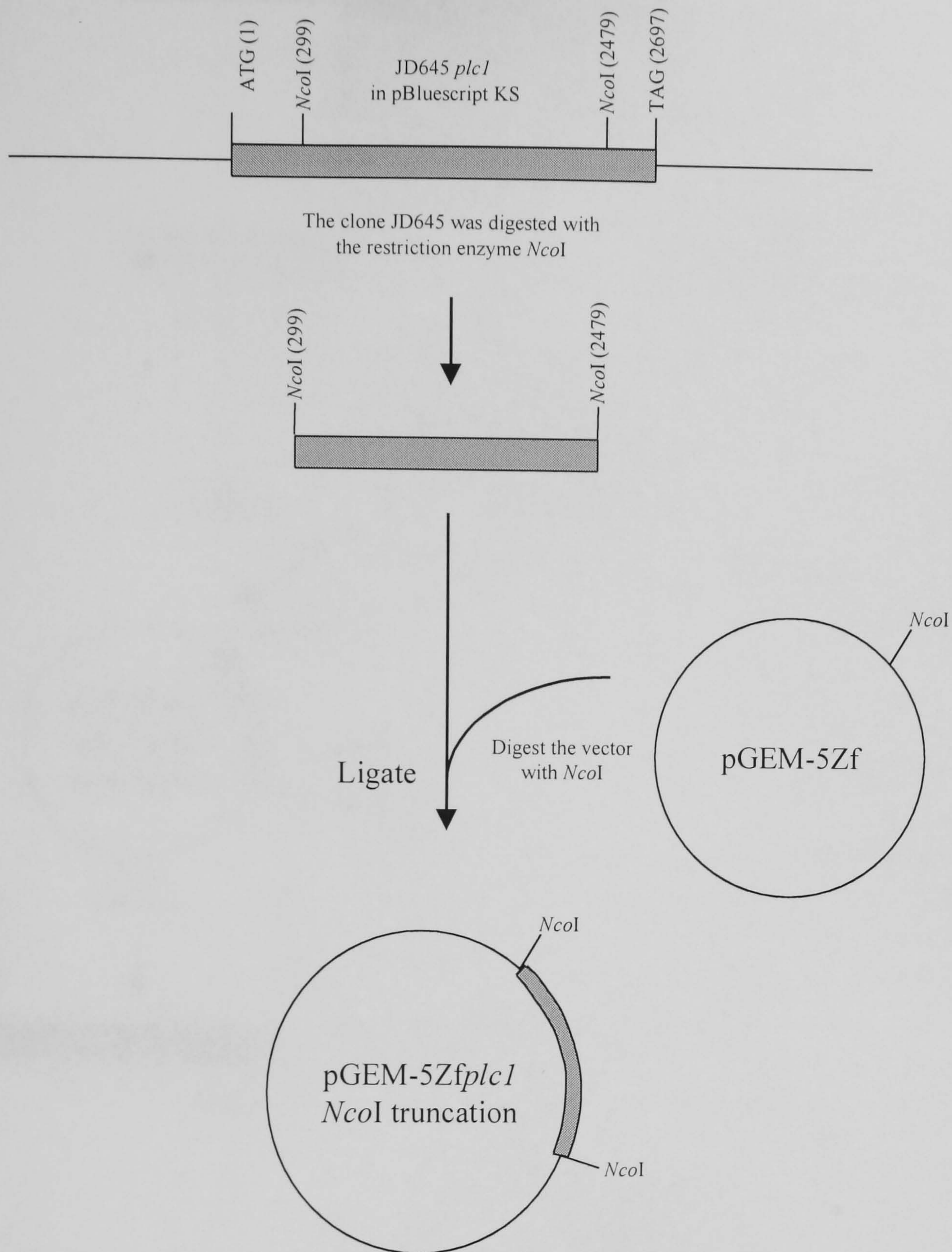


Figure 14 Removing the central *NcoI* portion of *plc1* for inverse PCR

The clone JD645 was digested with *NcoI*, this central portion was then ligated into *NcoI*-digested pGEM-5Zf. This truncated form of *plc1* allowed inverse PCR to be performed, thus creating the active site mutants (see Figures 15-18 for details).

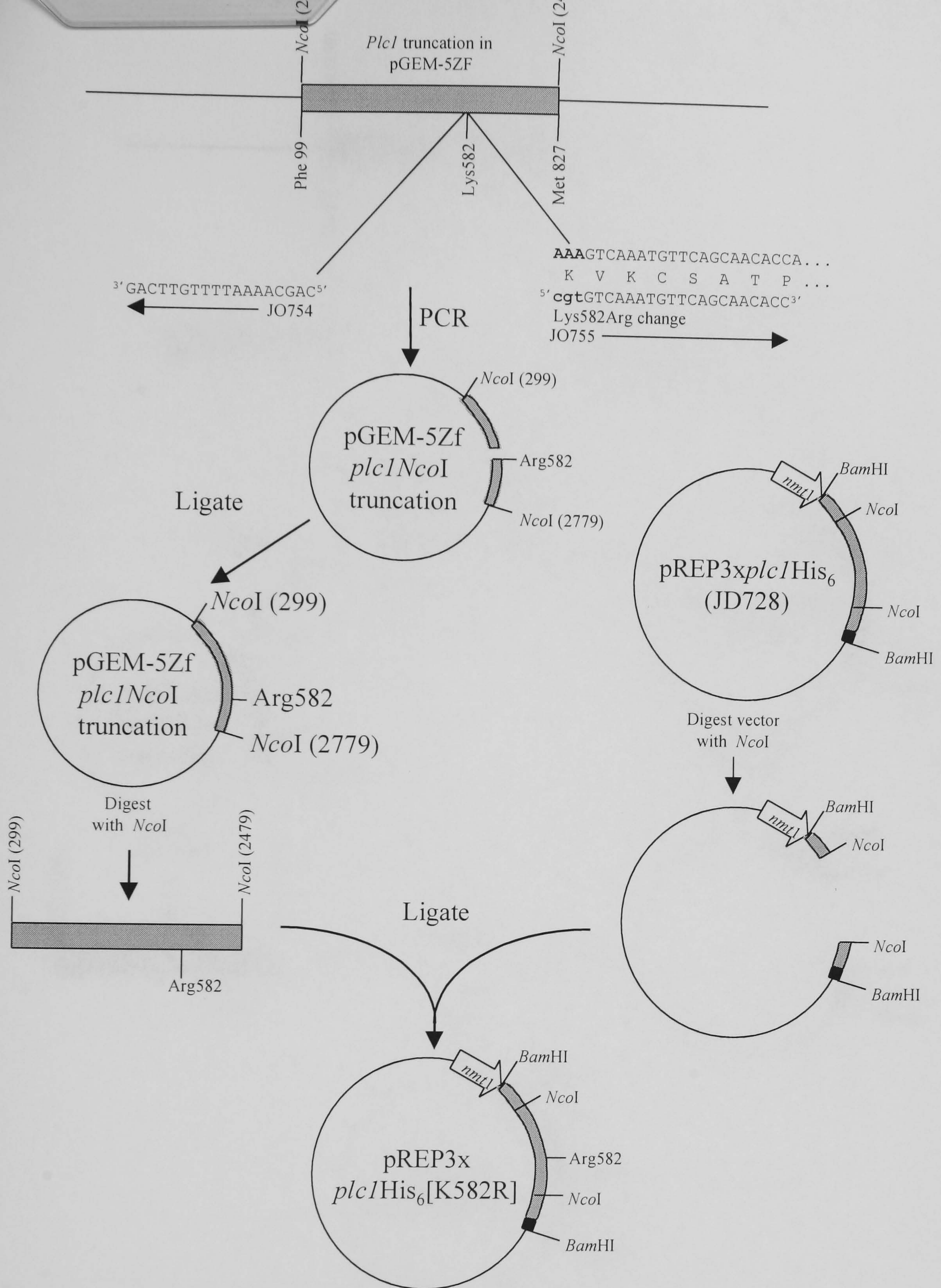


Figure 15 Replacing the lysine at position 582 with arginine

Inverse PCR was used to amplify the *NcoI* portion of *plc1* and the pGEM-5Zf vector using the primers JO754 and JO755. Primer JO755 contains three bases (lowercase, bold) that are different to the wild-type sequence. This replaces the lysine codon from AAA (Bold) to arginine CGT. The PCR fragment was ligated to circularise the pGEM-5Zf vector. This was subsequently digested with *NcoI* and was ligated into the *NcoI* sites of JD728 (which contains the flanking regions of *plc1* and the C-terminal His₆ tag).

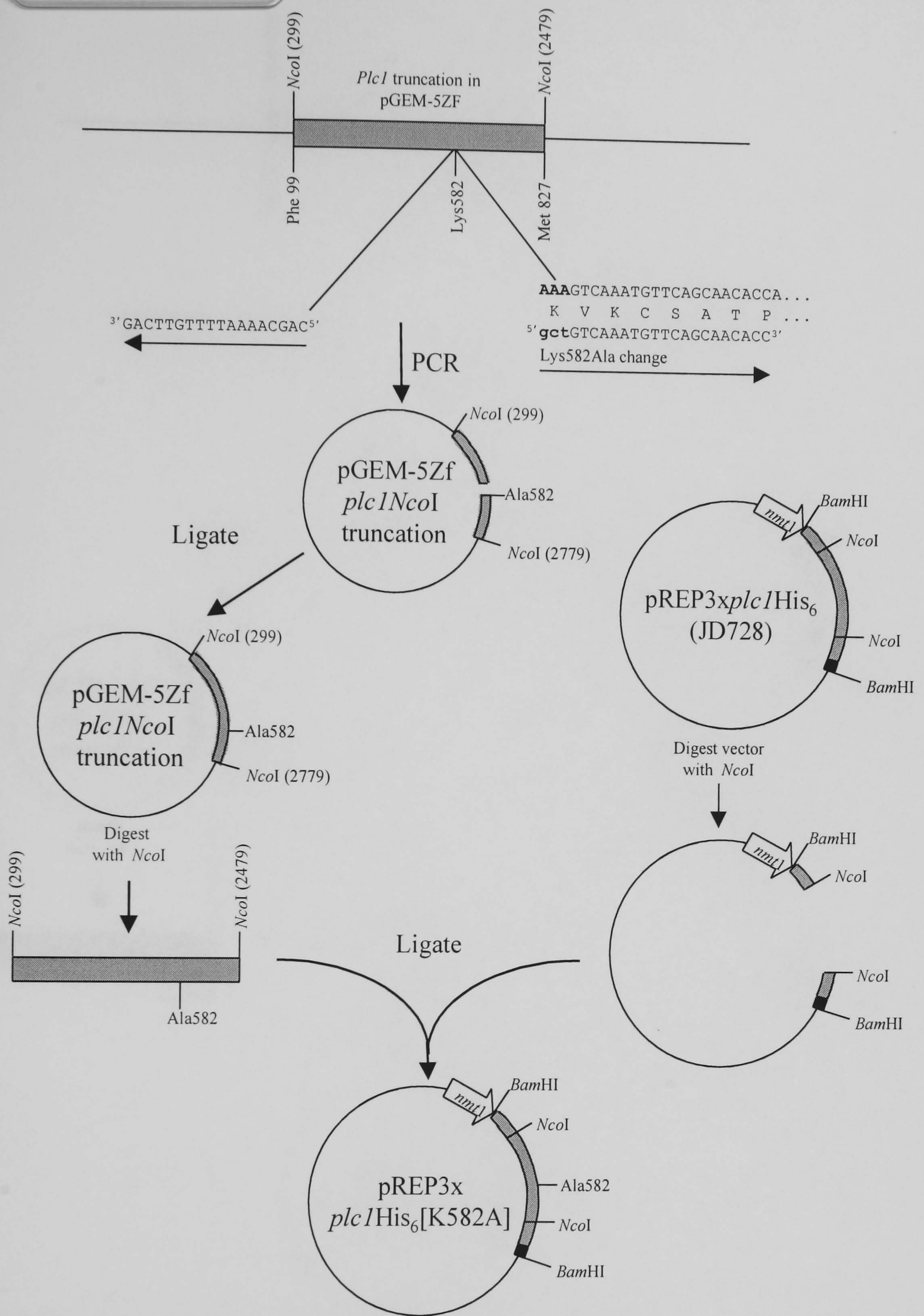


Figure 16 Replacing the lysine at position 582 with alanine

Inverse PCR was used to amplify the *NcoI* portion of *plc1* and the pGEM-5Zf vector using the primers JO754 and JO756. Primer JO756 contains three bases (lowercase, bold) that are different to the wild-type sequence. This replaces the lysine codon from AAA (Bold) to alanine GCT. The PCR fragment was ligated to circularise the pGEM-5Zf vector. This was subsequently digested with *NcoI* and was ligated into the *NcoI* sites of JD728 (which contains the flanking regions of *plc1* and the C-terminal His₆ tag).

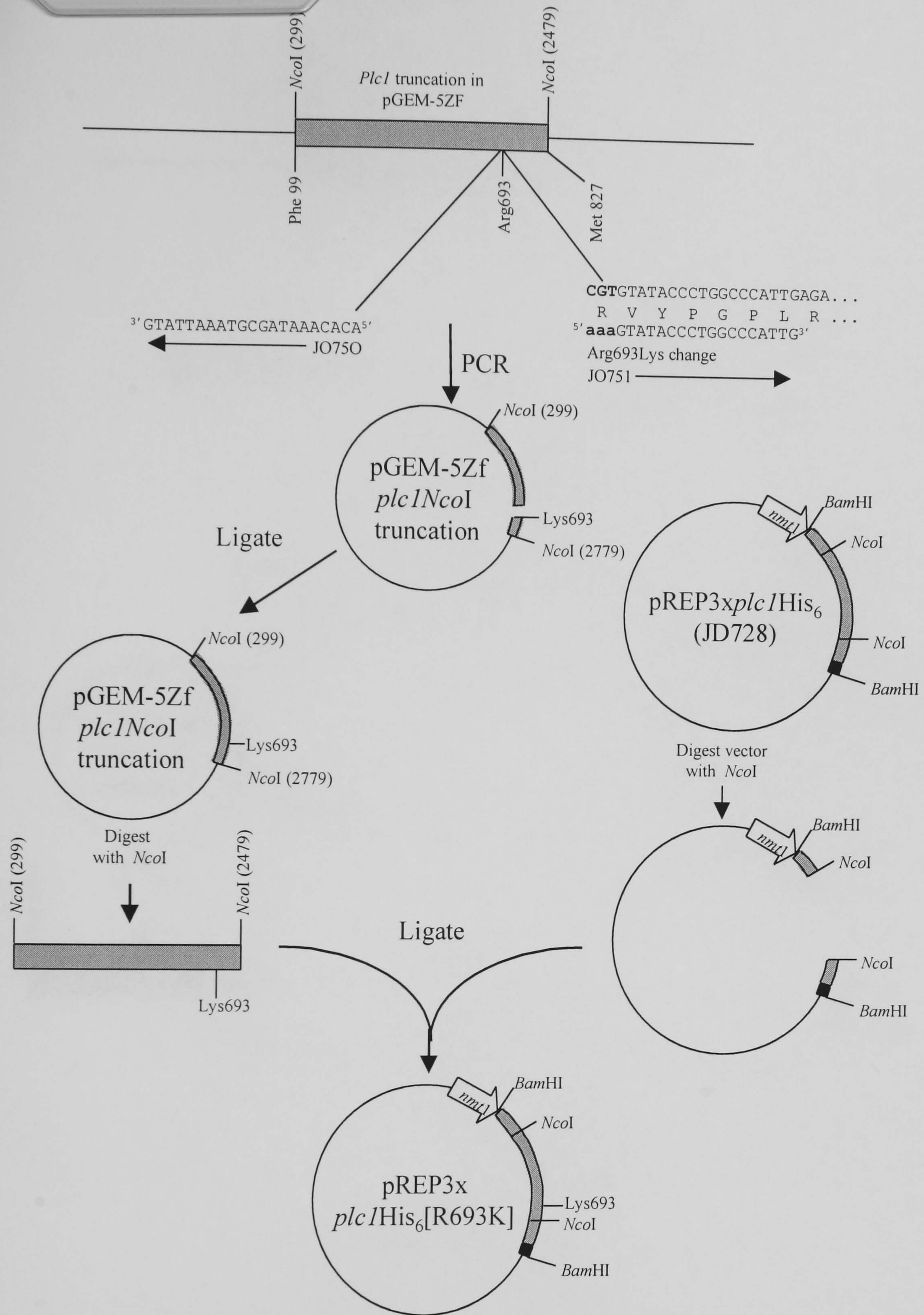


Figure 17 Replacing the arginine at position 693 with lysine

Inverse PCR was used to amplify the *NcoI* portion of *plcI* and the pGEM-5Zf vector using the primers JO750 and JO751. Primer JO751 contains three bases (lowercase, bold) that are different to the wild-type sequence. This replaces the Arginine codon from CGT (Bold) with Lysine AAA. The PCR fragment was ligated to circularise the pGEM-5Zf vector. This was subsequently digested with *NcoI* and was ligated into the *NcoI* sites of JD728 (which contains the flanking regions of *plcI* and the C-terminal His₆ tag).

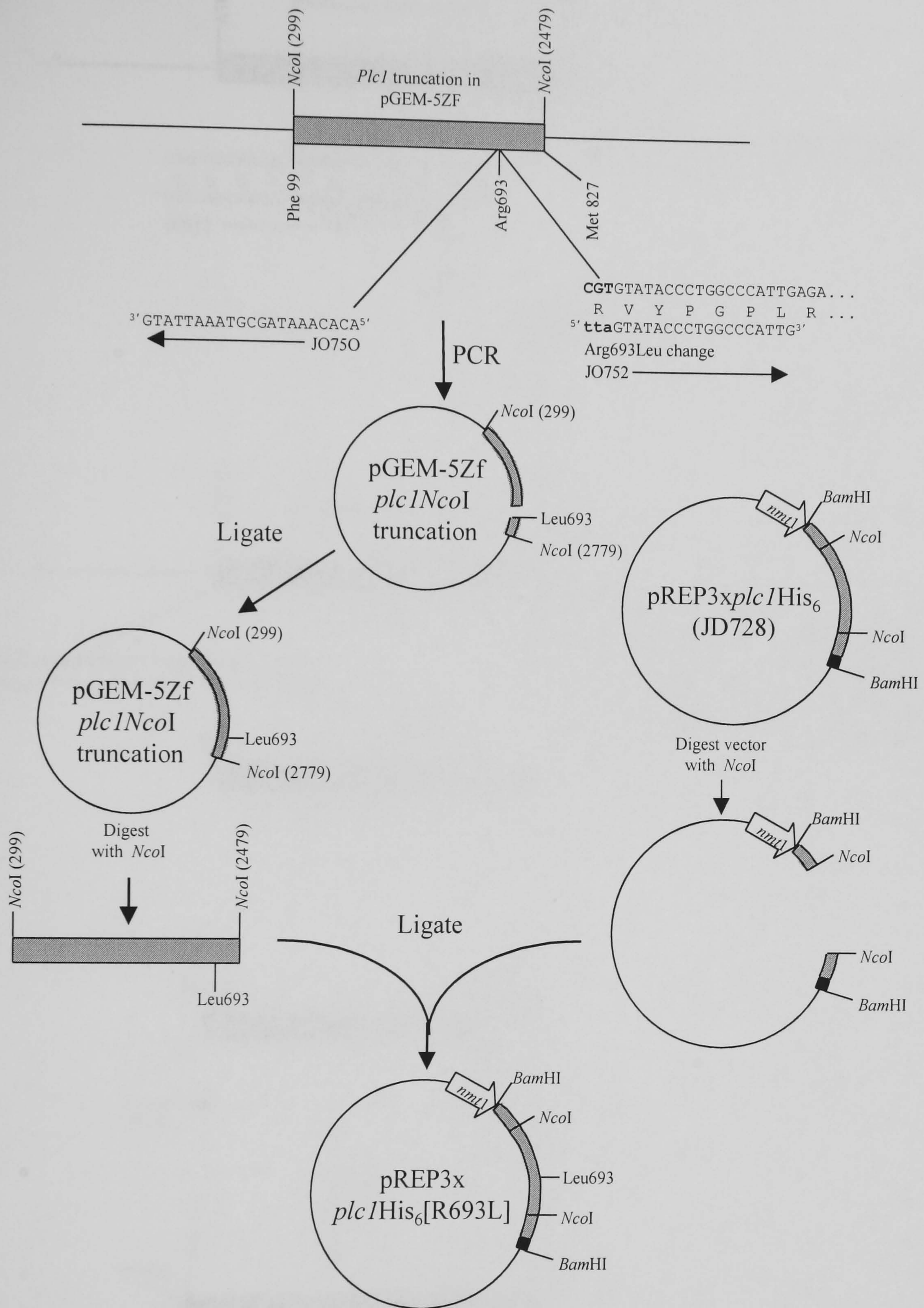
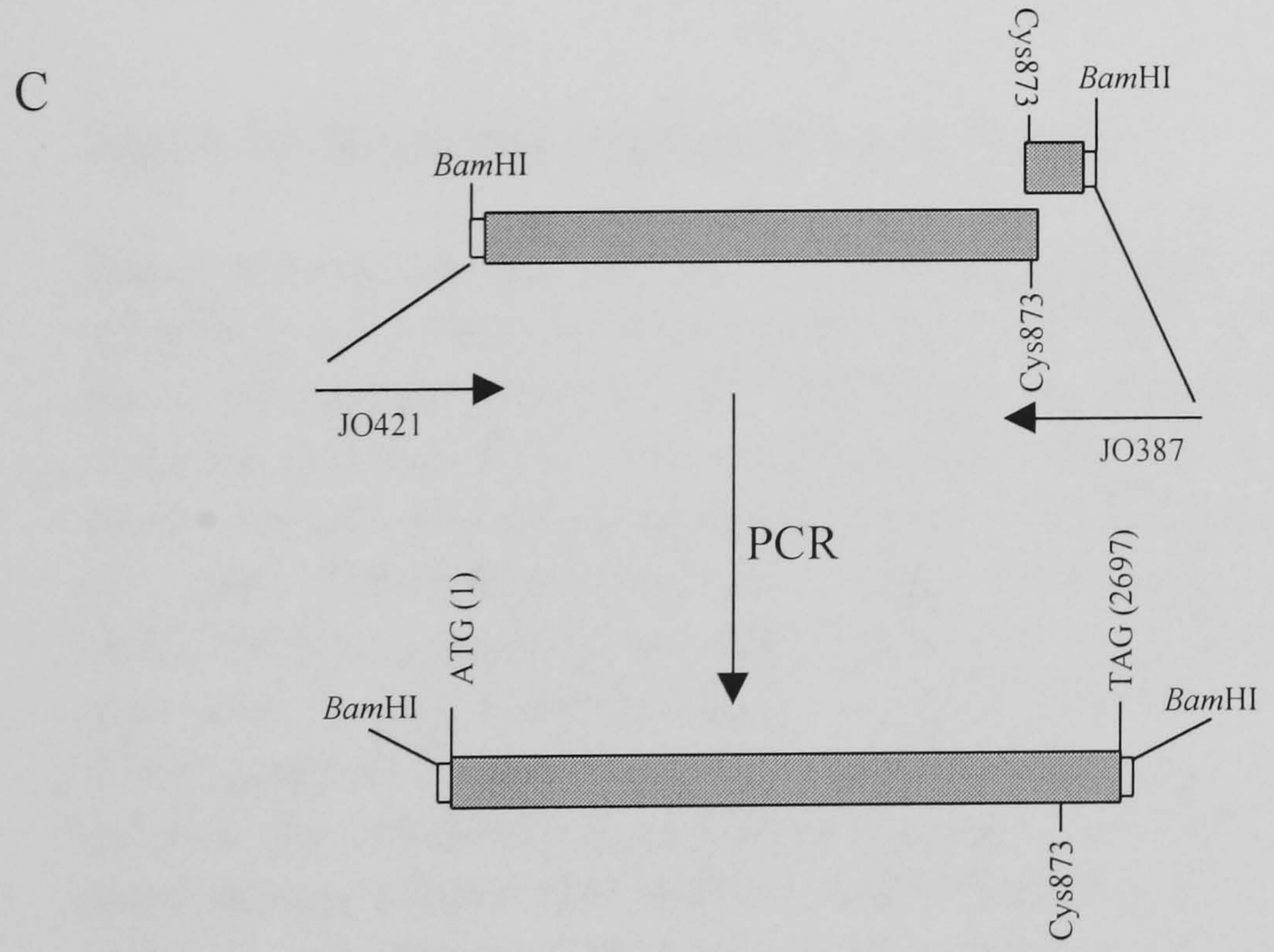
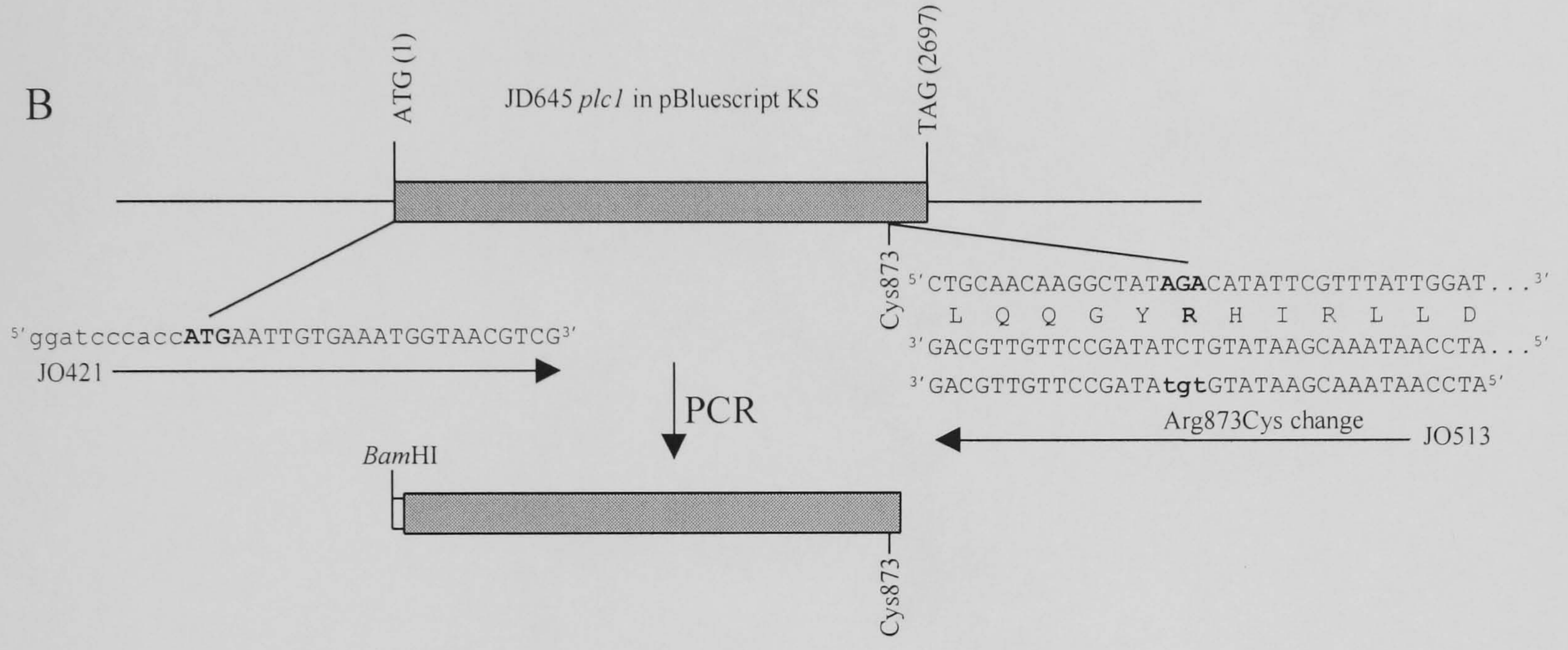
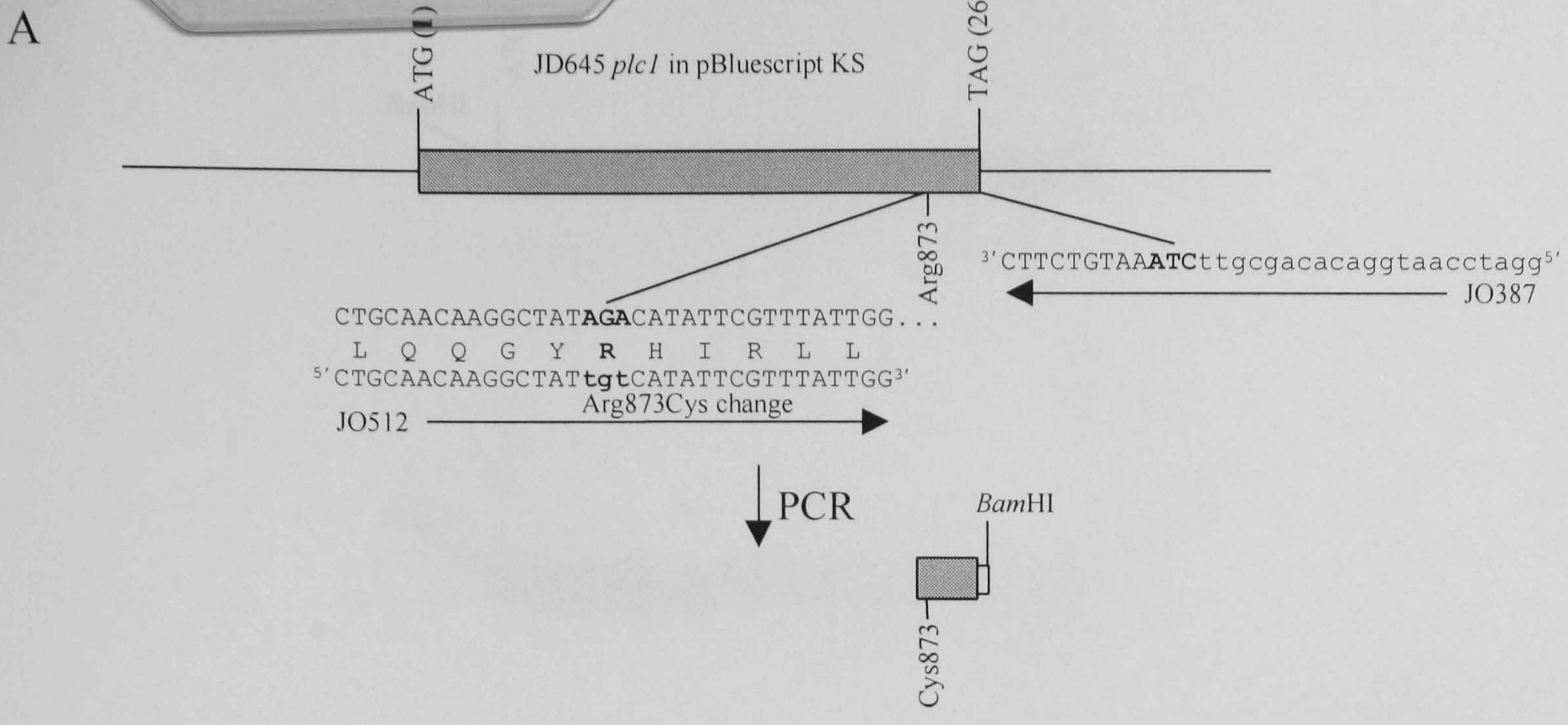


Figure 18 Replacing the arginine at position 693 with alanine

Inverse PCR was used to amplify the *NcoI* portion of *plc1* and the pGEM-5Zf vector using the primers JO750 and JO752. Primer JO752 contains three bases (lowercase, bold) that are different to the wild-type sequence. This replaces the Arginine codon from CGT (Bold) with Alanine TTA. The PCR fragment was ligated to circularise the pGEM-5Zf vector. This was subsequently digested with *NcoI* and was ligated into the *NcoI* sites of JD728 (which contains the flanking regions of *plc1* and the C-terminal His₆ tag).



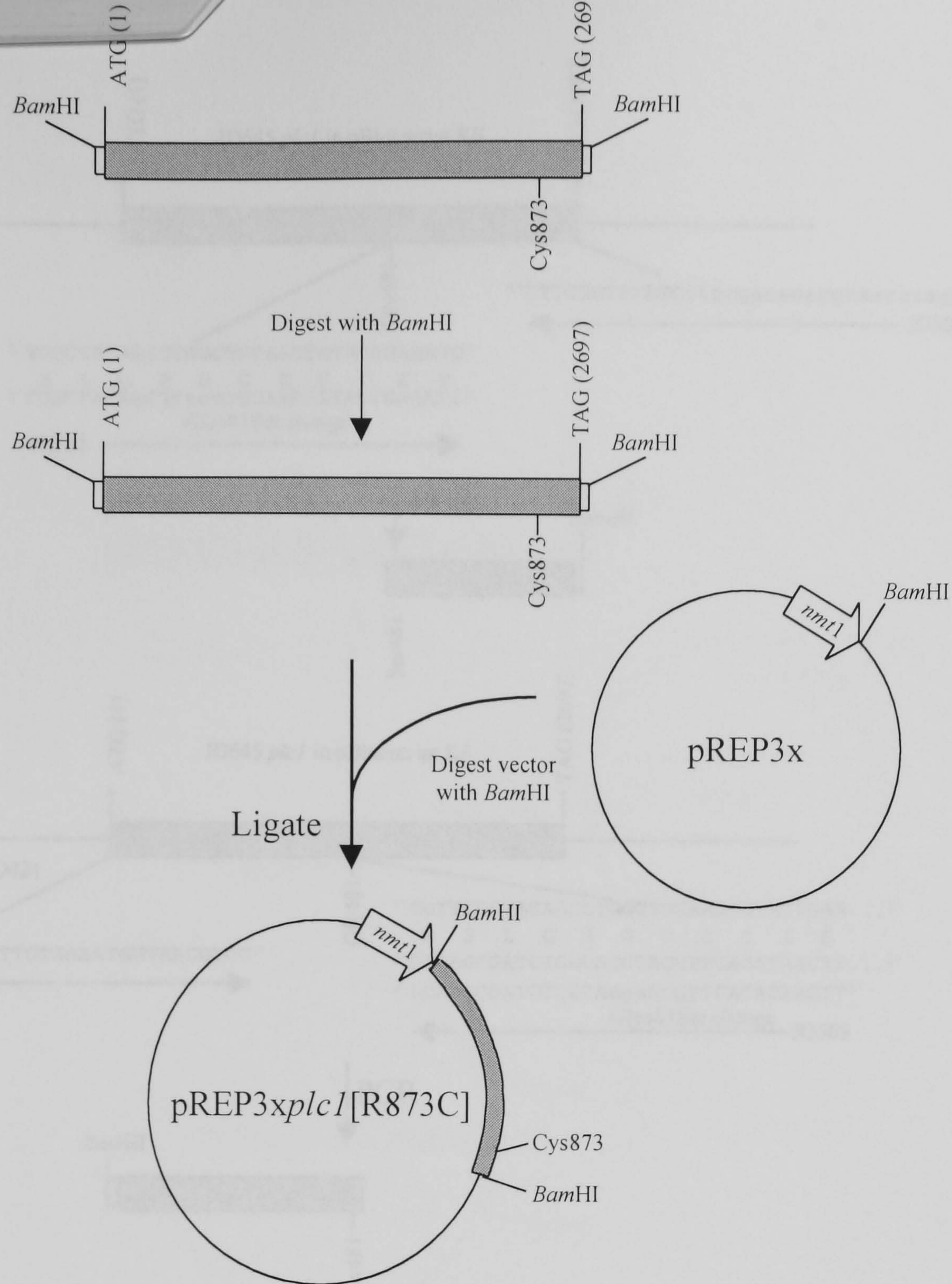
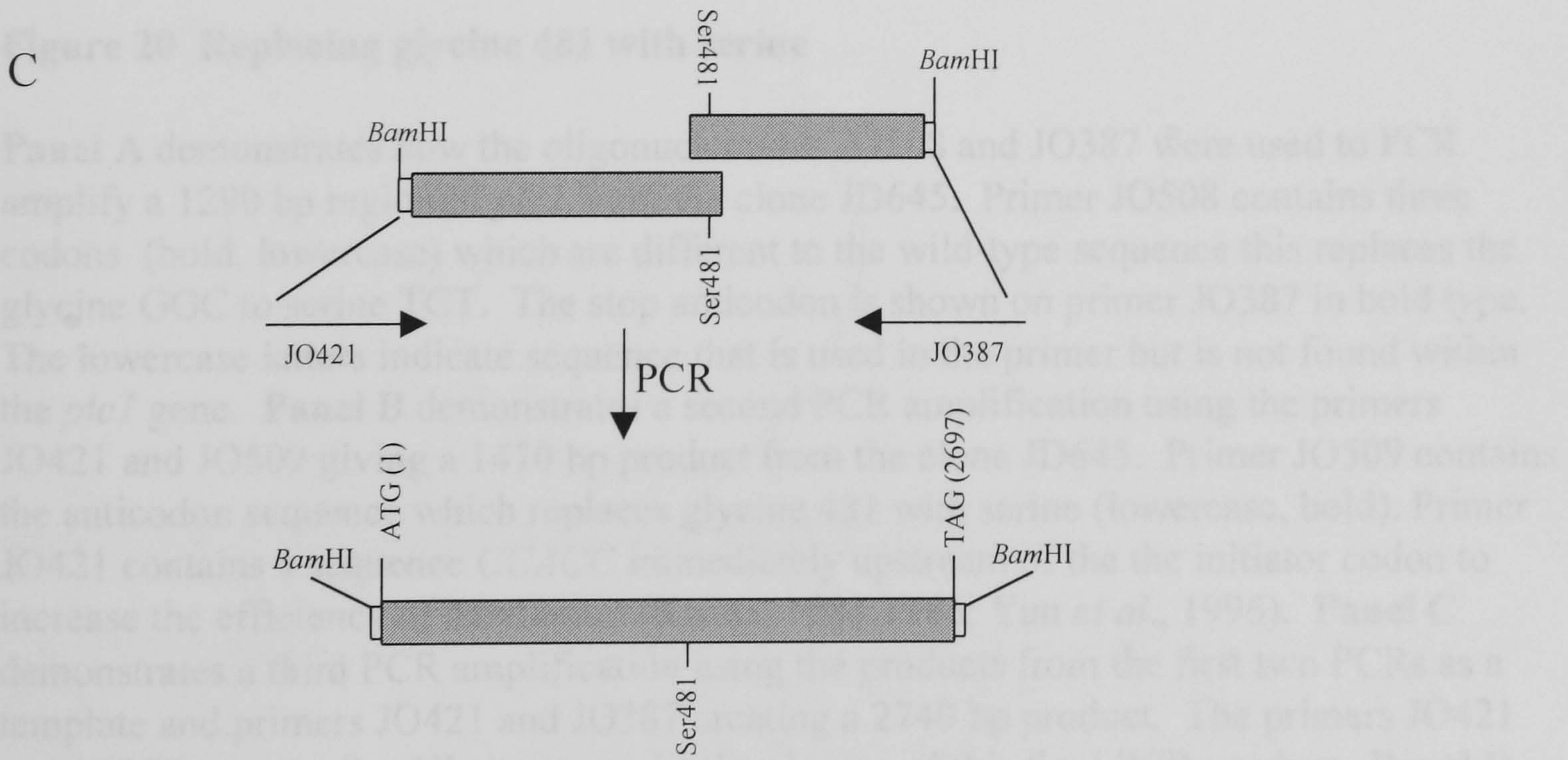
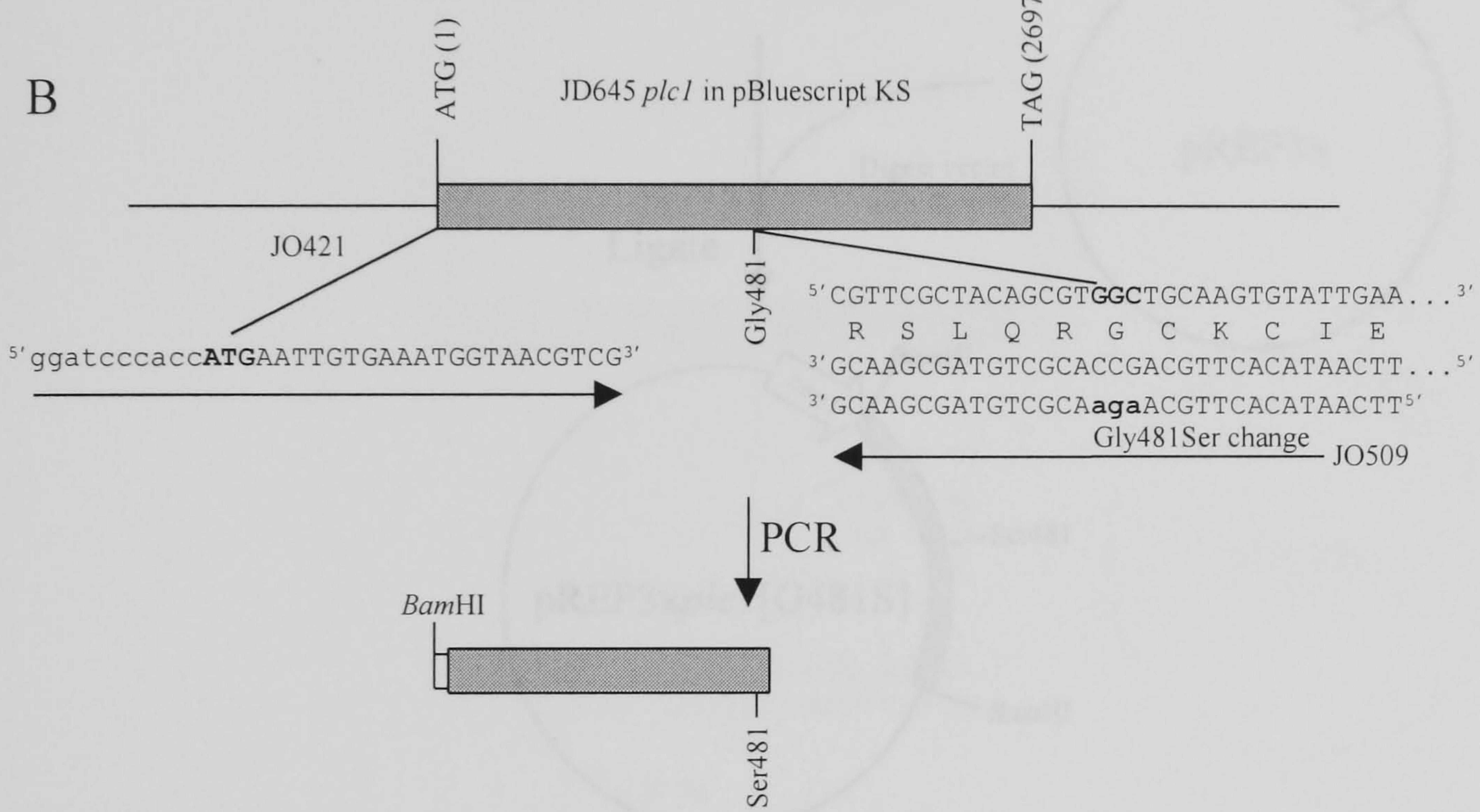
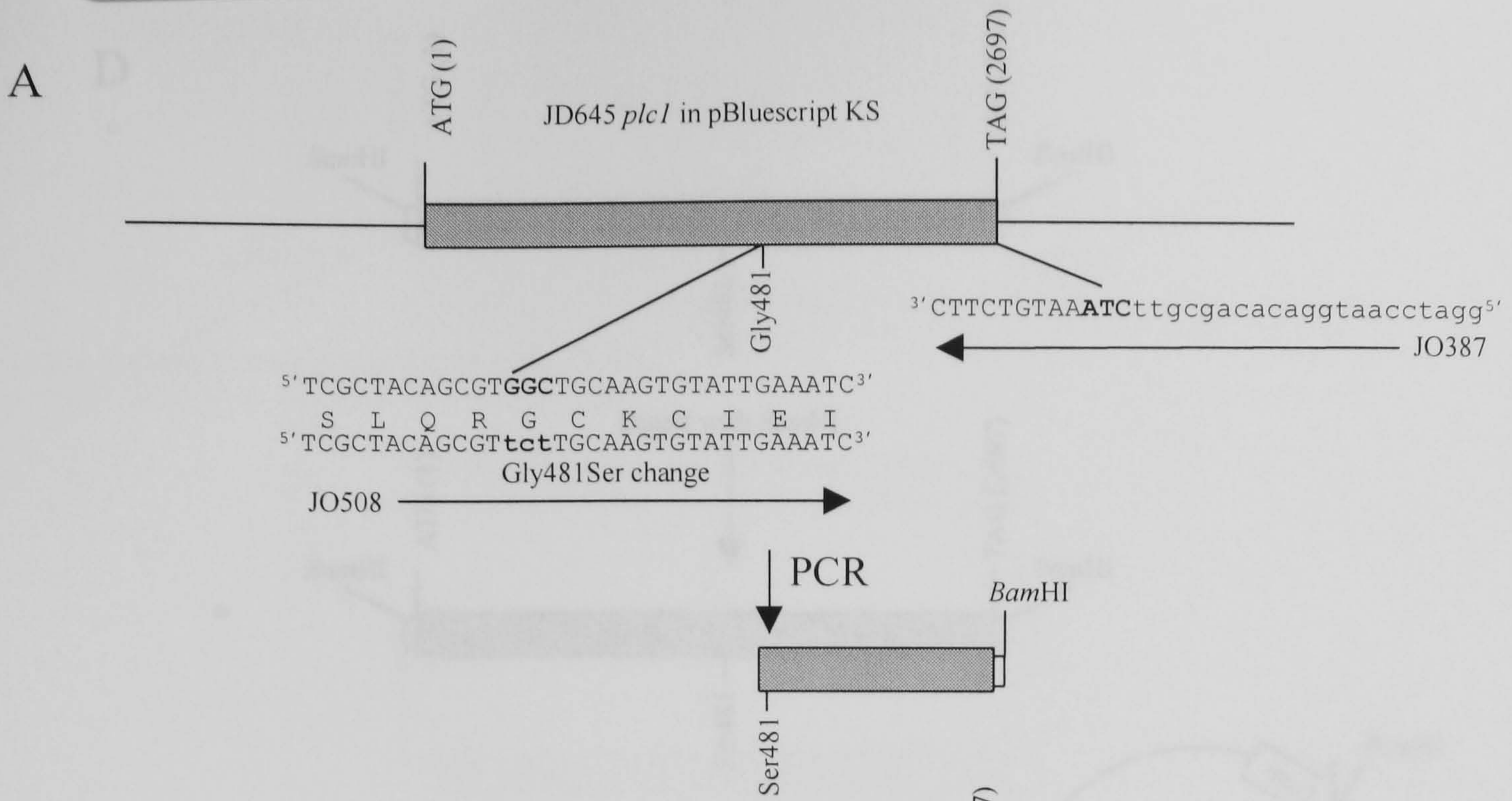


Figure 19 Replacing arginine 873 with cysteine

Panel A demonstrates how the oligonucleotides JO512 and JO387 were used to PCR amplify a 115 bp region of *plc1* from the clone JD645. Primer JO512 contains two codons (bold, lowercase) which are different to the wild-type sequence this replaces the arginine AGA for cysteine TGT. The stop anticodon is shown on primer JO387 in bold type. The lowercase letters indicate sequence that is used in the primer but is not found within the *plc1* gene. **Panel B** demonstrates a second PCR amplification using the primers JO421 and JO513 giving a 2640 bp product from the clone JD645. Primer JO513 contains the anticodon sequence which replaces arginine 873 with cysteine (lowercase, bold). Primer JO421 contains a sequence *CCACC* immediately upstream of the the initiator codon to increase the efficiency of translation (Kozac, 1984;1986; Yun *et al.*, 1996). **Panel C** demonstrates a third PCR amplification using the products from the first two PCRs as a template and primers JO421 and JO387 creating a 2740 bp product. The primers JO421 and JO387 contain *Bam*HI sites to assist the cloning of this final PCR product. **Panel D** demonstrates that this PCR product was digested with *Bam*HI and ligated into the appropriate vector.



