

Isolation And Characterisation Of Rck2p, A Putative Receptor Of Activated C Kinase In *Saccharomyces cerevisiae*

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Doctor of Philosophy
at the University of Leicester



by

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FOR MY MAM AND DAD....

FOR EVERYTHING

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ABSTRACT

Isolation and characterisation of Rck2p, a putative receptor of activated C kinase in *Saccharomyces cerevisiae*

Lee Jamieson

Activated protein kinase C (PKC) was shown to translocate from the cytosol to the particulate fraction in mammalian cells. Recent evidence has indicated that PKC translocation is more complex than originally thought and is mediated by receptors of activated C kinase (RACKs). *RCK2* is a putative RACK gene in the yeast *Saccharomyces cerevisiae* which was isolated using an immunological screen. The screen detected yeast polypeptides which interacted with exogenously added mammalian PKC. *RCK2*, predicted to encode a polypeptide of ~33kDa, is a novel gene/protein. Analysis of an $\Delta rck2$ mutant indicated that Rck2p is not an essential protein, and phenotype analysis failed to suggest an *in vivo* function for this protein. Overexpression of Rck2p, as a fusion protein in an *E. coli* host, revealed that it becomes tightly associated with the bacterial chaperone GroEL. The merits of Rck2p being a yeast homologue of a mammalian RACK and the physiological relevance of its interaction with GroEL are discussed.

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ABBREVIATIONS

APS	Ammonium persulphate
ATP	Adenosine diphosphate
Bisacrylamide	N',N'-methylene bisacrylamide
BLA	Bottom layered agar
bp	Base pair
BSA	Bovine serum albumin
CIP	Calf intestinal phosphatase
dNTP	Deoxynucleoside triphosphate
ddNTP	Dideoxynucleoside triphosphate
DG	Diacylglycerol
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
EGTA	Ethylene bis (oxyethylenitrilo) tetra acetic acid
GST	glutathione S-transferase
hrs	hours
IgG	Immunoglobulin G
IMS	Industrial methylated spirit
IPTG	Isopropyl β -D-thiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
LUA	Luria agar
MBP	Maltose binding protein
mins	minutes
MOPS	3-(N-morpholino) propanesulphonic acid, pH 6.5-7.9
NB	Nutrient broth
OD	Optical density
OLB	Oligo labelling buffer
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethyleneglycol
pfu	Plaque forming unit
PKC	Protein kinase C
PMSF	Phenylmethanesulphonyl fluoride
PS	Phosphatidylserine
RACK	Receptor of Activated C Kinase
RNA	Ribonucleic acid
SDS	Sodium dodecylsulphate
secs	seconds
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEMED	N,N,N',N' - tetramethylethylenediamine
TLA	Top layer agar
Tris	Tris-(hydroxymethyl)-methylamine
Triton X-100	Octyl phenoxy polyethoxyethanol
ts	temperature sensitive
UV	Ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside

CHAPTER 1.

INTRODUCTION

Most external stimuli such as hormones and growth factors activate target cells by binding to cell surface receptors. The effect of this binding has then to be communicated to different compartments within the cell. This transmission of signals is termed signal transduction. Unlike the relatively insulated, linear nature of biochemical pathways, signal transduction pathways are part of a highly complex and dynamic network of interactions constantly accommodating the changing extracellular and intracellular environments of the cell. While the individual components of signal transduction pathways are being characterised, the process of identifying and understanding their regulatory mechanisms intensify.

Protein kinases and phosphatases are well recognised as generating and transmitting signals which regulate the activity of proteins in a rapid and reversible manner. Protein kinase C (PKC) has become accepted as a major signal transduction protein which responds to receptor-mediated signals and co-ordinates the appropriate intracellular responses. Accordingly, the regulation of PKC function and translocation *via* specific receptors has been studied extensively in recent years.

1.1 SIGNAL TRANSDUCTION PATHWAYS INVOLVING PKC

Two major signal transduction pathways converge on PKC; one initiated by a family of receptors linked to G proteins and the other by receptors linked to tyrosine kinases (figure 1.1). These different receptor mechanisms both activate members of the phospholipase C (PLC) family. PLCs hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce diacylglycerol (DG) and inositol triphosphate (IP₃) which are secondary messengers involved in the activation of PKC.

A large family of protein tyrosine kinases (PTKs) have been identified over the past decade (for review see Cantley, et al., 1991). These kinases fall into two classes: the transmembrane receptors and the cytosolic non-receptors. All PTKs have sequence homology over a stretch of approximately 300 amino acids defined as the kinase domain. The non-receptor family, in addition, has regions of homology not found in the receptor family. These regions include a short amino-terminal sequence required for the addition of myristic acid and two additional domains named Src homology 2 and 3 (SH-2 and SH-3). The added myristic acid localises the kinase to the membrane, whilst the SH-3 domain, which has been found in many actin-binding proteins, is thought to mediate protein-protein interactions within the cytoskeleton. The SH-2 domain recognises specific peptide sequences containing phosphotyrosines (for review see Cantley, et al., 1991). SH-2 domains have also been found in proteins otherwise unrelated to PTKs, including Ras GTPase activating