D

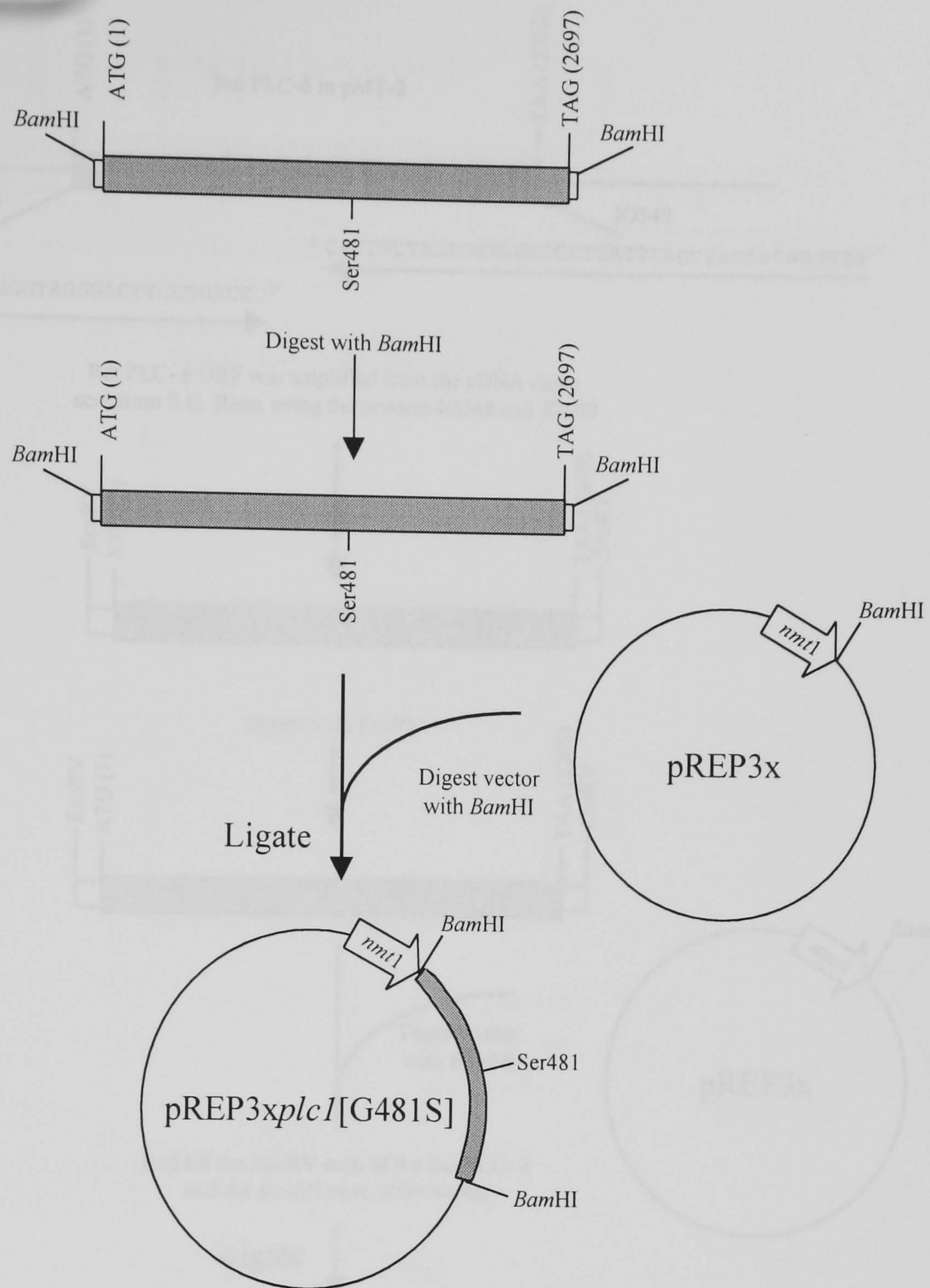


Figure 20 Replacing glycine 481 with serine

Panel A demonstrates how the oligonucleotides JO508 and JO387 were used to PCR amplify a 1290 bp region of *plc1* from the clone JD645. Primer JO508 contains three codons (bold, lowercase) which are different to the wild-type sequence this replaces the glycine GGC to serine TCT. The stop anticodon is shown on primer JO387 in bold type. The lowercase letters indicate sequence that is used in the primer but is not found within the *plc1* gene. **Panel B** demonstrates a second PCR amplification using the primers JO421 and JO509 giving a 1470 bp product from the clone JD645. Primer JO509 contains the anticodon sequence which replaces glycine 481 with serine (lowercase, bold). Primer JO421 contains a sequence *CCACC* immediately upstream of the the initiator codon to increase the efficiency of translation (Kozac, 1984;1986; Yun *et al.*, 1996). **Panel C** demonstrates a third PCR amplification using the products from the first two PCRs as a template and primers JO421 and JO387 creating a 2740 bp product. The primers JO421 and JO387 contain *Bam*HI sites to assist the cloning of this final PCR product. **Panel D** demonstrates that this PCR product was digested with *Bam*HI and ligated into the appropriate vector.

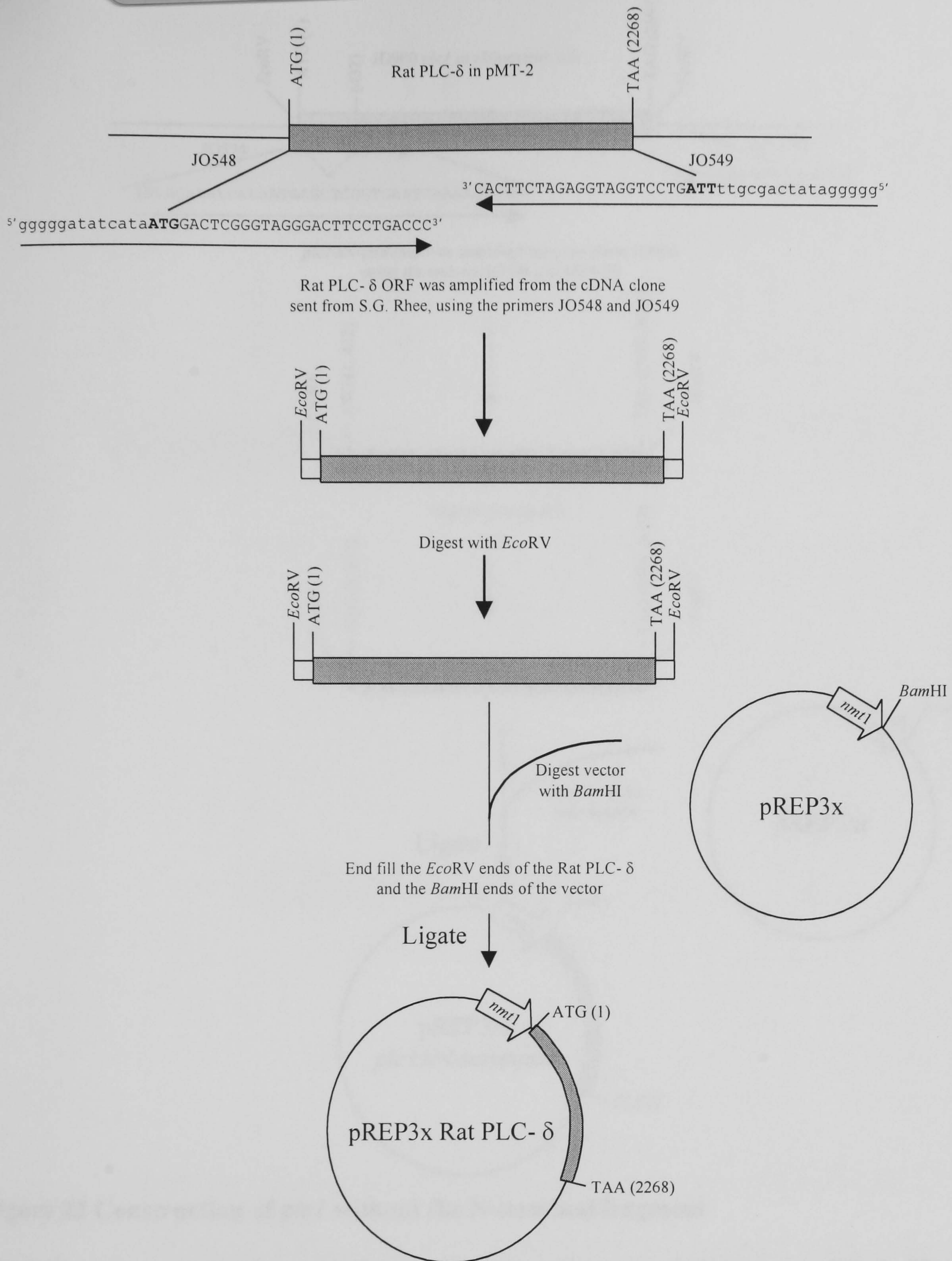


Figure 21 The cloning of Rat PLC- δ into the pREP3x vector

The oligonucleotides JO548 and JO549 were used to amplify the Rat PLC- δ gene, by PCR, from the clone JD??? (supplied by S.G. Rhee, National Institute of Health, Maryland). Both primers contained *EcoRV* sites to assist the cloning of the PCR product. Primer JO548 contains the initiator codon (bold type) and JO549 contains the stop codon (bold type). Lower case letters indicate sequence used in the primer but not found within the Rat PLC- δ gene. Due to the pREP vectors not containing an *EcoRV* site directly down stream of the *nmt1* promoter an end filled *Bam*HI site was used instead.

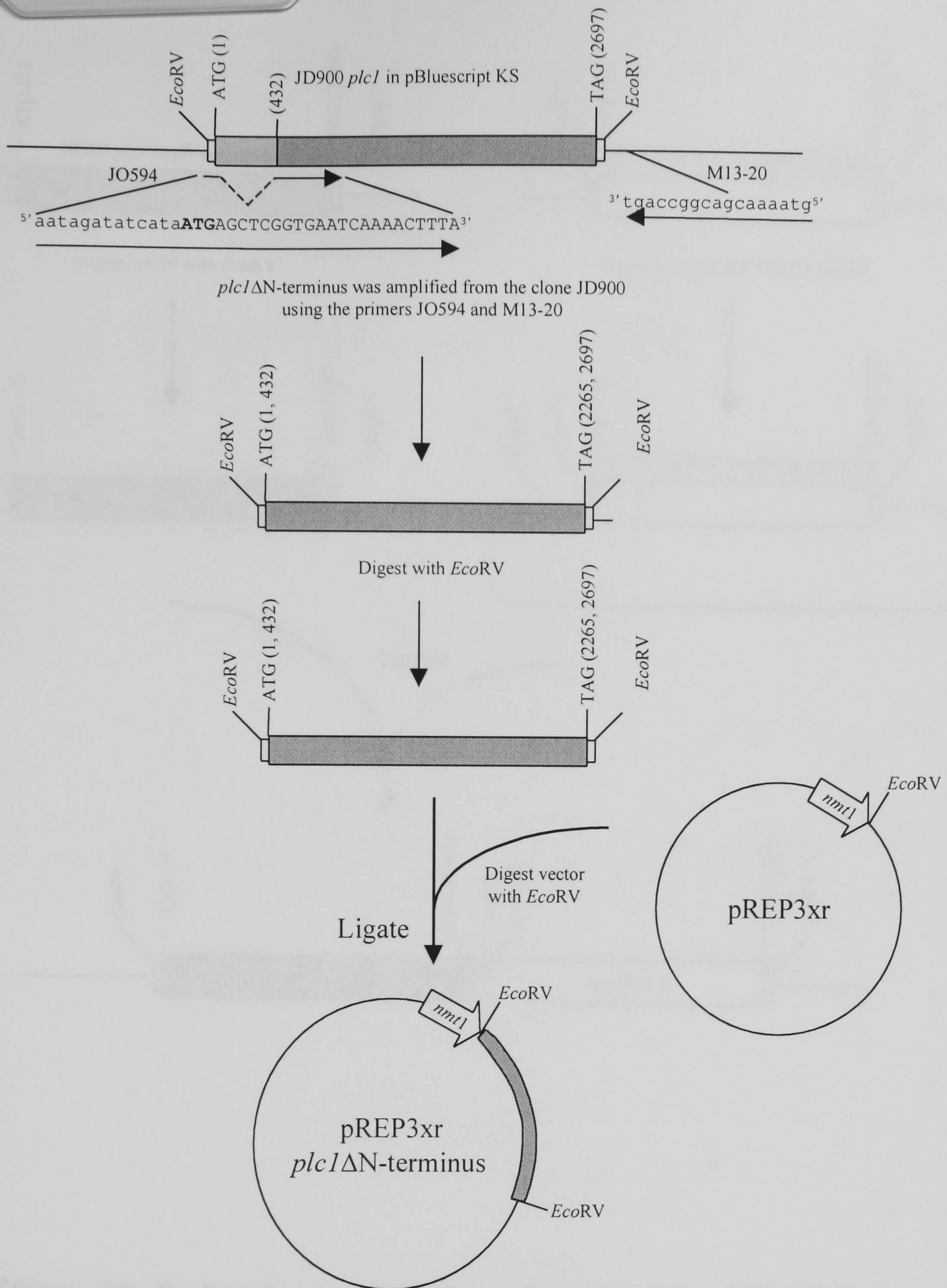


Figure 22 Construction of *plc1* without the N-terminal fragment

Using the primers M13-20 and JO594 the N-terminal fragment of *plc1* was amplified. The template used was the construct JD900 (*plc1* which has had the two internal *EcoRV* sites removed and *EcoRV* sites introduced onto the N and C-terminus in order to facilitate cloning). JO594 introduces an initiator codon Met ATG (bold) at position 144. This uses the A of Atg (Met1) and the TG of cTG (Leu 144) removing residues 2 to 144 inclusive. Once amplified and digested with *EcoRV* this construct, *plc1*ΔN-terminus was cloned into pREP3xr (pREP vectors which contain an *EcoRV* site directly downstream of the *nmt1* promoter, constructed by K. Davis, University of Warwick 1999).

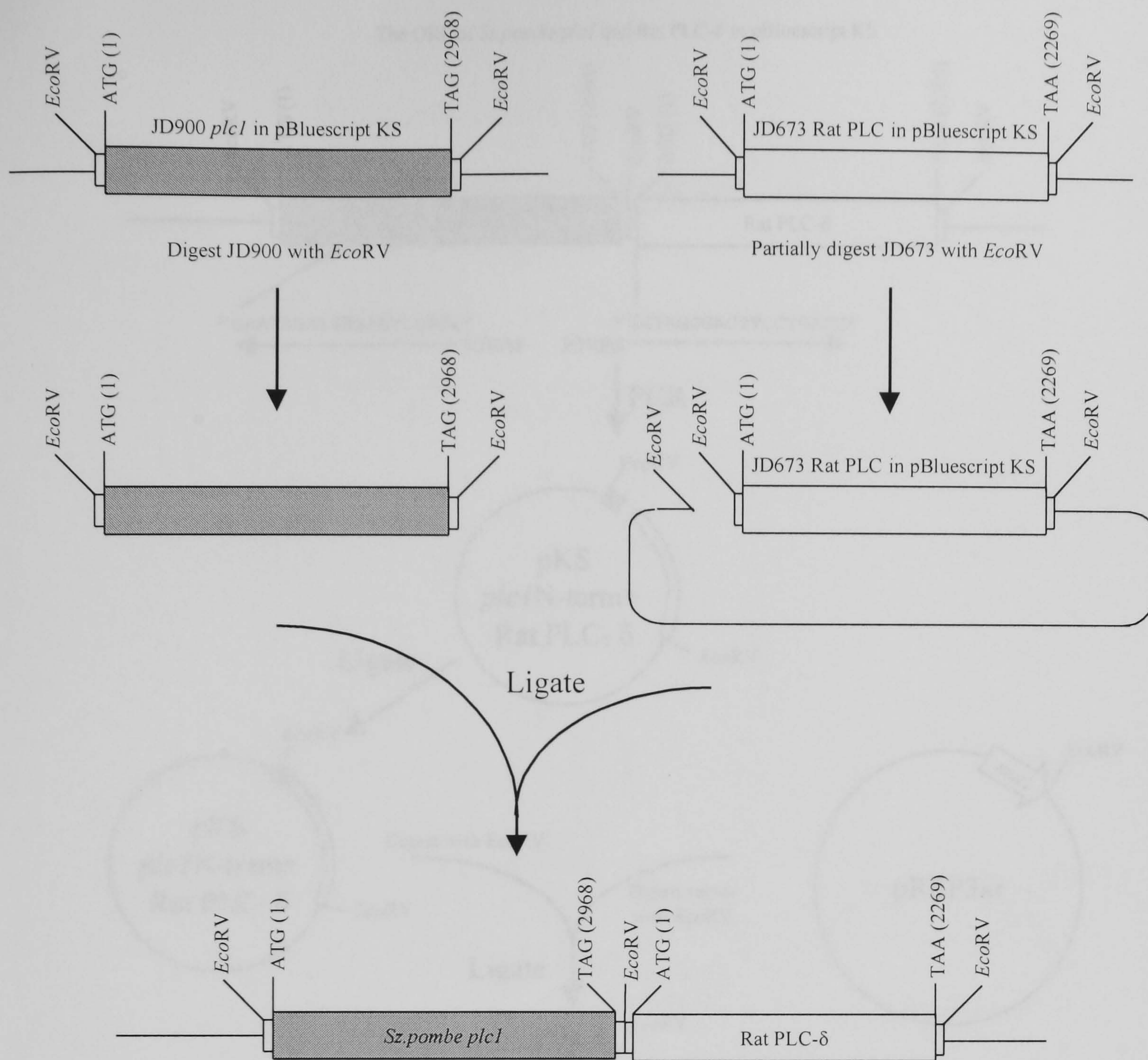


Figure 23a Exchanging domains between *Sz.pombe plc1* and Rat PLC- δ

JD900 was digested with *EcoRV*, the *plc1* gene was then ligated to JD673 which was partially digested with *EcoRV*. This meant that both *Sz.pombe plc1* and Rat PLC- δ were cloned into the same pKS vector.

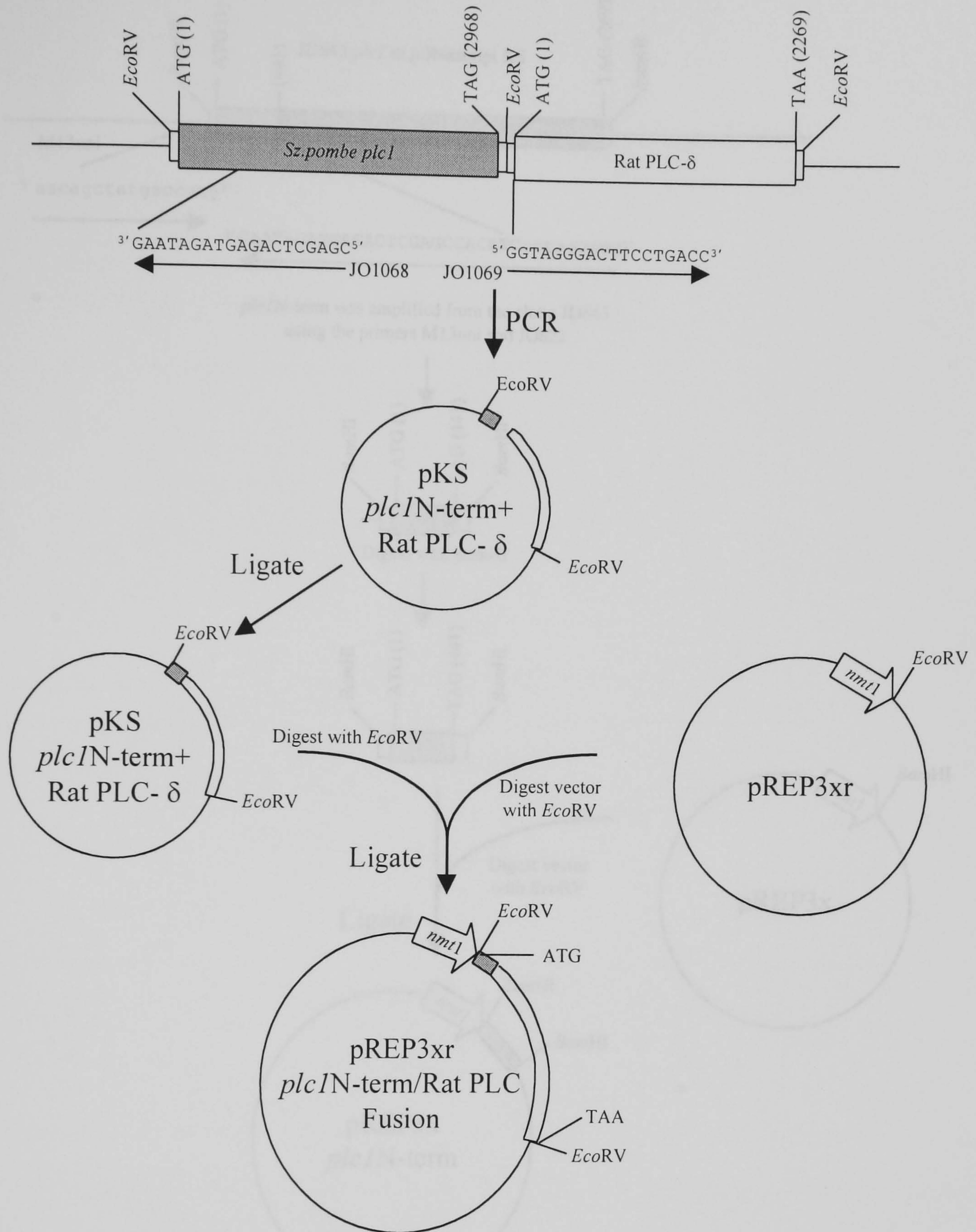


Figure 23b Exchanging domains between *Sz.pombe plc1* and Rat PLC- δ

The primers JO1068 and JO1069 were used to amplify the N-terminal section of *Sz.pombe plc1*, the Rat PLC- δ gene and the pKS vector through inverse PCR. This created a 5674 bp PCR product which was ligated to circularise the pKS vector. The fusion product could then be digested with *EcoRV* and ligated into pREP3xr vector.

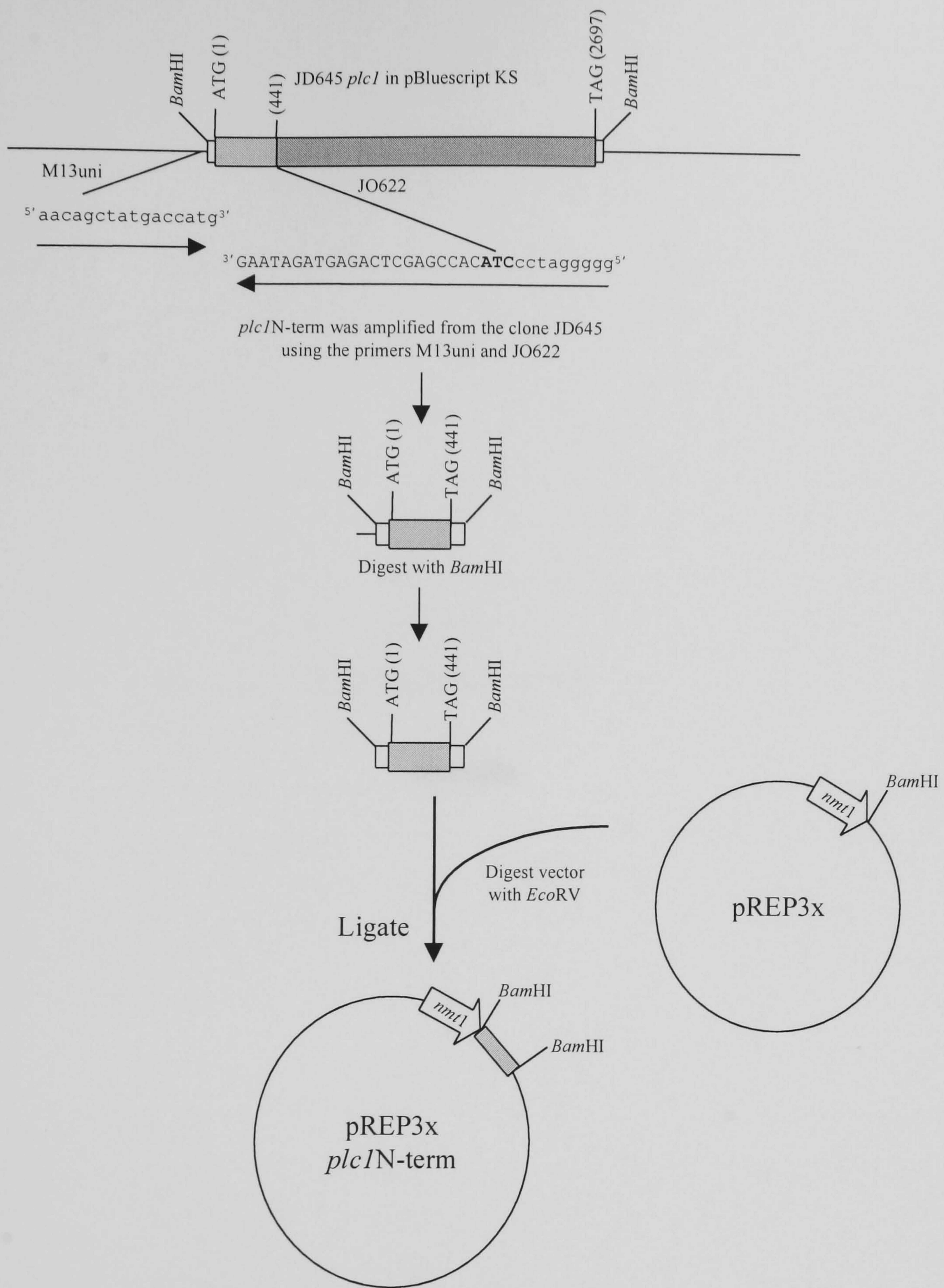


Figure 24 Amplification of the N-terminal domain of *plc1*

Using the primers M13uni and JO622 the N-terminal fragment of *plc1* was amplified. The template used was the construct JD645 (*plc1* in the vector pBluescriptKS⁺). JO622 introduces a stop codon ATC (bold) at amino acid position 148. Once amplified and digested with *Bam*HI this construct, *plc1*N-term was cloned into the yeast expression vectors pREP3x and pREP81x.

Results

Chapter 4
Disruption of *plc1*

Introduction

The *plc1* gene was identified in *Sz. pombe* by several groups simultaneously (Ando *et al.*, 1995; Fankhauser *et al.*, 1995; Slaaby and Davey, 1995). Slaaby and Davey, 1995, demonstrated that *Sz. pombe* deficient in Plc1 ($\Delta plc1$) grow slowly at low temperatures on rich media and exhibit swollen morphology. When cultured under unfavourable conditions, such as high temperature or on minimal media, the cells are unable to grow. This restrictive growth of $\Delta plc1$ cells will help to determine the functional role of Plc1 *in vivo*. The selective expression of mutants or varied expression levels within $\Delta plc1$ cells may induce changes in phenotype helping to identify the role of Plc1 within the cell. This unhealthy phenotype displayed by $\Delta plc1$ cells meant that the *plc1* disruptant strain created by Slaaby (1995) died in a freezer break down so a new *plc1* disruptant *Sz. pombe* cell needed to be produced.

This chapter will summarise the construct produced to disrupt the *plc1* gene and will describe the Southern blot analysis of *plc1::ura4⁺* disruptants. Following the conformation of the *plc1* disruptants through Southern blot it will discuss the studies performed helping to identify the role of Plc1 within the cell.

4.1.1 Creation of the *plc1::ura4⁺* disruption construct

The C-terminal section of the X catalytic domain and some of the Y catalytic domain were replaced with the selectable marker gene *ura4⁺* (see Chapter 3). Briefly, pK*Splc1* containing both the untranslated regions upstream (5') and downstream (3') was digested with *Hind*III. The *Hind*III digested 2.5 kb fragment containing three quarters of the ORF of *plc1* and some of the untranslated 3' region was sub-cloned into the *Hind*III site in pKS. An *Nru*I and *Bst*BI digest was performed on this new clone removing 670 bp which encompasses three-quarters of the X and some of the Y catalytic domain. The *Nru*I and the *Bst*BI sites were end-filled and phosphatase treated. The *ura4⁺* cassette contains *Bam*HI sites at the end for cloning purposes but it needed to be cloned into the end-filled *Nru*I and *Bst*BI sites in *plc1*. The *Bam*HI sites on the *ura4⁺* cassette were end-filled and cloned into *Nru*I and *Bst*BI sites of the *plc1* construct. The insertion of the *ura4⁺* cassette into the ORF of *plc1* created the disruption construct. This 3.67 kb fragment can be purified after digestion with *Hind*III (Figure 25) and used for transformation into JY 383 and JY 402 (see Materials for genotypes). The construct was transformed into the yeast strains using the lithium acetate method and the stable *ura4⁺* transformants were initially screened by PCR. Potentially correct strains were confirmed by Southern blot analysis.

4.1.2 Southern blot analysis of *plc1::ura4⁺* disruptants

The introduction of a linear *Hind*III fragment containing the disruption construct replaces three quarters of the X domain and a small region of the Y domain (*Nru*I to *Bst*BI) of Plc1. Removal of the X and Y catalytic region was found to inactivate mammalian PLCs (Ellis *et al.*, 1993; Yagisawa *et al.*, 1994; Emori *et al.*, 1989). By replacing a section of the X and Y catalytic region with the *ura4* cassette it will remove a functional Plc1 enzyme from the genome. Genomic

DNAs from selected transformants were digested with *EcoRI*. The DNA was separated on a standard 1% agarose gel and transferred onto a nitrocellulose filter. The filter was then probed with a radiolabelled 638 bp *plc1* fragment isolated from pKS*plc1* by digestion with *ClaI* and *NruI*.

The Southern blot (Figure 25) identified the strains that had a single *ura4*⁺ integration at the *plc1* locus. The integration of the *ura4* cassette was identified by a 3.95 kb band, compared to the 2.77 kb band observed from a wild-type strain. Once a *plc1* disruptant strain was identified the characterisation studies could be performed.

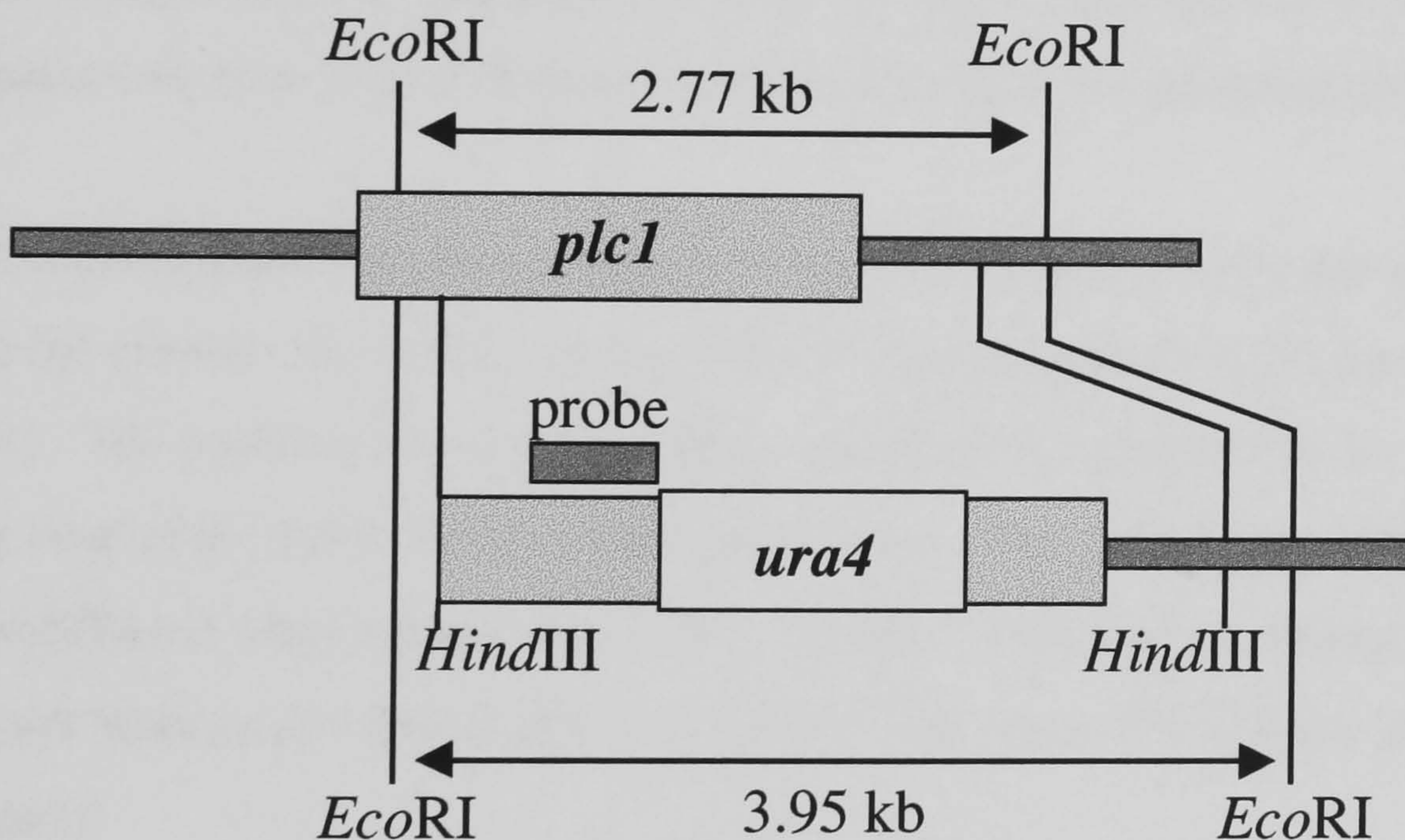
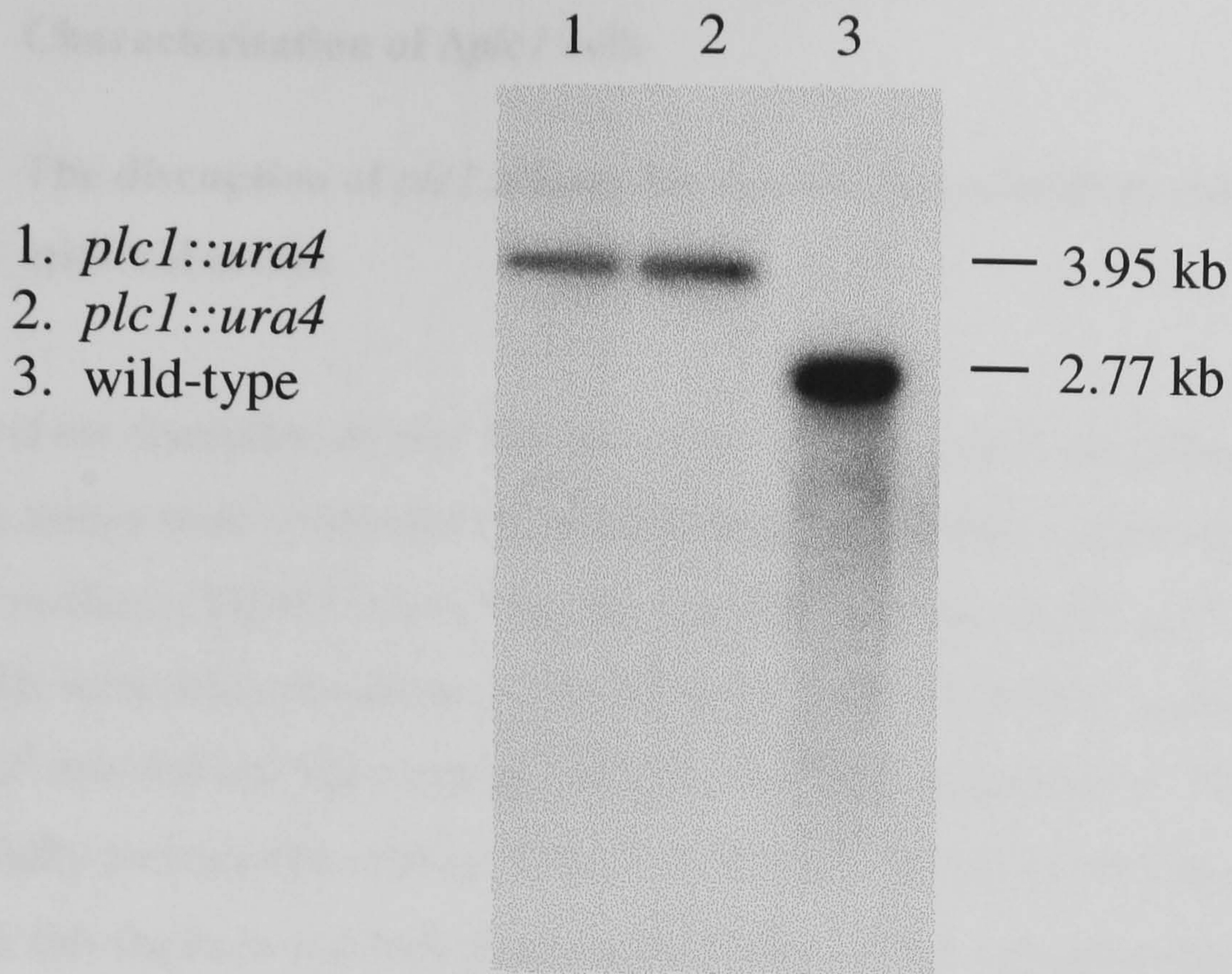


Figure 25 Southern blot analysis of $\Delta plc1$ strains

plc1 was disrupted in a *P* strain (JY383). Potential *plc1* disruptants were isolated by PCR and then confirmed through Southern blot. An *EcoRI* digest enabled the identification of the *ura4* intergrants as the probe binds to a 3.95 kb band. The strains where *plc1* has not been disrupted the probe binds to 2.77 kb band. The *HindIII* sites indicate the construct which was transformed into wild-type *Sz. pombe* cells (JY383) to disrupt *plc1*.

4.2 Characterisation of $\Delta plc1$ cells

4.2.1 The disruption of *plc1* affects the growth rate of cells in rich and minimal media

To see if the disruption of *plc1* had any effect on cell growth the following growth assays were conducted to characterise *plc1*. Briefly, cells were cultured in rich medium (YEALU) until they reached a concentration of 5×10^6 cells/ml. The cells were then sub-cultured into either YEALU or DMM at a concentration of 1×10^5 cells/ml and left overnight at 23°C in a shaking incubator. The reason for initially growing the cells in YEALU is because $\Delta plc1$ cells are known to grow in this medium and then they are transferred to the required medium to assay their growth rates. The cells were then counted after 12 hrs period. If the growth assay was concerned with cellular growth at 37°C then after 12 hrs the flask was moved to a 37°C shaking water bath. The cell concentration was determined every hour over a 10-hour period to determine the doubling time.

Figure 25 demonstrates that the disruption of the *plc1* ORF in *P*-cells had some effect on the growth rate of cells in rich (YEALU) medium at 23°C (Figure 26 top panel). The doubling time of *plc1*⁺ strain was 3 hours compared to the doubling time of the $\Delta plc1$ strain, which was 6 hours. The growth rate of these cells was different when measured at 37°C. The *plc1*⁺ cells had a doubling time of 2.5 hours whereas the $\Delta plc1$ cells were unable to grow at 37°C (Figure 26 lower panel).

When the doubling time of *plc1*⁺ is compared to $\Delta plc1$ in minimal (DMM) media it can be concluded that $\Delta plc1$ cells are unable to grow irrespective of the temperature (Figure 27 top and bottom panels). Even at a normally viable temperature of 23°C $\Delta plc1$ cells were unable to grow in DMM. Conversely, *plc1*⁺ cells are able to grow at both 23°C and 37°C with doubling times of 5

hours and 3.5 hours respectively. This suggests that $\Delta plc1$ cells are unable to grow in minimal media.

It appears that under stressful conditions such as high temperature or minimal media $\Delta plc1$ cells are unable to grow. Under less stressful conditions such as rich media and low temperatures there might be an alternative pathway that is able to compensate for the loss of Plc1 activity. When these cells are placed under more stressful conditions such as high temperature and minimal media these pathways may not now be able to compensate resulting in cell death. It has been shown in *S. cerevisiae* that mutant forms of PLC1 (PLC1-2 and PLC1-4) which had no detectable activity *in vitro* but was detectable through immunoblotting were able to complement for the loss of PLC1 activity at 25°C in $\Delta PLC1$ cells. This suggests that very small amounts of PLC1 activity is required for cell growth at 25°C (Yoko-o, *et al* 1995). At 37°C however, PLC1-2 and PLC1-4 were unable to complement and it was calculated that at least one seventh of the wild-type activity is required for cell growth (Yoko-o, *et al* 1995).

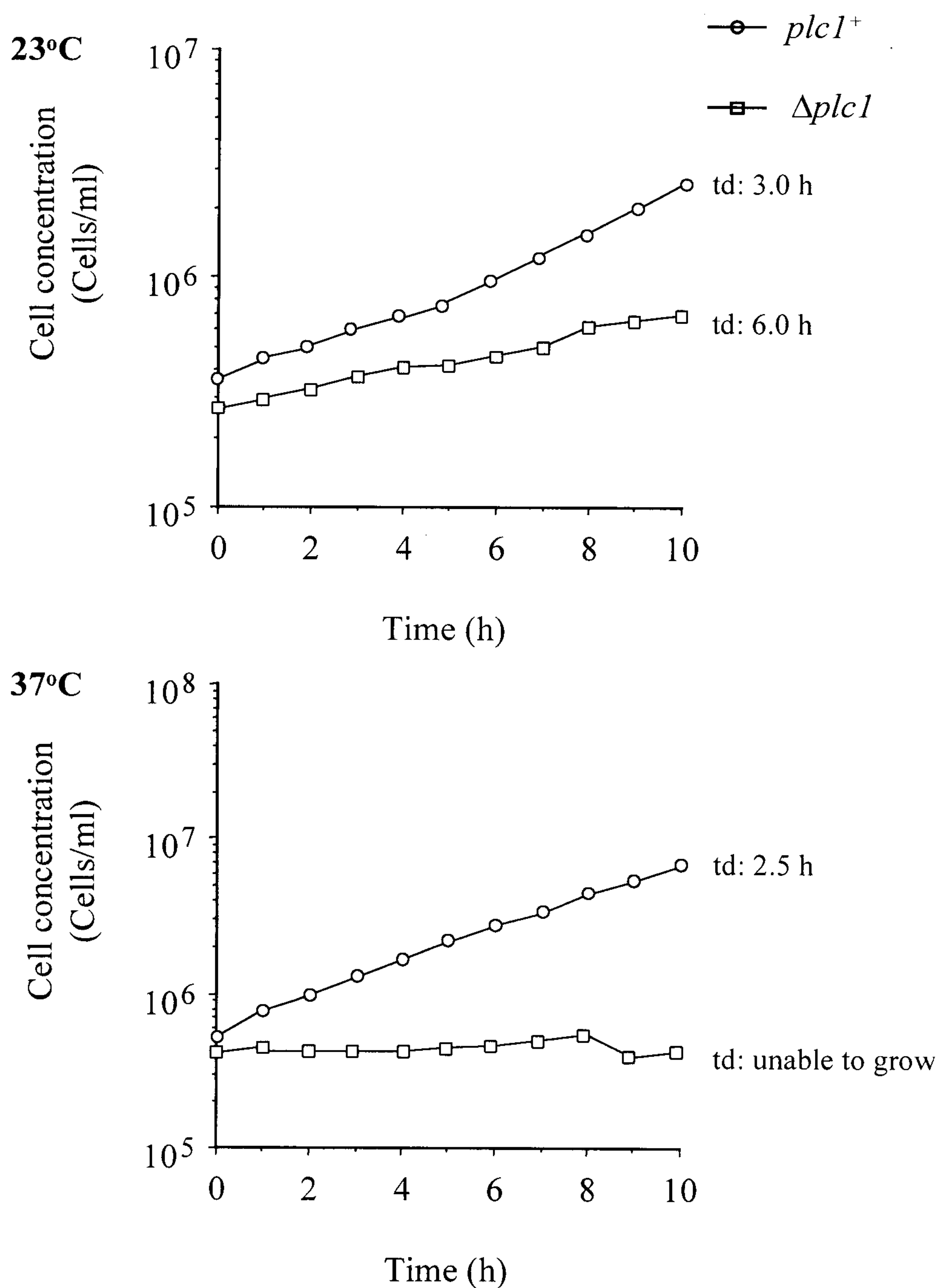


Figure 26 Growth rate of $\Delta plcl1$ in rich medium (YEALU)

Measurement of the growth rate of *plcl*⁺ (circles) and the $\Delta plcl1$ (squares) strains. The top panel demonstrates the ability of these cells to grow in rich media at 23°C. The bottom panel demonstrates when exponentially growing cells at 23°C were transferred to a 37°C shaking water bath at 0 hrs. The doubling time (td) of each strain is indicated to the right of the graph

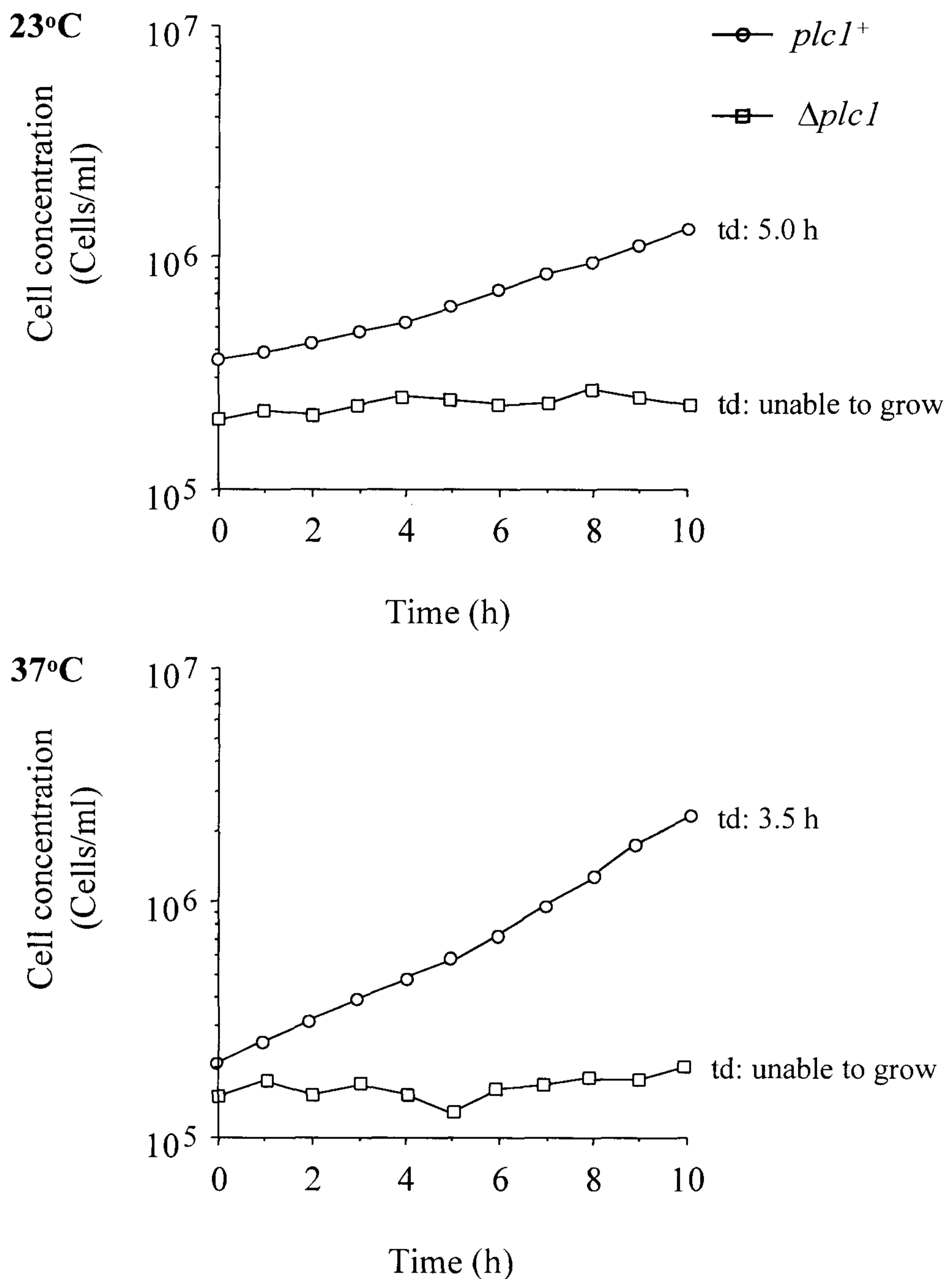


Figure 27 Growth rate of Δ *plc1* in minimal medium (DMM)

Measurement of the growth rate of *plc1*⁺ (circles) and the Δ *plc1* (squares) strains. Before the growth rate of Δ *plc1* cells were measured in DMM they were grown in YEALU, these cells were then collected through centrifugation and resuspended in DMM at a concentration of 1×10^5 cells/ml. The top panel demonstrates the ability of these cells to grow in DMM at 23°C. The bottom panel demonstrates when exponentially growing cells at 23°C were transferred to a 37°C shaking water bath at 0 hrs. The doubling time (td) of each strain is indicated to the right of the graph.

4.2.2 Expression of Plc1 *in vivo*

Wild-type *plc1* was cloned into the expression vectors pREP3x and pREP81x (for constructs see Chapter 3). The constructs pREP3x*plc1* pREP81x*plc1*, pREP3x and pREP81x were transformed into $\Delta plc1$ cells. Approximately five thousand of these transformed cells were plated onto DMM plates (see Methods 2.2.4) with or without thiamine. These plates were incubated at either 23°C or at 37°C and colony formation was recorded by photographing plates on the gel documentation system (see Methods, section 2.1.5).

$\Delta plc1$ cells are unable to grow on DMM plates irrespective of the thiamine content or the temperature at which they are incubated (Figure 28). $\Delta plc1$ cells that have been transformed with pREP3x or pREP81x alone are unable to grow on DMM plates. As expected, these cells display a phenotype similar to $\Delta plc1$ cells (Figure 28).

When pREP3x*plc1* and pREP81x*plc1* are expressed *in vivo* the cells display a phenotype similar to *plc1*⁺ cells. The $\Delta plc1$ cells expressing pREP3x*plc1* or pREP81x*plc1* are unaffected by temperature or minimal medium which normally results in $\Delta plc1$ cell death. Expression levels are controlled by the *nmt1* promoters contained on pREP3x (highest level of expression) and pREP81x (lowest level of expression). As discussed in Chapter 3, thiamine controls the expression levels of these promoters. In the absence of thiamine the promoter is switched on but in the presence of thiamine the expression level of the promoter is substantially reduced but not fully switched off. The strong growth of cells transformed with pREP81x*plc1* (Figure 28) indicates that only a small amount of Plc1 activity is required for $\Delta plc1$ cells to be rescued on DMM plates at 37°C.

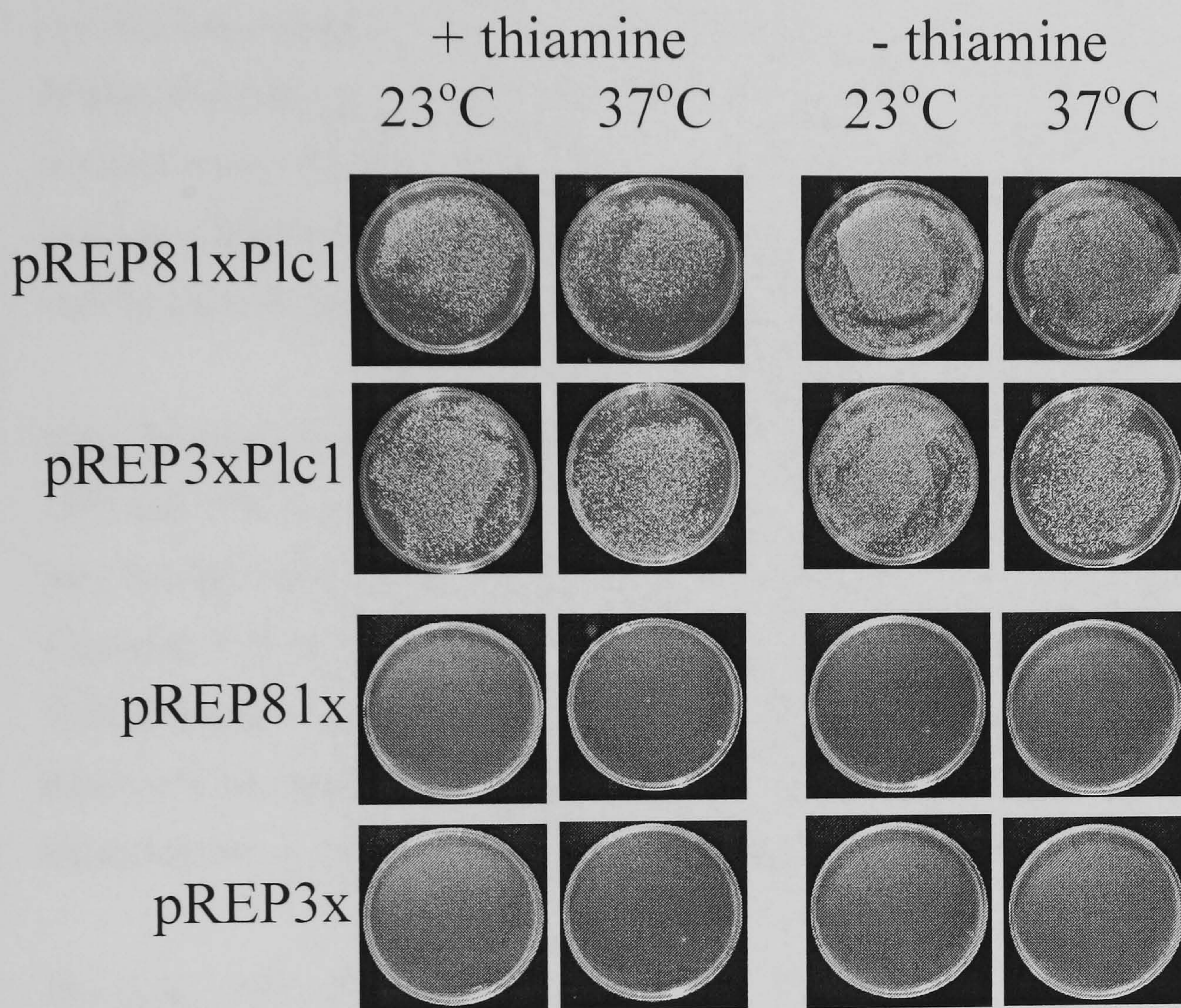


Figure 28 Expression of Plc1 *in vivo*

pREP3x*plc1*, pREP81x*plc1*, pREP3x and pREP81x alone were transformed into $\Delta plc1$ cells. Approximately five thousand of these transformants were spread onto DMM plates either with or without thiamine (see Methods 2.2.4). The plates were placed at either 23°C or at 37°C and colony formation was recorded.

4.2.3 Determining the cellular phenotype of $\Delta plc1$ cells

To determine the cellular phenotype of individual wild-type cells and $\Delta plc1$ cells, the cells were cultured on microscope slides (see Methods section 2.2.3).

Briefly, the cells are placed on a microscope slide containing a small amount of medium containing 1% LMP agarose. A cover slip is placed over the top and the cells are incubated at the required temperature for 24 hours. The cells are then viewed using the Nomarski digital camera (see Methods section 2.2.3).

From the growth assays it was shown that $plc1^+$ cells are able to grow at both 23°C and 37°C (see Figure 29). When the $plc1^+$ cells were viewed after 24 hours they formed colonies at both 23°C and at 37°C (Figure 29). The colonies that are formed at 23°C consist of about 10 to 15 cells and these cells can be described as ‘barrel shaped’. The colonies formed at 37°C consist of about 25 to 30 cells but these cells are smaller and fatter (Figure 29). This shape is probably due to the increased rate at which these cells are dividing due to the increased temperature.

The $\Delta plc1$ cells cultured at 23°C for 24 hrs divided in an irregular fashion (Figure 29) and colony formation was different to the wild-type cells. When $\Delta plc1$ cells were observed at 37°C after a 24 hrs period, no colonies were observed; instead there were elongated cells, which were unable to divide (Figure 29). Sometimes there were cells that were up to 4-fold longer than wild-type cells and occasionally bulbous-shaped cells were observed (Figure 29).

4.3 Summary

A construct was designed to produce a $\Delta plc1$ cell. This linear piece of DNA containing the *ura5* gene was transformed into a *plc1* strain and the cells were cultured on minimal media. The $\Delta plc1$ disruptant cells were unable to grow on minimal media. The $\Delta plc1$ cells were then transformed with a construct containing a *URA5* gene under the control of the *plc1* promoter. When the $\Delta plc1$ cells were cultured in rich medium they were able to grow at 37°C. When the growth rate of $\Delta plc1$ cells was determined in minimal media at 23°C and 37°C, the growth rate of $\Delta plc1$ cells was determined to be similar to wild-type cells at 23°C. This suggested that the $\Delta plc1$ cells were able to grow on minimal media at 23°C. The $\Delta plc1$ cells were able to grow on minimal media at 23°C. When *pK273-*plc1** or *pK274-*plc1** was transformed into $\Delta plc1$ cells they were able to rescue the phenotype. The $\Delta plc1$ cells were now able to grow on minimal media at 37°C.

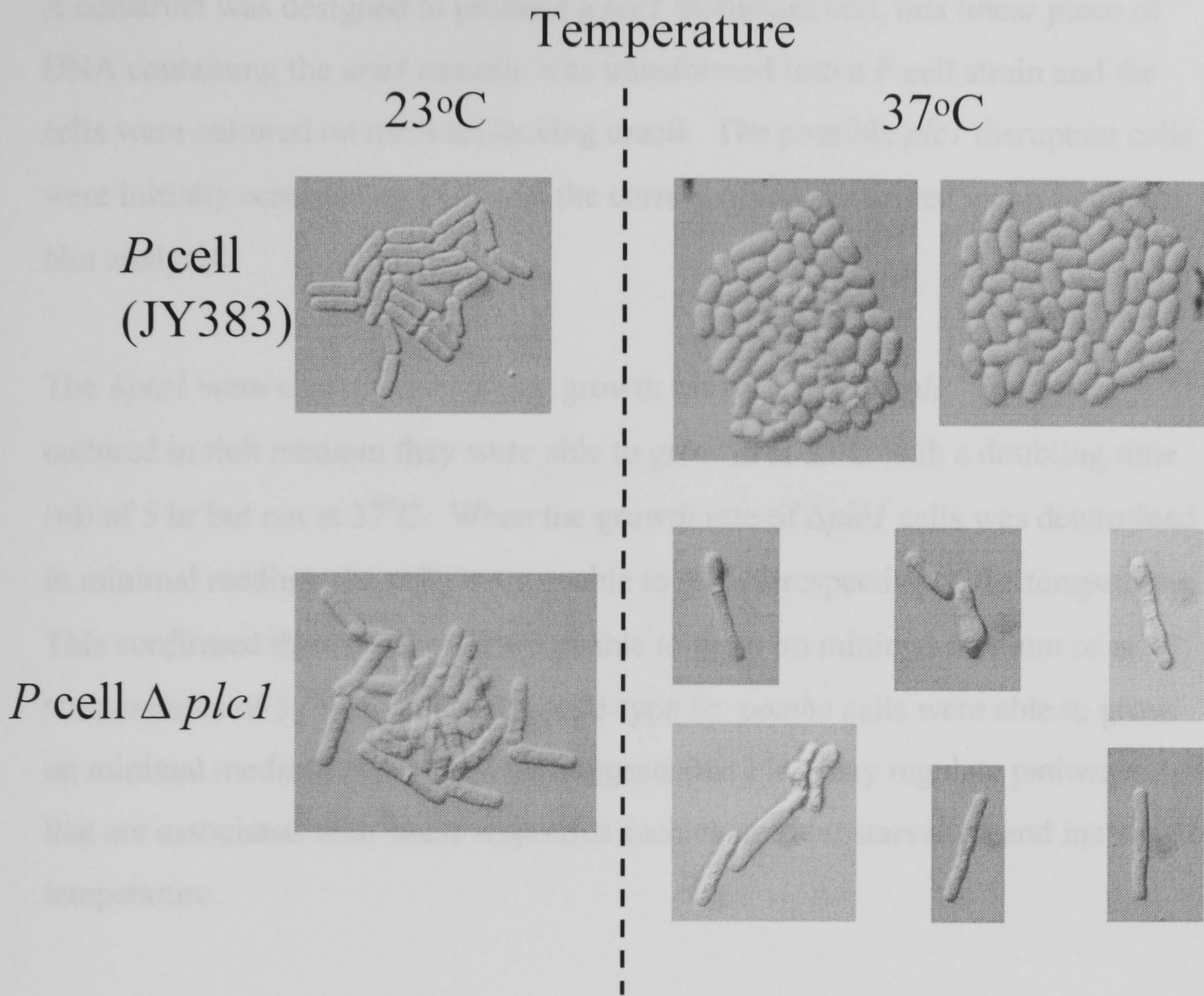


Figure 29 Phenotype of $\Delta plc1$ cells

Pictures showing the growth of *Sz. pombe* cells after 24 hrs incubation at 23°C or at 37°C in slide culture on rich media. The top panel displays wild-type *P*-cells, the bottom panel displays $\Delta plc1$ cells. These photographs were taken on a Nomarski digital camera.

4.3 Summary

A construct was designed to produce a *plc1* disruptant cell, this linear piece of DNA containing the *ura4* cassette was transformed into a *P* cell strain and the cells were cultured on medium lacking uracil. The possible *plc1* disruptant cells were initially screened by PCR and the correct $\Delta plc1$ confirmed using Southern blot analysis.

The $\Delta plc1$ were characterised using growth assays. When $\Delta plc1$ cells were cultured in rich medium they were able to grow in at 23°C with a doubling time (td) of 5 hr but not at 37°C. When the growth rate of $\Delta plc1$ cells was determined in minimal medium the cells were unable to grow irrespective of the temperature. This confirmed that $\Delta plc1$ cells are unable to grow on minimal medium or at temperatures of 37°C. Conversely, wild-type *Sz. pombe* cells were able to grow on minimal medium at 37°C. This suggests that Plc1 may regulate pathways that are associated with stress responses such as nutrient starvation and increased temperature.

When pREP3*xplc1* or pREP81*xplc1* was transformed into $\Delta plc1$ cells they were able to rescue the phenotype. The $\Delta plc1$ cells were now able to grow on minimal medium at 37°C.

These studies confirmed that the characteristic phenotypes of $\Delta plc1$ *Sz. pombe* cells, observed by other groups (Andoh *et al.*, 1995; Fankhauser *et al.*, 1995; Slaaby and Davey, 1995) were also detected in this *plc1* disruption strain.

Chapter 5
The characterisation of Plc1

5.1 Introduction

Plc1 activity is not detectable in wild-type *Sz. pombe* cells, so before the characterisation of Plc1 could be carried out, an assay needed to be developed where Plc1 activity could be measured *in vitro*. This chapter describes how *plc1* was expressed and once activity was detected how its activity was determined. A lot of different substrate mixtures are used to assay PLC activity so it also discusses which substrate mixture was selected and why. It finally describes the characterisation studies including temperature and pH profiles that allowed the optimum assay conditions for Plc1 to be identified. This assay is used to determine how active the Plc1 mutants are *in vitro* (discussed in Chapters 6-8).

5.1.1 An assay to determine the activity of Plc1

Sz. pombe cells expressing *plc1* are cultured in liquid culture, the cells are homogenised and a crude extract of Plc1 is produced (see Methods 2.2.2.1). The assay is outlined in detail in the Methods 2.2.2.2; briefly, the crude extract containing expressed Plc1 is added to a substrate containing $^3\text{H-PIP}_2$ and incubated at 30°C for the required length of time. After the required length of time a mixture of chloroform / methanol is added to the reaction mix to separate the substrate $^3\text{H-PIP}_2$ (which is hydrophobic) from the product $^3\text{H-InsP}_3$ (which is hydrophilic). The $^3\text{H-InsP}_3$ contained within the aqueous phase can be withdrawn and measured using a liquid scintillation counter (see Methods 2.2.2.2). The rate of PIP_2 hydrolysis under different assay conditions can be calculated by plotting IP_3 production against time. These linear sections of graphs can be used to determine the rate at which PIP_2 is hydrolysed to InsP_3 and DAG over a period of 20 min / mg of total protein (see appendix for an example of the calculations). Total protein concentration is used because the activity of Plc1 is determined in a crude extract prepared from cells and Plc1 is not purified (see Methods 2.1.4 for protein concentration determination)

5.1.2 The pREP expression vectors

The pREP vectors are based upon the thiamine-repressible *nmt1* promoter. The *nmt1* gene product is involved in thiamine biosynthesis and consequently the level of cellular thiamine tightly regulates the promoter. Therefore, in the absence of external thiamine it is highly expressed but is repressed by the addition of thiamine to media (Maundrell, 1990; Tommasino, and Maundrell, 1991). Different expression levels can be obtained by using different versions of the pREP vectors that contain mutations within their TATA boxes resulting in different expression from the *nmt1* promoter (Basi, *et al.*, 1993). There are three levels of expression that can be attained, the highest level of expression can be achieved by using pREP3x, the intermediate level through pREP41x and the lowest level is through pREP81x. The expression levels of these pREP vectors can be reduced by 10 fold with the addition of 167 μ g/ml of thiamine to the growth medium.

5.1.3 Expressing Plc1 in *Sz. pombe*

Since Plc1 activity in wild-type *Sz. pombe* cells is undetectable when measured *in vitro*, expression of *plc1* was required so that its activity could be measured *in vitro*. The *plc1* gene was therefore, cloned into pREP3x (JD627), pREP41x (JD628) and pREP81x (JD629) by Rita Slaaby. These plasmids were transformed into the *Sz. pombe* strains JY330 and JY402, mating type-stable *P* cell and *M* cell, respectively. These cells were inoculated into two cultures the first was DMM containing 167 μ g/ml of thiamine and the second was DMM in the absence of thiamine. A crude extract was made from these cells (see Methods 2.2.2.1) and activity was measured using the Plc1 assay 2.2.2.2). The rate at which PIP₂ was hydrolysed into InsP₃ and DAG was calculated from linear sections of a time course graph (see appendix for examples).

Figure 30 demonstrates the different levels of Plc1 activity detected *in vitro* using the three pREP vectors. It also displays the difference in levels of activity both in the presence and absence of thiamine. Figure 30 demonstrates that wild-type cells alone have undetectable levels of Plc1 activity, as do wild-type cells transformed with pREP3x alone. The bars on the graph represent the level of background activity probably caused by the natural breakdown of PIP₂. It can also be observed that Plc1 under the control of the *nmt1* promoter on pREP3x cultured in the absence of thiamine has the highest level of activity, with pREP81x being the lowest and pREP41x being the intermediate. Extract produced from cells cultured in the presence of thiamine demonstrates that there is very little difference in activity detected between the different pREP vectors (pREP3x-*plc1* and pREP81x-*plc1* have the same amount of activity, data not shown). Extracts prepared from cells cultured in the presence of thiamine (see Methods 2.2.2.1) have very low Plc1 activity levels irrespective of the *nmt1* promoter selected. This Plc1 activity however, is still higher than extracts prepared from wild-type cells. This suggests that even in the presence of thiamine the *nmt1* promoter is not fully switched off.

Figure 30 also demonstrates that *M* cell and *P* cells have comparable levels of Plc1 expression, so all other *in vitro* studies were carried out in *P* cells.

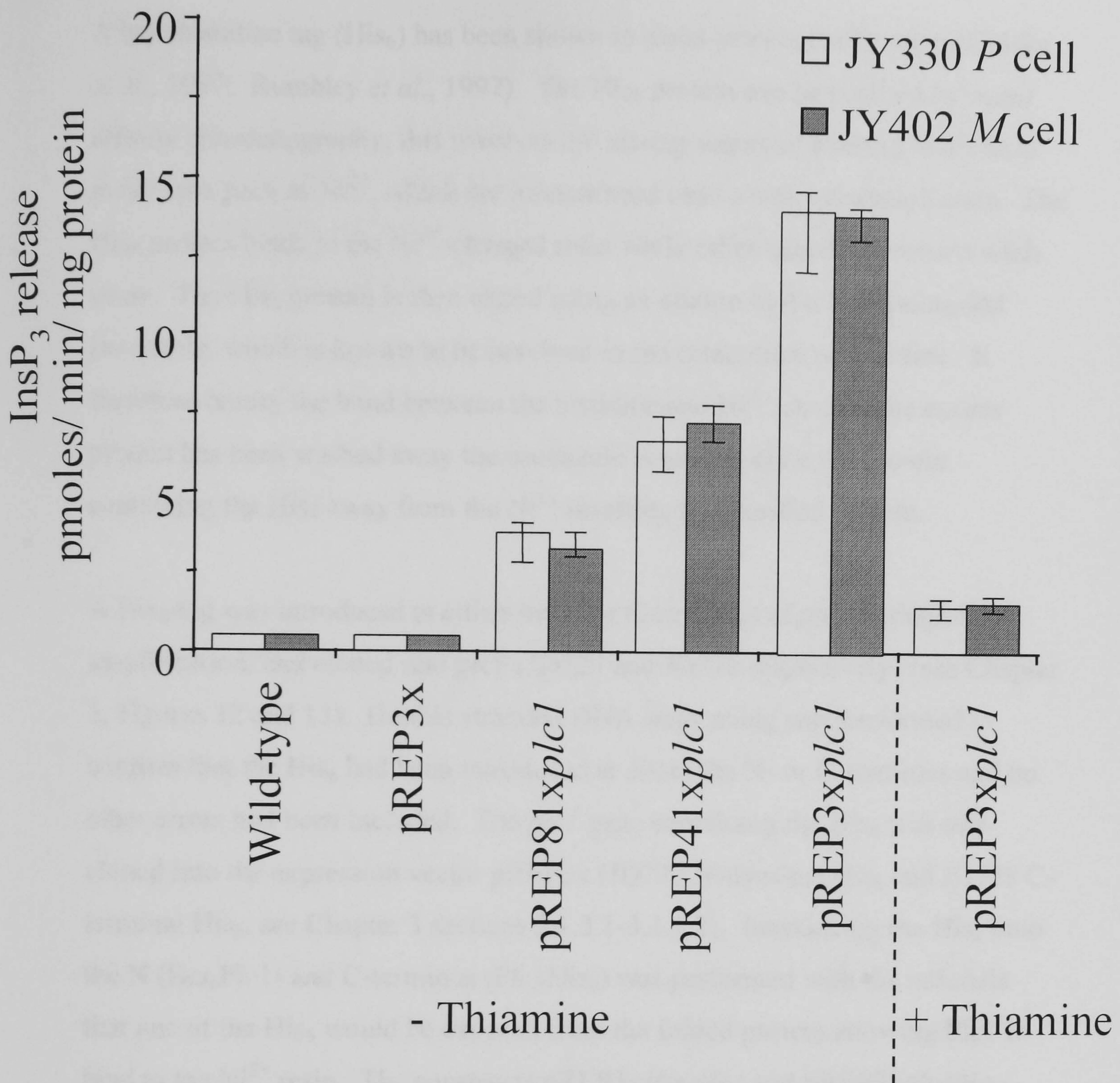


Figure 30 Activity levels of pREP $plc1$ in M and P cells

Wild-type cells and cells containing pREP3x alone have undetectable levels of Plc1 activity. Wild-type cells expressing Plc1 from the *nmt1* promoters in the absence of thiamine have different levels of expression, with pREP3x $plc1$ being the highest and pREP81x $plc1$ being the lowest. When pREP3x $plc1$ is expressed in the presence of thiamine, the levels of Plc1 activity reduced to almost wild-type cell levels. Activity of Plc1 was used to measure the expression levels of the *nmt1* promoters. The rate of InsP₃ production by Plc1 was calculated using the linear sections of graphs (see Appendix for an example of the calculations). The assay determining Plc1 activity under the control of different *nmt1* promoters was performed at pH 7.5, 30°C and using a sodium cholate concentration of 6.4% w/v. The bars represent the standard error of the mean for triplicate determinations.

5.1.4 Expression of Plc1 containing the Hexahistidine-tag

A hexahistidine tag (His₆) has been shown to assist protein purification (Hidaka *et al.*, 1997; Rumbley *et al.*, 1997). The His₆ protein can be purified by metal affinity chromatography, this involves the his-tag sequence binding to divalent metal ions such as Ni²⁺, which are immobilised onto a metal chelation resin. The His₆ protein binds to the Ni²⁺-charged resin while other unbound proteins wash away. The His₆ protein is then eluted using an elution buffer containing 4M imidazole, which is known to be involved in the catabolism of histidine. It therefore breaks the bond between the histidine and Ni²⁺, so after the excess protein has been washed away the imidazole is used to elute the protein containing the His₆ away from the Ni²⁺ resulting in a purified protein.

A His₆-tag was introduced at either the N or C-terminus of *plc1* through PCR amplification, and cloned into pKS (JD725 and JD726 respectively) (see Chapter 3, Figures 12 and 13). Double stranded DNA sequencing was performed to confirm that the His₆ had been introduced at either the N- or C-terminus and no other errors had been included. The *plc1* gene containing the His₆ was sub-cloned into the expression vector pREP3x (JD727 N-terminal His₆ and JD728 C-terminal His₆, see Chapter 3 sections 3.1.3.1-3.1.1.2). Introducing the His₆ onto the N (His₆Plc1) and C-terminus (Plc1His₆) was performed with the rationale that one of the His₆ would be exposed from the folded protein allowing Plc1 to bind to the Ni²⁺ resin. The constructs pREP3xHis₆*plc1* and pREP3x*plc1*His₆ were transformed into a *P* cell (JY330) (see Methods 2.2.1.4), since assays carried out on pREP vectors demonstrated there was no difference between activity levels of Plc1 expressed in *P* and *M* cells (Figure 30).

To confirm that the His₆ was not having an adverse effect upon Plc1 activity, the expressed proteins His₆Plc1 and Plc1His₆ were assayed *in vitro* using the Plc1 assay (see Methods 2.2.2.2). Figure 31 demonstrates that the activity of His₆Plc1 and Plc1His₆ was determined *in vitro* and the His₆ had no significant effect upon activity when compared to wild-type Plc1 expressed in a *P* cell. All further constructs produced contain the His₆ on the C-terminus of Plc1 (unless otherwise stated, see Chapter 3 Constructs) due to its activity *in vitro* appeared to be more similar to wild-type Plc1.

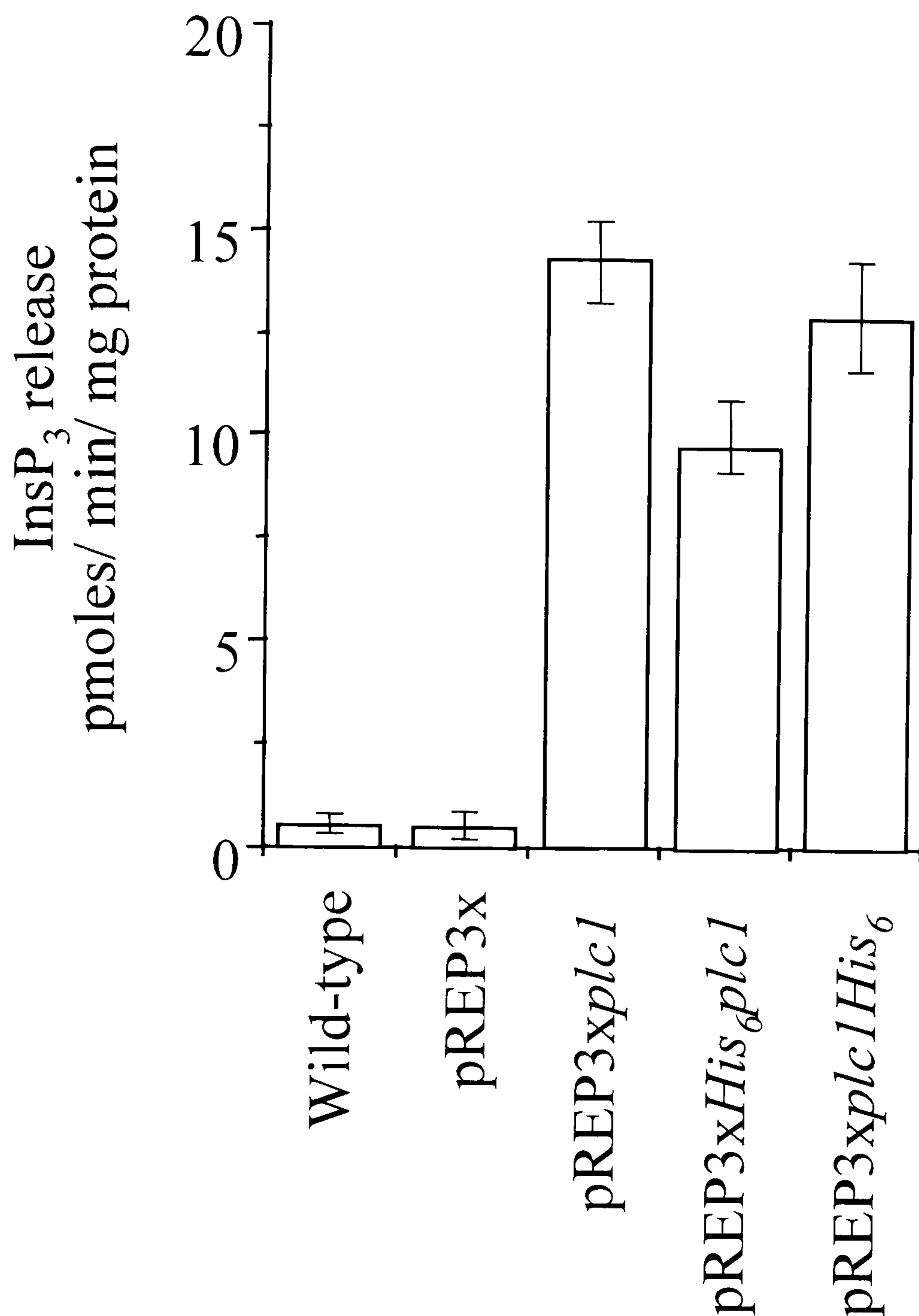


Figure 31 Determining the activity of His₆Plc1 and Plc1His₆

Expression of Plc1His₆ resulted in activity similar to wild-type Plc1. There was a small decrease in activity when the His₆ was on the N-terminus of Plc1 but the expression levels were still considerably higher than extract prepared from wild-type (JY330) cells. Activity of Plc1 was measured using the scintillation counter method (see Methods 2.2.2.2) using a substrate containing ³H-PIP₂, PIP₂ and a sodium cholate concentration of 6.4% w/v. The rate was determined at pH 7.5, and at 30°C. The bars represent the standard error of the mean for triplicate determinations.