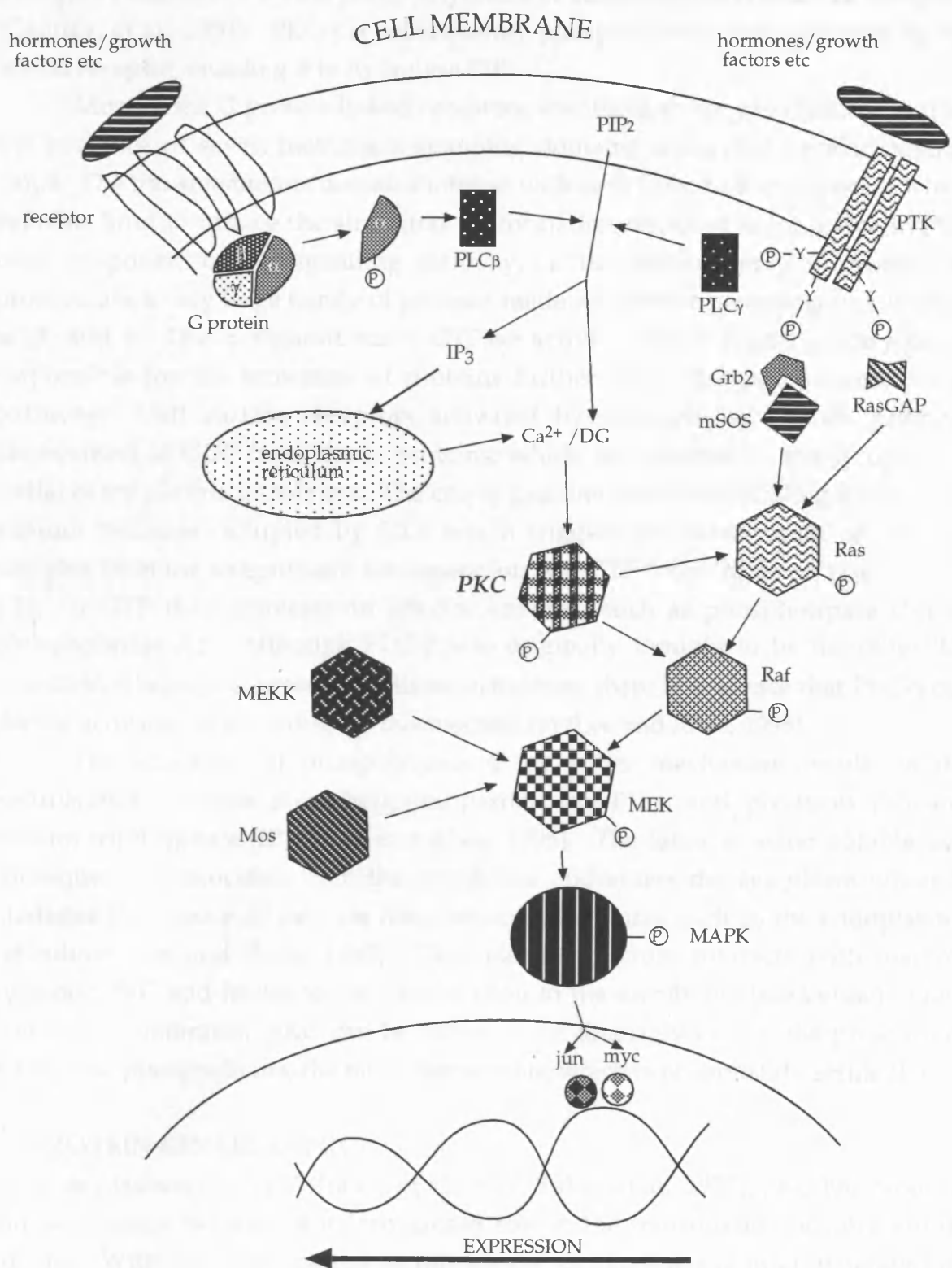


Figure 1.1
Summary of mammalian signal transduction pathways involving PKC. (See text for details).

proteins (*ras* GAPs) and phospholipase C- γ (PLC- γ). The SH-2 domain of PLC- γ is thought to associate with the phosphotyrosine of autophosphorylated PTK receptors (Cantley, et al., 1991). PLC- γ is subsequently phosphorylated and activated by the bound receptor, enabling it to hydrolyse PIP₂.

Most of the G protein-linked receptors, identified so far, are characterised by the presence of seven membrane-spanning domains connected by extracellular loops. The transmembrane domains interact with each other to form a pocket where agonists bind to induce the structural conformation responsible for activating the next component of the signalling pathway, *i.e.* the heterotrimeric G protein. G proteins are a very large family of proteins made up of three heterologous subunits, α , β , and γ . The α subunit has a GTPase activity and in higher eukaryotes is responsible for the activation of proteins further down the signal transduction pathway. Cell surface receptors activated by extracellular ligands promote dissociation of GDP from the G proteins, which are attached to the cytoplasmic leaflet of the plasma membrane. The empty guanine nucleotide binding site of the α subunit becomes occupied by GTP which triggers the dissociation of the $\alpha\beta\gamma$ complex from the receptor and the separation of α -GTP from the $\beta\gamma$ complex (figure 1.1). α -GTP then activates an effector enzyme, such as phospholipase C- β or phospholipase A₂. Although PLC- β was originally thought to be the only PLC stimulated through G protein-mediated activation, there is evidence that PLC- γ can also be activated in some cells by this mechanism (Lee and Rhee, 1995).

The activation of phospholipase C by either mechanism results in the hydrolysis of inositol phospholipids, particularly PIP₂, and produces DG and inositol triphosphate (IP₃) (Lee and Rhee, 1995). The latter is water soluble and consequently dissociates from the membrane and enters the cytoplasm where it mediates the release of calcium from intracellular stores such as the endoplasmic reticulum (Lee and Rhee, 1995). The released calcium interacts with inactive cytosolic PKC and facilitates its translocation to the membrane (see below). Once bound to membranes, PKC can be activated by diacylglycerol in the presence of membrane phospholipids, the most important of which is phosphatidylserine (PS).

1.2 PROTEIN KINASE C (PKC)

Since its discovery in 1977 (Inoue, et al., 1977, Takai, et al., 1977), PKC has received much attention because of its recognised role in the transmission of intracellular signals. With the participation of this kinase, extracellular stimuli generated by hormones and growth factors result in intracellular responses such as muscle contraction, modulation of gene expression, cell proliferation, cell differentiation and many others (Nishizuka, 1992). The studies of PKC have greatly expanded since the discovery that several components of signal transduction pathways involving PKC

are products of oncogenes and that disruption of signals mediated by PKC can promote tumourigenesis.

1.2.1 PKC Structure

Protein kinase C was originally defined biochemically as a phospholipid-dependent serine/threonine kinase, activated by diacylglycerol (DG) in the presence of calcium ions (Ca^{2+}), or by tumour-promoting phorbol esters. PKC was defined genetically by sequence homology and the presence of recognised functional domains. Its basic structure is represented by the classical PKC isozymes which were initially identified (for review see Nishizuka, 1992). Their single polypeptide chain is divided into regulatory and catalytic domains (figure 1.2). They are made up of four conserved domains (C_1 - C_4) which are separated by five variable domains (V_1 - V_5). The C_1 domain contains two zinc-finger repeats, each made up of a conserved (cysteine)₆ (histidine)₂ pattern of residues- C,X,H,C,[X₁₃],C,[X₂],C,[X₄],H,[X₂],C,[X₇],C- (where X represents any residue). These domains bind with high affinity to DG and phorbol ester in a co-operative manner with respect to PS (Quest, et al., 1994b). The C_2 domain contains the Ca^{2+} -interacting site, the C_3 includes the ATP-binding site implicated in the phosphorylating activity and the C_4 domain is required for substrate recognition (figure 1.2).

A sequence found in the C_1 domain, the pseudo substrate sequence, displays homology to consensus PKC phosphorylation sites but lacks the serine/threonine residue which is the phosphorylation target (House and Kemp, 1987). Although the pseudo substrate is not phosphorylated, it is thought to be tightly associated with the catalytic domain promoting a 'closed', inactive PKC conformation (figure 1.8; see later). According to this model, the binding of PKC activators to the regulatory domain removes the pseudo substrate sequence leading to an 'open' PKC conformation with an unmasked catalytic site (House and Kemp, 1987).

1.2.2 PKC Isozymes

Gene cloning and protein purification confirmed that PKC activity represents several distinct isozymes (Nishizuka, 1992). These isozymes have recently been assigned into three classes namely: classical, novel and atypical (figure 1.2). Their classification reflects the genetic structure of their respective genes and their biochemical properties. Four classical PKC isozymes (cPKC): PKC α , β I, β II and γ , display the basic structure described above (figure 1.2). These isozymes were originally designated type I (γ), type II (β I and β II), and type III (α) according to the three peaks of their biochemical activities observed during their purification (Nishizuka, 1988). The novel (nPKC) isozymes δ , ϵ , η , and θ , lack the C_2 region and consequently display Ca^{2+} -independent activation. In addition to the absence of the C_2 region, the atypical (aPKC) isozymes ζ and λ (1) have only one of the two cysteine-