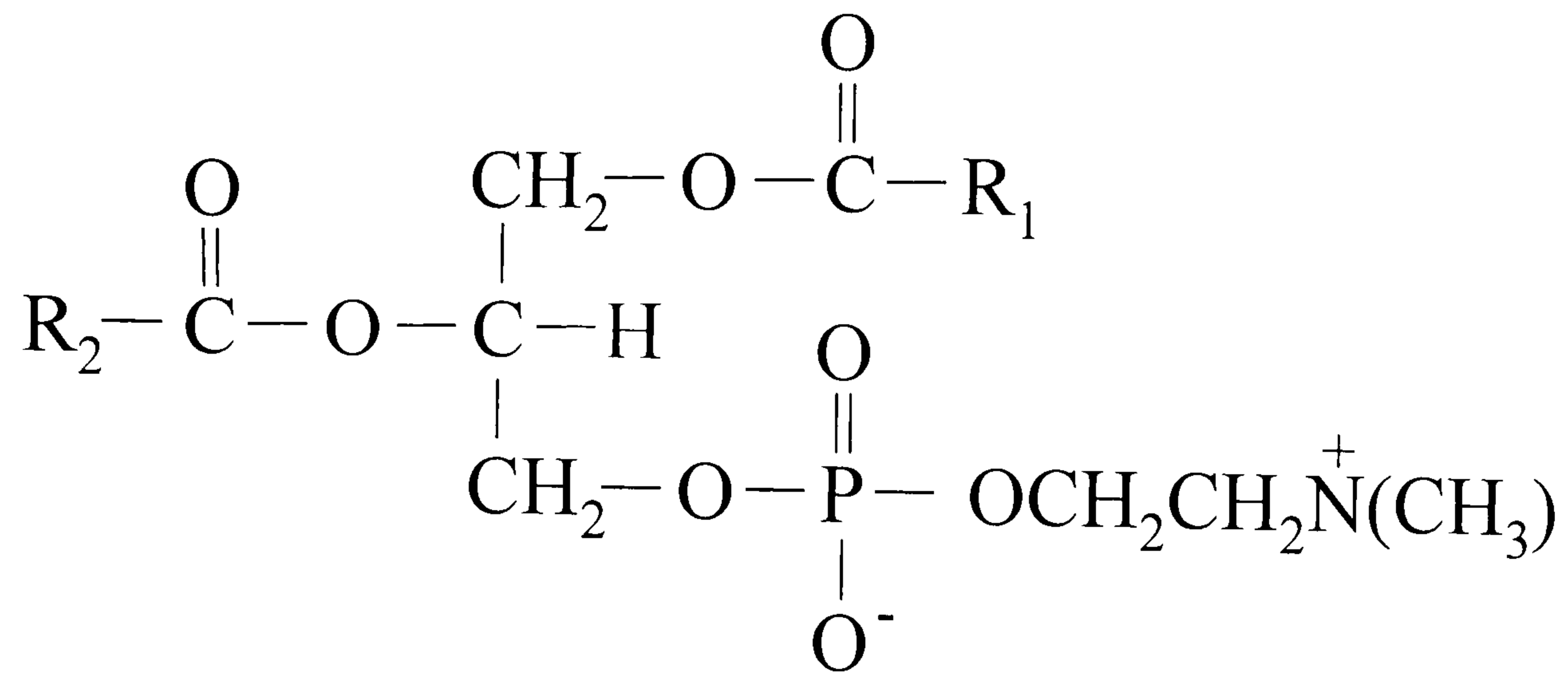
5.2 Possible substrate mixtures for Plc1

The substrate for phospholipase C has already been discussed in Chapter 1 (section 1.3), and it is also known from previous work performed by Rita Slaaby that Plc1 has the ability to hydrolyse PIP₂ into InsP₃ and DAG (discussed in Chapter 1 section 1.4.2). A common assay has been developed by many different research groups to determine the activity of certain phospholipases *in vitro*. The difference between these research groups, however, is the different compounds they mix with PIP₂, to create a substrate that allows the phospholipase to bind to and hydrolyse PIP₂. For example, phospholipids often found in mammalian membranes include phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are mixed with PIP₂ to create a substrate (Martin *et al.*, 1996). Lipid substitutes are also used such as sodium cholate (Katan and Parker, 1987) and dodecylmaltoside (Wang *et al* 1996). When sonicated all these substrate mixtures create a micellar structure that will permit phospholipase C to bind to their structure and facilitate PIP₂ hydrolysis.

5.2.1 Phosphatidylcholine and Phosphatidylethanolamine

PC and PE are phospholipids commonly found in eukaryotic cell membranes. They consist of a polar head group; for example PC has a polar head group comprising an inorganic phosphate attached to choline (Figure 32). They have a glycerol backbone which generally have two side chains composed of esterified fatty acids and alcohols (R₁ and R₂ Figure 32). These phospholipids (including PIP₂) are amphipathic so they contain both polar (hydrophilic) and non-polar regions (hydrophobic). When placed in an aqueous environment it causes these phospholipids to spontaneously arrange into ordered structures including, micelles, vesicles and membrane bilayers. It is possible that after sonication the PC, PE and PIP₂ form structures similar to the ones suggested above, permitting Plc1 to bind and hydrolyse PIP₂

Phosphatidylcholine (PC)



Phosphatidylethanolamine (PE)

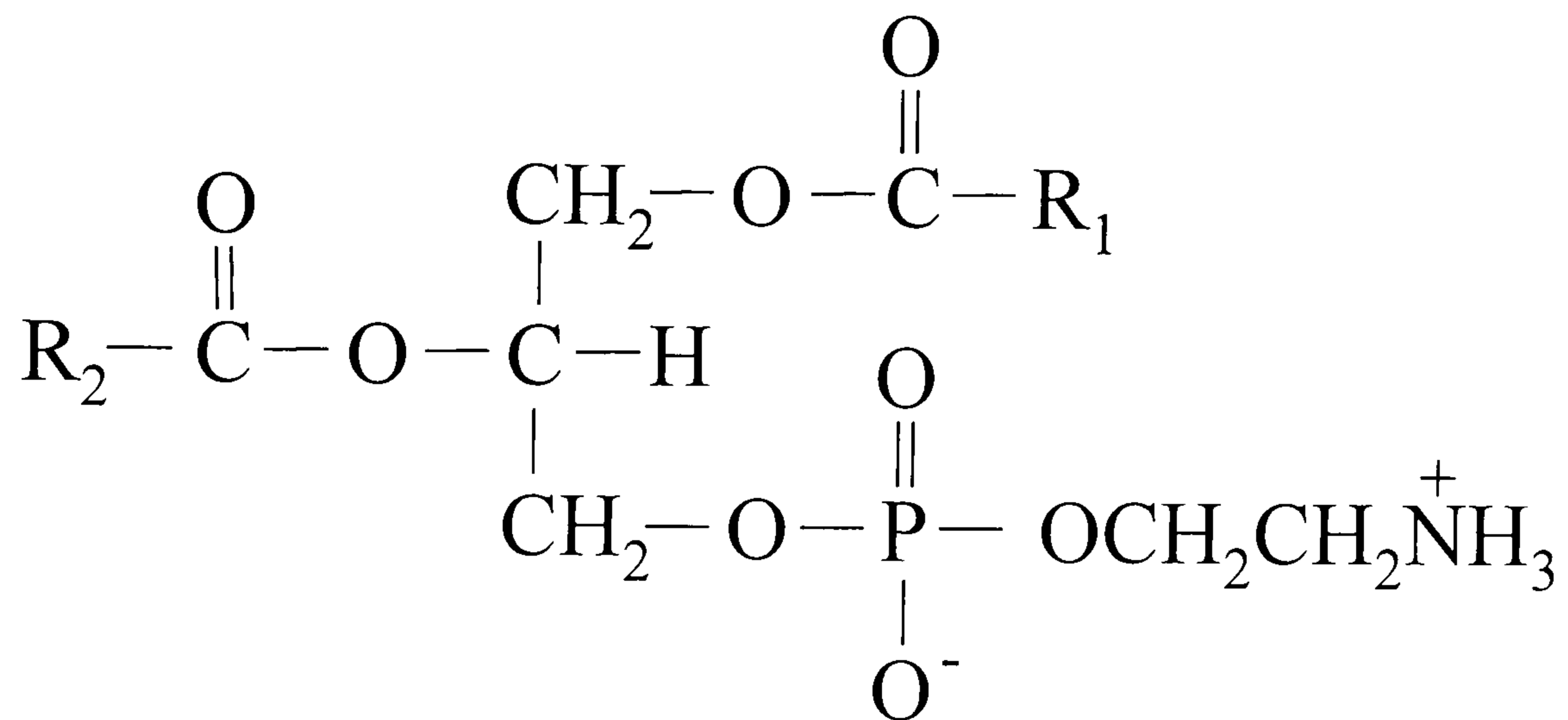


Figure 32 The chemical structures of PC and PE

PC and PE have very similar structures and they are common phospholipids that are found in eukaryotic membranes. They contain a polar head group which is hydrophilic and also contain hydrophobic side chains (R_1 and R_2). This results in them creating bilayers within cellular membranes, the polar head groups are exposed to the cytosol and external surface.

5.2.2 Dodecylmaltoside and sodium cholate

It has been demonstrated that the presence of increasing concentrations of non-substrate phospholipids reduces PLC activity indicating that these lipids may inhibit enzyme action (James *et al.*, 1995). PIP₂ can be mixed with alternative substrate mixtures such as dodecylmaltoside and sodium cholate (Wang *et al.*, 1996; Katan and Parker, 1987). Dodecylmaltoside and sodium cholate behave as an inert diluent of PIP₂ (Hepler *et al.*, 1993; James *et al.*, 1995; Wu *et al.*, 1997), which is able to create a mixed micellar system. Since PLCs operate with several lipid binding and then catalytic steps, enzyme activity is dependent upon the amount of PIP₂ that is exposed on the surface.

There are four possible substrate mixtures that can be used to determine the activity of Plc1; these include, PIP₂ alone or alternatively PIP₂ mixed with PC and PE or with dodecylmaltoside or with sodium cholate. Figure 33 demonstrates that when sodium cholate is mixed with PIP₂ it creates a micelle that could effectively exposes PIP₂ on the surface allowing Plc1 to bind and hydrolyse PIP₂. Dodecylmaltoside however, does not appear to create a micelle that exposes sufficient quantities PIP₂ on the surface. When Plc1 was assayed *in vitro* with this substrate mixture, undetectable levels of Plc1 activity were observed (Figure 33). When PC and PE were incorporated into the substrate mixture (Figure 33) the activity of Plc1 was ten-fold lower than when PIP₂ was mixed with sodium cholate, perhaps this is due to the inhibiting nature of these non-substrate phospholipids (James *et al.*, 1995). When PIP₂ was used alone as the substrate Plc1 activity was indistinguishable from background levels (Figure 33), this suggests that PIP₂ on its own is unable to form a substrate which allows Plc1 to bind and hydrolyse the PIP₂.

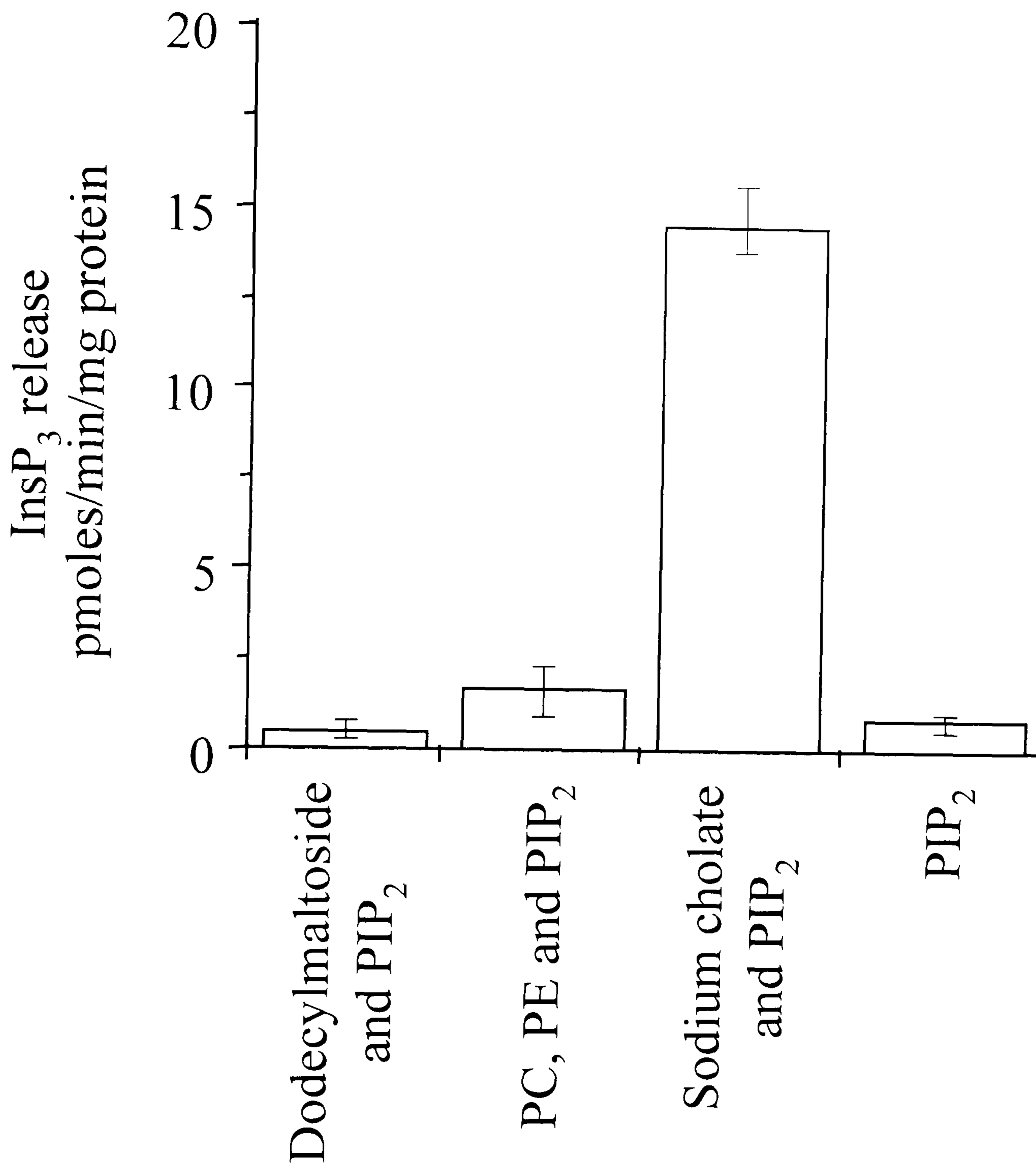


Figure 33 Possible substrate mixtures for Plc1

The ability of Plc1 to hydrolyse PIP₂ in the presence of other lipids (PC and PE) or lipid substitutes such as dodecylmaltoside and sodium cholate. The rate was calculated from linear plots as described in the text (see appendix for an example of the calculations). The assay conditions include pH 7.5 and at 30°C. The bars represent the standard error of the mean for triplicate determinations.

5.3 Optimising the conditions for Plc1 activity

5.3.1 A precise ratio of sodium cholate to PIP₂ is necessary for Plc1 activity

Since the concentration of sodium cholate added to the substrate may affect PIP₂ hydrolysis. It has been shown that if the concentration of PIP₂ is either too high or too low then Plc1 has a reduced binding and processing affinity for the substrate (James *et al.*, 1995), therefore, an optimum ratio of sodium cholate to PIP₂ was identified. *Sz. pombe* cells were transformed with pREP3x *plc1*His₆ and the resulting transformants were cultured in DMM minus thiamine. The *nmt1* promoter contained on the pREP vectors is switched on in the absence of thiamine but repressed in the presence of thiamine. Extracts were prepared from cells expressing Plc1His₆ and assays were completed in which the concentration of PIP₂ remained constant while the concentration of sodium cholate added to the PIP₂ substrate was increased from 0.16% to 9.6% w/v. The assays were performed at 30°C and in 50mM Tris buffer pH 7.5 (see Methods section 2.2.2.2). This demonstrated that there were significant differences in the ability of Plc1 to hydrolyse PIP₂ depending upon the concentration of sodium cholate within the substrate mixture. Figure 34 demonstrates that the optimum concentration of sodium cholate that is required for PIP₂ hydrolysis is 6.4%. Any deviation from this concentration of sodium cholate results in a rapid decrease in Plc1 activity. Values for the rate of reaction were calculated using a time course, measuring the production of IP₃ over a 20 min period. Linear sections of the graph were used to calculate the amount of InsP₃ produced /min /mg protein (see appendix for an example of the calculation).

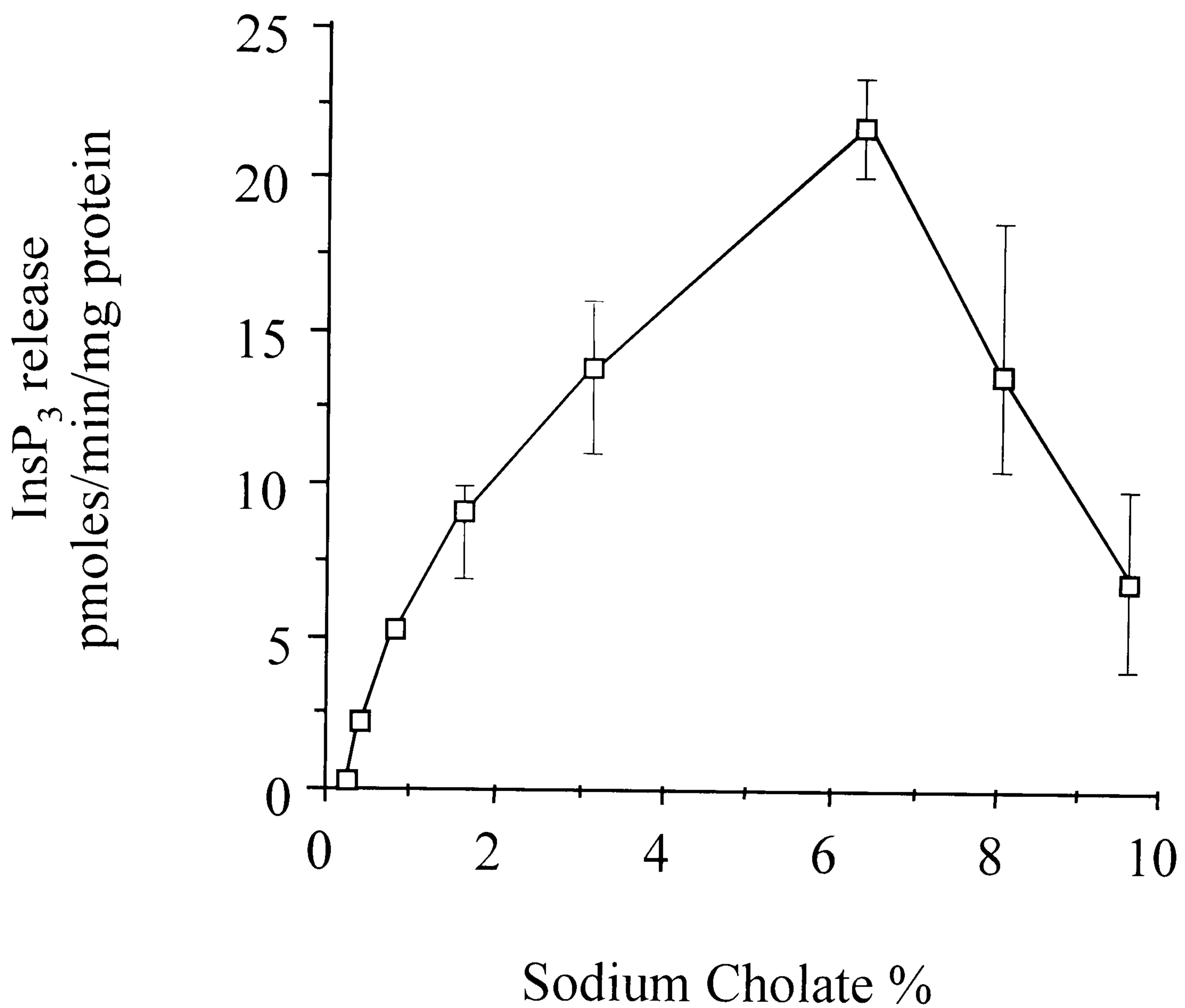


Figure 34 Identifying the optimum sodium cholate concentration when assaying Plc1 activity *in vitro*

The ability of Plc1 to hydrolyse PIP₂ under different concentrations of sodium cholate was investigated using the assay described in the Methods section 2.2.2.2. The rate was calculated using linear sections of time course graphs as described in the text (see appendix for examples). Activity is expressed in pmoles/min/mg protein at a temperature of 30°C and a pH of 7.5. The bars represent the standard range of the mean for triplicate determinations.

5.3.2 Plc1 activity is dependent upon temperature

Crude extracts of Plc1 were incubated and assayed at a range of temperatures to determine if Plc1 activity was dependent upon temperature. A crude extract was prepared from *Sz. pombe* cells transformed with pREP3xplc1His₆ (see Methods section 2.2.2.1) which were cultured in DMM minus thiamine (the *nmt1* promoter is switched on in the absence of thiamine, see Section 3.1.2). The crude extract containing the expressed Plc1 was incubated at temperatures ranging from 4°C to 50°C for a 20 min period and the level of Plc1 activity was detected using the scintillation counter (see Methods section 2.2.2.2). The rate was calculated from linear sections of time course graphs to give a temperature profile (calculations are described in the appendix). The temperature profile (Figure 35) displays a rapid increase in Plc1 activity from 4°C to 30°C and then a rapid decrease in activity as the temperature is increased to 42°C. Plc1 has a specific working temperature of 30°C in a 50mM Tris buffer pH 7.5 and a substrate containing 6.4% w/v of sodium cholate. Inactivation of Plc1 at high temperatures is irreversible (Table 1); extracts preincubated at temperatures above 42°C are unable to hydrolyse PIP₂ once the extracts are returned to 30°C. This indicates that Plc1 has been heat inactivated.

The substrate, a sodium cholate / PIP₂ mixture was not so sensitive to heat. When the substrate was preincubated at a range of temperatures it appeared to be intact up to 42°C (Table 1). After a 20 min preincubation the substrate was returned to 30°C and incubated with the extract. At this temperature Plc1 could still hydrolyse PIP₂ suggesting that the substrate had not been denatured by heat. Preincubation of the substrate at 50°C or above resulted in a small decrease in PIP₂ hydrolysis. This could be due to some of the micellular structures breaking down but as Plc1 was inactivated at this temperature this slight reduction would not have any effects on the assay.

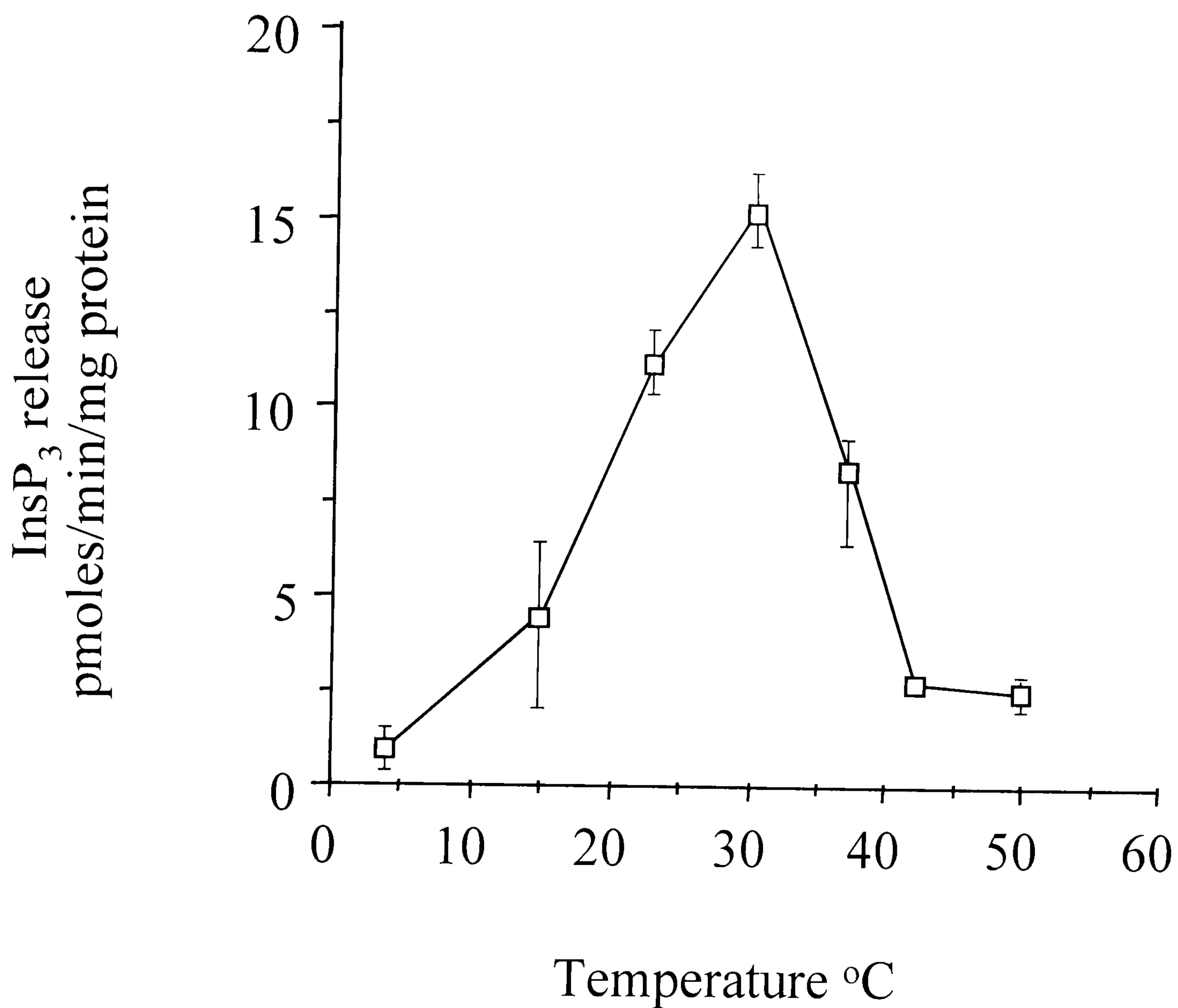


Figure 35 The temperature profile of Plc1

The ability of Plc1 to hydrolyse PIP₂ under different temperatures was investigated using the assay described in the Methods section 2.2.2.2. The rate was calculated using linear sections of time course graphs as described in the text (see appendix for examples). Activity is expressed in pmoles/min/mg protein at a pH of 7.5 and a sodium cholate concentration of 6.4% w/v. The bars represent the standard range of the mean for triplicate determinations.

Assay conditions	Activity pmoles/min/mg protein pH 7.5	Activity of extract pmoles/min/mg protein pH7.5 returned to 30°C	Activity of substrate pmoles/min/mg protein pH7.5 returned to 30°C
30°C	14.6	13.7	15.8
37°C	6.0	10.8	15.2
42°C	0.7	6.5	17.2
50°C	0.3	0.1	8.3

Table 1 Determining the nature of temperature inhibition

When Plc1 was preincubated in temperatures above 37°C for 20 min it was irreversibly inhibited. Extracts that were preincubated at 37°C, 42°C and 50°C were returned to 30°C but the enzyme did not retain its full activity, suggesting that the activity had not returned due to Plc1 being denatured. Substrate preincubated at 50°C showed a decrease in activity when returned to 30°C. All preincubations were for 20min before readjustment to 30°C.

5.3.3 Plc1 activity is affected by pH

Extracts containing expressed Plc1His₆ were prepared in the same way as described in Method section 2.2.2.1. The extract was then incubated at different pHs over a period of 20 min and Plc1 activity was determined using the scintillation counter (see Methods section 2.2.2.2). Linear sections of the graphs were used to determine the rate of PIP₂ hydrolysis over a pH range of 5.5 to 8.5. Using different buffers (50mM concentration for all) with overlapping ranges, the effect of pH on Plc1 activity was investigated. The different buffers were shown to have no effect on the activity of the enzyme (Table 2). The pH profile (Figure 36) for Plc1 is proportionately symmetrical with a relatively tight range of enzyme activity, with the enzyme being inactive at pHs 5.5 and 8.5. Plc1 has an optimum working pH of 7.5, at a temperature of 30°C and a sodium cholate concentration of 6.4% w/v.

The pH value at which the enzyme has 50% of the maximal rate corresponds to the pKa values of amino acids within the active site of the enzyme. These amino acids that correspond to the pKa values are concerned with the binding and selecting of substrates. The pKa values of Plc1 correlate to pH 6.5 and pH 8.1. The amino acid that has a pKa value in the region of pH 6.5 is histidine (pKa 6.04). Cysteine has a pKa value of 8.33, which is comparable to the value of 8.1 seen in Figure 36. Figure 45 (Chapter 7) demonstrates that there are both histidines and cysteine amino acid residues present within the active site of Rat PLC- δ . As there is a high percentage of amino acid similarity conserved between the X and Y catalytic regions (Figures 37 and 44) it may be possible that these amino acid residues are involved in the activity of Plc1.

Even though Plc1 is inactive at the extreme pH of the curve (pH 5.5 and pH 8.5), this inactivity is reversible (Table 3). Preincubation of the extracts in either pH 5.5 or pH 8.5 for 20 min before replacing them back to pH 7.5 results in the enzyme regaining most of its activity, suggesting it is reversibly inhibited.

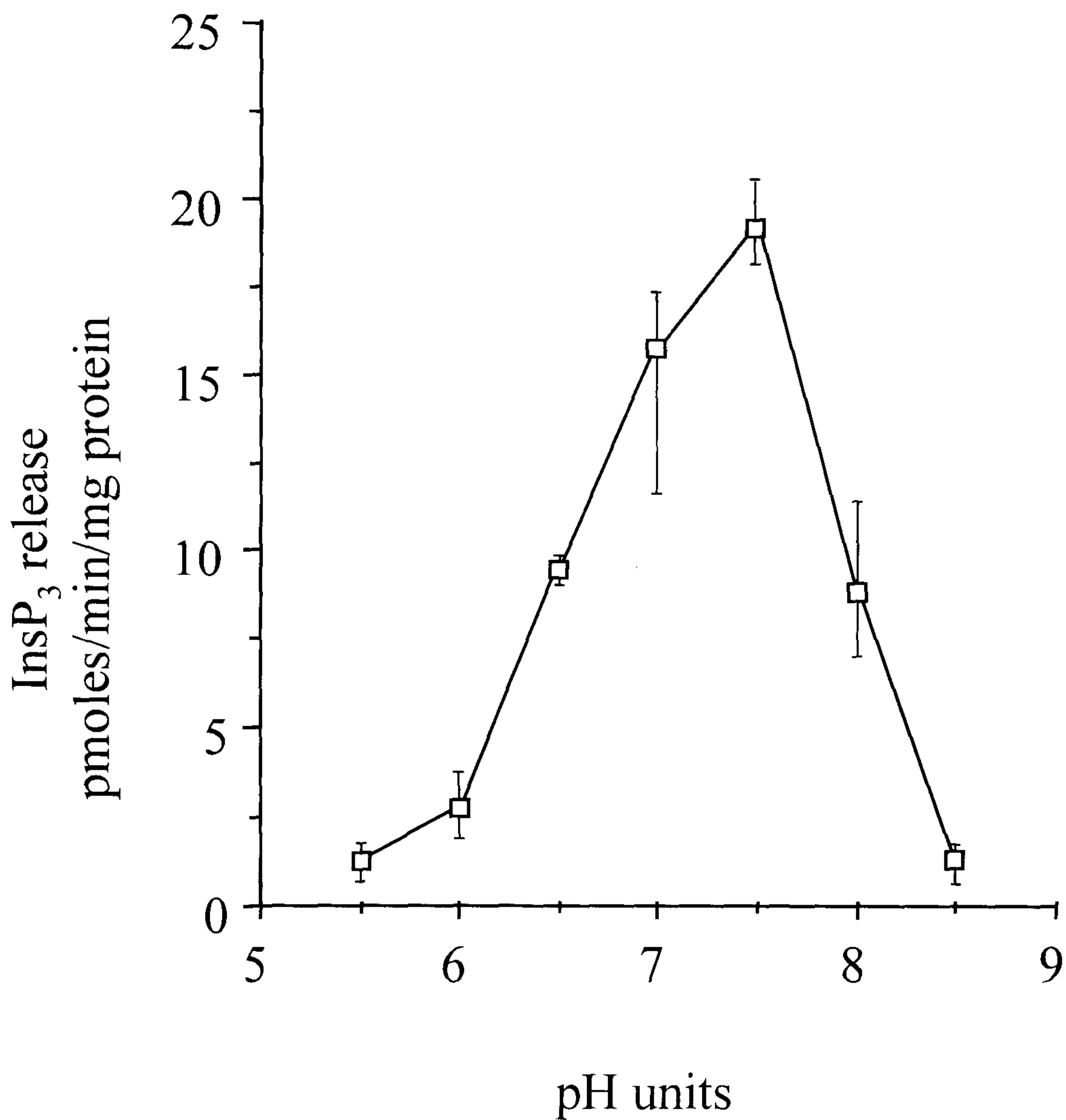


Figure 36 pH profile of Plc1

The ability of Plc1 to hydrolyse PIP₂ under different pH conditions was investigated using the assay described in the Methods section 2.2.2.2. Two buffers (MES and Tris) at a concentration of 50mM were used to assay Plc1 activity over this pH range. Activity is expressed in pmoles/min/mg protein at 30°C and a sodium cholate concentration of 6.4% w/v. The bars represent the standard range of the mean for triplicate determinations.

Buffer type (50mM)	pH of the assay	Plc1 activity pmoles/min/mg protein
MES	5.5	0.9
MES	6.8	13.1
Tris	6.8	11.6
Tris	8.5	1.6

Table 2 Control assays to determine Plc1 activity using different buffers

Two overlapping pH buffers were used to determine the pH profile of Plc1. The overlaps of the buffers (pH 6.8) were assayed to justify that the buffer composition was not having any effect upon Plc1 activity. The assays were performed at 30°C and the substrate contained a sodium cholate concentration of 6.4% w/v.

Assay conditions	Activity of extract pmoles/min/mg protein	Activity of extract pmoles/min/mg protein returned to pH7.5
pH 5.5	0.9	17.2
pH 7.5	19.2	18.4
pH 8.5	1.6	16.3

Table 3 Determining the nature of pH inhibition

After a 20 min preincubation at pH 5.5 and pH 8.5, the extract is returned to pH 7.5 where Plc1 activity is restored. This suggests that Plc1 is reversibly inhibited by pH. The assay was carried out at 30°C and with a substrate containing 6.4% w/v sodium cholate, using the Plc1 assay (see Methods 2.2.2.2).

5.4 Summary of the characterisation of Plc1

Using a crude extract prepared from *Sz. pombe* cells expressing Plc1, characterisation has been accomplished. Different levels of Plc1 activity were obtained by using the three different pREP expression vectors. The expression vector pREP3x*plc1* had the highest level of activity, pREP81*plc1* had the lowest level of Plc1 activity with pREP41*plc1* being somewhere in the middle. This result was expected as the *nmt1* promoter found on pREP3x is supposed to give the highest level of protein expression whereas the *nmt1* promoter on pREP81x should give the lowest. This indicates that there is a positive correlation between Plc1 expression levels and Plc1 activity. The addition of a His₆ to either the N or C-terminus of Plc1 resulted in no change in activity when assayed *in vitro*.

From Figure 33 it was determined that PIP₂ mixed with sodium cholate appeared to create a structure that allowed Plc1 to bind and hydrolyse PIP₂. It was then determined that the optimum concentration of sodium cholate, which was mixed with PIP₂, was 6.4% w/v. Deviation from this concentration appears to result in a decrease in Plc1 activity.

Activity of Plc1 increases with increasing temperature up to 30°C but above this temperature Plc1 is irreversibly inactivated. The substrate mixture of PIP₂ and sodium cholate is unaffected by the temperature range used in this study.

The optimum pH for Plc1 was established to be pH 7.5, and the pKa values obtained from the pH profile suggest that histidine and cysteine may be involved in catalytic activity. After preincubation at pH 5.5 and pH 8.5 the extract is still active when returned to pH 7.5. This suggests that Plc1 is reversibly inhibited by the extreme pHs investigated in this study.

Chapter 6

Investigating the role for Plc1 signalling in *Sz. pombe*

6.1 Introduction

It is known that Plc1 is involved in the hydrolysis of phosphoinositide lipids to create second messengers but the role of these second messengers and thus the role of Plc1 within *Sz. pombe* cells is unknown. The disruption of *plc1* in *Sz. pombe* cells resulted in slow-growing cells and a distinctive phenotype (see Chapter 4). One way in which the role of Plc1 could be investigated is by creating strains where the activity of Plc1 could be controlled. This chapter will describe how temperature sensitive mutations in *S. cerevisiae* PLC1 were used to introduce mutations in *Sz. pombe* Plc1 that may result in a temperature sensitive enzyme. Once the mutations have been introduced this chapter will discuss how these mutants were characterised. This included determining their level of activity at different temperatures using an *in vitro* assay (described in Chapter 2, section 2.2.2.2) and assessing their activity when expressed *in vivo*. When the mutants were expressed *in vivo*, cell growth on DMM plates (described in Chapter 2, section 2.2.4) and cell culture doubling times at different temperatures were used to confirm their level of temperature sensitivity. Analysis of these mutants using the above methodology may indicate possible roles for Plc1 and the second messengers it produces within *Sz. pombe* cells.

6.1.1 The role of Plc1 might be discovered through controlled expression

If Plc1 activity could be switched on and off the initial effects of a cell lacking Plc1 could be observed and this might suggest a possible role for Plc1. One way of achieving this is to create a temperature sensitive mutation within the open reading frame of *plc1*. Cells growing at low temperatures would contain an active Plc1 but when incubated at a higher temperature Plc1 activity would cease.

6.1.2 Using *S. cerevisiae* PLC1 as a model

In the yeast *S. cerevisiae*, five temperature sensitive *PLC1* mutants were identified following *in vitro* mutagenesis (Yoko-o *et al.*, 1995). These temperature sensitive mutants were created by amino acid substitution within the X or the Y catalytic domains (Yoko-o *et al.*, 1995). There were two mutants that demonstrated true temperature sensitive phenotypes when assayed *in vitro* whereas, the others had no detectable activity *in vitro* but were detectable through immunoblotting. This indicates that the mutations resulted in a major change in the enzymes active site without affecting its tertiary structure domains (Yoko-o *et al.*, 1995).

One of the mutations within *PLC1* was identified in the X catalytic domain; a glycine residue was substituted for a serine, a second mutation was in the Y catalytic domain where an arginine was substituted for a cysteine (Yoko-o *et al.*, 1995). The catalytic domain is conserved among PLC isozymes so the X and Y regions of *Sz. pombe*, *S. cerevisiae* and mammalian PLCs can be compared to show regions of sequence similarity within these domains (Figure 37). These same mutations created in *S. cerevisiae PLC1* can be introduced into *Sz.pombe* Plc1, [serine was substituted for glycine in the X catalytic domain (Plc1[G481S]) and arginine was substituted for cysteine in the Y catalytic domain (Plc1[R873C])] through PCR amplification and then sub-cloned into the pREP vectors (see Chapter 3, Figures 19 and 20). Double stranded DNA sequencing (see Chapter 2 section 2.2.1.5) was used to confirm that the mutation had been introduced and that the rest of the sequence was correct. These mutants could be used to help identify the role of Plc1 within the cell.

X region

		S
<i>Sz. p plc1</i>	463	QFGGESSIEGYIRSLQRGCKCIEIDCWDG
<i>S. c PLC1</i>	385	QIAETPSVEGYIQVLQQGCRCVEIDIWDG
Rat PLC- β 1	321	QLAGNSSVEMYRQVLLSGCRCVELDCWKG
Human PLC- β 2	317	QFSGLSSAEMYRQVLLSGCRCVELDCWKG
Rat PLC- β 3	303	QLAGTSSVEMYRQALLWGCRCVELDVWKG
Rat PLC- β 4	318	QFGGKSSVEMYRQVLLAGCRCVELDCWDG
Rat PLC- γ 1	325	QFSSESSLEAYARCLRMGCRCIEIDCWDG
Rat PLC- γ 2	317	QLRSESSTEAYIRCLRAGCRCIEIDCWDG
Rat PLC- δ 1	301	QLTGPSSSTEAYIRALCKGCRCLEIDCWDG
Bovine PLC- δ 2	295	QLCGQSSVEGYIRALKRGRCVEVDIWDG

Y region

		C
<i>Sz. p plc1</i>	859	FANFTCPIDRLQQGYRHIRLLNMQGENL
<i>S. c PLC1</i>	830	IASVCLKLNYLRMGYRHIPLFNMRGEQY
Rat PLC- β 1	757	IGHRILPVQAIRPGYHYICLRNERNQPL
Human PLC- β 2	759	LGHRIIPINALNSGYHHLCLHSESNMPL
Rat PLC- β 3	788	VGHRILPVSAIRSGYHYVCLRNEANQPL
Rat PLC- β 4	782	IGQRILPLDGLQAGYRHISLRNEGNKPL
Rat PLC- γ 1	1173	LAQATFPVKGLKTGYRAVPLKNNYSEDL
Rat PLC- γ 2	1148	LAHATYPIKGIKSGFRSVPLKNGYSEDI
Rat PLC- δ 1	716	IGQSTIPWNSLKQGYRHVHLLSKNGDQH
Bovine PLC- δ 2	717	IGQYTLPWSCMQQGYRHIIHLLSKDGLSL

Figure 37 Comparisons of the amino acid sequence in eukaryotic PLCs

Comparisons of the amino acid sequence of the X and Y regions of *Sz. pombe*, *S. cerevisiae* and mammalian PLCs. The positions at which the amino acids are identical to *Sz. pombe* are coloured red. The position of the substitution creating a temperature sensitive *S. cerevisiae PLC1* is coloured blue and the substituted amino acid is shown above the blue amino acids. These substitutions were made in *Sz. pombe plc1* by PCR (see constructs Chapter 3, Figures 19 and 20). The position of the amino acid residues is indicated to the left of each sequence.

6.2 Expression of the temperature sensitive mutants *in vitro*

The open reading frames of Plc1[G481S] and Plc1[R873C] were subcloned into the expression vector pREP3x (see Chapter 3 for constructs). *Sz. pombe* cells transformed with these constructs were cultured at 23°C in DMM minus thiamine. The *nmt1* promoter found on the pREP vectors is switched on when cells are cultured in the absence of thiamine resulting in increased expression levels of *plc1*. Crude extracts were prepared from cells expressing the Plc1 mutants (see Methods section 2.2.2.1) so that the activity of these Plc1 mutants could be measured *in vitro* using the Plc1 assay (see Methods section 2.2.2.2).

6.2.1 Plc1 mutants are more sensitive to temperature when assayed *in vitro*

Crude extracts of cells expressing wild-type Plc1, Plc1[G481S] and Plc1[R873C] were incubated and assayed at a range of temperatures to determine if the activity of the mutants was sensitive to temperature. The temperature profile of wild-type Plc1 (Figures 38 and 39) demonstrates that there is a rapid increase in Plc1 activity from 4°C to 30°C. When wild-type Plc1 was assayed at 37°C the activity was reduced by almost 50% and when assayed at 42°C there was a further reduction in Plc1 activity. The temperature profile of Plc1[R873C] (Figure 38) shows an increase in activity from 4°C to 30°C. This increase in activity corresponds to the increase in activity seen by wild-type Plc1. When the temperature is increased above 30°C a decrease in activity is seen; this reduction in activity is comparable to the wild-type Plc1. When assayed *in vitro* at a range of temperatures, Plc1[R873C] and wild-type Plc1 appear to have similar activities.

The temperature profile of Plc1[G481S] (Figure 39) was investigated over a range of temperatures, from 4°C to 30°C. Over this temperature range, the activity of Plc1[G481S] shows a similar profile to that of wild-type Plc1. When

the temperatures were increased to 37°C the Plc1[G481S] extract was inactive, while the wild-type Plc1 retained approximately 50% of the maximum activity. When assayed *in vitro* it would appear that Plc1[G481S] is more temperature sensitive than wild-type Plc1; it is inactivated at 37°C which is normally a permissive temperature for wild-type Plc1.

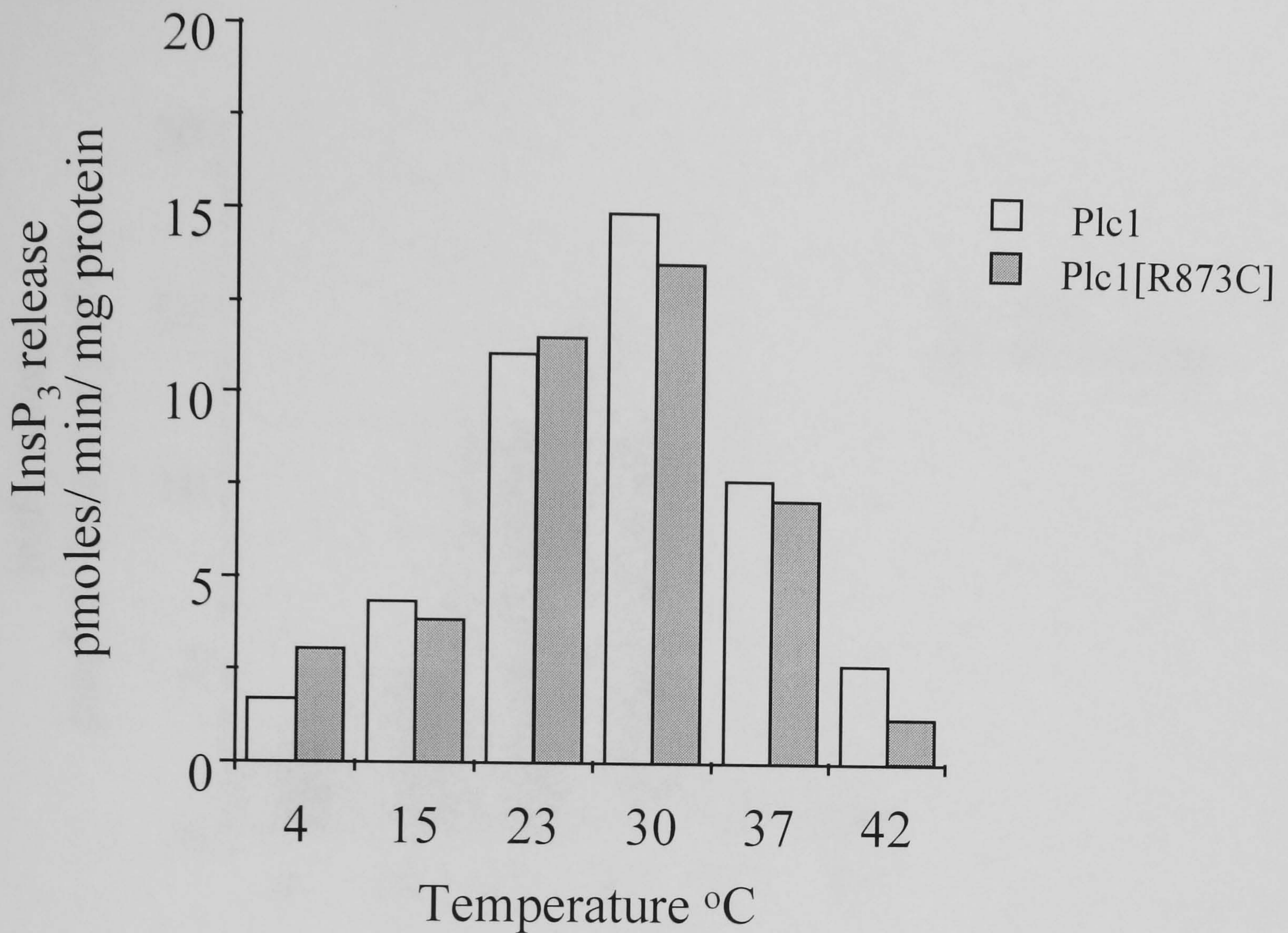


Figure 38 The temperature profile of Plc1[R873C]

The activity of Plc1[R873C] was compared to wild-type Plc1 over a range of temperatures. This was investigated using the assay described in the Methods section 2.2.2.2. Plc1 activity is expressed in pmoles/min/mg protein at a pH of 7.5 and a sodium cholate concentration of 6.4% w/v.

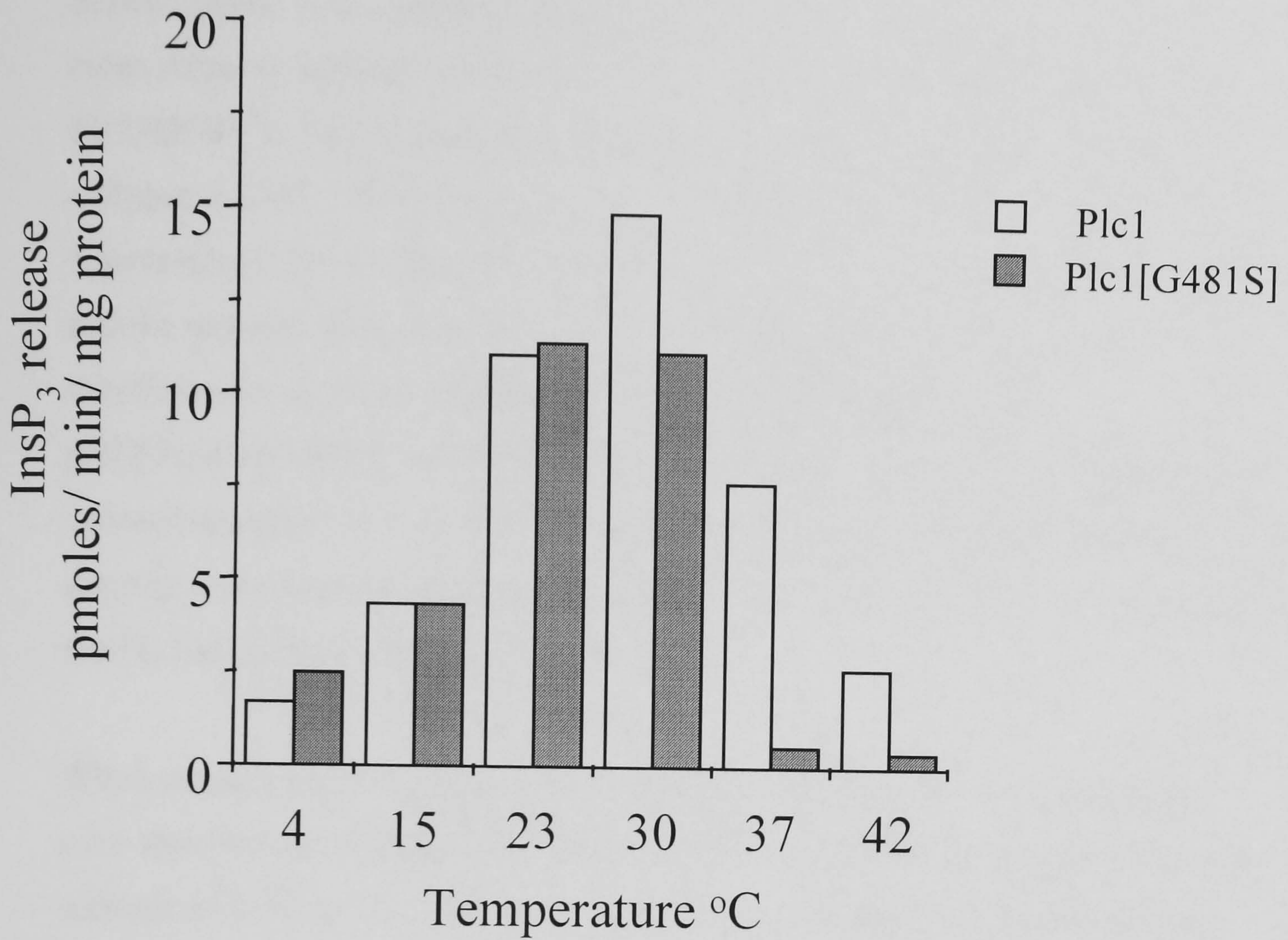


Figure 39 The temperature profile of Plc1[G481S]

The activity of Plc1[G481S] was compared to wild-type Plc1 over a range of temperatures. This was investigated using the assay described in the Methods (section 2.2.2.2). Plc1 activity is expressed in pmoles/min/mg protein at a pH of 7.5 and a sodium cholate concentration of 6.4% w/v.

6.2.2 Preparation of the extract is vital in producing functional Plc1 mutants

In this chapter it has already been demonstrated that the mutant Plc1[G481S] is more sensitive to temperatures above 30°C than wild-type Plc1 and the mutant Plc1[R873G]. The extracts used in these assays were produced from cells cultured at 23°C. The next step was to discover if cells cultured at high temperatures (for example 37°C) affected the activities of both wild-type Plc1 and the mutants when assayed *in vitro*. The following constructs were transformed into *P* cells; pREP3*plc1*, pREP3*plc1*[R873C] and pREP3*plc1*[G481S] (see Chapter 3 for constructs). The transformed cells were cultured at either 23°C or at 37°C. Crude extracts containing the expressed enzyme were prepared from these cells and were assayed at 30°C *in vitro* using the Plc1 assay (see Methods section 2.2.2.2).

When extracts prepared from cells expressing wild-type Plc1 were assayed *in vitro* there was no difference in activity between the extracts prepared from cells cultured at 23°C or 37°C (Table 4). Extracts of wild-type Plc1 prepared from cells cultured at 23°C and assayed at 30°C had 14.9 units of activity and wild-type extracts prepared from cells cultured at 37°C but assayed at 30°C had 14.6 units of activity. Extract prepared from cells cultured at 23°C expressing pREP3*plc1*[R873C] and assayed at 30°C resulted in a Plc1 which had a level of activity similar to the wild-type Plc1. Extract prepared from cells cultured at 37°C and assayed at 30°C resulted in a decrease in activity of about 70% when compared to Plc1[R873] extract prepared from cells cultured at 23°C (Table 4). Extract prepared from cells cultured at 23°C expressing pREP3*plc1*[G481S] and assayed at 30°C produced a Plc1 that had similar activity to the wild-type Plc1. Extracts of Plc1[G481S] prepared from cells cultured at 37°C and assayed at 30°C resulted in an inactive Plc1 (Table 4).

This data suggests that the mutant Plc1[G481S] is probably temperature sensitive but the mutant Plc1[R873C] is not as sensitive to temperature as its temperature profile closely resembles wild-type Plc1

Plc1 construct	Activity IP ₃ release pmoles/min/mg protein Cells cultured at 23°C, extracts assayed at 30°C	Activity IP ₃ release pmoles/min/mg protein Cells cultured at 37°C, extracts assayed at 30°C
pREP3xplc1	14.9	14.6
pREP3xplc1[R873C]	17.2	5.0
pREP3xplc1[G481S]	15.4	0.1

Table 4 Demonstrating cellular growth temperature is vital in producing functional Plc1 mutants.

Wild-type Plc1 expressed in cells cultured at either 23°C or 37°C has the same activity when measured *in vitro*. Cells cultured at 37°C expressing the mutated forms of Plc1 resulted in an inactive mutant (pREP3xplc1[G481S]) and a mutant which had less activity (pREP3xplc1[R873C]) than wild-type Plc1 when assayed at 30°C *in vitro*. Activity is expressed as pmoles/ min/ mg protein at 30°C, pH 7.5 and a sodium cholate concentration of 6.4% w/v.

6.3 Expression of the Plc1 mutants *in vivo*

Chapter 4 demonstrated *Sz. pombe* cells lacking Plc1 are only able to grow on rich media such as YE and at low temperatures such as 23°C. Figure 28 (Chapter 4) indicates that $\Delta plc1$ expressing the pREP vectors alone are unable to grow on DMM plates irrespective of the temperature or the thiamine content. $\Delta plc1$ expressing wild-type Plc1 are able to grow at 23°C and 37°C. This growth is not dependent upon the expression levels of Plc1 (pREP3x and pREP81x) as growth was seen irrespective of the promoter or the thiamine concentration in the plates.

6.3.1 Cell growth is not dependent upon the expression levels tested

Wild-type Plc1, Plc1[R873C] (under the control of the *nmt1* promoters on pREP3x and pREP81x), pREP3x and pREP81x vector alone were transformed into *P* cells $\Delta plc1$ (see Chapter 3 Figures 19 and 20). Cells transformed with these constructs were then cultured in culture overnight and 5×10^3 cells were spread on each of a series of DMM plates:

DMM plus thiamine	incubated at 23°C
DMM minus thiamine	incubated at 23°C
DMM plus thiamine	incubated at 37°C
DMM minus thiamine	incubated at 37°C

Replicate plates were then placed at 23°C or 37°C and colony formation was recorded (see Methods 2.2.4) (Figure 40).

Plc1[R873C] expressed *in vivo* displays a phenotype similar to the wild-type. Within the specific parameters that this study was investigating the $\Delta plc1$ cells expressing Plc1[R873C] were unaffected by temperature or level of expression (Figure 41). This suggests that the mutant Plc1[R873C] does not demonstrate the temperature sensitive phenotype that was discovered from a similar sequence mutation identified in *S. cerevisiae* PLC1 (Yoko-o *et al.*, 1995).

Figure 40 demonstrates that $\Delta plc1$ cells expressing pREP vector alone are unable to grow on DMM plates, $\Delta plc1$ cells expressing wild-type Plc1 are able to grow at either temperature both in the presence and in the absence of thiamine. The mutant Plc1[G481S] which is under the control of the *nmt1* promoters on pREP3x and pREP81x was transformed into $\Delta plc1$ cells. When this mutant is expressed *in vivo* (Figure 41) it can be seen that cells are able to grow at 23°C but are unable to grow at 37°C irrespective of the thiamine concentration. This strongly suggests that Plc1[G481S] is a temperature sensitive mutation.

6.3.2 *In vivo* growth assays

The growth characteristics of the temperature sensitive Plc1 strains were investigated (pREP81x*plc1*[R873C] and pREP81x*plc1*[G481S]). The mutant strains had doubling times (td) of 5 hrs and 5.5 hours respectively (Figure 42, panel A), which is similar to the wild-type strain (JY 383) incubated at 23°C in DMM (Chapter 4, Figure 27). The $\Delta plc1$ strain is unable to grow in selective medium such as DMM but is able to grow very slowly in a.a. medium. Although a.a. medium has been shown to isolate auxotrophic mutants, it contains more nutrients than DMM thus, $\Delta plc1$ cells are able to grow very slowly in a.a. medium at 23°C (td: 6 hr). When cells growing exponentially were shifted to 37°C (Figure 42, panel B), the $\Delta plc1$ strain stopped growing; this is because under stressful conditions such as high temperature and minimal medium $\Delta plc1$ cells are unable to grow (see Chapter 4). The mutant strains behaved differently when compared to the $\Delta plc1$ cells, pREP81x*plc1*[R873C] continued to grow over the 10 hrs assay period with a doubling time of 4 hrs but this was at a slower rate than wild-type strain which had a doubling time of 3.5 hrs in DMM (see Figure 27, Chapter 4). The other mutant, pREP81x*plc1*[G481S] when incubated at 37°C grew for the first 4hrs but no further growth was observed over the next six hours. This confirmed that the mutation G481S was temperature sensitive and that the mutation R873C was not.

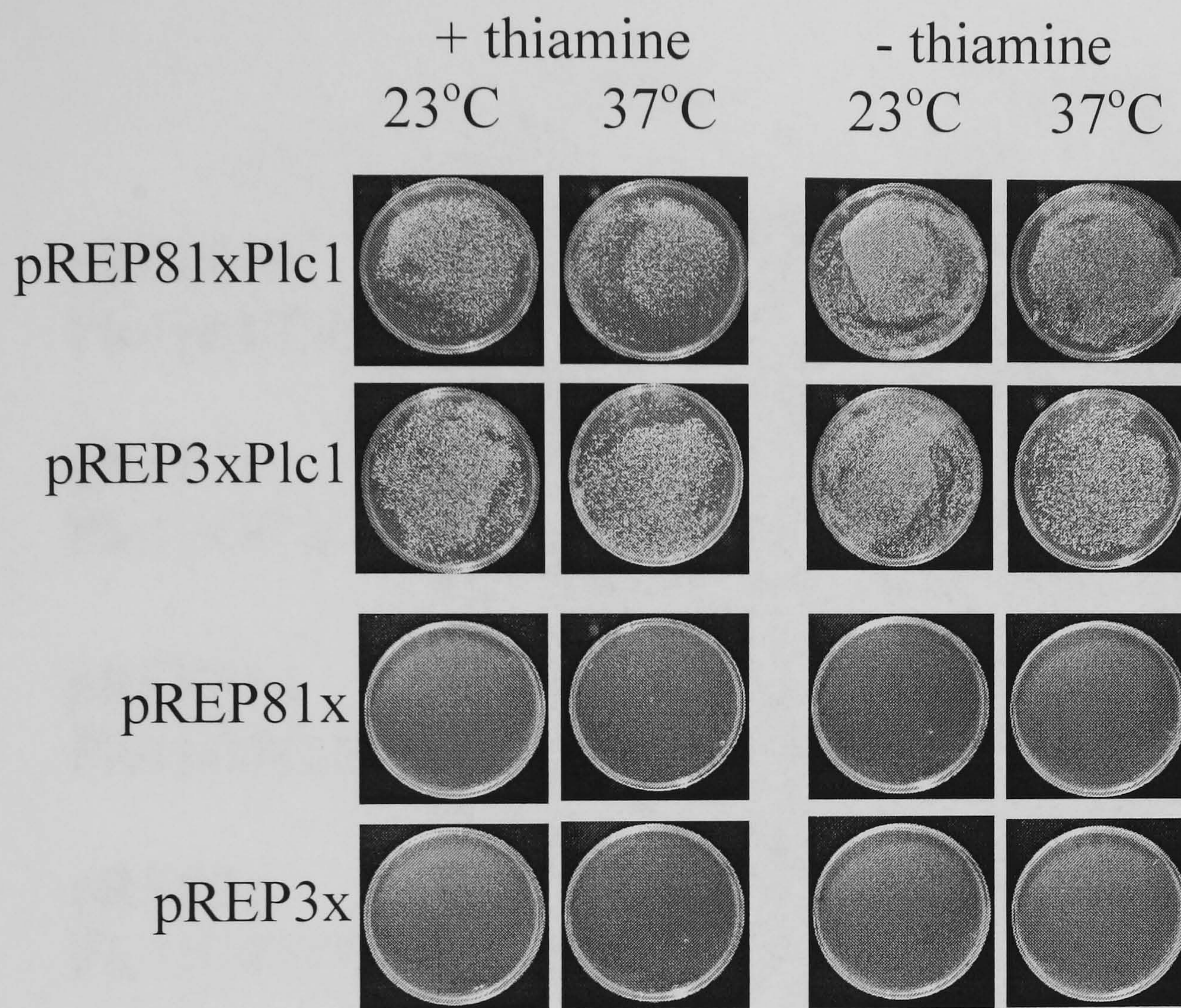


Figure 40 Expression of the Plc1 and the pREP vectors in $\Delta plc1$ cells

Plc1 under the control of pREP3x and pREP81x *nmt1* promoters, pREP3x and pREP81x alone were transformed into $\Delta plc1$ cells. Five thousand of these transformants were plated out on DMM plates either containing thiamine or without thiamine, these plates were incubated at either 23°C or at 37°C and colony formation was noted after 4 days (see Methods 2.2.4).

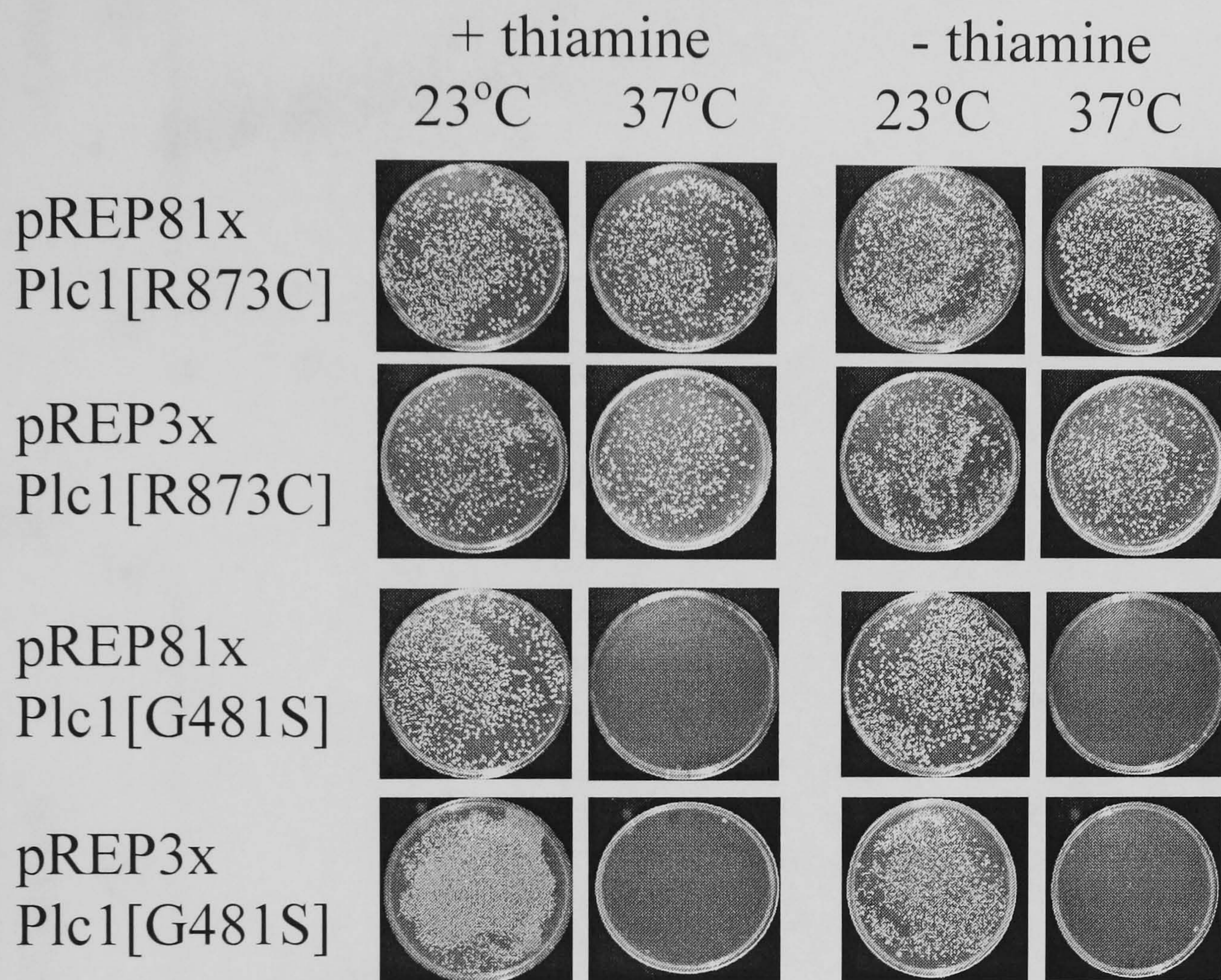


Figure 41 Expression of the Plc1 mutants *in vivo*

Plc1[R873C], Plc1[G481S] (under the control of pREP3x and pREP81x *nmt1* promoters), were transformed into $\Delta plc1$ cells. Five thousand of these transformants were plated out on DMM plates either containing thiamine or without thiamine, these plates were incubated at either 23°C or at 37°C and colony formation was noted after 4 days (see Methods 2.2.4).

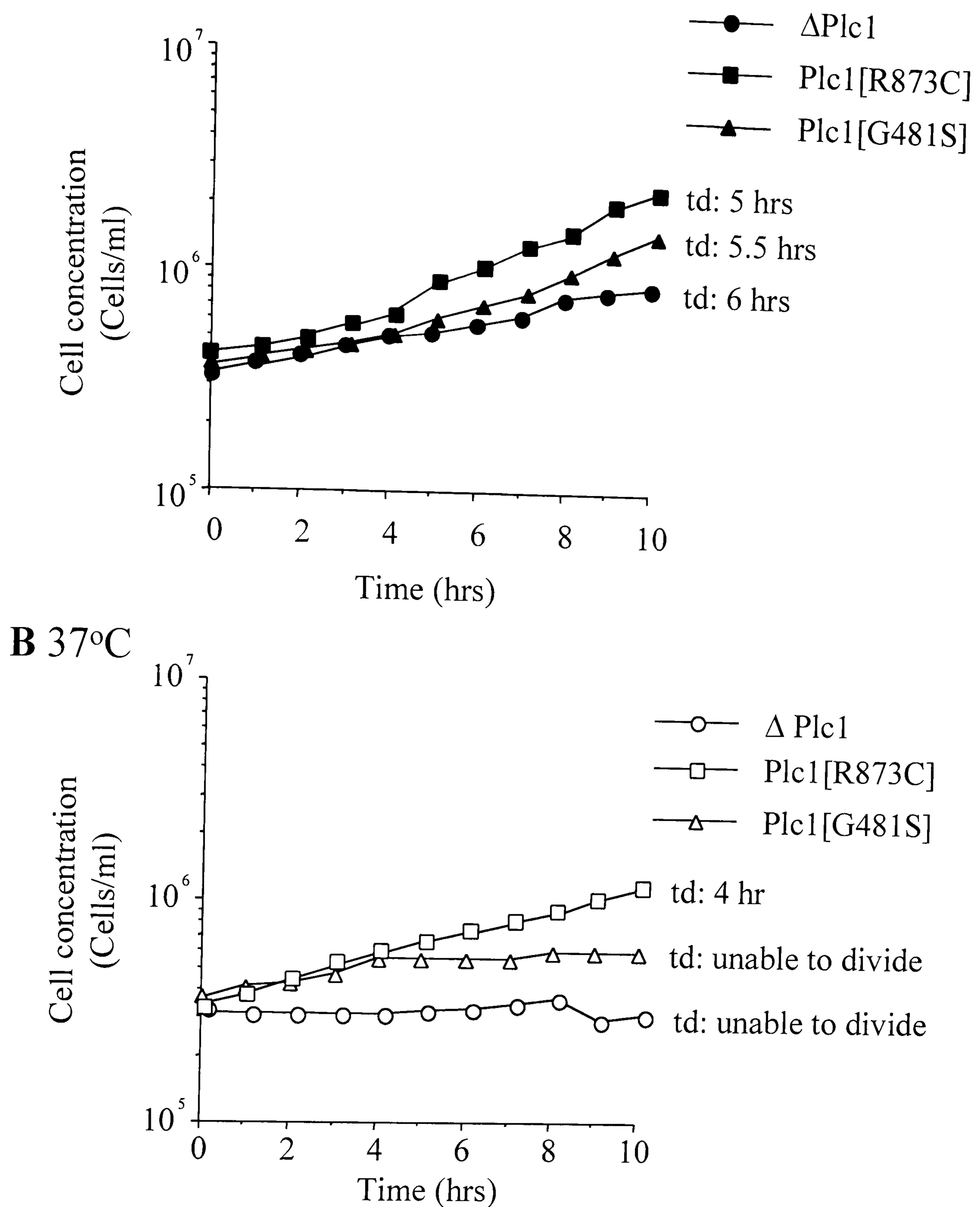


Figure 42 Growth assays

Growth curve of temperature sensitive mutants Plc1[R873C] (squares), Plc1[G481S] (triangles) and $\Delta plc1$ cells (circles). Panel A demonstrates these cells grown in at 23°C in AA media. Exponentially growing cells were transferred to a 37°C shaking water bath (Panel B) at 0 hrs. Cell numbers were determined using a Coulter Channelyser (see Methods 2.1.7).

6.4 Cells lacking Plc1 display a very distinct phenotype

It is known that $\Delta plc1$ cells display a very distinct phenotype that was described in Chapter 4. Assays performed on Plc1[G481S] *in vitro* and *in vivo* confirmed this to be a temperature sensitive mutation which was inactivated at 37°C. Now that a temperature sensitive Plc1 had been produced, the next step was to observe cells expressing a functional Plc1 at 23°C, then shift them to 37°C switching off Plc1 activity. This will demonstrate the initial effects of a cell without Plc1 activity and may give an insight into the role of Plc1 within *Sz. pombe* cells.

Cells cultured on microscope slides were used (see Methods section 2.2.3) to observe single $\Delta plc1$ cells expressing Plc1, Plc1[R873C] and Plc1[G481S] in either a 23°C or 37°C environment. Briefly the cells were cultured in culture at 23°C and were then transferred to a slide containing 1% LMP agarose plus the appropriate media. After a 24 hrs period photographs of the cells were taken using the Nomarski digital camera. Wild-type *Sz. pombe* cells are able to grow equally well at both 23°C and 37°C, forming small colonies after 24 hrs (Figure 43). This same phenotype is observed by $\Delta plc1$ cells expressing Plc1[R873C] (Figure 43). The $\Delta plc1$ cells however, form irregular colonies at 23°C after a 24 hrs period, no colonies are seen when cells taken from the same culture and incubated at 37°C. Instead there are elongated cells, which are unable to divide across the septum, and sometimes-small bulbous-shaped cells can be seen suggesting perhaps cell wall or membrane defects within the cell (Figure 43). The same phenotype is seen in cells expressing Plc1[G481S] (Figure 43) except that at 23°C the formation of colonies is more regular (similar to wild type cells). At 37°C these cells are unable to divide forming elongated structures and small bulbous cells. These pictures do not confirm the role of Plc1 but they do indicate that Plc1 is involved in functions such as cell growth and that it may play a role in osmoregulation.

Temperature

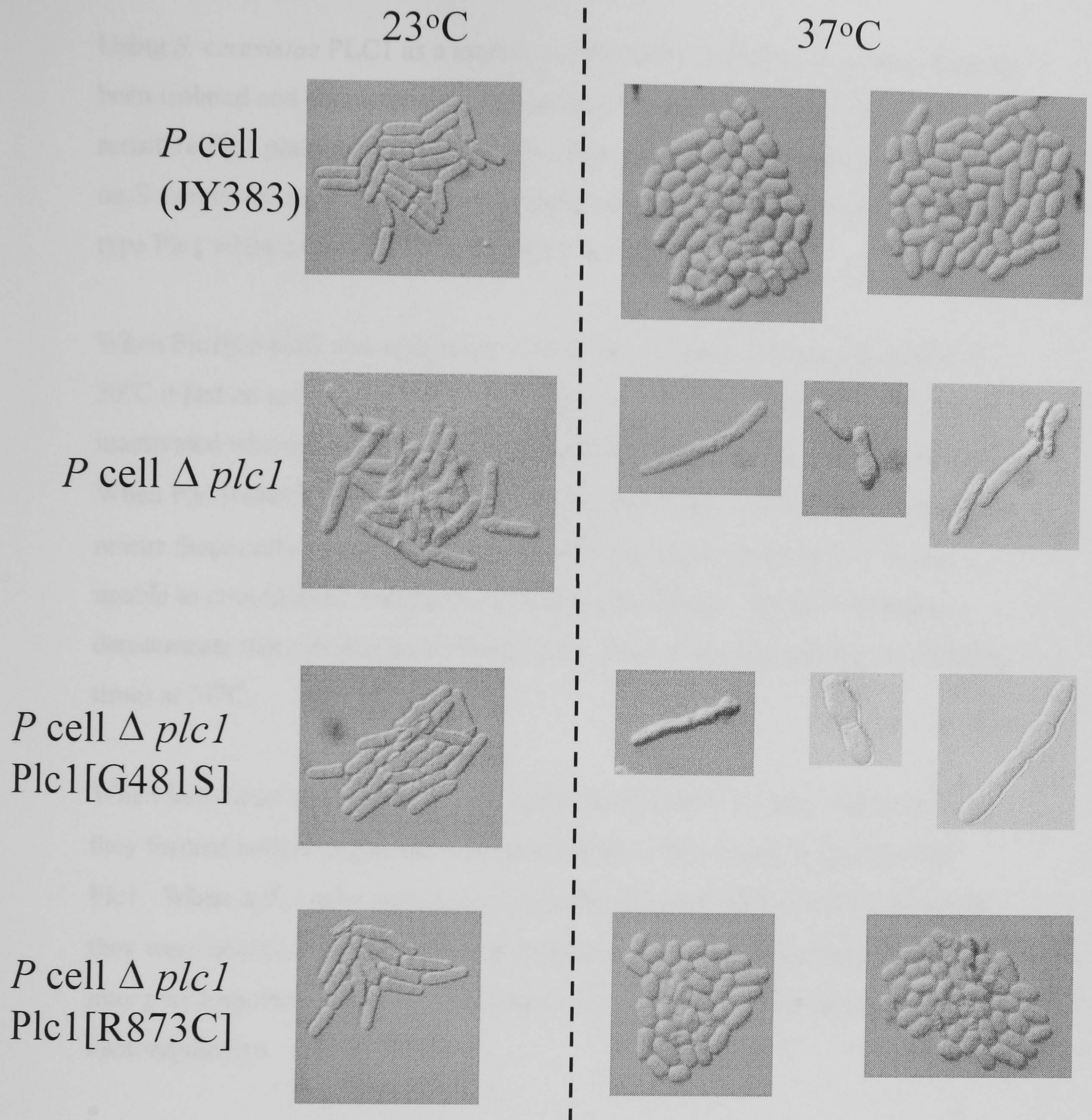


Figure 43 Phenotype of $\Delta plc1$ cells

Pictures showing the growth of *Sz. pombe* cells after 24 hrs incubation at 23°C or at 37°C in slide culture on rich media. The top panel displays wild-type *P*-cells, the next panel displays $\Delta plc1$ cells. The bottom two panels display the mutants Plc1[G481S] and Plc1[R873C]. It demonstrates that at 23°C $\Delta plc1$ cells expressing Plc1[G481S] grows in a similar fashion to wild-type cells, but when incubated at 37°C these cells are unable to divide displaying a phenotype similar to $\Delta plc1$ cells. The mutant Plc1[R873C] when expressed in $\Delta plc1$ cells displays a phenotype similar to wild-type *Sz. pombe* cells. These photographs were taken using a Nomarski digital camera.

6.5 Summary

Using *S. cerevisiae* PLC1 as a model, a temperature sensitive *Sz. pombe* Plc1 has been isolated and characterised. The mutation that results in the temperature sensitive Plc1 phenotype is G481S. Another mutant, R873C was created based on *S. cerevisiae* PLC1 but this was found to exhibit a phenotype similar to wild-type Plc1 when expressed from the pREP vectors.

When Plc1[G481S] was assayed *in vitro* over a temperature range from 4°C to 30°C it had an activity similar to wild-type Plc1. At 37°C Plc1[G481S] became inactivated whereas wild-type Plc1 retained about 60% of the maximum activity. When Plc1[G481S] was expressed *in vivo* in $\Delta plc1$ *Sz. pombe* cells it was able to rescue these cells on minimal media at 23°C but when placed at 37°C it was unable to complement irrespective of expression levels. The growth assays demonstrate that cellular growth stops after about 4 hrs incubation (one doubling time) at 37°C.

When individual $\Delta plc1$ cells expressing Plc1[G481S] were analysed, at 23°C they formed normal organised colonies similar to cells expressing wild-type Plc1. When $\Delta plc1$ cells expressing Plc1[G481S] were shifted to 37°C however, they were unable to divide and created bulbous structures suggesting that Plc1 may play important roles in cell division, cell organisation and perhaps even osmoregulation.

Chapter 7
Identifying the possible substrates for Plc1

7.1 Introduction

The substrates for *Sz. pombe* Plc1 are unknown. Assays have shown that it has the ability to hydrolyse PIP₂ efficiently but this may not be its only substrate or even its major substrate. If PIP₂ is the primary substrate of Plc1 then the products of its hydrolysis (InsP₃ and DAG) may play an important role within the *Sz. pombe* cells. Searches carried out on the *S. cerevisiae* and *Sz. pombe* genomes were unable to identify an InsP₃ receptor within yeast cells. InsP₃ in yeast is probably not involved in Ca²⁺ release from intracellular stores. Instead its principal function could be as a precursor for the production of alternative inositol polyphosphates. Conversely, DAG has been shown to be an important activator of PKC which regulates other signal transduction pathways (Nishizuka, 1988).

Mutations identified in mammalian PLCs resulted in a change in specificity for its substrate. Instead of the PLCs preferentially hydrolysing PIP₂>PIP>PI they were now only able to hydrolyse PI. If similar mutations could be introduced into Plc1, this may help identify the role of Plc1. These mutations might also indicate the importance of PIP₂ concentration or the production of InsP₃ and DAG, it may even highlight the importance of all of these factors.

This chapter will initially describe the mutations that resulted in altered substrate specificity within mammalian PLCs. It will demonstrate which mutations were introduced into *Sz. pombe* Plc1 and why those changes were made. Finally this chapter will describe the experiments performed on the Plc1 mutants attempting to elucidate a possible role for Plc1.

7.1.1 Mammalian PLCs are able to hydrolyse PIP₂, PIP and PI

Although mammalian PLC isozymes differ in the way that they are regulated, they have similar enzymatic properties. The three members of the PLC family; β , γ and δ contain both an X and Y catalytic domains, enabling them to recognise PI, PIP and PIP₂ (Cheng *et al.*, 1995). Once bound to the substrate, PLCs can carry out Ca²⁺-dependent hydrolysis of these phosphoinositide lipids. Prokaryotic PLCs do not contain the conserved Y catalytic domain that is seen in mammalian PLCs (Kuppe *et al.*, 1989). The active site of bacterial PLCs therefore are unable to recognise PIP or PIP₂ but are able to bind and hydrolyse PI. PI hydrolysis by bacteria is independent of Ca²⁺, another factor that distinguishes prokaryotic PLCs from eukaryotic PLCs (Cheng *et al.*, 1995).

7.1.2 The substrate of Plc1

From comparisons of amino acid sequences (Figure 44) it can be seen that Plc1 from *Sz. pombe* contains an X and Y catalytic domains with significant sequence similarity to mammalian PLCs. This suggests that Plc1 displays more similarity to eukaryotic PLCs than prokaryotic PLCs. The products of PIP₂ hydrolyses by mammalian PLCs result in the production of InsP₃, which is hydrophilic, and a hydrophobic product, DAG. It is known that when Plc1 activity is measured *in vitro* the scintillation counter is used to detect a hydrophilic radioactive product (see Methods 2.2.2.2). This product was likely to be an inositol phosphate, so the hydrophilic products were passed through a dowex column and then loaded onto a HPLC column. Fractions were collected and were compared to the elution time of the standards inositol polyphosphates (Work done by Rita Slaaby). This confirmed that Plc1 hydrolysed PIP₂ into Ins 1,4,5-P₃ (InsP₃) and DAG. As Plc1 contains the X and Y catalytic domain and it is able to hydrolyse PIP₂ it is likely that it also has the ability to hydrolyse PIP and PI similar to other higher

eukaryotic PLCs. Alternatively, it may have a single specificity as demonstrated by bacterial PLCs.