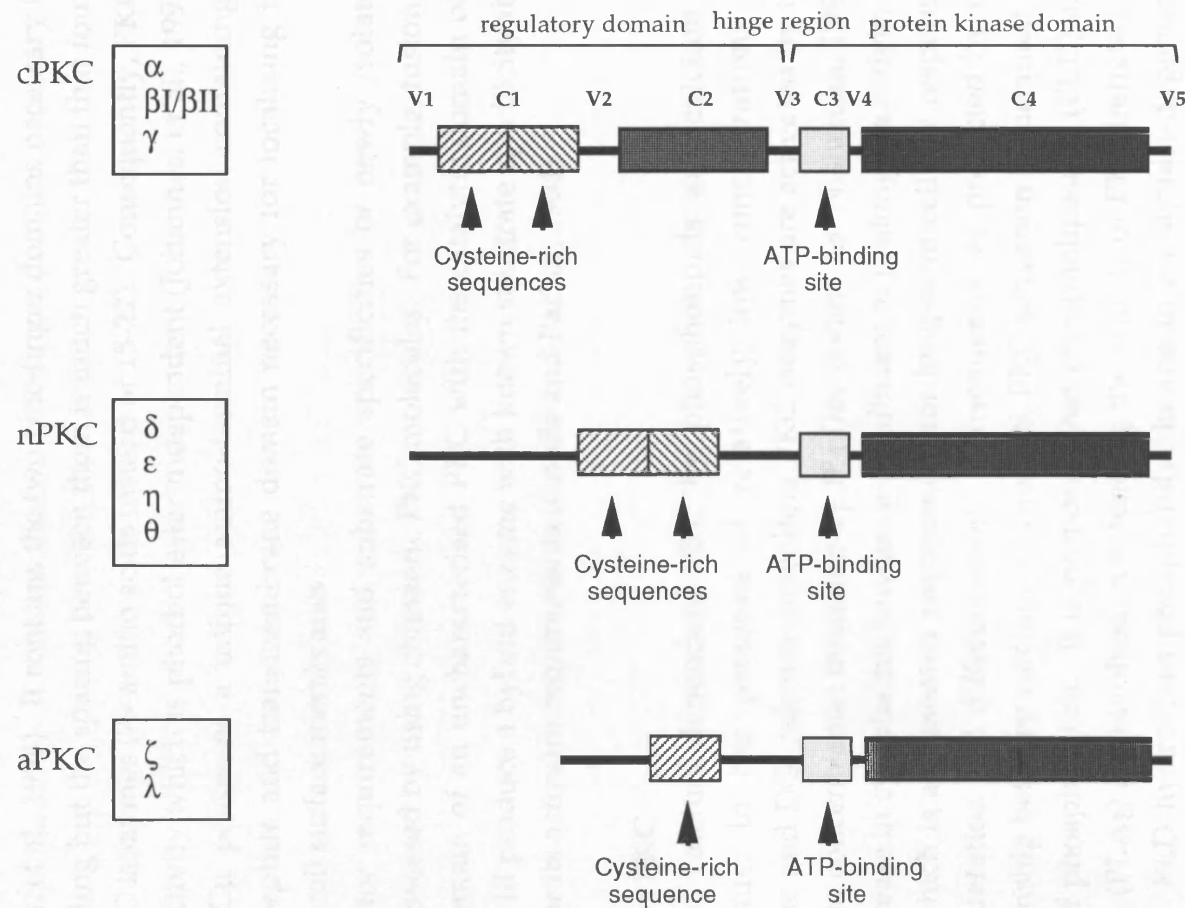


Figure 1.2

Structure of PKC. The classical isozymes (cPKC) consist of four conserved domains (C1-4) and five variable domains (V1-5). The cysteine-rich C1 region is involved in phospholipid and phorbol ester binding, C2 is associated with Ca^{2+} . The C3 region is the ATP-binding catalytic site with the C4 domain necessary for substrate recognition. The novel isozymes (nPKC) lack the C2 domain and are consequently Ca^{2+} -independent. The atypical isozymes (aPKC), in addition to the lack of a C2 domain, also lack one of the cysteine-rich sequences of the C1 domain and consequently have altered activator requirements. (Figure based on Nishizuka, 1992).

rich sequences found in the C₁ domain of the other isozymes. Consequently, these isozymes have altered requirements for activation; for example, PKC ζ does not appear to be activated by DG or phorbol esters but responds to PS and free fatty acids (Ways, et al., 1992). Table 1.1 (Nishizuka, 1992) describes ten of the isozymes found in mammalian cells.

The latest aPKC isozyme to be identified, PKC μ , exhibits several interesting features (Johannes, et al., 1994). It contains the two zinc-finger domains necessary for phorbol ester binding but the spacing between them is much greater than that found in cPKC and nPKC isozymes (79 amino acids instead of 15-22). Consequently, PKC μ displays kinase activity which is phorbol ester-independent (Johannes, et al., 1994). Furthermore, PKC μ possesses a unique amino-terminal extension containing a putative signal peptide and transmembrane domain necessary for localising the mature protein at cell surface membranes.

The activator requirements and substrate specificities of newly isolated isozymes can be assessed by using chimaeric PKC molecules. For example, fusion of the regulatory domain of an uncharacterised PKC with the catalytic domain of a known isozyme will produce a hybrid enzyme with known substrate specificity that can be analysed for its activator requirements (Goode and Parker, 1994).

1.2.3 Activation Of PKC

The activation of calcium-dependent PKC by phospholipids and calcium is synergistic. Thus, in the presence of relatively low concentrations of phosphatidylserine and DG, calcium-dependent PKC isozymes are activated by the increase in calcium concentration mediated by IP₃ (for review see Nishizuka, 1992). This transient increase in cytoplasmic calcium is insufficient to prolong the activity of PKC, however, which is an essential requirement for long-term cellular responses such as cell proliferation and differentiation. Consequently, as predicted by the synergistic relationship between calcium, DG and PS, PKC activation is sustained by the metabolism of phospholipids. It is proposed that phospholipase D (PLD) and phospholipase A₂ (PLA₂) are involved in extending the activity of PKC initiated by PLC (figure 1.3). PLD hydrolyses phosphatidylcholine in an agonist-dependent manner, resulting in the formation of phosphatidic acid, which is then converted to DG by the activity of phosphatidic acid phosphomonoesterase (Nishizuka, 1992). Phosphatidic acid itself has also been implicated in stimulating the activity of PKC *in vitro* (Toker, et al., 1994 and references therein). PLD may also convert phosphatidylcholine into phosphatidylinositol which is subsequently hydrolysed by phospholipase C to produce DG and inositol phosphate. It is believed that activated PKC may itself take part in the direct activation of PLD in a positive feedback mechanism, thus prolonging its own activation (Nishizuka, 1992).