X region

		R/A
<i>Sz. p plc1</i>	567	VLPSPEDLLNKILLKVKCSATP
<i>S. c PLC1</i>	499	TRTDKLPSPRELKHKILLKSKK
Rat PLC- β 1	444	ESGVPLPSPMDLMYKILVKNKK
Human PLC- β 2	440	KPGVPLPSPEDLRGKILIKNKK
Rat PLC- β 3	426	SAGTPLPSPQDLMGRILVKNKK
Rat PLC- γ 1	441	IAADGLPSPNQKRRKIIKHKK
Rat PLC- γ 2	433	ASADQLPSPSQLREKIIKHKK
Human PLC- δ 1	417	GVTNSLPSPSQLKKGKILLKGKK

Y region

		K/L
<i>Sz. p plc1</i>	679	MVPRLSKHNLRYLCRVYPGPLRVG
<i>S. c PLC1</i>	630	LKLSLDKHNRRYLMRVYPHVLRYK
Rat PLC- β 1	583	SPVEFVEYNKMQLSRIYPKGTRVD
Human PLC- β 2	585	SNVQFVDYNKRQMSRIYPKGTRMD
Rat PLC- β 3	615	SPMEFVEYNKQQLSRIYPKGTRVD
Rat PLC- γ 1	996	KGKKFLQYNRLQLSRIYPKGQRLD
Rat PLC- γ 2	973	KPVDLLRYNQKGLTRVYPKGQRVD
Human PLC- δ 1	535	SGNGFVRHNVGHL SRIYPAGWR TD

Figure 44 Comparison of the amino acid sequences within the X and Y regions of eukaryotic PLCs

Comparison of the amino acid sequences within the X and Y regions of *Sz. pombe*, *S. cerevisiae* and mammalian PLCs. The positions at which the amino acid sequence is identical to *Sz. pombe* is marked in red. The position of the substitution creating a substrate specificity mutant in Human PLC- δ (Y catalytic domain), Human PLC- β (X catalytic domain) are marked in blue. The amino acids substituted in *Sz. pombe plc1* are shown above the blue amino acids in black. The amino acids marked in green in the X catalytic region are a basic amino acid motif [(K/R)xxxKxK(K/R)]; x denotes any amino acid, this motif has been shown to be present in most PLCs and PIP₂ actin binding proteins but is not found in *Sz. pombe* Plc1. The number of the amino acid is indicated to the left of each amino acid sequence.

7.2 The active site of phospholipase C

The X and Y catalytic domains are highly conserved among eukaryotic PLCs (Figures 37 and 44). Presence of the conserved X and Y domains is essential for PLC activity, deletion of either the X (Ellis *et al.*, 1993 and Yagisawa *et al.*, 1994) or the Y domain (Emori *et al.*, 1989) inactivates the enzyme.

7.2.1 Precise amino acid configuration within the active site is essential for PLC activity

The X catalytic domain of most PLCs and actin binding proteins such as gelsolin contain a basic amino acid motif (Figure 44 green letters) [(K/R)xxxKxK(K/R); x denotes any amino acid], which represents a possible PIP₂ binding site. Replacing one or several residues within this motif results in reduced enzyme activity (Simoes *et al.*, 1995). Replacement of lysine 461 (Lys461) within this motif results in complete loss of PLC-β activity (Simoes *et al.*, 1995). Lys 461 is conserved among most eukaryotic PLCs including *Sz. pombe plc1* but *plc1* is the only eukaryotic PLC that does not contain this basic amino acid motif seen in all other PLCs (Figure 44 green letters). This suggests that Lys461 may be a catalytically important residue within the X catalytic domain. The homologue of lys461 in *Sz. pombe Plc1* is lysine 582 (Lys 582) and can be identified in the X catalytic domain (Figure 45). Other residues that may be catalytically important in the X domain include arginine 338 and histidine 356. Replacement of these residues with leucine results in no detectable PIP₂ or PI hydrolysis in human PLC-δ1 (Cheng, *et al.*, 1995). Replacement of histidine 311 and tyrosine 314 with alanine resulted in a thousand-fold and 10-fold reduction in rat PLC-δ1 activity respectively (Ellis, *et al.*, 1995).

Within the Y catalytic domain of human PLC-δ there are four arginine residues (Arg527, Arg549, Arg556, and Arg701) which are highly conserved amongst eukaryotic PLCs. The mutant PLC-δs containing a substituted arginine resulted in them being either moderately defective or fully active in their abilities to

hydrolyse PIP₂ and PI (Wang, *et al.*, 1996). When Arg549 was replaced with glycine or histidine in PLC- δ it could no longer hydrolyse PIP₂ but still retained the ability to hydrolyse PI. Replacing Arg549 with lysine had only a small effect on PIP₂ and PI hydrolysis this suggests that the positive charge at residue 549 is important for the specific binding of PIP₂ or PI (Wang, *et al.*, 1996). Arginine 549 is conserved throughout all eukaryotic PLCs and therefore an equivalent arginine 693 can be identified in the Y catalytic domain of *Sz. pombe* Plc1 (Figure 45). This evidence gained from human PLC- δ suggests that Arg693 may play an important role in the catalytic activity of Plc1.

The amino acids Lys461 and Arg549 were identified in mammalian PLC- δ , to have an important role in binding and hydrolysing the substrates, PIP₂ and PI. It has been hypothesised from a 2.4-Å resolution crystal structure of rat PLC- δ (Essen *et al.*, 1996) that Lys461 forms a salt bridge with the 4-phosphoryl group of the substrate (Simoes *et al.*, 1995). The residue Arg549 on the other hand with its positively-charged guanidinium group may form a salt bridge with the negatively-charged phosphate at position 4 and 3'-hydroxyl group of the inositol ring (Wang, *et al.*, 1996) (Figure 45).

Equivalent amino acids have been identified in *Sz. pombe* Plc1, Lysine 582 and Arginine 693. If these similar amino acid replacements that were performed in mammalian PLCs were now introduced into *Sz. pombe* Plc1 it may help determine its true substrate. These mutant forms of Plc1 may identify if the generation of the second messenger Ins 1,4,5-P₃ is important, as similar mutations in mammalian PLCs were unable to bind or hydrolyse PIP₂ thus there was no generation of Ins 1,4,5-P₃. This reduction of Ins 1,4,5-P₃ production in *Sz. pombe* cells may implicate a role for Plc1.

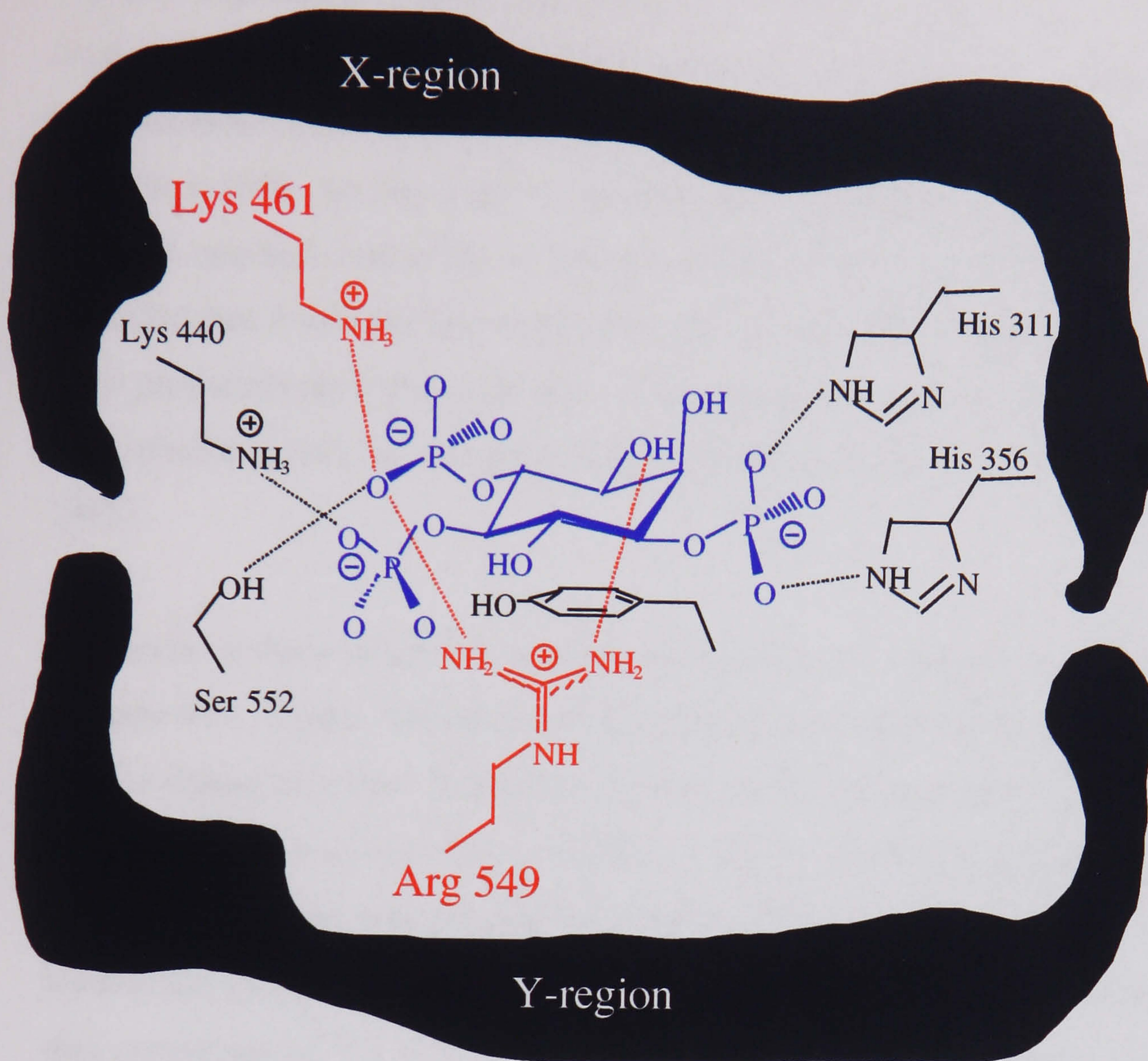


Figure 45 The active site of PLC- δ

A schematic diagram of the network of substrate and amino acid interactions within the active site. Coloured in blue in the centre of the diagram is the substrate PIP₂. The amino acid residues coloured in red (Lys 461 and Arg 549) are the ones important in binding and hydrolysing the substrate. The corresponding amino acids in *Sz. pombe* Plc1 were identified as Lys 582 and Arg 693. Other amino acids that are important in binding the substrate are also indicated on the diagram in black.

7.2.2 Amino acid charge and shape is important for Plc1 activity

Previous experiments carried out by numerous groups (Wang *et al.*, 1996; Cheng *et al.*, 1995; Simoes *et al.*, 1995; Ellis and Katan, 1995) on mammalian PLCs have demonstrated it is not just the charge of amino acids that is important for catalytic activity but the shape of the amino acid is also important. Mutations of the basic residues seen in the X catalytic domain (Figure 44 highlighted in green) indicated that it was not just charge that was important for enzyme activity but other properties were also important. These properties include the hydrophobicity and / or correct spacing of the basic amino acids (Simoes *et al.*, 1995).

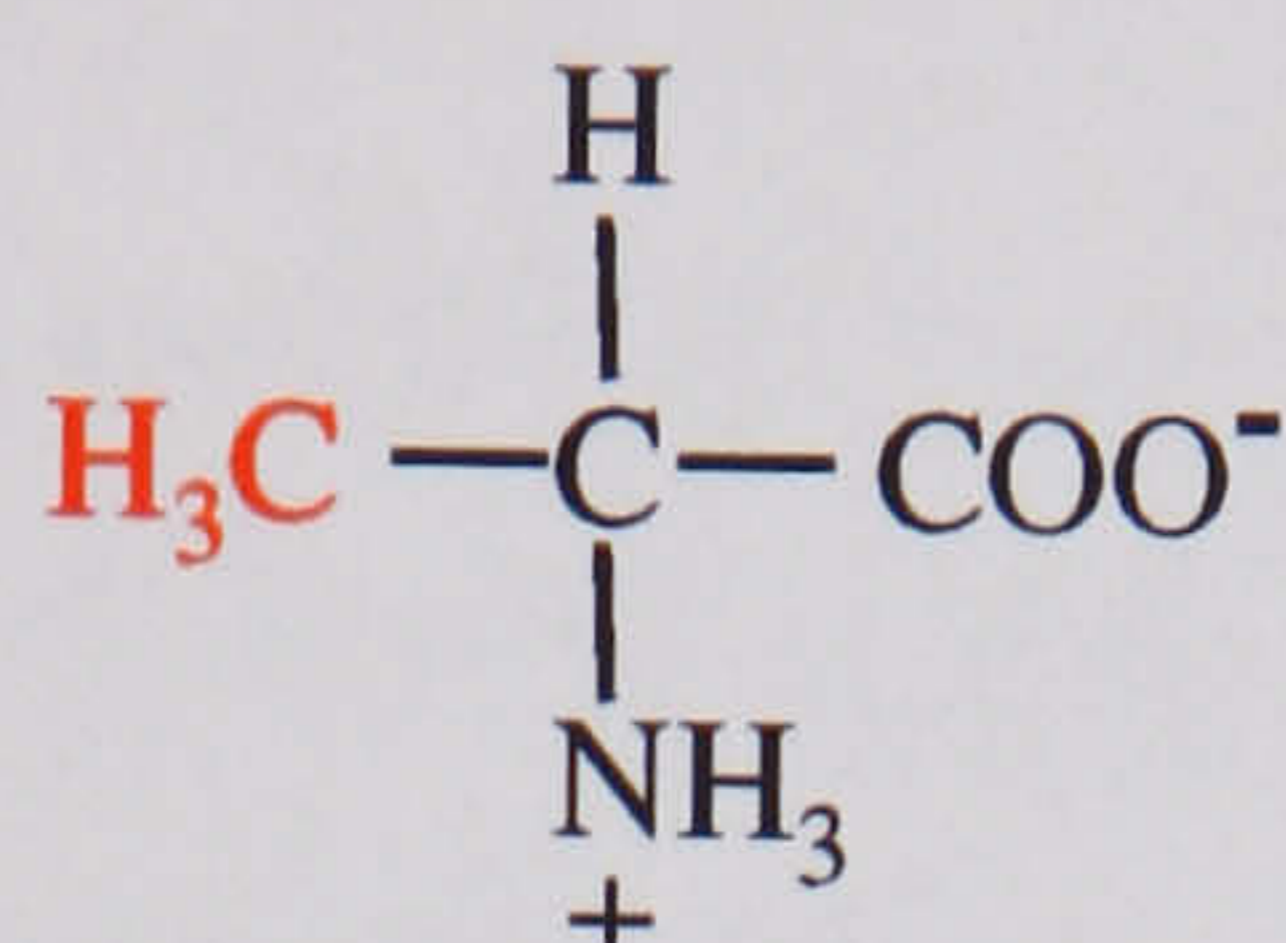
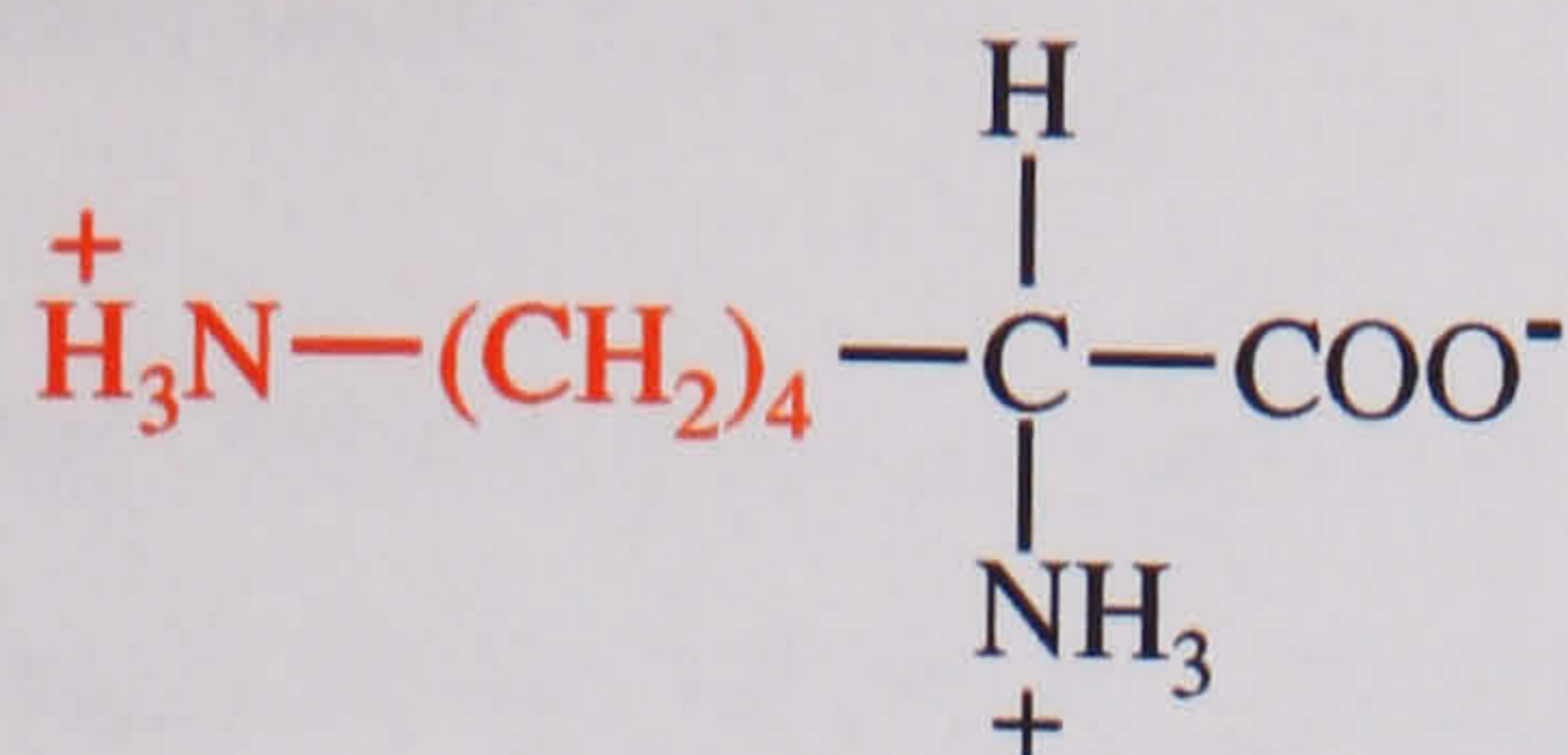
The amino acids selected for mutation in *Sz. pombe plc1* were lysine 582 and arginine 693. Lysine 582 was replaced by arginine (Figure 46) because it had a similar charge to lysine. When this same mutation was carried out on human PLC- β , it led to a complete loss of PIP₂ hydrolysis activity (Simoes *et al.*, 1995). Lysine 582 was also replaced alanine (Figure 46) because it had no charge but reduces the chances of structural modification within the active site. When this was carried out in PLC- δ it resulted in loss of both PIP₂ and PI hydrolysis activity but did not affect the binding of the substrate (Cheng *et al.*, 1995).

Arginine 693 was replaced by lysine (Figure 46), when this was carried out on Human PLC- δ there was no effect on its ability to hydrolyse PIP₂ or PI (Wang *et al.*, 1996). The other substitution was arginine for leucine (Figure 46); when this was performed on Human PLC- δ it was unable to hydrolyse PIP₂ but was still able to hydrolyse PI (Wang *et al.*, 1996). These amino acid replacements may indicate whether charge or shape is important for catalytic activity at this active site residue.

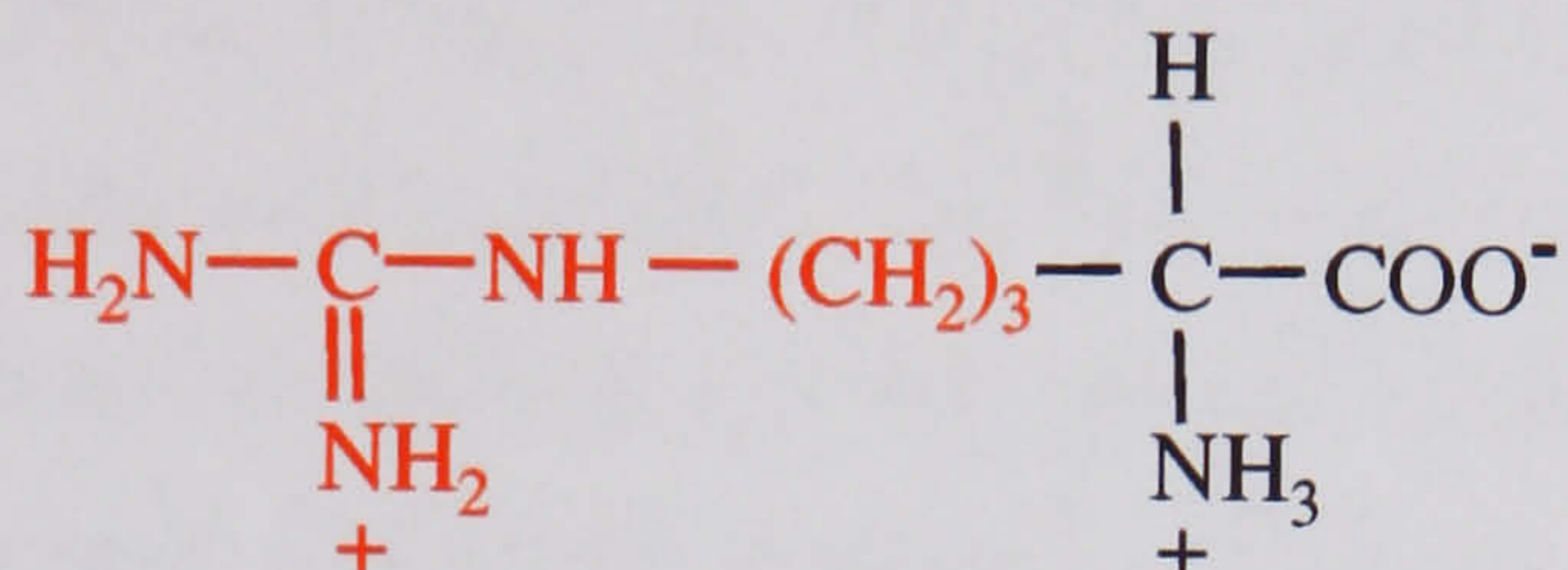
Original amino acid

Replaced by

Lysine

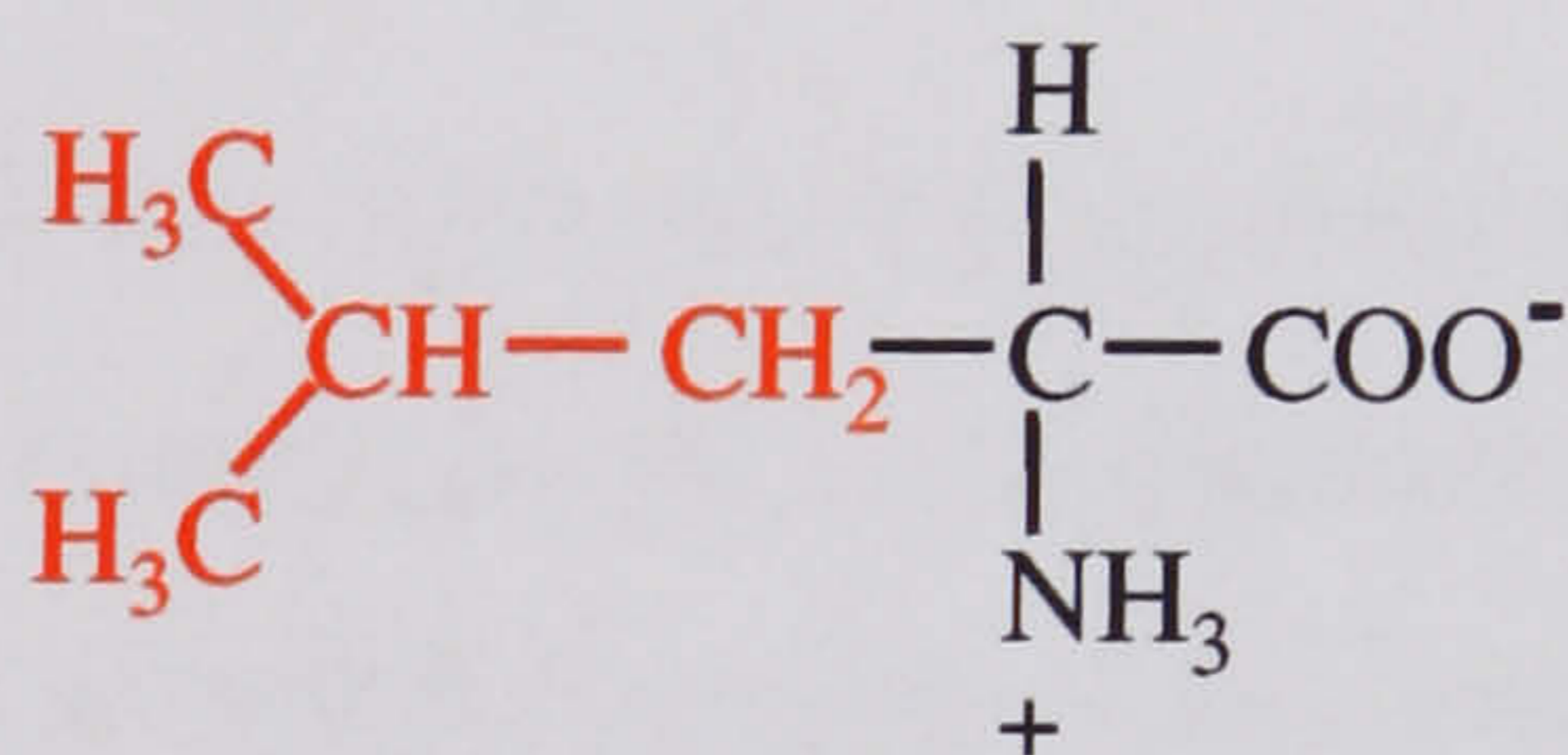
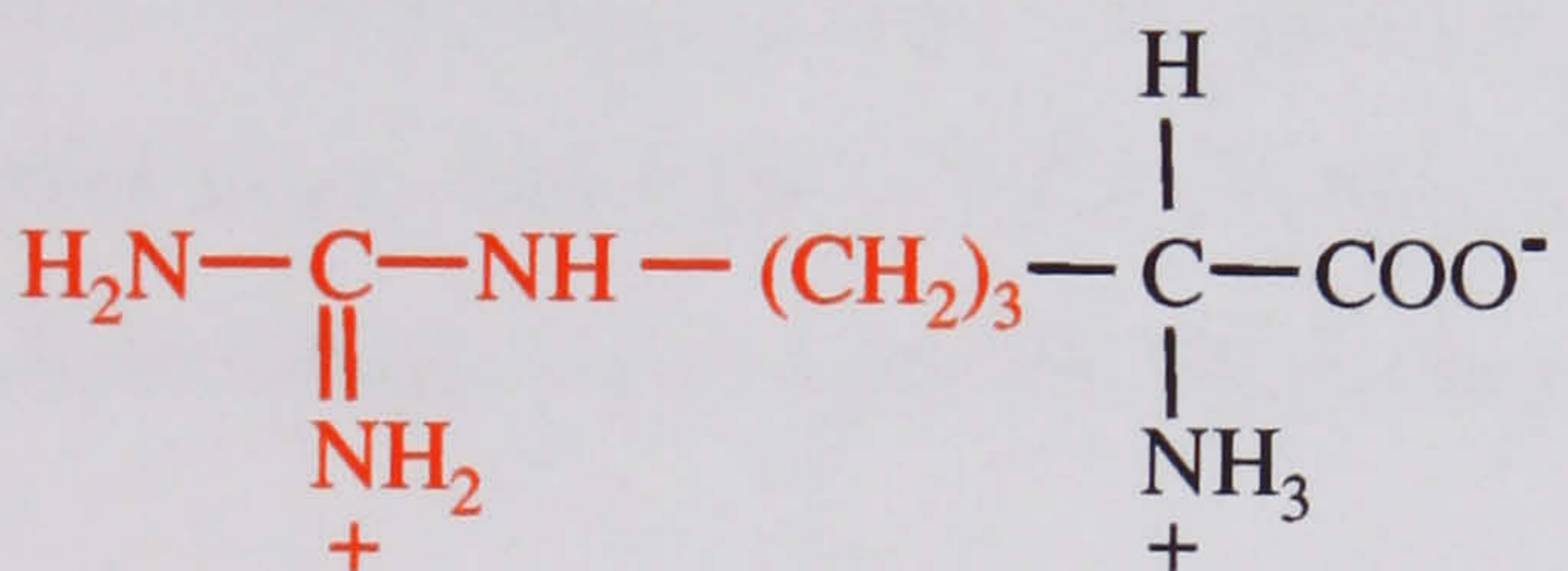


Alanine

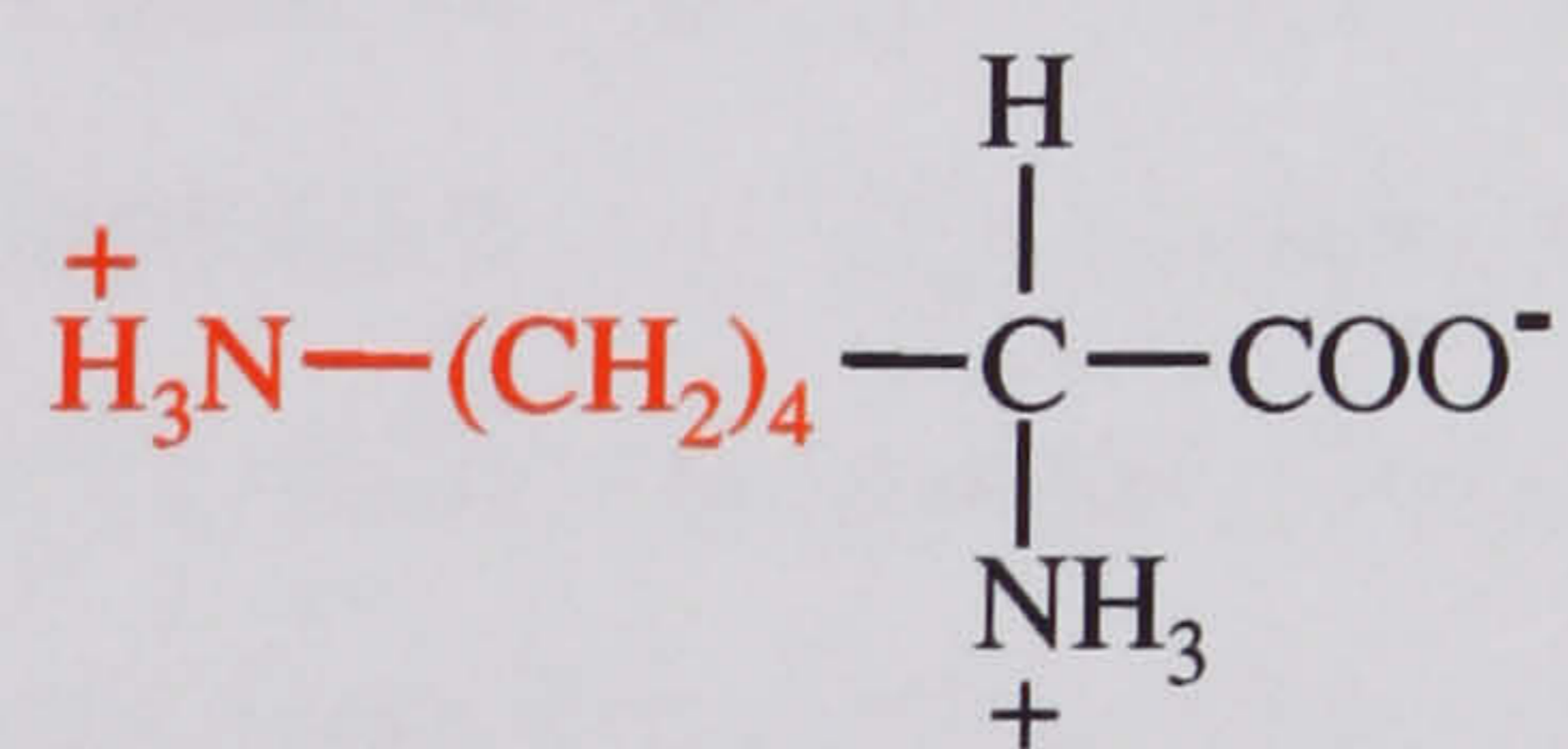


Arginine

Arginine



Leucine



Lysine

Figure 46 The amino acid replacements carried out in the active site of Plc1

This illustrates the amino acid replacements carried out within the active site of Plc1. Lysine 582 was replaced by alanine because it had no charge but this change results in less structural disruption. Lysine was also exchanged for arginine because it had a charge similar to lysine. Arginine 693 was replaced by leucine because it was structurally similar but had no charge. Arginine was also exchanged for lysine because of the similar charge they carry on their side chain.

7.3 Determining the hydrolysis activity of the active site mutants *in vitro*

The amino acid replacement was introduced into the ORF of *plc1* by using inverse PCR (see Chapter 3, Figure 14). Double stranded DNA sequencing (see Methods section 2.2.1.5) was then performed on the inverse PCR products confirming no errors other than the required amino acid replacement had been introduced into the ORF. The ORF of Plc1[R693K], Plc1[R693L], Plc1[K582R] and Plc1[K582A] were sub-cloned into the expression vector pREP3x (see Chapter 3, Figures 15, 16, 17 and 18). The pREP vectors are yeast expression vectors that contain the *nmt1* promoter. The *nmt1* promoter is expressed in the absence of thiamine and is repressed in the presence of thiamine. Wild-type *Sz. pombe* cells transformed with pREP3x containing wild-type *plc1* or one of the mutant forms of Plc1. The transformed cells were cultured in cultures at 30°C in DMM minus thiamine. Crude extracts were prepared from cells (see Methods section 2.2.2.1) expressing mutant forms of Plc1 so that the activity of these mutants could be determined *in vitro* using the Plc1 assay (see Methods section 2.2.2.2).

The activity of the Plc1 mutants *in vitro* was determined against the substrates PIP₂ and PI. This was to determine whether the substrate specificity of the Plc1 mutants had been modified in a similar manner to the mammalian PLC mutants (see Sections 7.2.1-7.2.2). The method that uses PIP₂ or PI as a substrate *in vitro* is stated in the methods (see Methods 2.2.2.2) but briefly the PI substrate mixture consists of unlabelled PIP₂, ³H-PI and 6.4% sodium cholate. Without PIP₂ in the substrate mixture the PH domain of Plc1 would not be able to bind to the substrate and hydrolyse PI (Wang, *et al.*, 1996). A high concentration of PIP₂ was added to the substrate, because saturating concentrations of PIP₂ appear not to affect PI hydrolysis (Wang *et al.*, 1996).

Wild-type Plc1 hydrolysed PIP₂ and PI, but appeared to have a ten-fold lower rate of PI hydrolysis compared to PIP₂ hydrolysis (Figure 47). All of the mutant

forms of Plc1 (Plc1[R693K], Plc1[R693L], Plc1[K582R] and Plc1[K582A]) had lost the ability to hydrolyse PIP₂ *in vitro* but still retained some ability to hydrolyse PI (Figure 47). The mutations that retained the charge at position 582 or 693 had greater PI hydrolysing activity than mutants maintaining an uncharged amino acid that was structurally similar to the original residue. This result confirmed that when these mutants are assayed *in vitro* no PIP₂ activity can be detected above the normal background levels but they retain varying abilities to hydrolyse PI. This suggests that the two amino acid residues investigated here, Lys582 and Arg693 may play an important role when selecting and hydrolysing PIP₂ but not PI.

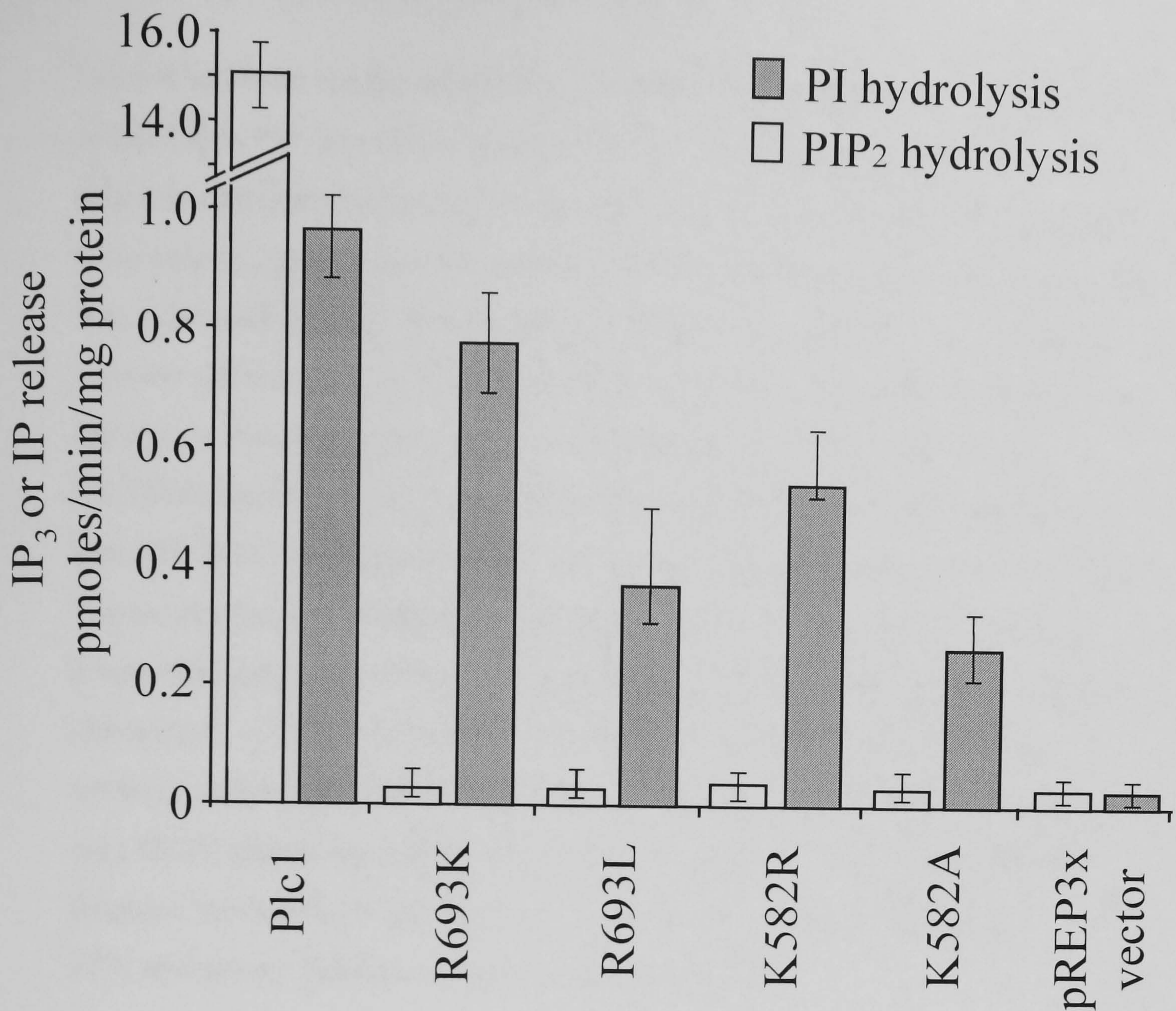


Figure 47 Activity of the active site mutants *in vitro*

Wild-type Plc1 and the mutants Plc1[R693K], Plc1[R693L], Plc1[K582R] and Plc1[K582A] were sub-cloned into pREP3x and transformed into *P* cells. Crude extracts were prepared from these cells and assayed *in vitro*. 1mg/ml of crude protein extract was mixed with a substrate comprising of PIP₂, ³H-PIP₂ or ³H-PI (depending on which substrate the enzyme is being assayed against) and 6.4% w/v sodium cholate. The buffer consisted of 50mM Tris pH 7.5 and reaction was incubated at 30°C. The bars represent the standard range of the mean for triplicate determinations.

7.4 Expression of the active site mutants *in vivo*

Once it had been confirmed that these mutant forms of Plc1 were no longer able to hydrolyse PIP₂ but retain varying levels of PI hydrolysis *in vitro*. The next step was to express these mutants in $\Delta plc1$ *Sz. pombe* cells to see if the mutants were able to suppress their temperature and nutritional sensitive phenotype. This may give some insights into the catalytic role of Plc1 in *Sz. pombe* and might indicate whether Ins 1,4,5-P₃ production is important. In order to express these mutants *in vivo* they were firstly cloned in to pREP81x. Wild-type Plc1, Plc1[R693K], Plc1[R693L], Plc1[K582R] and Plc1[K582A] were sub-cloned into pREP81x (see Chapter 3 for constructs) and transformed into $\Delta plc1$ *P* cells. The expression vector pREP81x has the lowest level of expression due to the lower efficiency with which RNA polymerase can bind to the promoter region (Maundrell, 1990) (see Methods for information on the pREP expression vectors). Approximately five thousand of these transformed cells were plated onto DMM plates containing 167µg/ml of thiamine or DMM plates without thiamine (see Methods section 2.2.4). Plates were incubated at either 23°C or 37°C and colony formation was recorded (Figure 48).

The active site mutants when expressed *in vivo* are under the control of the *nmt1* promoter incorporated in pREP81x. Figure 48 demonstrates that the mutant forms of Plc1 (Plc1[R693K], Plc1[R693L], Plc1[K582R] and Plc1[K582A]) when expressed *in vivo* display growth that is identical to wild-type cells. The transformed cells are able to grow on DMM plates incubated at 23°C and 37°C both in the presence or absence of thiamine. $\Delta plc1$ cells when transformed with pREP vector alone are unable to grow on DMM medium regardless of the temperature or the thiamine content (Figure 40).

From the results of the *in vitro* analysis of the Plc1 active site mutants it would seem reasonable to assume that all of the PIP₂ hydrolysis activity had been abolished. When these mutants were expressed *in vivo* under the control of pREP81x in both the presence and absence of thiamine they were able to

suppress the temperature and nutritional sensitive phenotype. This would suggest that very low levels of PI hydrolysis might be required for $\Delta plc1$ cells to function normally. This suppression may be due to the production of DAG or IP may be an important precursor for another inositol polyphosphate signalling molecule. Alternatively if all the PIP₂ hydrolysis has not been abolished then undetectable levels of PIP₂ hydrolysis may be responsible for $\Delta plc1$ suppression on minimal media and high temperatures.

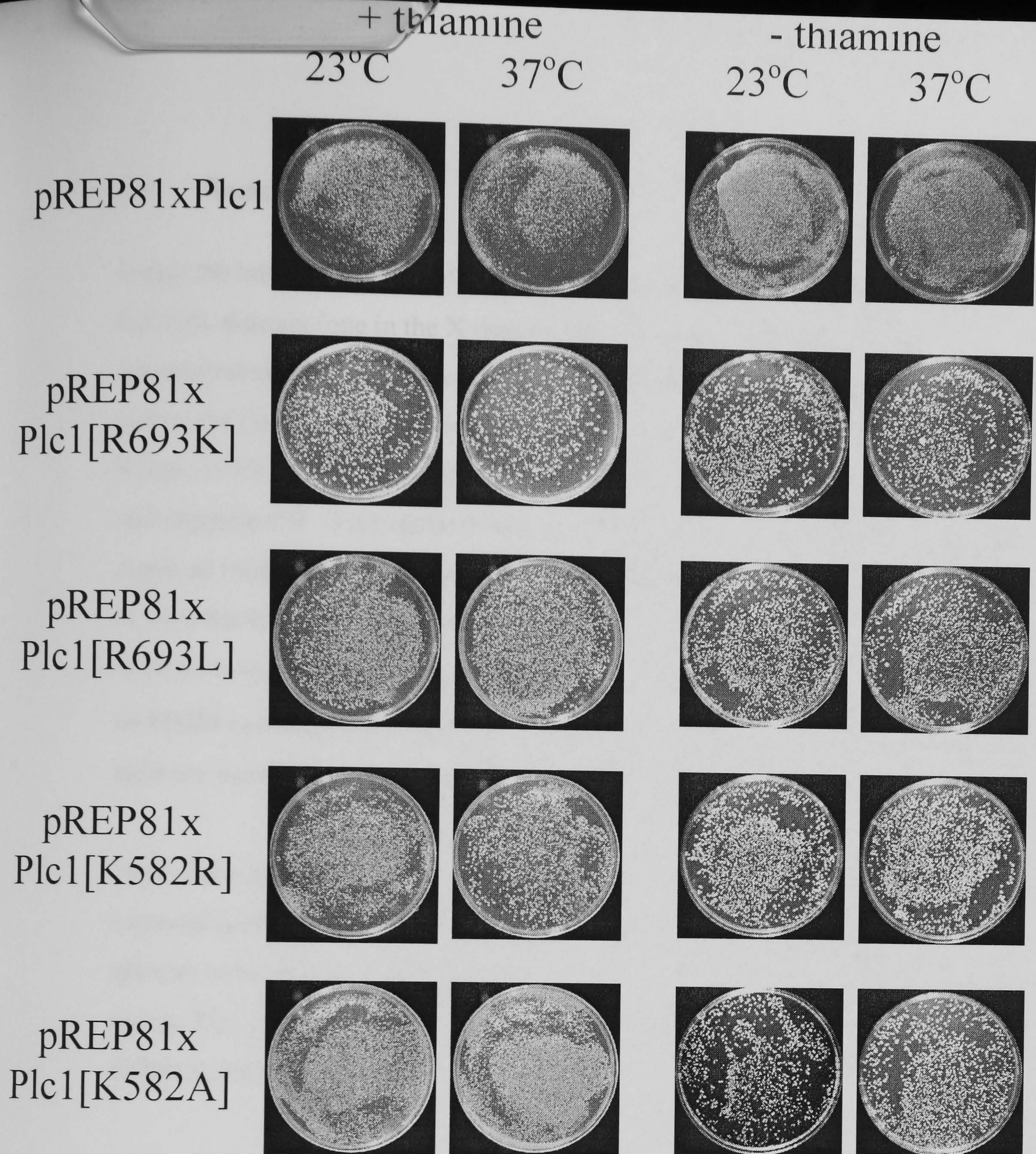


Figure 48 Expression of the active site mutants *in vivo*

Plc1, Plc1[R693K], Plc1[R693L], Plc1[K582R] and Plc1[K582A] were subcloned into pREP81x (see Chapter 3) and transformed into Δ Plc1 cells. Five thousand of these transformants were plated out on DMM plates either containing thiamine or without thiamine (see Methods section 2.2.4). These plates were incubated at either 23°C or at 37°C and colony formation was noted after 4 days.

7.5 Summary

Using the information gained from mammalian PLCs, two residues within the catalytic domain (one in the X domain and one in the Y domain) have demonstrated their importance in binding and selecting substrates within the active site (Wang *et al.*, 1996; Cheng *et al.*, 1995; Simoes *et al.*, 1995; Ellis and Katan, 1995). Equivalent residues were identified in *Sz. pombe plc1*, lysine 582 and arginine 693. Plc1 mutants were constructed in which arginine and alanine replaced lysine 582 and lysine and leucine replaced arginine 693. When assayed *in vitro* these mutants were unable to hydrolyse PIP₂ but still retained some level of PI hydrolysis. Expression of the mutants in $\Delta plc1$ cells resulted in cell growth on DMM media irrespective of the temperature and the thiamine content ($\Delta plc1$ cells are normally unable to grow on DMM media even at low temperatures).

From these experiments the following conclusions can be drawn. Similar to mammalian PLCs which preferentially hydrolyse PIP₂ to PI, wild-type Plc1 appears to recognise, bind and hydrolyse both PIP₂ and PI. This confirms that *Sz. pombe* Plc1 is more similar to mammalian PLCs than prokaryotic PLCs but has different amino acid requirements at the active site.

$\Delta plc1$ cells are unable to grow on DMM or at temperatures above 29°C but this is suppressed by the expression of the Plc1 active site mutants, which are unable to hydrolyse PIP₂ but able to hydrolyse PI. This suggests that PI hydrolysis is sufficient for cell growth. In *Sz. pombe* cells PI hydrolysis probably results in the production of DAG, which may activate either PKC or another protein kinase. PI hydrolysis also results in the production of InsP, which may be an important precursor for the production of other inositol polyphosphates.

Chapter 8
Investigating the N-terminal domain of Plc1

8.1 Introduction

8.1.1 The regulation of mammalian PLCs

The regulators of mammalian PLC- β and PLC- γ have been identified. PLC- β and PLC- γ contain extra domains that are known to interact with GTP binding proteins, tyrosine kinase receptors or nonreceptor tyrosine kinases.

8.1.1.1 The regulation of PLC- β

The extended C-terminal domain of PLC- β (Figure 49) is known to interact with heterotrimeric G-proteins. Activation of a receptor that is coupled to a heterotrimeric G-protein results in GTP binding to the α subunits. The G-protein then dissociates into three subunits; the α and $\beta\gamma$ subunits. Once dissociated either the α subunit of the G-protein (G_q family) (Taylor, *et al.*, 1991; Hepler, *et al.*, 1993) or the $\beta\gamma$ subunits (G_o family) (Katz, *et al.*, 1992; Hepler, *et al.*, 1993) interact with the C-terminal domain of PLC- β facilitating its activation.

8.1.1.2 The regulation of PLC- γ

PLC- γ has an extra five domains encoding 400 amino acids (two PH domains, two SH2 domains and an SH3 domain) that are not found in other PLC isoforms (Figure 49). The activation of PLC- γ is by the phosphorylation of identical tyrosine residues identified within the SH2 domains (Kim, *et al.*, 1991). This is thought to create a structural change in PLC- γ allowing the SH3 domain to bind to proteins such as profilin (Goldschmidt-Clermont, *et al.*, 1991) which is associated with the membrane, bringing PLC- γ in close proximity to the substrate. This phosphorylation step is regulated by receptor or nonreceptor tyrosine kinase activities. Ligands such as epidermal growth factor (EGF) bind

to the receptor which results in auto-phosphorylation of the receptor. The tyrosine kinase activity found on the intracellular portion of the receptor phosphorylates specific tyrosine residues on PLC- γ facilitating its interaction with proteins on the membrane. The non-receptor tyrosine kinase activation of PLC- γ is essentially the same but a cytosolic tyrosine kinase phosphorylates specific residues on PLC- γ aiding its interactions with proteins on the membrane.

8.1.1.3 The regulation of PLC- δ

Unlike PLC- β and PLC- γ , PLC- δ does not contain any extra domains (Figure 49). The regulator of PLC- δ therefore, is unknown but it has been suggested that it may be regulated through a GTP-binding protein known as $G\alpha_h$ (Feng, J-F *et al.*, 1996). Reconstitution studies with PLC- δ , the α 1-adrenergic receptor and G_h resulted in PIP₂ hydrolysis (Das *et al.*, 1993). Co-immunoprecipitation of PLC- δ in the α 1-adrenergic system demonstrates that PLC- δ only associates with the GTP-bound form of G_h (Das *et al.*, 1993). It is also known that all eukaryotic PLCs require calcium for activation so regulation of PLC- δ may be facilitated by fluctuations in cytosolic Ca²⁺ concentrations. It appears however that PLC- δ is more sensitive to cytosolic Ca²⁺ concentrations than PLC- β and PLC- γ . Increases in Ca²⁺ concentration within the range of 0.1-10 μ M was sufficient to stimulate PLC- δ activity but insufficient to stimulate PLC- β or PLC- γ (Allen *et al.*, 1997).

8.1.2 The N-terminus of yeast PLCs

The PLC isozymes from *S. cerevisiae* and *Sz. pombe* appear to have an extended N terminal domain (Figure 49). This extended N-terminal domain is not a lengthened part of the PH domain and bears no resemblance to the extended C-terminal domains seen in PLC- β or any of the extra domains observed in PLC- γ . The extended N-terminus of *Sz. pombe plc1* is longer than *S. cerevisiae PLC1*

and there is no sequence similarity between them. Payne and Fitzgerald-Hayes (1993) reported that the N-terminal end of *S. cerevisiae* PLC1 contains a possible nuclear localisation signal (KKLRK) as well as five potential sites for phosphorylation by protein kinase C. Expression of the PLC1 open reading frame starting from the second methionine (79th codon) complemented the loss of the *PLC1* gene suggesting that the 78 N-terminal residues are not essential for activity (unpublished data mentioned in Yoko-o *et al.*, 1993). Flick and Thorner (1993) inferred that this extended N-terminal section of PLC1 might reflect some distinctions in regulatory properties or subcellular localisation between yeast and mammalian PLCs.

8.1.3 The N-terminus of *Sz. pombe* Plc1

It has been shown that the N-terminus of *Sz. Pombe* Plc1 is approximately 147 amino acids in length that constitutes one sixth of the total protein size. This N-terminal section of Plc1 might have a role associated with regulation, cellular localisation, protein folding or binding of Plc1 to the membrane. Since PLC- β and PLC- γ contain extra domains involved in their regulation and the N-terminal domain of PLC1 from *S. cerevisiae* may be involved in regulation (Flick and Thorner, 1993), it would seem reasonable to suggest that this N-terminal section of Plc1 might also be involved in regulation. Although the N-terminal domain in Plc1 displays no sequence similarity to the extra regulatory domains seen in PLC- β and PLC- γ , there may be alternative regulators that bind specifically to the N-terminal domain. As the N-terminus of *Sz. pombe* Plc1 and *S. cerevisiae* PLC1 display no sequence similarity it is unlikely that they regulated by similar methods.

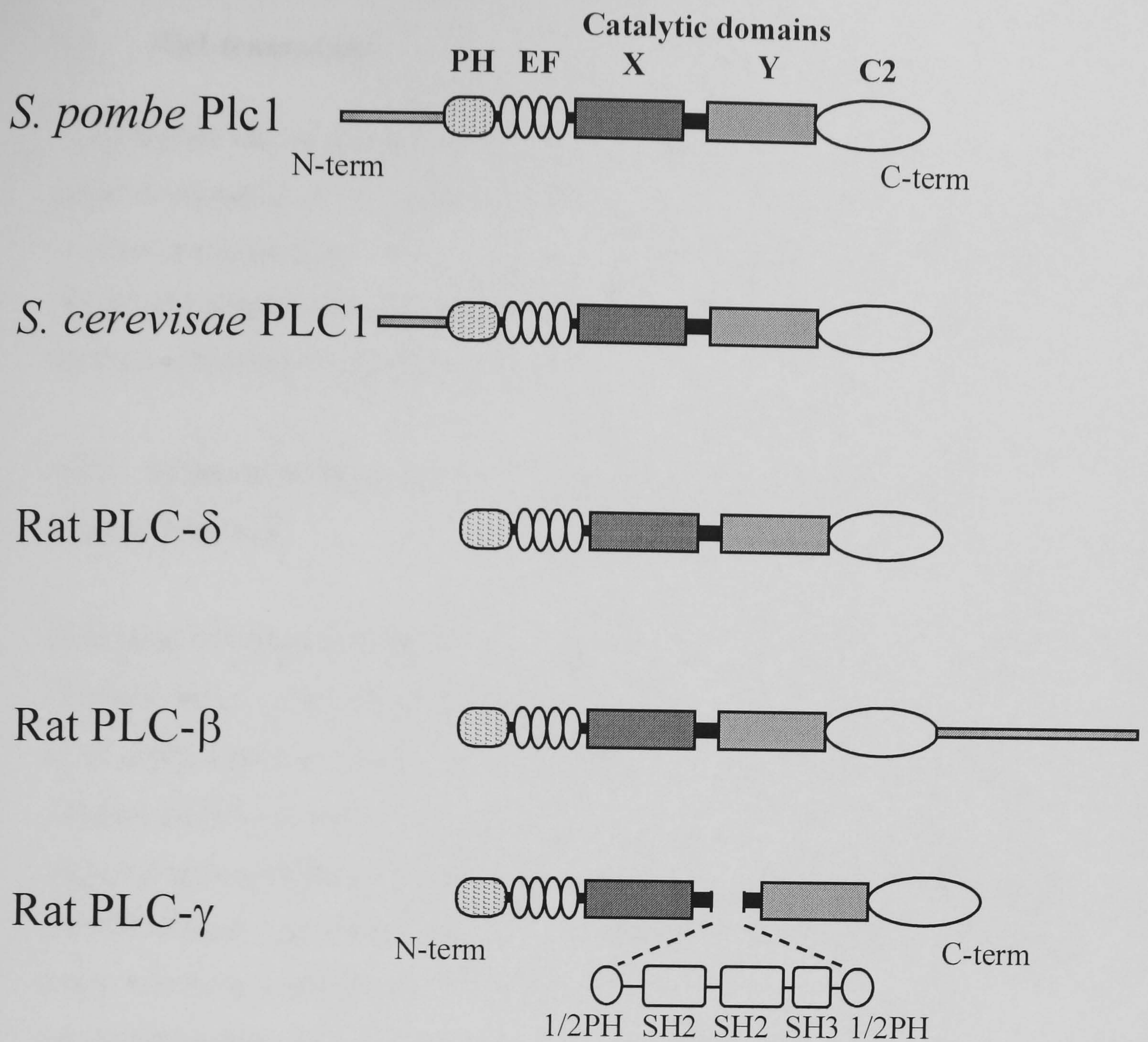


Figure 49 The structure of eukaryotic PLCs

PLCs contain four conserved domains these include the Pleckstrin Homology domain (PH), the EF hand domain (EF), the X and Y catalytic domain and the C2 domain. PLC- β has an extended C-terminal domain and PLC- γ has an extra four domains, these extra domains are involved in their regulation. The yeast PLCs have an extended N-terminus, which is not seen in any other eukaryotic PLCs. The function of this N-terminal domain is unknown.

8.2 Plc1 truncations

The simplest way to identify the importance of this N-terminal domain is to create a truncation of Plc1 that removes the first 144 amino acids. When assayed *in vitro* or expressed *in vivo* this truncated form of Plc1 may reveal whether or not this domain plays an important role in regulation, interaction with other proteins, or perhaps membrane binding.

8.2.1 Removal of the N-terminus results in a PLC structurally more similar to PLC- δ

If the first 144 amino acids are removed from the N-terminus of Plc1 by PCR (Plc1 Δ N-term₁₄₄) (see Chapter 3 Figure 22) it results in a PLC that looks structurally similar to Rat PLC- δ 1 (Figure 50). Plc1 Δ N-term₁₄₄ now simply contains the four domains that are common to all PLCs. Double stranded DNA sequencing (see Methods, section 2.2.1.5) was performed on the PCR product of Plc1 Δ N-term₁₄₄ without the N-terminus at this stage to confirm that no unwanted errors had been incorporated. This construct was sub-cloned into the yeast expression vectors pREP3x and pREP81x (see Chapter 3, Figure 22).

8.2.2 Expressing the N-terminus of *Sz.pombe* Plc1

The N-terminus of *Sz. pombe* Plc1 might be a site that interacts with other proteins regulating its activity. If the N-terminal domain does interact with regulatory proteins, then expression in wild-type cells may display possible growth defect due to competitive inhibition. The wild-type cells expressing the N-terminus might display a phenotype that is more similar to Δ *plc1* cells because proteins that normally interact with the N-terminus of the full length protein could now also recognise and bind to this short N-terminal peptide. To test this hypothesis, the first 147 amino acids of Plc1 (N-term) (Figure 50) were amplified using PCR (see Chapter 3, Figure 24) and was sequenced using double stranded

DNA sequencing (see Methods section 2.2.1.5). The N-term was then sub-cloned into pREP3x and pREP81x.

8.2.3 Expressing Rat PLC- δ in *Sz. pombe* cells

Since *Sz. pombe* Plc1 Δ N-term₁₄₄ looked structurally similar to a PLC- δ (Figure 50) it would be interesting to discover if rat PLC- δ was able to complement for the loss of Plc1 activity in Δ *plc1* cells. Rat PLC- δ was shown to complement in *S. cerevisiae* Δ *PLC1* cells but it is not known whether rat PLC- δ is able to complement in Δ *plc1* cells. The Rat PLC- δ gene was amplified by using PCR and was sequenced to confirm that no errors had been introduced during the PCR process (see Chapter 3, figure 21). Rat PLC- δ was then cloned into the yeast expression vectors pREP3x and pREP81x.

8.2.4 Fusion of the Plc1 N-terminus to PLC- δ results in a mammalian PLC structurally similar to *Sz. pombe*

Fusion of the N-terminal 144 amino acids of Plc1 to the amino terminus of rat PLC- δ results in a PLC that looks structurally similar to *Sz. pombe* Plc1 (Figure 50). The N-terminus of *Sz. pombe* was introduced to see if this Plc1/ Rat PLC- δ fusion (Plc1₁₄₄-PLC- δ) was active and see if it was able to suppress the loss of Plc1 activity in Δ *plc1* cells. Plc1₁₄₄-PLC- δ was produced through PCR amplification. Double stranded DNA sequencing was performed on the Plc1₁₄₄-PLC- δ to confirm no errors had been introduced into the sequence. The Plc1₁₄₄-PLC- δ was subsequently cloned into the yeast expression vectors pREP3x and pREP81x (see Chapter 3, Figure 23).

The open reading frames of *Plc1*

were sub-cloned into

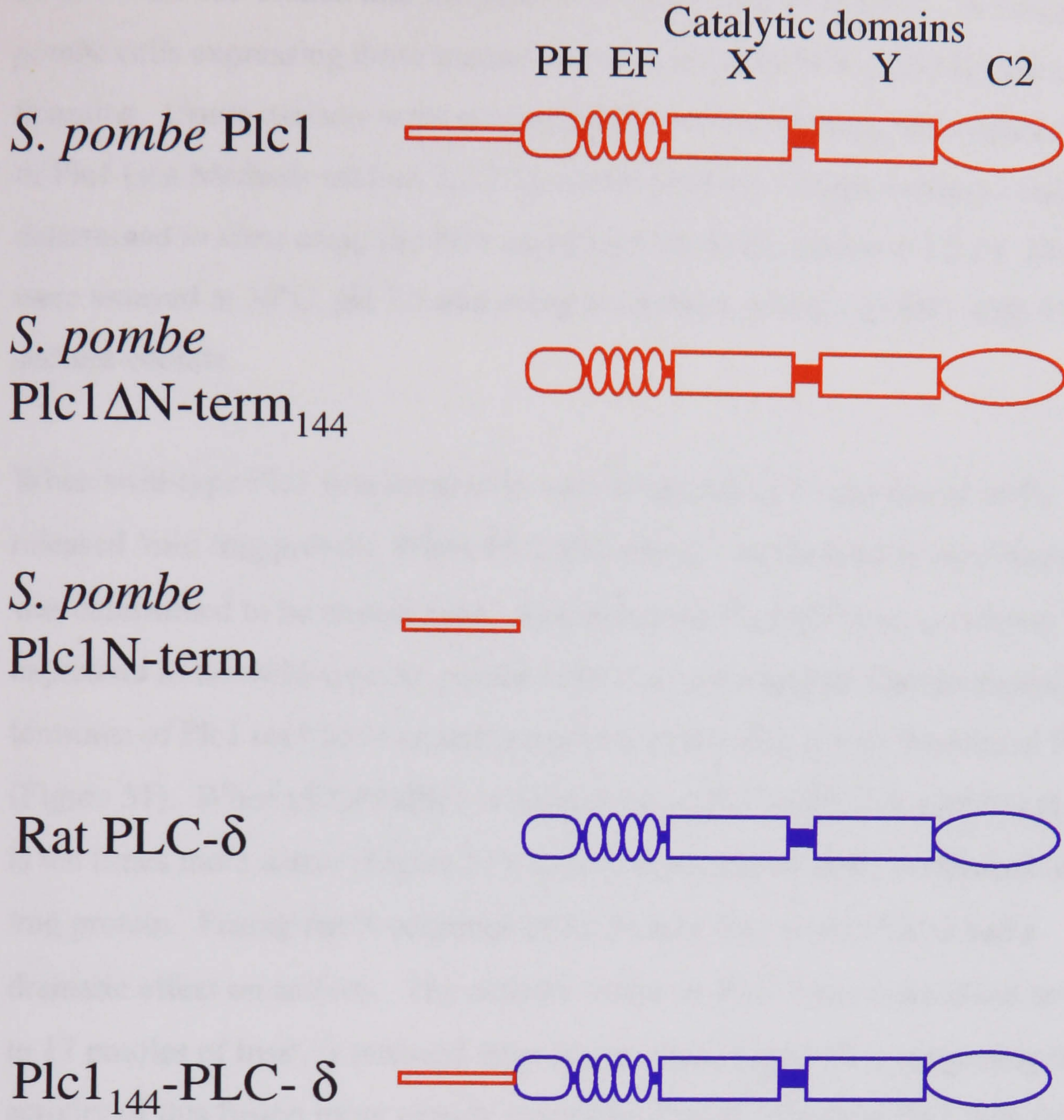


Figure 50 Constructs which may help identify the role of the Plc1 N-terminus

A diagram displaying the mutants constructed to investigate the N-terminal domain of Plc1. Highlighted in red is *Sz. pombe* Plc1 and highlighted in blue is Rat PLC- δ . This figure demonstrates the clones produced, the removal of the N-terminus from *plc1* to make Plc1 look structurally similar to PLC- δ . The N-terminus of Plc1 was fused to Rat PLC- δ to make it look structurally similar to Plc1.

8.3 Activity of the N-terminal domain mutants *in vitro*

The open reading frames of Plc1 Δ N-term₁₄₄, Rat PLC- δ , N-term and Plc1₁₄₄-PLC- δ were sub-cloned into the yeast expression vector pREP3x. Wild-type *Sz. pombe* cells expressing these mutants were cultured at 30°C in DMM minus thiamine. Crude extracts were prepared from cells expressing the mutant forms of Plc1 (see Methods section 2.2.2.1), so the activity of these mutants could be determined *in vitro* using the Plc1 assay (see Methods section 2.2.2.2). Mutants were assayed at 30°C, pH 7.5 and using a substrate containing PIP₂ with 6.4% sodium cholate.

When wild-type Plc1 was assayed *in vitro* it had about 15 pmoles of InsP₃ released /min /mg protein. When Plc1 Δ N-term₁₄₄ was assayed *in vitro* its activity was determined to be almost zero. Assuming that Plc1 Δ N-term₁₄₄ is being expressed in the wild-type *Sz. pombe* cells it would suggest that the extended N-terminus of Plc1 may have an important role in forming a fully functional Plc1 (Figure 51). When pREP3xPlc1 is compared to pREP3xPLC- δ , pREP3xPLC- δ is ten times more active (Figure 51), over 150 pmoles of InsP₃ is released /min /mg protein. Fusing the N-terminus of *Sz. pombe* Plc1 to rat PLC- δ had a dramatic effect on activity. The activity of the rat PLC- δ decrease about ten fold to 17 pmoles of InsP₃ is released /min /mg protein (Figure 51), suggesting the activity of this fusion more closely resembles that of wild-type Plc1 than rat PLC- δ . The extracts prepared for assaying PLC activity are very crude and the rate of PIP₂ hydrolysis per minute is based on the total amount of protein within the extract. This assay assumes that the level of expression by the *nmt1* promoter contained on pREP3x is the same for Plc1 Δ N-term₁₄₄, rat PLC- δ and Plc1₁₄₄-PLC- δ and that large differences in expression levels are not causing these contrasting levels of activity.

When the Plc1 N-term₁₄₇ was expressed in wild-type *Sz. pombe* cells, growth defects were seen. The wild-type cells expressing N-term were able to grow at 37°C in DMM media; if they were displaying a phenotype similar to Δ *plc1* cells

then reduced or no growth should have been observed. This could be for one of three reasons the first that the N-term may not be involved in regulating the activity of Plc1. The second is that the N-term is not being expressed resulting in no competitive inhibition. Finally the N-term is being expressed but the expression level is not high enough to affect the Plc1 activity of wild-type cells. When the activity of the N-term was investigated *in vitro* no activity was seen which is unsurprising considering this is only a short section of the Plc1 protein which does not contain any catalytic domains.

From these results it suggests that the N-terminal domain may have an important function in perhaps regulating Plc1 activity. This is because Plc1 Δ N-term₁₄₄ was unable to hydrolyse PIP₂ as efficiently as Plc1 and rat PLC- δ which does not contain this N-terminal domain had extremely high levels of activity ten-times higher than wild-type Plc1. When the activity of the mutant Plc1₁₄₄-PLC- δ was assayed *in vitro* it demonstrated levels of activity closer to wild-type Plc1. This N-terminal domain of Plc1 and the mutant Plc1₁₄₄-PLC- δ may have a regulatory protein that interacts with it controlling the level of PLC activity, without it rat PLC- δ had uncontrollable levels of PLC activity and Plc1 Δ N-term₁₄₄ was inactive.

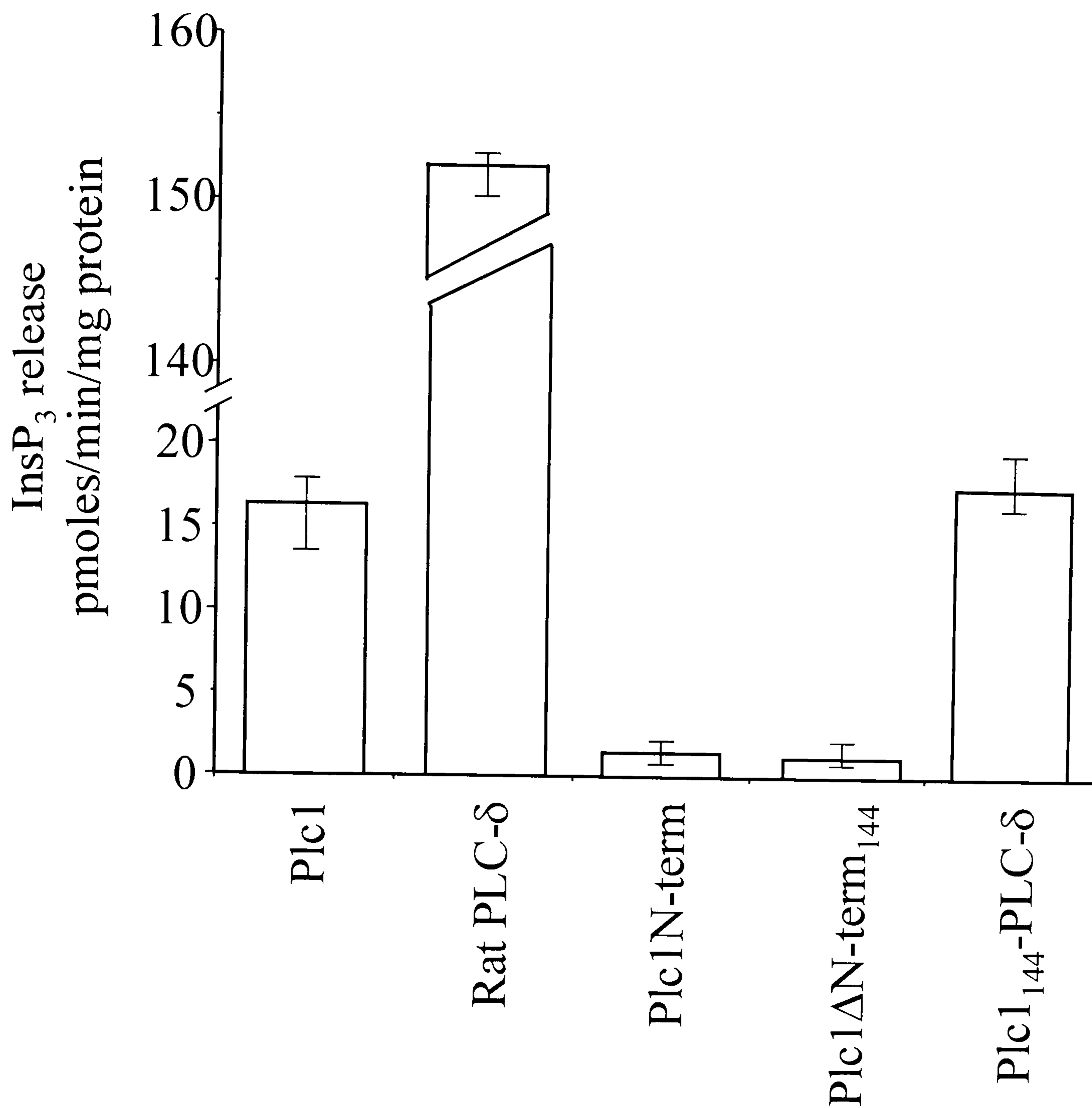


Figure 51 Determining the activity of the PLCs and their mutants *in vitro*

Wild-type Plc1, Rat PLC- δ and the mutants Plc1N-term, Plc1 Δ N-term₁₄₄ and Plc1₁₄₄-PLC- δ were sub-cloned into pREP3x and transformed into a *P* cell. Crude extracts were prepared from these cells and assayed *in vitro*. The substrate comprised PIP₂, ³H-PIP₂ and 6.4% w/v sodium cholate. The assay conditions included a 50mM Tris buffer pH 7.5 and incubation temperature was 30°C. The bars represent the standard range of the mean for triplicate determinations.

8.4 Expression of the N-terminal mutants *in vivo*

Wild-type Plc1, rat PLC- δ , Plc1 Δ N-term and Plc1₁₄₄-PLC- δ were sub-cloned into the expression vectors pREP3x and pREP81x. These constructs were transformed into *P* cells Δ *plc1*; approximately five thousand of these transformed cells were plated onto DMM plates either with or without thiamine. Plates were then incubated at either 23°C or 37°C and colony formation was recorded (see Methods, section 2.2.4).

When pREP3x*plc1* or pREP81x*plc1* is expressed in Δ *plc1* cells, growth is observed on all plates irrespective of the temperature or thiamine content. When pREP3x*plc1* Δ N-term or pREP81x*plc1* Δ N-term is expressed *in vivo* no growth is detected on any of the plates (Figure 52). When Δ *plc1* cells are expressing wild-type Plc1, they are able to grow under normally non-permissive conditions such as temperatures above 29°C or on DMM. When this N-terminal domain is removed and Plc1 Δ N-term is subsequently expressed in Δ *plc1* cells, no growth is observed. This suggests that this N-terminal domain may have an important role in producing a functional Plc1.

When pREP3xPLC- δ or pREP81xPLC- δ is expressed in Δ *plc1* cells no cell growth is detected under any of the conditions studied (Figure 52). This is unexpected because when rat PLC- δ was assayed *in vitro* it displayed ten times more activity than wild-type Plc1, but when expressed in Δ *plc1* cells rat PLC- δ was unable to complement for the loss of Plc1 activity.

When pREP3xPlc1₁₄₄-PLC- δ and pREP81xPlc1₁₄₄-PLC- δ were expressed *in vivo* growth was seen on all plates irrespective of temperature or the presence of thiamine (Figure 52). This suggests that this N-terminus of Plc1 might have an important role, because without it both Plc1 Δ N-term and rat PLC- δ are unable to suppress the loss of Plc1 activity under non-permissive conditions. When the N-terminus of Plc1 is fused to the amino terminus of rat PLC- δ it is able to

complement for the loss of Plc1 activity under non-permissive conditions such as temperatures above 29°C or on DMM. In the *in vitro* experiments rat PLC- δ activity was extremely high, more than ten-fold greater than wild-type Plc1. One possible reason why the clone pREP3xrat PLC- δ may not complement for the loss of Plc1 activity may be due to this high level of activity observed *in vitro*. In contrast extract prepared from cells expressing the clone pREP81xPLC- δ in the presence of thiamine had very low levels of PLC activity. When measured *in vitro* activity was indistinguishable from background levels but was still unable to complement for the loss of Plc1 activity (data not shown). As demonstrated in Chapter 7, the minimum level of Plc1 activity is required to rescue $\Delta plc1$ cells so the level of expression may not be the only factor preventing cell growth.

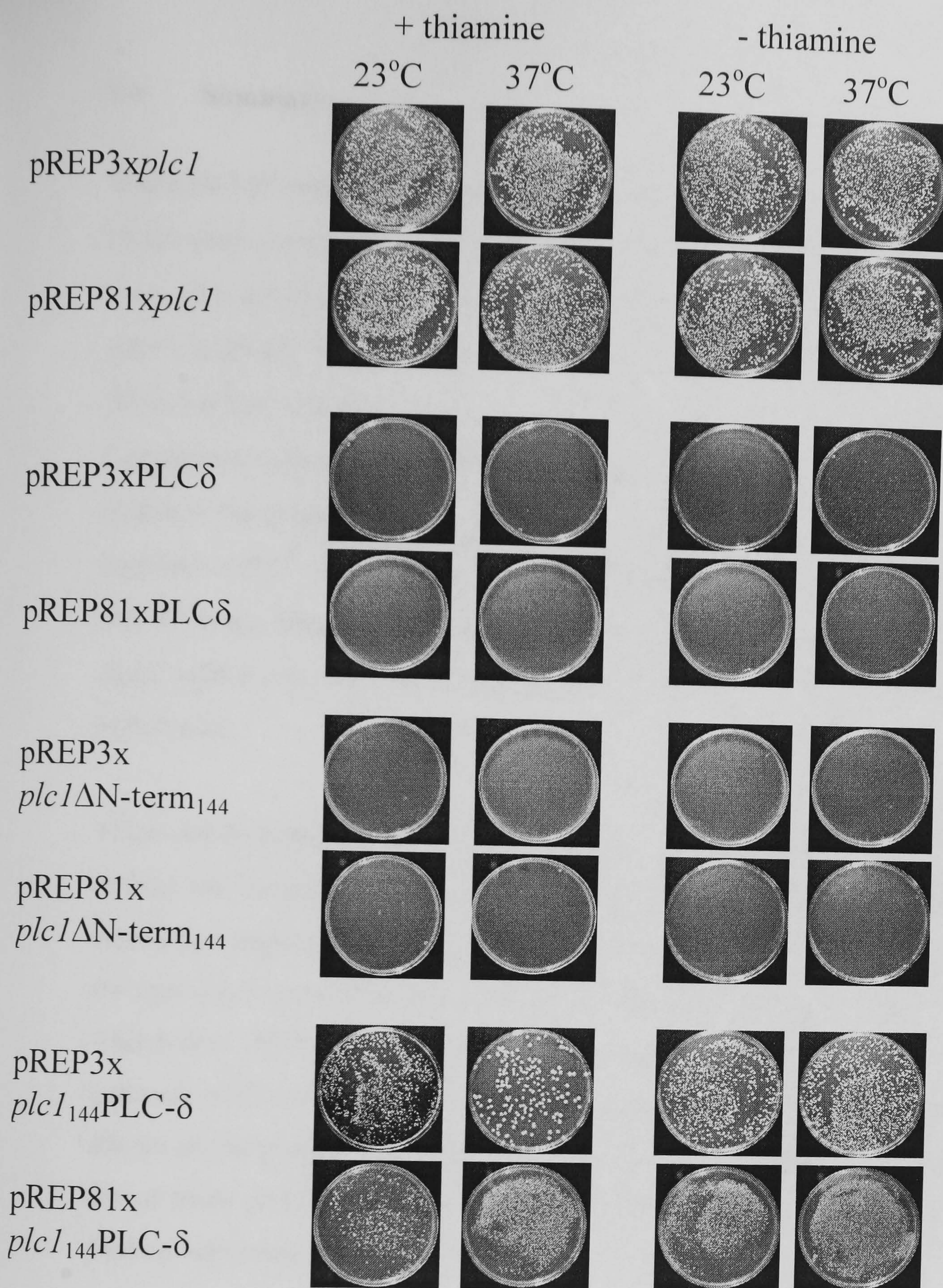


Figure 52 Expression of the N-terminal mutants *in vivo*

Plc1, Plc1 ΔN-term₁₄₄, rat PLC-δ and Plc1₁₄₄-PLC-δ were sub-cloned into pREP3x, pREP81x and transformed into Δ*plc1* cells. Five thousand of these transformed cells were plated out on DMM plates either containing thiamine or without thiamine (see Methods section 2.2.4). These plates were incubated at either 23°C or at 37°C and colony formation was observed.

8.5 Summary

When Plc1 Δ N-term is expressed *in vitro* activity was reduced to undetectable levels when compared to wild-type Plc1. The activity of Plc1 Δ N-terminus is lower than detectable levels using this assay but when this is expressed in Δ *plc1* cells it is unable to complement at any temperature or expression level.

When rat PLC- δ was expressed *in vitro* it displayed ten times more activity when compared to wild-type Plc1. When expressed in Δ *plc1* cells however, it was unable to complement irrespective of the expression levels. When the N-terminus of Plc1 was fused to rat PLC- δ and its activity was investigated *in vitro*, this construct had an activity similar to the wild-type Plc1. When expressed in Δ *plc1* cells it was able to complement irrespective of the temperature or level of expression.

These results suggest that that the N-terminus of Plc1 may have an important role as Plc1 lost its ability to hydrolyse PIP₂ without it and rat PLC- δ activity is unable to complement for the loss of Plc1 activity without it. This N-terminal domain may interact with other proteins regulating its activity in some way. Andoh *et al.*, (1998) identified that a 14-3-3 protein interacted with the N-terminus of Plc1 in response to UV light but unfortunately they did not elaborate further on the precise interaction points within the N-terminus. This could be one of many proteins that interact with Plc1 regulating its activity resulting in the desired responses within the cell.

Chapter 9
Discussion

9.1 Overview

The loss of Plc1 activity in *Sz. pombe* resulted in a very distinct cellular phenotype. $\Delta plc1$ cells demonstrated a temperature sensitive phenotype on rich medium; these cells grew at 23°C but not at 37°C. On DMM $\Delta plc1$ cells were unable to grow, possibly due to the increased stresses enforced on the cells as a result of the limiting nutrients available. Previous studies have demonstrated that Plc1 is a phospholipase C enzyme that is able to hydrolyse PIP₂ into the second messengers InsP₃ and DAG (Slaaby, 1996). In this thesis I have utilised three different approaches (Chapters 6, 7 and 8) to help identify possible regulators of Plc1 and also signalling pathways that might require the activity of Plc1 within *Sz. pombe* cells.

Interpretation of these results was difficult due to the difficulty in trying to quantify the amount of Plc1 present in the *in vitro* and *in vivo* assays. Numerous attempts were made to quantify Plc1 concentrations using Western blots but no bands were detected. A second approach was used to quantify Plc1 concentration in *Sz. pombe* cells by purifying Plc1 using the hexahistidine-tag introduced on to the N- or C-terminus of the protein. Plc1 activity was observed after elution away from the nickel column but no bands were seen on the protein gels that corresponded to the predicted size of Plc1.

9.2 Regulation of Plc1

9.2.1 The N-terminus may be a site for Plc1 regulation

Yeast PLCs have an extended N-terminal domain, which is not seen in mammalian PLCs. The N-terminal domains of *S. cerevisiae* PLC1 and *Sz. pombe* Plc1 bear no sequence similarity. It would seem reasonable to assume that if the N-terminus does have a role in regulating yeast PLCs, then *S. cerevisiae* and *Sz. pombe* will have different regulatory mechanisms which

control PLC activity through this N-terminal domain. Wild-type Plc1 when expressed in $\Delta plc1$ cells was able to overcome the loss of Plc1 activity. Conversely, when the mutant Plc1 Δ N-term was expressed in $\Delta plc1$ cells it was unable to suppress the loss of Plc1 activity. This could possibly be a result of protein not correctly folding creating an inactive Plc1. Alternatively without the presence of the N-terminus the activity of Plc1 might be switched off because a regulatory influence was unable to bind. When the activity of rat PLC- δ was assayed *in vitro* it had ten-times the amount of activity compared to wild-type Plc1 but when rat PLC- δ was expressed in $\Delta plc1$ cells it was unable to suppress the loss of Plc1 activity on DMM plates incubated at either 23°C or 37°C. In contrast the mutant Plc1₁₄₄-PLC- δ had lower PLC activity (similar to wild-type Plc1) but was able to complement for the loss of Plc1 activity in $\Delta plc1$ cells. The mutant Plc1₁₄₄-PLC- δ demonstrated a ten-fold decrease in activity compared to PLC- δ . This reduction in activity could be due to the Plc1 N-terminus not allowing the protein to fold correctly or alternatively the N-terminal domain may have a regulatory protein that binds to this domain controlling rat PLC- δ activity. It has been demonstrated that the UV checkpoint proteins may interact with the N-terminus of Plc1 in *Sz. pombe* and this is discussed in more detail in section 9.2.2.