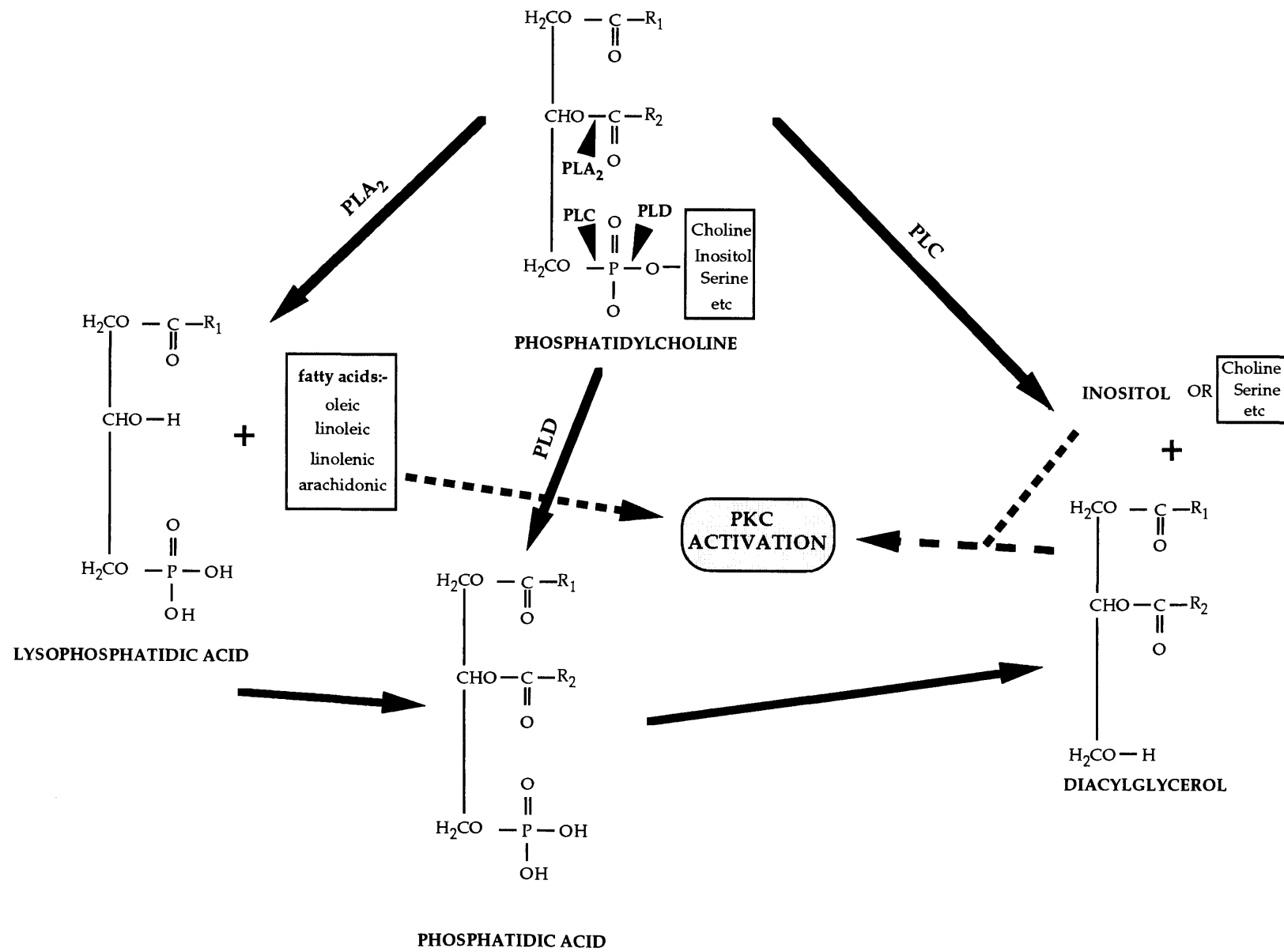
Sub-species	Mol. Size (Da)	Activators	Tissue Expression
Classical PKC Isozymes			
α	76,799	PS, Ca ²⁺ , DG, FFA, LysoPC	Universal
β I	76,790	PS, Ca ²⁺ , DG, FFA, LysoPC	Some Tissues
β II	76,933	PS, Ca ²⁺ , DG, FFA, LysoPC	Many Tissues
γ	78,366	PS, Ca ²⁺ , DG, FFA, LysoPC	Brain Only
Novel PKC Isozymes			
δ	77,517	PS, DG	Universal
ϵ	83,474	PS, DG, FFA	Brain And Others
η	77,972	?	Lung, Skin, Heart
θ	81,571	?	Skeletal Muscle (mainly)
Atypical PKC Isozymes			
ζ	67,740	PS, FFA	Universal
λ	67,200	?	Ovary, Testis, etc

Table 1.1

PKC isozymes in mammalian cells. DG, diacylglycerol; PS, phosphatidylserine; FFA, cis unsaturated fatty acid; LysoPC, lysophosphatidylcholine. (Table based on Nishizuka 1992).

Figure 1.3

Factors influencing the prolonged activation of PKC. Phospholipase A₂ (PLA₂), and phospholipase D (PLD) mediate the prolonged activation of PKC necessary for certain cellular responses such as cell differentiation and proliferation. Phospholipase C (PLC) is responsible for a more immediate, short term activation of PKC. (Figure based on Nishizuka, 1992.



Phospholipase A₂, which is present ubiquitously in mammalian tissues, hydrolyses phospholipids to release free fatty acids and lysophospholipids (figure 1.3). As with PLC, PLA₂ is thought to be activated in a receptor-mediated manner involving G proteins. Agonists that lead to the hydrolysis of inositol phospholipids also cause the release of arachidonic acid, which itself can promote other messengers in the signal transduction pathway. *Cis* unsaturated fatty acids including oleic, linoleic, linolenic, and arachidonic acids, which are all produced from phospholipids through the activity of a PLA₂, enhance the DG-dependent activation of PKCs α , β , γ , ϵ , and ζ , allowing them to exhibit almost full enzymatic activity in the presence of low calcium concentrations (for review see Nishizuka, 1992).

Phosphoinositide-3,4-bisphosphate (PI-3,4-P₂) and phosphoinositide-3,4,5-triphosphate (PI-3,4,5-P₃) are not normally detected in unstimulated mammalian cells. Cell stimulation, which promotes the metabolism of membrane lipids and activates phosphoinositol kinases, results in the rapid appearance of PI-3,4-P₂ and PI-3,4,5-P₃. *In vitro* studies indicated that these two phospholipids stimulate calcium-independent PKC isozymes, especially PKC ϵ and PKC η , but have little effect on the calcium-dependent isozymes or PKC ζ (Toker, et al., 1994). As these phosphoinositides are not substrates for any known phospholipase C, it is likely that they function themselves as second messengers for PKC activation, rather than being precursors of second messengers, as is the case for PI-4,5-P₂ (see earlier).

Three main features contribute to the activation of PKC; i) activator molecule binding, ii) specific modification, and iii) removal of the inhibitory regulatory domain.

i) Activators of PKC

Diacylglycerol (DG), phosphatidylserine (PS), and Ca²⁺

The C₁ domain of PKC (figure 1.2) was shown to bind phorbol esters, and therefore assumed to bind DG (Ono, et al., 1989). As mentioned above, the cysteine-rich motifs in this domain are thought to mediate this binding, especially as *n*-chimaerin and unc-13, two non-PKC proteins which also contain these motifs, bind phorbol esters. To further assess the importance of the cysteine-rich motifs in phorbol ester binding, the second cysteine-rich motif from PKC γ (Cys2) was expressed in *E. coli* as a fusion protein with GST (Quest, et al., 1994b). This fusion protein exhibited high affinity binding to phorbol ester (PDBu) and the binding was co-operative with PS. Interestingly, the fusion protein was also found to translocate to liposomes containing phosphatidylcholine/phosphatidylserine in a PS-dependent manner, and this translocation was enhanced by the presence of PDBu (see below). These results indicate that a single cysteine-rich motif is necessary and sufficient for the binding of phorbol ester and DG to PKC, and is required for PKC translocation.

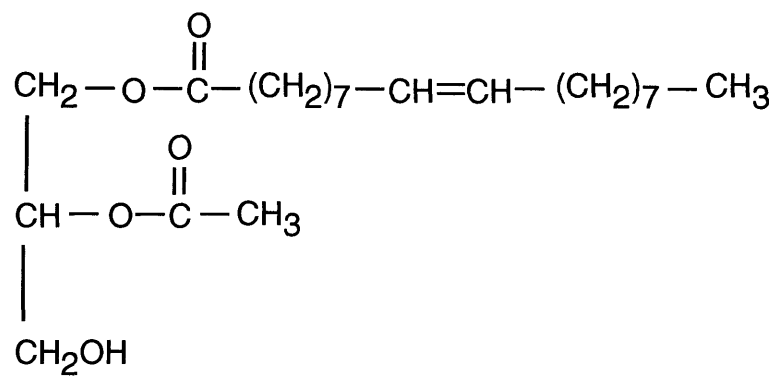
The Cys2-GST fusion binds 2 zinc atoms per molecule of protein implying that the full length PKC γ , which contains two cysteine-rich domains, could bind a total of four zinc atoms (Quest, et al., 1994a). The conserved pattern of (cysteine)₆(histidine)₂ found in all PKCs was shown to be important for the binding of zinc. Three cysteines and one histidine residue are thought to make up a pocket which binds one molecule of zinc. The presence of any of the three cysteines and one histidine, out of the eight conserved residues, appears to be the minimum requirement for the binding of a single zinc atom, although all eight are required for the binding of both zinc atoms. Binding of zinc to the first pocket is necessary for PDBu binding (Quest, et al., 1994a). It is proposed that the binding of zinc to the first pocket stabilises the protein to allow PDBu to bind to the second pocket.

PS in membranes is essential for the activation of PKC. An anti-idiotypic monoclonal antibody (Id8F7), raised against the combining site of a PS-specific antibody, was found to bind to a PS-specific binding site on PKC (Igarashi, et al., 1995). The binding of Id8F7 to PKC was enhanced by the presence of DG suggesting that an 'open' conformation of PKC was important for the antibody binding. Analysis of Id8F7 binding to various synthetic peptides, based on their sequence homologies to PKC and phosphatidylserine decarboxylase (PSD), revealed that the consensus sequence motif, in PKC, responsible for the PS interaction, is FXFXLKXXXXKXR (Igarashi, et al., 1995). This consensus sequence represents a basic structural motif required for a specific interaction with PS and forms the necessary binding sites in both PKC and PSD.

The role of Ca²⁺ in PKC activation is less clear, especially as the majority of isozymes display Ca²⁺-independent activity. Ca²⁺-dependent PKC isozymes require Ca²⁺ for the 'opening' of their folded conformation, necessary for activation. In addition, Ca²⁺ promotes the electrostatic interaction of all PKC isozymes (irrespective of the calcium requirement for activation by the isozyme) with membrane anionic phospholipids during the initial stages of PKC translocation (see translocation section below).

Phorbol esters

Phorbol esters are plant metabolites which have a DG-like structure (see figure 1.4) and can therefore replace DG, the natural activator of the enzyme (Blumberg, 1988). Unlike DG, however, phorbol esters are not metabolised by mammalian cells; their presence, therefore, results in a prolonged activation of PKC which leads to a variety of cellular responses, including the formation of tumours. The use of phorbol esters as 'PKC-specific' activators has been influential in understanding the biological activity of PKC. However, the recent identification of proteins other than PKC, such as *n*-chimaerin and unc-13, which bind phorbol-esters calls for careful analysis when interpreting 'PKC-specific' function (Wilkinson and Hallam, 1994).



A

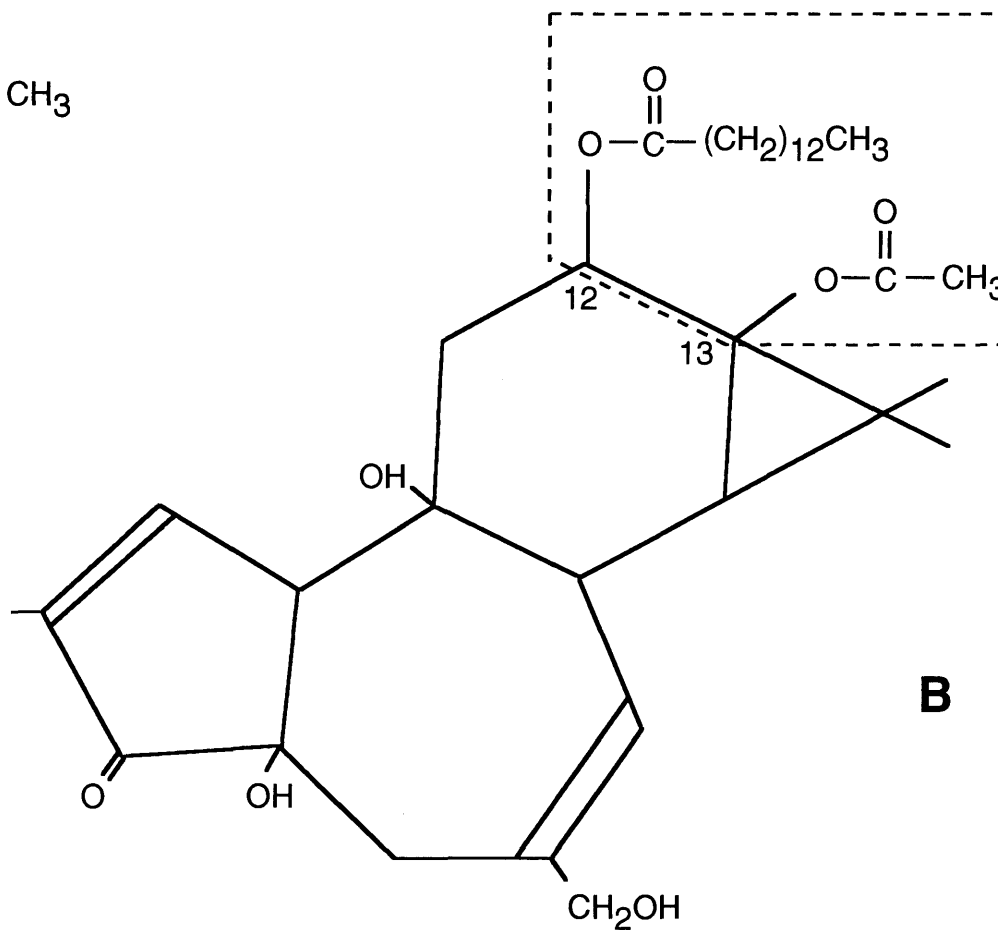


Figure 1.4

Structures of synthetic diacylglycerol (1-oleoyl-2-acetylglycerol; A) and tumour-promoting phorbol ester (1-O-tetradecanoylphorbol-13-acetate, TPA; B). TPA contains a diacylglycerol-like structure in its molecule, as shown in the dotted square. (Based on Nishizuka, 1984)

ii) Modifiers of PKC

'PKC kinase'

It was observed that while a functional, effector-dependent PKC was purified from tissues as an 80kDa phosphoprotein, expression of PKC in bacterial cells produced an inactive, 76kDa protein (Cazaubon, et al., 1990, Kikkawa, et al., 1982). In addition, analysis of newly synthesised PKC α from human breast cancer cells indicated that the primary translation product (74kDa) is converted into two larger forms (77- and 80kDa) by a two-step phosphorylation (Borner, et al., 1988). In line with these observations, it was reported that the primary translation product of PKC β 1 is an unphosphorylated protein associated with the triton-insoluble fraction (Zhang, et al., 1994). Upon phosphorylation, the mobility of PKC β 1 on SDS-PAGE changed, presumably due to phosphorylation, and the enzyme was released into the Triton-soluble fraction (Zhang, et al., 1994). The working model, based on several groups' work, suggests that the newly synthesised, inactive PKC (observed as Borner's 74kDa protein) is membrane-bound. Phosphorylation by a putative PKC kinase (observed as Borner's 77kDa protein) 'primes' the activity of PKC and mediates autophosphorylation (observed as Borner's 80kDa protein). This final, modified PKC, is released from the membrane into the cytosol where it can be regulated by PKC activators.

The highly conserved Thr⁶⁴² of PKC has been singled out as being the target for the PKC kinase (Zhang, et al., 1994 and references therein). The Ser/Thr⁶⁴²-Pro⁶⁴³ target sequence is the minimal consensus sequence recognised by members of the proline-directed kinases, including the MAP kinases and the cyclin-dependent protein kinases, although further research is required to identify the actual PKC kinase(s) (Zhang, et al., 1994).