9.2.2 14-3-3 proteins interact with the N-terminus of Plc1

Two-hybrid interaction assay was performed and discovered that Plc1 interacted with the proteins Rad24 and Rad25 which are members of the 14-3-3 protein family (Andoh *et al.*, 1998). Mammalian 14-3-3 proteins have been suggested to regulate signal transduction pathways through MAP kinase cascades (reviewed by Aitken, 1995) and it is possible that yeast use a similar mechanism (Delgols and Russell, 1997)(see Section 9.3.2). The genes *rad24* and *rad25* that encode for 14-3-3 proteins are reported to be DNA damage checkpoint genes. These genes are required for *Sz. pombe* cells to recover from DNA damage brought about by UV radiation (Ford *et al.*, 1994). The two-hybrid analysis carried out

by Andoh, *et al.*, (1998) suggested that the N-terminal region of Plc1 but not the catalytic (C-terminal) region was required for the interaction of the 14-3-3 proteins. Unfortunately Andoh, *et al.*, (1998) do not elaborate further on the precise interaction points within the N-terminus. The interaction of the 14-3-3 proteins with the N-terminus of *Sz. pombe* Plc1 might be a regulatory role controlling the enzymes activity. Wild-type rat PLC- δ and Plc1 Δ N-term were unable to complement the loss of Plc1 activity but the Plc1₁₄₄-PLC- δ mutant was able to complement. This suggests that the N-terminal domain might interact with the 14-3-3 proteins, which subsequently may not control its activity. Andoh *et al.*, (1998) demonstrated that *Sz. pombe* cells over-expressing Plc1, Δ rad24 mutant cells and cells over-expressing Rad24 showed almost the same Plc1 activity. This confirmed that although 14-3-3 proteins may interact with the N-terminus of Plc1 they do not appear to directly regulate its activity.

9.2.3 [Ca²⁺] might play a role in regulating Plc1 activity

It was demonstrated that the PIP₂ hydrolysing activity of Plc1 is Ca²⁺-dependent (Slaaby, 1996), similar to mammalian PLCs. It has been shown that Ca²⁺ ion concentration within a physiological range of 0.1-10 μ M was sufficient to stimulate PLC- δ activity, but not PLC- β and PLC- γ (Allen *et al.*, 1997). As Plc1 bears closest resemblance to PLC- δ changes in Ca²⁺ concentration might be enough to activate Plc1. The role of Ca²⁺-mediated hydrolysis of PIP₂ needs to be investigated in order to identify whether or not Ca²⁺ concentrations may regulate Plc1 activity.

9.2.4 Growth conditions that might regulate Plc1 activity

It has been confirmed that Δ plc1 cells are unable to grow under conditions that may cause the cell stress, this includes temperatures above 30°C and growth on minimal medium. It has been demonstrated in many experiments that Plc1 activity in wild-type cells appears to increase when placed under stressful

conditions. This includes UV light (Andoh *et al.*, 1998), hyper-osmotically stressed cells (Ongusaha *et al.*, 1998) and incubation at 37°C on DMM (York *et al.*, 1999). These studies demonstrated that PIP₂ hydrolysis increased over the stress-induced period, resulting in increased IP₆ production (Ongusaha *et al.*, 1998; York *et al.*, 1999) or possibly acting on a MAP kinase cascade (Andoh *et al.*, 1998). It appears that these external factors are able to regulate the activity of Plc1. This may either take place directly through a receptor or perhaps by a novel mechanism whereby proteins interact with Plc1, controlling its activity.

9.3 Pathways which may require Plc1 activity

9.3.1 Plc1 activity may be required for IP₆ production

The *S. cerevisiae* genome lacks a recognisable Ins-P₃ sensitive receptor or Ca²⁺ channel (Ongusaha *et al.*, 1998). It therefore seems reasonable to assume Ins-P₃ in yeast does not regulate the concentration of Ca²⁺ in a manner that has been identified in mammalian cells. It is more likely that the released Ins-P₃ is either dephosphorylated and recycled into phosphatidylinositols or it could be phosphorylated into Ins-P₆. The functions of Ins-P₆ in eukaryotic cells were reviewed in section 1.2.3.3. The roles of Ins-P₆ briefly include stimulating Ca²⁺ entry into neurones; serine-threonine phosphatases are inhibited by increased concentrations of Ins-P₆ stimulating the activity of L-type Ca²⁺ channels. Ins-P₆ also regulates endocytosis and exocytosis of clathrin coated vesicles through the inhibition of assembly proteins.

Concentrations of Ins-P₆ within yeast cells are thought to range from μM to mM and when cells are osmotically stressed there is a rapid increase in Ins-P₆ concentration probably synthesised from Ins 1,4,5-P₃ (Ongusaha *et al.*, 1998). ΔPLC1 *S. cerevisiae* cells were unable to produce Ins-P₆, thus demonstrating further evidence that Ins-P₆ is synthesised directly from Ins 1,4,5-P₃ and not from some other InsPP source (York *et al.*, 1999). In *Sz. pombe* Ins-P₆ production is probably carried out by a multifunctional kinase that is capable of phosphorylating the spare OH residues on the inositol ring of Ins 1,4,5-P₃ (Ongusaha *et al.*, 1998). In *S. cerevisiae* cells it appears that a multifunctional kinase phosphorylates Ins 1,4,5-P₃ to Ins 1,3,4,5,6-P₅, which is followed by Ins-P₅ 2-kinase, phosphorylating it into Ins-P₆ (York *et al.*, 1999).

It has been demonstrated that *S. cerevisiae* cells cultured under stressful conditions such as synthetic medium or high growth temperatures synthesise Ins-P₆, which subsequently results in enhanced mRNA export (York *et al.*, 1999). It is not fully understood how Ins-P₆ promotes the export of mRNA but it has been suggested that Ins-P₆ may be a positive regulator of GLE1-mediated mRNA

export (York *et al.*, 1999). GLE1 is an essential protein in the nuclear pore complex (NPC) that is required for mRNA export in both yeast and mammalian cells (Murphy and Wente, 1996). The NPC is an essential protein complex that spans the nuclear envelope allowing the selective exchange of proteins, RNAs and ions (York *et al.*, 1999). Conformational changes in the NPC induced by Ins-P₆ regulating GLE1 may facilitate the translocation of heterogeneous nuclear ribonucleoprotein complexes (Daneshmandi, 1998). Alternatively Ins-P₆ may facilitate the removal of an export inhibitor resulting in mRNA export (Ohno *et al.*, 1997).

It has been demonstrated in *S. cerevisiae* that InsP₆ is produced directly from Ins 1,4,5-P₃ resulting from PIP₂ hydrolysis by PLC1 (York *et al.*, 1999). If *Sz. pombe* cells use the same method of InsP₆ production then the Plc1 active site mutants described in chapter 7 could be used. The active site mutants were able to hydrolyse PI but not PIP₂. These mutants may identify if InsP₆ is only produced directly from Ins 1,4,5-P₃ using the multifunctional kinase described by Ongusaha *et al.*, (1998) or if there is an alternative pathway which can phosphorylate InsP₆ from IP. Inositol polyphosphate production in *Sz. Pombe* cells could be identified using HPLC analysis that distinguishes between the different inositol polyphosphates produced within the cell.

9.3.2 Plc1 is part of a complex that is involved in UV resistance

It has been illustrated that Plc1 is responsible for resistance to UV irradiation but is not involved in the DNA damage checkpoint pathway in co-operation with Rad24 and Rad25 (Andoh *et al.*, 1998). Rad24 and Rad25 are 14-3-3 proteins, which are necessary for cells to recover from DNA damage induced by UV irradiation (Ford *et al.*, 1994). Immunoprecipitation techniques were used to confirm that the N-terminus but not the catalytic region of Plc1 appeared to form a complex with the 14-3-3 proteins (Andoh *et al.*, 1998).

In mammalian cells, UV irradiation causes oxidative stress rather than DNA damage. Oxidative stress initiates the signalling mechanism that results in the induction of genes encoding metallothioneins. These work as scavengers of free radicals that appear to be the activator of the MAP kinase cascade (Devary *et al.*, 1992; 1993). Reports indicate that Sty1 (also known as Phh1), an *Sz. pombe* MAP kinase, is activated by UV irradiation in a MAPKK-dependent manner. This is similar to the MAP kinase cascades that are activated in mammalian UV response (Degols and Russell, 1997). Since Plc1 and Sty1 appear to belong to the same epistasis group with reference to UV resistance, Plc1 may therefore be a regulator of the MAP kinase cascade in the UV response pathway (Andoh *et al.*, 1998).

9.3.3 DAG may stimulate PKC in *Sz. pombe*

Mutations were introduced into the active site of Plc1 so that these mutants were unable to hydrolyse PIP₂ but still retained some ability to hydrolyse PI. When expressed in $\Delta plc1$ cells the active site mutants could suppress the loss of Plc1 activity even on DMM plates incubated at 37°C; normally a non-permissive growth condition. This suggested that DAG might be performing an important signalling function in *Sz. pombe* cells as the other second messenger Ins-P is probably recycled into phosphatidylinositols. In mammalian cells DAG is known to activate PKC and *Sz. pombe* has two PKC related genes named *pck1* and *pck2*. When a $\Delta pck2$ *Sz. pombe* cell was created it displayed a distinct phenotype displaying irregular cell shapes such as small round cells or pear-like shapes and $\Delta pck2$ cells were unable to grow on minimal media (Toda *et al.*, 1993). A double disruptant of *pck1* and *pck2* in a haploid cell is lethal suggesting that these genes together perform an essential function in maintaining cell viability (Toda *et al.*, 1993). The *pck2* disruptant cell displays a similar phenotype to $\Delta plc1$ cells which suggests that they may be a part of the same signalling pathway which regulates cytokinesis, cell morphology and cell wall structure.

In mammalian cells PKCs are known to be connected to MAP kinase pathways (Marshall, 1995; Li *et al.*, 1996). In *S. cerevisiae* MPK1 was identified as a kinase involved in cell wall integrity that worked downstream of the PKC homologue (Errede and Levin, 1993). The homologue of MPK1 in *Sz. pombe* is Pmk1 and may interact with Pck1 and Pck2. Two models have been considered for the interaction of Pck1/Pck2 with Pmk1 (Toda *et al.*, 1996). The first of these models is that if Pck1/Pck2 acts upstream of Pmk1 then they must have a bifurcated role where one branch acts through Pmk1 whereas the other is through an alternative pathway (Toda *et al.*, 1996). This is because the double *pck1/pck2* disruptant is lethal whereas cells lacking *pmk1* are viable. The second model is that Pmk1, Pck1 and Pck2 pathways are related but they regulate different aspects of the pathway (Toda *et al.*, 1996). If DAG does activate either Pck1 or Pck2 which subsequently acts through the Pmk1 MAP kinase cascade then a change in the phosphorylation states of the MAP kinase would be observed. This could be measured using assays described in Toda *et al.*, (1996) and it could be determined from this how much involvement DAG has in this signalling pathway.

This suggests that DAG produced as a result of PIP₂ or PI hydrolysis by Plc1 may be sufficient to regulate this MAP kinase pathway which is involved in maintaining cell wall integrity, cytokinesis and cell morphology. Expression of Pck1 or Pck2 in $\Delta plc1$ cells may give an indication if they are part of the same pathway. Increased expression of Pck1 or Pck2 may suppress the loss of Plc1 activity in $\Delta plc1$ cells resulting in growth on DMM incubated at 37°C. Elimination of one or more of these regulators in the pathway and the cells either display aberrant morphology or ultimately it could result in cell death.

9.4 Summary

Although the regulator of Plc1 has not been identified, from the investigation of the N-terminal domain it seems reasonable to assume that this may play an

important role in regulating the enzyme's activity. Plc1 without this domain had no detectable activity whereas wild-type rat PLC- δ which does not contain this domain had ten fold higher activity. When the N-terminal domain of Plc1 was added onto the N-terminus of PLC- δ the activity dropped by ten-fold, closely resembling wild-type Plc1 activity levels. Assuming that the expression levels of the proteins are roughly the same this evidence suggests that a regulator might interact with this N-terminal domain controlling the activity of Plc1.

A summary of the possible regulatory roles of Plc1 in *Sz. pombe* can be seen in Figure 53. It demonstrates that external signals such as UV light, temperature and osmotic stress may lead to the activation of Plc1 which hydrolyses PIP₂ into the second messengers InsP₃ and DAG. DAG in mammalian cells is known to activate PKC and in *Sz. pombe* two PKC homologues have been identified Pck1 and Pck2 (Toda *et al.*, 1993). Pmk1 is a MAP kinase that is involved in controlling cell structure and might be regulated by the activity of Pck2. Alternatively Pck1 or Pck2 may act independently of Pmk1 resulting in responses in cell morphology, cell wall integrity and cytokinesis (Toda *et al.*, 1996). Pck1 or Pck2 may also regulate another MAP kinase cascade; it has been suggested that UV light activates a MAP kinase cascade through the activity of Plc1 (Andoh *et al.*, 1998). The MAP kinase cascade which includes the MAP kinase Sty1 (Phh1) which is known to respond to UV light and could be regulated through Plc1 activity (Toda *et al.*, 1996).

The other second messenger produced from PIP₂ hydrolysis is InsP₃. It is believed that InsP₆ is produced directly from InsP₃, as $\Delta plc1$ cells are unable to produce InsP₆. Analysis of *Sz. pombe* or *S. cerevisiae* cells that are either placed under osmotic stress or are incubated at 37°C have increased levels of InsP₆ (Ongusaha *et al.*, 1998; York *et al.*, 1999). Increased levels of InsP₆ may activate GLE1 an essential protein in the NPC that is required for mRNA export or InsP₆ may remove an inhibitor of mRNA export in *S. cerevisiae* cells (York *et al.*, 1999). Alternatively if InsP₆ production is due to an osmotic response it

might activate the MAP kinase cascade containing Sty1 as this is known to be associated with maintaining an osmotic balance.

Obviously this speculation is based upon research performed on mammalian PLCs and on *S. cerevisiae* PLC1, but there may be many alternative roles for the phosphatidylinositols (PI) and the inositol polyphosphates (InsPP) in *Sz. pombe*. The roles of PIs and InsPPs were discussed in the general introduction but, briefly, they are known to regulate vesicle traffic such as endocytosis and exocytosis, bind cytoskeletal proteins such as gelsolin and profilin and InsPPs are known to regulate cytosolic Ca^{2+} concentration in cells.

This demonstrates the complexities of PLC signalling not only in mammalian cells but also in simple eukaryotes such as yeast cells. In *Sz. pombe* cells it suggests that Plc1 might regulate many pathways, which have not yet been identified, and suggests that small changes in Plc1 activity may have exaggerated outcomes due to many pathways being influenced. Perhaps this explains why cells lacking Plc1 activity need precise conditions for growth such as rich growth medium and growth temperatures below 29°C. Any deviation from these conditions results in *Sz. pombe* cells being put under stress and eventually results in cellular death.

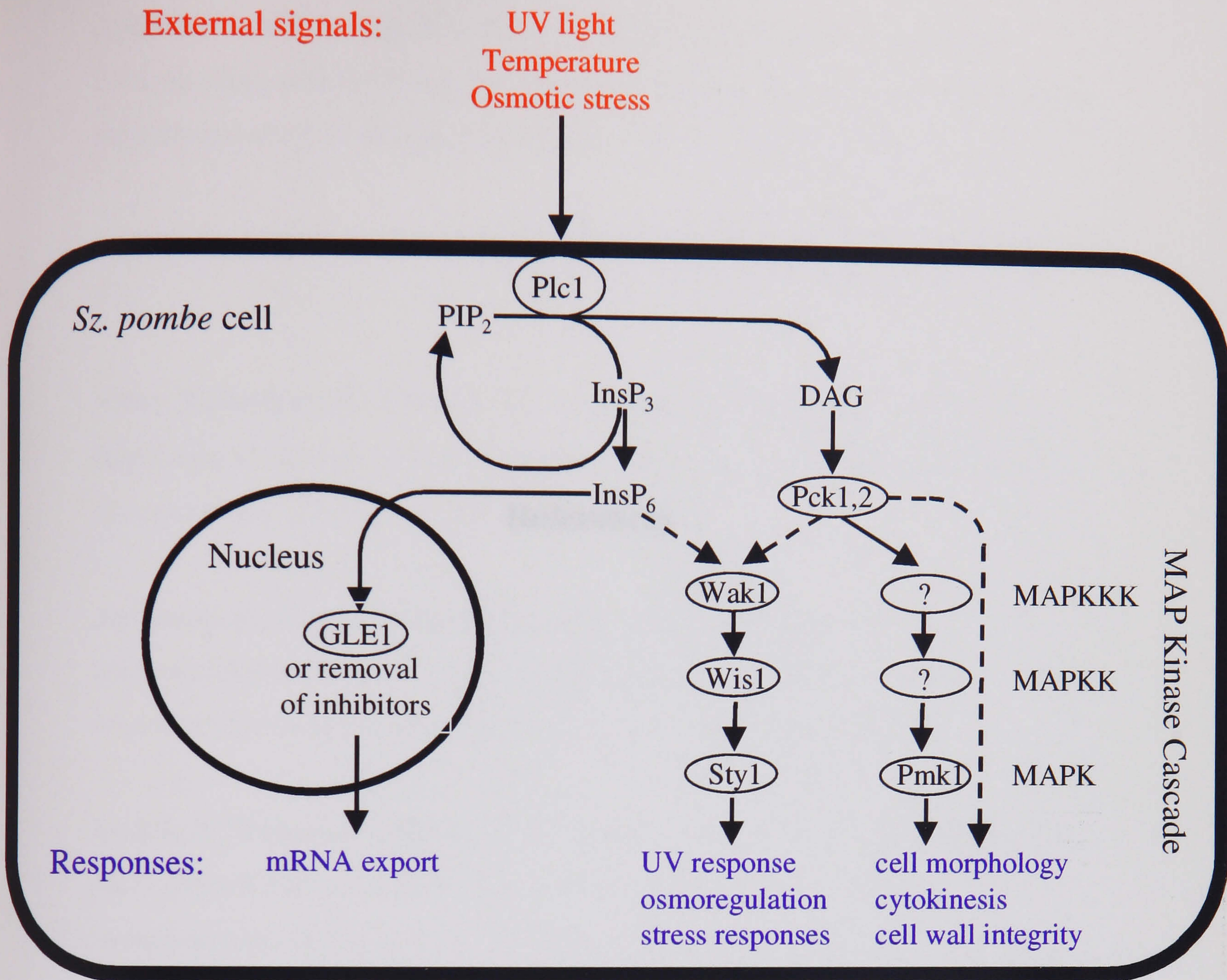


Figure 53 A summary of the possible Plc1 responses in *Sz. pombe*

A schematic diagram of an *Sz. pombe* cell which displays the external signals (red writing) which result in Plc1 activation. Plc1 hydrolyses PIP₂ into the second messengers InsP₃ and DAG. InsP₆ is known to be produced from InsP₃ in response to stress signals such as osmotic stress and increased temperature. InsP₆ then either acts directly on GLE1 facilitating mRNA export or it removes export inhibitors this pathway was identified in *S. cerevisiae*. InsP₆ is also produced in response to osmotic stress it may also have some regulatory influence on the MAP kinase cascade (dotted line) which regulates osmoregulation. In mammalian systems DAG is known to activate PKC, two PKC homologues have been identified in *Sz. pombe* (Pck1 and Pck2) which may regulate the MAP kinase cascade that regulates cell morphology and cytokinesis. It is possible that Pck1,2 may regulate cell wall integrity independently of the MAP kinase cascade (dotted line) As Plc1 is required for UV resistance it may be possible that by activating Pck1,2 through DAG it may activate the MAP kinase cascade (dotted line) known to be required for UV response, osmoregulation and stress responses. The ? indicate kinases that have not been identified in the MAP kinase cascade.

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Appendix

1 ATGACTGAAAGTGCTATAGATGACCAAAGGTTTAAATCTTACAAAGGAGTT
1 M T E S A I D D Q R F N L T K E L
51 ACAACGACACTCATGCAGAGATCAAGGGAAAATAACACAAAAGGATGATG
18 Q R H S C R D Q G K I T Q K D D
101 CTTTAGATTTTATAAGTTACAGTTCTTTTCAATCCTCTTTTAATACTGAC
34 A L D F I S Y S S F Q S S F N T D
151 CAAAAAAGCGCGAACAATGGAAGTACTGTGAGAAGAAGCATAAGGTCGAT
51 Q K S A N N G S T V R R S I R S I
201 TTTTAGAAGAGCAGCCGAATTGCCTAGAGTCCATATGGGGCCTCTTACTT
68 F R R A A E L P R V H M G P L T
251 ATTCACATGGGATAAATGAGCTTGTTAACAAGAAATTAAGAAAAGACTGT
84 Y S H G I N E L V N K K L R K D C
301 GATCTCAGCACGCTATGTCGCGTATTGCAAAGAGGAATCAGGATGATTAG
101 D L S T L C R V L Q R G I R M I R
351 GATGACAAGAAGAAGAAGGAAGTTCTATGAATTTAAATTAATCAATAACA
118 M T R R R R K F Y E F K L I N N
401 ACGGGCAAATAATATGGAAAGATGGTTCGAAGTATCTGGAATTAGACTCC
134 N G Q I I W K D G S K Y L E L D S
451 GTTAAAGACATCAGAATCGGTGATACGGCGAGTACTTATCAAGAGGAAGT
151 V K D I R I G D T A S T Y Q E E V
501 AGATCCGAAAAGATTGAGATCGGACAGTAAACTTTGGATTGCTATTATTT
168 D P K R L R S D S K L W I A I I
551 ATAAGGTTTCCAACAAATTGAAAGCGTTACACGTAGTTGCTTTGAATGAA
184 Y K V S N K L K A L H V V A L N E
601 TTGGACTTTAACACATTCTTAAGCTGCATTTGCGGTTTAGTGAAGTTAAG
201 L D F N T F L S C I C G L V K L R
651 GAGGGAATTAATGGAAAGCATACTTTTGCCTGATAATTCGCAATTCGCTA
218 R E L M E S I L L P D N S Q F A
701 GGATACATTGGCAAATTACTGTTTCTGAAAAGAGGAAGATGAGAAAAAG
234 R I H W Q I T V S E K E E D E K K
751 GACACATTGAGCTTTGCAGATGTGAAGAACTATGTGATAAATTTTCATAT
251 D T L S F A D V K K L C D K F H I
801 TTATGTGTCAACTGGTCAATTGTTAGAGTTTTTCCAATTAGCAGACATCA
268 Y V S T G Q L L E F F Q L A D I
851 ATCATAACGGACTATTAAATTATTTTGAATTTGAAAAGTTCATTAAAATC
284 N H N G L L N Y F E F E K F I K I
901 TTAAAGAATAGGAAAGAGGTAATATGATTTGGTCTAAATTTACAAAACC
301 L K N R K E V N M I W S K F T K P
951 TCCACACTCCCCTTATCATTGAAAATTTTTTTTCAATTTTAAATTACAG
318 P H S H L S F E N F F Q F L I T
1001 AGCAGCATGAGCAAGTGGATCGTCAAACCTGCATGGTCATATTTTATTAAG
334 E Q H E Q V D R Q T A W S Y F I K
1051 TACAGAGAACCGACTCAGCTGACGATGGGGCAAGATGGTTTTTACCAAGTT
351 Y R E P T Q L T M G Q D G F T K F
1101 CTTGAAGGAGCAACCATATTTGGTGGAGGTTAAAGAGGAGTTATATTCAA
368 L K E Q P Y L V E V K E E L Y S
1151 AGCCATTAACCATTATTTTATTGCATCTTCACATAATACTTATTTATTG
384 K P L N H Y F I A S S H N T Y L L
1201 GGGAAACAAATTGCAGAACTCCATCTGTGGAGGGATATATTCAAGTCCT
401 G K Q I A E T P S V E G Y I Q V L
1251 ACAACAAGGTTGTAGGTGCGTAGAAATTGATATATGGGATGGTGAAAATG
418 Q Q G C R C V E I D I W D G E N
1301 GGCCAGTGGTATGCCATGGATTTTTTAACGTCAGCTATTCCTTAAAAACA
434 G P V V C H G F L T S A I P L K T
1351 GTTATACGCGTGATAAAGAAGTACGCTTTTATAACGTCCTCCGTATCCTTT
451 V I R V I K K Y A F I T S P Y P L

1401 GATTATTTCACTGGAAATAAACTGTAACAAGGATAATCAAAAATTGGCAA
468 I I S L E I N C N K D N Q K L A
1451 GCCTGATAATGAGAGAGGTTTTAGCAGAACAGCTGTACTTTGTTGGCACA
484 S L I M R E V L A E Q L Y F V G T
1501 AGAACTGATAAACTGCCATCACCAAGGGAATTTAAAACATAAAATACTGTT
501 R T D K L P S P R E L K H K I L L
1551 GAAATCCAAGAAGACGAGTGAAGCAACTAGAGGTCTTAGCGTTAATGAGC
518 K S K K T S E A T R G L S V N E
1601 CATTTCCATCTTCTTTTTAGTTCTTCTTACGAATCAGCTAATGAACAGGAA
534 P F P S S F S S S Y E S A N E Q E
1651 TTAAGAATGAAAGATGACTCCACGAACAGTAGTAGCGCAACAAATTCTTC
551 L R M K D D S T N S S S A T N S S
1701 TAGTATGCAACGCATCAAAAGAATTGGATTAAAAAAGCACGCTGATATAA
568 S M Q R I K R I G L K K H A D I
1751 TTAATGATGTTTCTAACATTTCTGGAATACATGGCATCAAGTTCAGGAAT
584 I N D V S N I S G I H G I K F R N
1801 TTCTCTTTACCAGAGTCCAAAACGATAGCGCATTGTTTTTCACTGAATGA
601 F S L P E S K T I A H C F S L N E
1851 GCGCAAAGTAGAATACATGATAAAAGACAAACACTTAAAATTATCGTTGG
618 R K V E Y M I K D K H L K L S L
1901 ATAAACATAATAGAAGATATTTGATGAGAGTCTATCCTCACGTTTTACGA
634 D K H N R R Y L M R V Y P H V L R
1951 TACAAATCATCAAATTTTAACCCAATACCTTTTTTGGAAAGGCCGGTGTACA
651 Y K S S N F N P I P F W K A G V Q
2001 AATGGTGGCTACGAATTGGCAAACATAATGATATTGGACAGCAACTTAATC
618 M V A T N W Q T N D I G Q Q L N
2051 TGGCAATGTTTCAAATATTAGATCACCAACCTGATGGAAGTTTTAAATCA
684 L A M F Q I L D H Q P D G S F K S
2101 GGATACGTGCTAAAACCAAAAAAGTTGCTACCTGTAGTAACAAAGGCGAA
701 G Y V L K P K K L L P V V T K A K
2151 AATGATTCCATTAATTTACGAACATTTTGAATAATGGTAGTGATCCTGTTA
718 M I P L I Y E H F E N G S D P V
2201 CTGTAAAATTCGAATTTTATCTACACAGTTATTGCCAGATTGAATGAT
734 T V K I R I L S T Q L L P R L N D
2251 ACATCGCCAAGCAGAAATAACACCAATTCCTTTGTTAAAGTGGAGTTTCA
751 T S P S R N N T N S F V K V E F H
2301 TACCGATGACGAGCCGACAATGCCCATCTCAATCGATAAGGGTACAAGGA
768 T D D E P T M P I S I D K G T R
2351 TTTCTGCCACTGAAGCATCCACTAAGAGTTCTCAAGGGAATGGATTCAAC
784 I S A T E A S T K S S Q G M G F N
2401 CCAATATGGGATGCTGAAGTTTCTATCACATTGAAAGATACAGACCTCAC
801 P I W D A E V S I T L K D T D L T
2451 ATTTATAAAATTCATGGTTATCTCTGAAGAGACCCAGATTGCATCTGTTT
818 F I K F M V I S E E T Q I A S V
2501 GCCTTAAGCTGAACTATTTGAGGATGGGATATCGTCACATTCCGTTGTTT
834 C L K L N Y L R M G Y R H I P L F
2551 AACATGGAGGGGGAACAATACATATTTTGCCTTTATTTATTCATACACA
851 N M E G E Q Y I F C T L F I H T Q
2601 AATTTTATAG
868 I L •

Sequence of *plc1*

The amino acid sequence of the *plc1* gene. The gene was predicted to encode a sequence of 869 amino acids. Numbering of the nucleotide sequence and amino acids are from the start of the *plc1* ORF.

Oligonucleotides for the sequencing of *plc1*

Primer (5' to 3')	Direction Relative to <i>plc1</i> open reading frame	Position relative to <i>plc1</i> ATG
JO208 CCGCTAACGAATCAGTTC	Sense	1698-1705
JO213 GCCACATATTCTCATTT	Sense	1985-2001
JO218 CATATCTGGGCAACAAC	Sense	2322-2339
JO224 CTTATTTACTAGGTAAG	Sense	1373-1384
JO300 GGTGCGCCAACGTAAAGTC	Sense	534-552
JO318 CGTCGCCTGAATCGGTC	Sense	20-36
JO319 CGTAAACGATCAAATTC	Sense	232-249
JO321 GATTATGGCTTTAGAAG	Sense	792-808
JO322 TCAGCATTTTGTAAGCC	Sense	1065-1081
JO324 GGCTATAGACATATTCG	Sense	2611-2627
JO204 CCATTCGGTCCATCCCAGC	Antisense	1484-1466
JO219 AGTGCTGTAATGGACAC	Antisense	2723-2707
JO220 GAGAAGTTGATAGAAAGTGACG	Antisense	1183-1162
JO221 CGGTAACGGGTTTCATC	Antisense	1690-1674
JO222 GAATATGTGGCTATACG	Antisense	1995-1979
JO223 GGAAGTCCTTCTTCGCC	Antisense	2294-2278
JO299 CTATCGCATGAGTAAGGG	Antisense	410-393
JO301 CCAACTGTGCACTTTGCTCC	Antisense	943-924
JO306 CCTATTGCCAACATATGG	Antisense	165-147
JO320 CGAGCATCGCGGCCCTG	Antisense	662-646
JO323 GTCATCACTAATTATAC	Antisense	2511-2495

Oligonucleotides for the PCR amplification of *plc1*

Primer

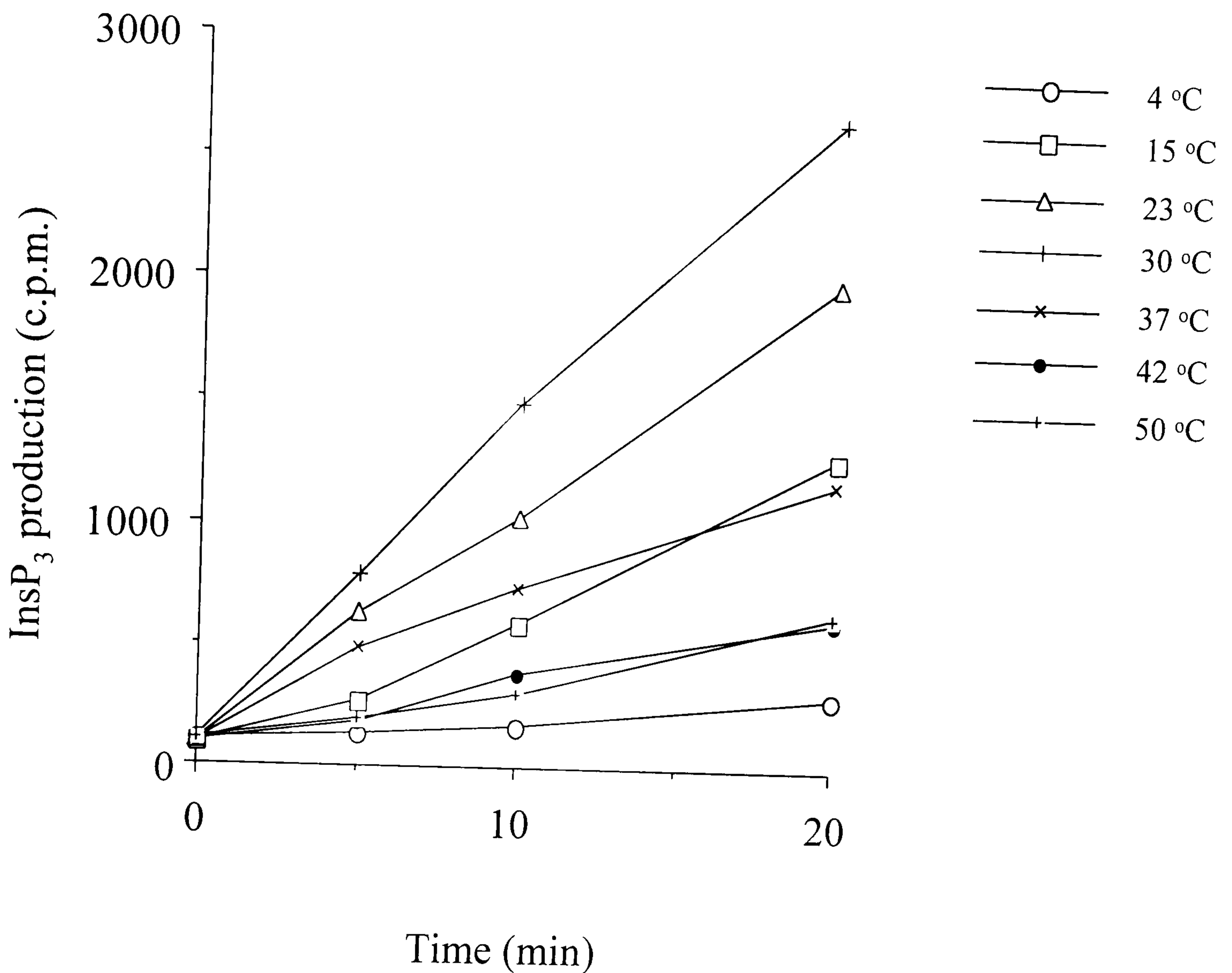
JO508 TCGCTACAGCGTtctTGCAAGTGTATTGAAATC
JO509 TTCAATACACTTGCAagaACGCTGTAGCGAACG
JO512 CTGCAACAAGGCTATtgtCATATTCGTTTATTGG
JO513 ATCCAATAAACGAATATGtgtATAGCCTTGTTGCAG
JO514ggatcccaccatgcatcatcatcatcatGGCGGCAATTGTGAAATGGTAAC
GTCG
JO515gggatccaatggacacagcgttctagatgatgatgattagGGCCGCC
AATGTCTT
JO594 aatagatatcataATGAGCTCGGTGAATCAAACTTTA
JO622 gggggatccCTACACCGAGCTCAGAGTAGATAAG
JO750 ACACAAATAGCGTAAATTATG
JO751 aaaGTATACCCTGGCCCATTG
JO752 ttaGTATACCCTGGCCCATTG
JO754 GACTTGTTTTAAAACGAC
JO755 cgtGTCAAATGTTTCAGCAACACC
JO756 gctGTCAAATGTTTCAGCAACACC

Oligonucleotides for the PCR amplification of Rat PLC- δ

Primer

JO548 gggggatatacataATGGACTCGGGTAGGGACTTCCTGACCC
JO549 gggggatatacagcgttTTAGTCCTGGATGGAGATCTTCAC
JO1068 GAATAGATGAGACTCGAGC
JO1069 GGTAGGGACTTCCTGACC

A list of oligonucleotide PCR primers used within this study. Nucleotides shown in capital text indicate sequence shared with the *plc1* gene. Nucleotides shown in lower case indicate sequence not found within the *plc1* gene.



Determining the rate of production of InsP₃

An example of a graph from which the rate of InsP₃ was calculated. Extracts were prepared from *Sz. pombe* cells expressing Plc1 (see Section 2.2.2.1) and samples were assayed at various temperatures using the scintillation counter method (see Section 2.2.2.2). The production of InsP₃ is linear over a period of 20 min, so the rate could be calculated (see overleaf for calculations associated with this graph).

An example of the calculations taken from the linear plots of the graph shown on the previous page. They demonstrate how the rates of InsP_3 production were calculated. All rates shown in this thesis were calculated in the same way.

Calculations:

For 4°C

After 0 min incubation: 96 cpm. produced
After 20 min incubation: 302 cpm produced

$$302 - 96 = 206 \text{ cpm } 20\text{min}^{-1}$$
$$206 / 20 = 10.3 \text{ cpm min}^{-1}$$

1mg protein extract incubated per assay
 $10.3 / 1 = 10.3 \text{ cpm min}^{-1} \text{ mg protein}^{-1}$

1 cpm = 110 fmoles of InsP_3 produced
 $10.3 \times 110 = 1133 \text{ fmoles of } \text{InsP}_3 \text{ produced min}^{-1} \text{ mg protein}^{-1}$
or
 $1.133 \text{ pmoles of } \text{InsP}_3 \text{ produced min}^{-1} \text{ mg protein}^{-1}$

For 15°C

After 0 min incubation: 96 cpm produced
After 20 min incubation: 1299 cpm produced

$$1299 - 96 = 1203 \text{ cpm } 20\text{min}^{-1}$$
$$1203 / 20 = 60.2 \text{ cpm min}^{-1}$$

1mg protein extract incubated per assay
 $60.2 / 1 = 60.2 \text{ cpm min}^{-1} \text{ mg protein}^{-1}$

1 cpm = 110 fmoles of InsP_3 produced
 $60.2 \times 110 = 6622 \text{ fmoles of } \text{InsP}_3 \text{ produced min}^{-1} \text{ mg protein}^{-1}$
or
 $6.622 \text{ pmoles of } \text{InsP}_3 \text{ produced min}^{-1} \text{ mg protein}^{-1}$

For 23°C

After 0 min incubation: 104 cpm produced
After 20 min incubation: 2014 cpm produced

$$2014 - 104 = 1910 \text{ cpm } 20\text{min}^{-1}$$
$$1910 / 20 = 95.5 \text{ cpm min}^{-1}$$

1 mg protein extract incubated per assay
 $95.5 / 1 = 95.5 \text{ cpm min}^{-1} \text{ mg protein}^{-1}$

1 cpm = 110 fmoles of InsP_3 produced
 $95.5 \times 110 = 10,505 \text{ fmoles of } \text{InsP}_3 \text{ produced min}^{-1} \text{ mg protein}^{-1}$
or $10.505 \text{ pmoles of } \text{InsP}_3 \text{ produced min}^{-1} \text{ mg protein}^{-1}$

For 30°C

After 0 min incubation: 102 cpm produced
After 20 min incubation: 2698 cpm produced

$$2698 - 102 = 2596 \text{ cpm } 20\text{min}^{-1}$$
$$2596 / 20 = 129.8 \text{ cpm min}^{-1}$$

1 mg protein extract incubated per assay
 $129.8 / 1 = 129.8 \text{ cpm min}^{-1} \text{ mg protein}^{-1}$

1 cpm = 110 fmoles of InsP_3 produced
 $129.8 \times 110 = 14,278 \text{ fmoles of } \text{InsP}_3 \text{ produced min}^{-1} \text{ mg protein}^{-1}$
or $14.278 \text{ pmoles of } \text{InsP}_3 \text{ produced min}^{-1} \text{ mg protein}^{-1}$

For 37°C

After 0 min incubation: 83 cpm produced
After 20 min incubation: 1195 cpm produced

$$1195 - 83 = 1112 \text{ cpm } 20\text{min}^{-1}$$
$$1112 / 20 = 55.6 \text{ cpm min}^{-1}$$

1 mg protein extract incubated per assay
 $55.6 / 1 = 55.6 \text{ cpm min}^{-1} \text{ mg protein}^{-1}$

1 cpm = 110 fmoles of InsP_3 produced
 $55.6 \times 110 = 6,116 \text{ fmoles of } \text{InsP}_3 \text{ produced min}^{-1} \text{ mg protein}^{-1}$
or $6.116 \text{ pmoles of } \text{InsP}_3 \text{ produced min}^{-1} \text{ mg protein}^{-1}$

For 42°C

After 0 min incubation: 83 cpm produced
After 20 min incubation: 720 cpm produced

$$720 - 83 = 637 \text{ cpm } 20\text{min}^{-1}$$
$$637 / 20 = 32 \text{ cpm min}^{-1}$$

1mg protein extract incubated per assay
 $32 / 1 = 32 \text{ cpm min}^{-1} \text{ mg protein}^{-1}$

1 cpm = 110 fmoles of InsP_3 produced
 $32 \times 110 = 3,520 \text{ fmoles of InsP}_3 \text{ produced min}^{-1} \text{ mg protein}^{-1}$
or $3.520 \text{ pmoles of InsP}_3 \text{ produced min}^{-1} \text{ mg protein}^{-1}$

For 50°C

After 0 min incubation: 127 cpm produced
After 20 min incubation: 487 cpm produced

$$487 - 127 = 360 \text{ cpm } 20\text{min}^{-1}$$
$$360 / 20 = 18 \text{ cpm min}^{-1}$$

1mg protein extract incubated per assay
 $18 / 1 = 18 \text{ cpm min}^{-1} \text{ mg protein}^{-1}$

1 cpm = 110 fmoles of InsP_3 produced
 $18 \times 110 = 1,980 \text{ fmoles of InsP}_3 \text{ produced min}^{-1} \text{ mg protein}^{-1}$
or $1.980 \text{ pmoles of InsP}_3 \text{ produced min}^{-1} \text{ mg protein}^{-1}$