Tyrosine kinases

Activation of PKC by phorbol esters induces tyrosine phosphorylation of an 82kDa protein (Li, et al., 1994). Genistein, a tyrosine kinase inhibitor, interferes with the phosphorylation of this protein. Surprisingly, the 82kDa protein was shown to be PKC δ , the tyrosine phosphorylation of which enhances the kinase activity of this isozyme *in vivo*. The physiological significance of this phosphorylation is still unclear.

iii) Removal of the PKC regulatory domain

PKC can be activated by limited proteolysis with calcium activated calpains which cleave the enzyme at its hinge region (Inoue, et al., 1977, Nishizuka, 1984; figure 1.2). The resultant catalytic component is constitutively active as it is independent of the

inhibitory, regulatory region (figure 1.2). Membrane-bound PKC is more susceptible to this proteolysis, but the physiological significance of this remains unclear.

1.2.4 Translocation Of PKC

Early studies emphasised the importance of calcium for the membrane association of PKC (Bazzi and Nelsestuen, 1990). Cytosolic PKC binds little or no calcium while at least eight calcium ions bind to the enzyme-membrane complex. It was originally proposed that calcium-binding sites were generated at the interface between the protein and the membrane so that calcium effectively forms a 'bridge' that holds PKC and the phospholipid complex together. The identification of the Ca^{2+} -independent isozymes altered these views. It is now thought that a low affinity association of PKC with anionic lipids in the membrane initially occurs by a non-specific electrostatic interaction promoted by high calcium concentrations. A more stable association occurs when either DG or PS bind to the C_1 or C_2 domains of PKC, respectively. The presence of both DG and PS leads to the release of the pseudo substrate domain from the catalytic site and thus activates the enzyme (Newton, 1995). It is proposed that Ca^{2+} affects the non-specific affinity of PKC for anionic phospholipids by exposing an 'acidic lipid-binding domain' in the enzyme when it adopts an active conformation. Such a lipid binding domain in non-classical isozymes could be constantly exposed as these isozymes show an affinity for anionic phospholipids in the absence of Ca^{2+} (Newton and Keranen, 1994).

PKC was initially thought to bind irreversibly to phorbol ester-associated membranes (Kraft and Anderson, 1983; Nelsestuen and Bazzi, 1991). This property of PKC reflects its high affinity to such membranes, but like the association of PKC with DG-associated membranes, can be reversed. Specifically, phorbol ester (PMA) is 250-fold more potent than DG in increasing the affinity of PKC to membranes (Mosior and Newton, 1995).

Although phorbol ester treatment of some cells sometimes leads to cytoplasmic alkalinization, similar treatment of epithelial cells mediates the acidification of the cytoplasm and slightly increases the calcium concentration, promoting the translocation of PKC (Vaaraniemi, et al., 1994). Interestingly, PKC translocation was inhibited if the cytoplasmic pH was clamped neutral (pH7.2). PKC translocation also failed to occur when the increase in the intracellular concentration of Ca^{2+} was caused by a calcium ionophore (A23187) suggesting that the calcium increase had to be stimulus-mediated. In contrast, acidification of the cytoplasm to below pH6.5 by the addition of nigericin, brought about the rapid translocation of PKC (Vaaraniemi, et al., 1994). This drop in pH is also associated with a large increase of intracellular free calcium and it has been suggested that the membrane lipids are one source of this calcium. This suggestion is strengthened by the observation that phosphatidylethanolamine binds more calcium at higher pH. It is

likely, therefore, that the translocation of PKC is mediated by cytoplasmic acidification, and the subsequent increase in hydrophobicity and protonation of lipids and proteins (Vaaraniemi, et al., 1994).

1.2.5 Down-Regulation Of PKC

The consequence of a sustained phorbol ester treatment is an eventual decrease in PKC activity, termed down-regulation. Although the mechanism responsible for the down-regulation is unclear, it is believed that Ca^{2+} -activated calpain proteases specifically degrade membrane-bound PKC.

Recent evidence suggests that certain PKC isozymes affect, in *trans*, the down-regulation of other isozymes. A strain of *Schizosaccharomyces pombe* expressing endogenous PKC ϵ was transformed with vectors expressing either PKC ζ or PKC δ . A PKC ζ -containing strain exhibited no down-regulation of either itself or PKC ϵ , while a PKC δ -containing strain exhibited its own down-regulation as well as a marked decrease in the protein levels of the endogenous PKC ϵ (Goode, et al., 1995). Kinase activity was later shown to be required for this down-regulation. As PKC ζ does not translocate to membranes, a positive correlation between the down-regulation of an enzyme and its translocation was established. Such a correlation was confirmed when a chimaeric isozyme consisting of the catalytic domain of PKC ζ and the regulatory domain of PKC δ was translocated and down-regulated. It is possible that the translocation of PKC to membranes up-regulates membrane transport processes, such as vesicle accumulation and endocytosis, which presumably play a central role in the down-regulation of PKC (Goode, et al., 1995).

1.2.6 PKC Distribution And Developmental Expression

Isozyme-specific antibodies initially helped to determine the tissue distribution and the developmental expression of PKC isozymes. In mammals, classical isozymes are highly enriched within various regions of the brain. PKC γ appears to be brain-specific, while PKC β I and β II are widely distributed in most tissues (Yoshida, et al., 1988). PKC α was also found in the retina and pineal gland (both light stimulated) and in rat olfactory bulbs (responsive to odour), indicating its probable participation in signal transduction pathways connected to sensory functions (Yoshida, et al., 1988).

Isozyme-specific antibodies were used recently in indirect immunofluorescence microscopy and Western blot analysis to characterise the cellular distribution of PKC isozymes in NIH 3T3 fibroblasts (Goodnight, et al., 1995). Table 1.2 illustrates the findings which indicate that various isozymes are specifically localised to particular cellular compartments. This study also mirrored the findings of other researchers which indicate that PKC isozymes not only

PKC	Localisation In Untreated Cells	Relocalisation After TPA Treatment
α	Pancytoplasmic	ER, cell membrane, focal adhesions
β I	Pancytoplasmic	Cell membrane, membrane ruffles
β II	Cytoplasmic (perinuclear)	Actin cytoskeleton, cell membrane, membrane ruffles
γ	Pancytoplasmic	Golgi, cell membrane
δ	Cytoplasmic (Golgi)	Cell membrane, cell junctures, perinuclear membranes
ϵ	Cytoplasmic (punctate ER-like))	Cell membrane, cell junctures, perinuclear membranes
ζ	Pancytoplasmic	No change
η	Cytoplasmic (Golgi)	Golgi, cell membrane, nuclear pores

Table 1.2

Characterisation of the cellular distribution of PKC isozymes in NIH 3T3 fibroblasts. Indirect immunofluorescence microscopy and Western blot analysis identified the specific localisation of PKC isozymes to intracellular locales. Localisation was observed both in untreated cells and cells activated by phorbol esters (TPA). (Based on Goodnight, 1995).

translocate upon activation but this translocation is specific to particular cellular sites (see below).

Finally, the expression of PKC isozymes is differentially regulated during development (Yoshida, et al., 1988). In rat brain, for example, the expression of the α , β I, and β II isozymes increases progressively from 3 days before birth up to 2-3 weeks of age and remains constant thereafter. The expression of the γ isozyme, however, is very low during the first week of birth, and increases abruptly between 2 and 3 weeks of age.

1.3 PKC-INTERACTING PROTEINS

There are at least three types of PKC-interacting protein that should be considered: i) substrates, ii) inhibitors, and iii) receptors.

i) Substrates:

Activated PKC mediates the activation of at least two members of a well characterised signal transduction pathway that includes the mitogen activated protein kinase (MAPK) cascade (see below). The two oncoproteins believed to be activated by PKC are Ras and Raf (figure 1.1; figure 1.9). Ras oncoproteins are among the most potent mitogenic polypeptides known; constitutively active mutations of Ras are found in nearly one third of all human cancers (for review see Avruch, et al., 1994). Several studies have revealed that Ras, TPA, growth factors and serum, each activate Raf (a Ser/Thr kinase), and that a dominant-negative Ras mutant blocked Raf activation (Avruch, et al., 1994). Although there was much data strongly suggesting that Raf exerts its activity downstream of Ras, recent studies, utilising immunoprecipitation and the yeast two-hybrid system, have pointed to a direct interaction between these two proteins (for review see Avruch, et al., 1994).

PKC regulates Ras GTPase activating proteins (Ras-GAPs), either directly or *via* an inhibitor protein of GAP (Downward, et al., 1990). The result of this regulation is a reduction in GAP activity and the subsequent increase of 'active' GTP-bound Ras proteins. Recent data using a PKC ζ -GST fusion indicated that PKC also binds directly to Ras *in vitro*, and particularly, to GTP-Ras (Diaz-Meco, et al., 1994). Immunoprecipitated Ras was also found to associate with PKC ζ -GST *in vivo* to an extent comparable to that of the Ras-Raf association. Furthermore, it was shown that both PKC ζ and Raf kinase activities were necessary for the full activation of Ras and the mitogenic response (Diaz-Meco, et al., 1994). Other studies pointed to the direct phosphorylation of Raf by PKC. However, there is still uncertainty over the placement of PKC in the MAPK pathway. For example, phorbol ester activates PKC, but not Ras; in addition, Ras-mediated phospholipid metabolism produces DG and promotes PKC activation. These observations are difficult to accommodate with the findings that PKC activates Ras/Raf (for review see Avruch, et al., 1994).

Raf is one of a series of protein kinases which are highly conserved throughout eukaryotes (Avruch, et al., 1994). Together, these kinases make up a module termed the mitogen activated protein kinase (MAPK) cascade (figure 1.9). It was observed that insulin stimulates Ser/Thr phosphorylations in adipose and liver cells (Avruch, et al., 1994 and references therein). Efforts to identify the kinases responsible for this activity and the targets of these Ser/Thr phosphorylations revealed the p42/p44 mitogen activated protein kinases (MAPKs), also known as extracellular-signal-regulated kinases (ERKs). The ability of MAPK/ERKs to regulate other protein kinases as well as a variety of proto-oncogenic transcription factors, such as c-Jun and c-Myc, established the MAPKs as important elements in mitogenic signal transduction (for review see Avruch, 1994). Subsequent studies revealed that the activator of the MAPKs was another cytosolic kinase, MAPK/ERK kinase (MEK) which activates p42/p44 MAPK/ERK by phosphorylating it on both tyrosine and Ser/Thr residues (Avruch, et al., 1994). Raf was subsequently shown to directly phosphorylate and activate MEK *in vitro*. Complete pathways which link cell surface receptors (PTK and G protein-linked) with Ras/Raf, the MAP kinase cascade, and results in the mitogen-mediated effect, such as the activation of proto-oncogenic transcription factors have consequently been derived (figure 1.9). PKC was found to play a pivotal role in co-ordinating these pathways.

Despite its central role in several cascades *in vivo*, including the MAPK cascade described above, PKC appears to be a relatively non-specific kinase *in vitro* and phosphorylates numerous substrates (Nishizuka, 1986, Nishizuka, 1988). This lack of specificity underlines the importance of regulating PKC if substrates have to be phosphorylated specifically *in vivo*. Efforts to identify *in vivo* substrates of PKC has proven difficult; a few substrates that have been characterised are described below.

MARCKS

Despite its coding sequence, which predicts a protein of 31kDa, the mobility of this prominent *in vivo* PKC substrate on SDS-PAGE was indicative of a protein of 80-87kDa (Stumpo, et al., 1989). This discrepancy was found to be due to the amino-terminal myristoylation of the protein. As the protein also contains 28.4% alanine residues it was termed myristoylated alanine rich C kinase substrate (MARCKS) (Stumpo, et al., 1989).

Dephosphorylated MARCKS binds to filamentous (F) actin and in so doing leads to the aggregation of F-actin (Hartwig, et al., 1992). The binding of calcium-calmodulin to dephosphorylated MARCKS prevents the phosphorylation of MARCKS by PKC as well as the ability of F-actin to aggregate. The actin binding, calmodulin-binding, and PKC phosphorylation sites of MARCKS are therefore functionally linked and provided the first evidence of a direct link between PKC and

the regulation of microfilament organisation (Hartwig, et al., 1992). It is possible that an intracellular calcium surge promotes both a rapid calmodulin response and activation of calcium-dependent PKC. As the calmodulin interaction with MARCKS occurs before that of PKC, its interaction would inhibit the phosphorylation of MARCKS by PKC. However, when the cell stimulus does not lead to the increase of intracellular calcium concentration, the calcium-independent PKC isozymes would still be able to phosphorylate MARCKS as calmodulin would be inactive (Chakravarthy, et al., 1995).

In addition to its actin binding ability, MARCKS can translocate between the cytoplasmic and particulate fractions. This translocation is mediated by the N-terminal myristoyl moiety of MARCKS which provides its weak affinity to membranes. The positively charged phosphorylation domain of MARCKS, which associates with anionic, PS-containing, membranes is believed to contribute to the binding activity (Taniguchi, et al., 1994). According to this hypothesis, phosphorylation of this domain would alter its charge to negative and be sufficient to remove the hydrophobic head of MARCKS from the membrane through electrostatic repulsion. During the purification of MARCKS from bovine brain, a non-myristoylated form of MARCKS was isolated and a demyristoylation activity was found in the cytoplasmic fraction (Manenti, et al., 1995). Membrane associated MARCKS was protected from demyristoylation suggesting that the myristoyl moiety was inaccessible to the demyristoylation enzyme when inserted into the lipid bilayer. These results not only question the established notion that myristoylation is an irreversible modification, but suggests that the control of MARCKS myristoylation could regulate MARCKS function.

So as to investigate the role of MARCKS, its respective gene was disrupted in mice using homologous recombination techniques in embryonic stem cells (Stumpo, et al., 1995). Heterozygous mice behaved normally but homozygous mutant mice were defective in the normal development of the central nervous system leading to prenatal or perinatal death. These observations may not just be due to the lack of MARCKS function but could reflect the aberrant activities of PKC and calmodulin due to their imbalanced cellular concentrations.

The cellular concentrations of MARCKS can be altered by oncogene expression and the exposure to phorbol esters (Stumpo, et al., 1995). A mechanism for regulating the concentration of MARCKS has recently been proposed. It is based upon the observation that activation of cells with phorbol ester leads to the disappearance of the two cleavage products of MARCKS which co-exist with the intact protein in unstimulated cells (Spizz and Blackshear, 1996). It was thus suggested that the activation of PKC by phorbol esters, and the subsequent phosphorylation of MARCKS inhibits its sensitivity to a specific protease which regulates MARCKS turn-over (Spizz and Blackshear, 1996).

Vimentin

Ectopically (plasmid-borne) expressed PKC phosphorylates vimentin *in vivo*, whereas activated endogenous PKC does not (Ogawara, et al., 1995). This discrepancy implies that although vimentin is a potential substrate of PKC *in vivo*, such phosphorylation fails to occur in nature. Vimentin phosphorylation, however, is linked to the re-organisation of intermediate filaments during mitosis and recent studies have demonstrated that mitosis-specific PKC phosphorylation of vimentin *in vivo* does occur (Takai, et al., 1996). The normal cellular compartmentalisation of PKC could be disrupted during the dramatic re-organisation of the mitotic membranes facilitating the association of membrane-bound vimentin with activated PKC.

Lamin B

The nuclear lamina, which imparts structural integrity to the nucleus and provides an attachment site for interphase chromatin, is composed of the nuclear lamins. Lamina disassembly, which occurs at the onset of mitosis, is thought to be mediated by hyperphosphorylation of the nuclear lamins. Lamin B, for example, is extensively phosphorylated *in vivo* at multiple sites, including the consensus PKC phosphorylation site of Ser⁴⁰⁵. PKC β II phosphorylates lamin B *in vitro* and, in addition, translocates to the nucleus at the G₂/M phase transition in intact cells. There is a strong possibility, therefore, that PKC β II directly phosphorylates lamin B in the interphase nuclear envelope *in vivo* (Goss, et al., 1994).

Proteins containing pleckstrin homology (PH) domains

The PH domain, made up of approximately 100 loosely conserved amino acids, was originally characterised in pleckstrin, a prominent *in vitro* PKC substrate. Platelet agonists, such as thrombin, trigger the phosphorylation of pleckstrin by PKC *in vivo*, although its exact cellular role is still unclear (Tyers, 1988). Although the primary sequence homology observed between different PH domains is low, significant structural similarity is displayed. Many of the PH-containing proteins are involved in signal transduction pathways and their possible association with PKC has been noted (for review see Lemmon, et al., 1996).

RAC (related to A and C kinases) protein kinase (RAC-PK) has a PH domain believed to regulate the enzyme's serine/threonine kinase activity. A 76kDa protein, identified as PKC ζ , was found to bind specifically to the PH domain of RAC-PK when expressed as a GST fusion (Konishi, et al., 1994a; Konishi, et al., 1994b).

Similarly, the PH domain of bruton tyrosine kinase (Btk), when expressed as a GST fusion, binds to both Ca²⁺-dependent (α , β I and β II) and Ca²⁺-independent (ϵ and ζ) PKC isozymes (Yao, et al., 1994). The observation that the

autophosphorylation of Btk is inhibited in the presence of PKC led to the suggestion that PKC negatively regulates the tyrosine kinase activity of Btk both *in vitro* and *in vivo*.

Dynamin I (96-94kDa) is a microtubule-stimulated GTPase which controls endocytosis and synaptic vesicle formation (Vallee and Shpetner, 1993). Not only does this protein contain a PH domain, it is also reported to bind SH3 domains. PKC phosphorylation activates dynamin's GTPase activity and thus maintains dynamin in an inactive state. The depolarisation of synaptosomes and the subsequent Ca^{2+} influx lead to the translocation of both dynamin and PKC from the cytosol to the particulate fraction. Under these conditions, which promote PKC-mediated phosphorylation of several known PKC substrates including MARCKS, dynamin is rapidly dephosphorylated (Liu, et al., 1994). This observation could be due to the masking of dynamin phosphorylation sites by its association to phospholipids. Moderate increases in ionic strength (Na^+ , K^+ , and Mg^{2+}) or the presence of GTP or ATP lead to the dissociation of dynamin from the particulate fraction to the cytoplasm where phosphorylation by PKC was observed (Liu, et al., 1994). It is proposed that the transition of dynamin between a dephosphorylated, membrane-associated protein to a phosphorylated, cytosolic protein could act as a switch for the GTPase activity of dynamin (Liu, et al., 1994). The model proposes that in the absence of electrical stimulation, when there would be little need for the production of synaptic vesicles, GTP hydrolysis by dynamin is mediated by PKC phosphorylation maintaining the protein in an inactive state. Depolarisation of synaptosomes, and the subsequent dephosphorylation of dynamin, causes a subsequent increase in the lifetime of activated GTP-bound dynamin (Vallee and Shpetner, 1993).

ii) Inhibitors:

14-3-3 proteins (Aitken, 1995 and references therein)

14-3-3 proteins are a highly conserved family of proteins found in a broad range of organisms, including higher eukaryotes, plants, invertebrates and yeast. They have been thought to participate in several signalling pathways, although their exact physiological role is unclear. A protein kinase C inhibitor protein (KCIP-1), was shown to be a 14-3-3 protein through sequence homology (Aitken, et al., 1992). It is thought that the inhibitory effect of 14-3-3 proteins on the activity of PKC can be overcome by a conformational change induced by phorbol esters or DG (Robinson, et al., 1994).

iii) PKC receptors

It has widely been accepted that the *in vivo* activation of PKC leads to the translocation of the enzyme to the particulate fraction (Kraft and Anderson, 1983).

As PKC activation depends on lipids (DG and PS), this translocation was thought to reflect the binding of PKC to the plasma membrane, where the enzyme activators are present (Bell, 1986). PKC, however, is involved in many diverse cellular processes including cell morphology, secretion, cell contraction, and gene expression. Its substrates, responsible for these diverse effects, are localised throughout the cell, for example, on the plasma membrane, nuclear membrane, in the cytosol, and in cytoskeletal structures. Such diverse localisation generated a paradox: how could membrane-restricted PKC phosphorylate substrates throughout the cell?

This paradox began to be resolved by the observation that treatment of membranes with either trypsin or phospholipases abolished the association of PKC with membranes (Gopalakrishna, et al., 1986). The results indicated the importance of both proteins and lipids for the translocation of PKC. Wolf and colleagues (Wolf and Sahyoun, 1986) further investigated whether different cellular proteins could mediate PKC translocation. Soluble or membrane-bound polypeptides were separated by SDS-PAGE and transferred to nitrocellulose. The binding of PKC to these polypeptides was determined by anti-PKC antibodies or by radiolabelling the kinase directly. This 'overlay assay' (figure 1.5) revealed that two polypeptides (110- and 115kDa) enriched in the cytoskeletal fraction, bound PKC in the presence of PS (Wolf and Sahyoun, 1986). Phosphorylation of these polypeptides by PKC diminished their binding to both PS and PKC, indicating that phosphorylation regulated this binding.

Finally, indirect immunofluorescence microscopy of cardiac myocytes using an isozyme-specific monoclonal antibody (recognising a non-classical isozyme) indicated that cytosolic PKC translocates to myofibrils upon its activation (Mochly-Rosen, et al., 1990). Conversely, the use of specific antibodies raised, against PKC β , suggested that this isozyme translocates to the plasma membrane and the perinuclear area under similar conditions. It was therefore concluded that distinct PKC isozymes could be translocated and targeted to different cellular sites.

1.4 RECEPTORS OF ACTIVATED C KINASE (RACKs)

The suggestion that activated PKC may bind to membrane-associated proteins but is localised to distinct cellular locations initiated the search for PKC-binding proteins (Mochly-Rosen, et al., 1987). A modified overlay assay (figure 1.5) was used to demonstrate that polypeptides present in the Triton-insoluble fraction bound PKC in the presence of DG, PS and calcium (Mochly-Rosen, et al., 1991a). These polypeptides, of approximately 30- and 33kDa, showed properties characteristic of a receptor-ligand interaction; their binding to PKC was specific, concentration-dependent, and saturable. In addition, the binding of PKC to these polypeptides was not mediated by the substrate binding site of PKC. A synthetic peptide, homologous to the PKC pseudo substrate sequence, could bind to the substrate binding site of

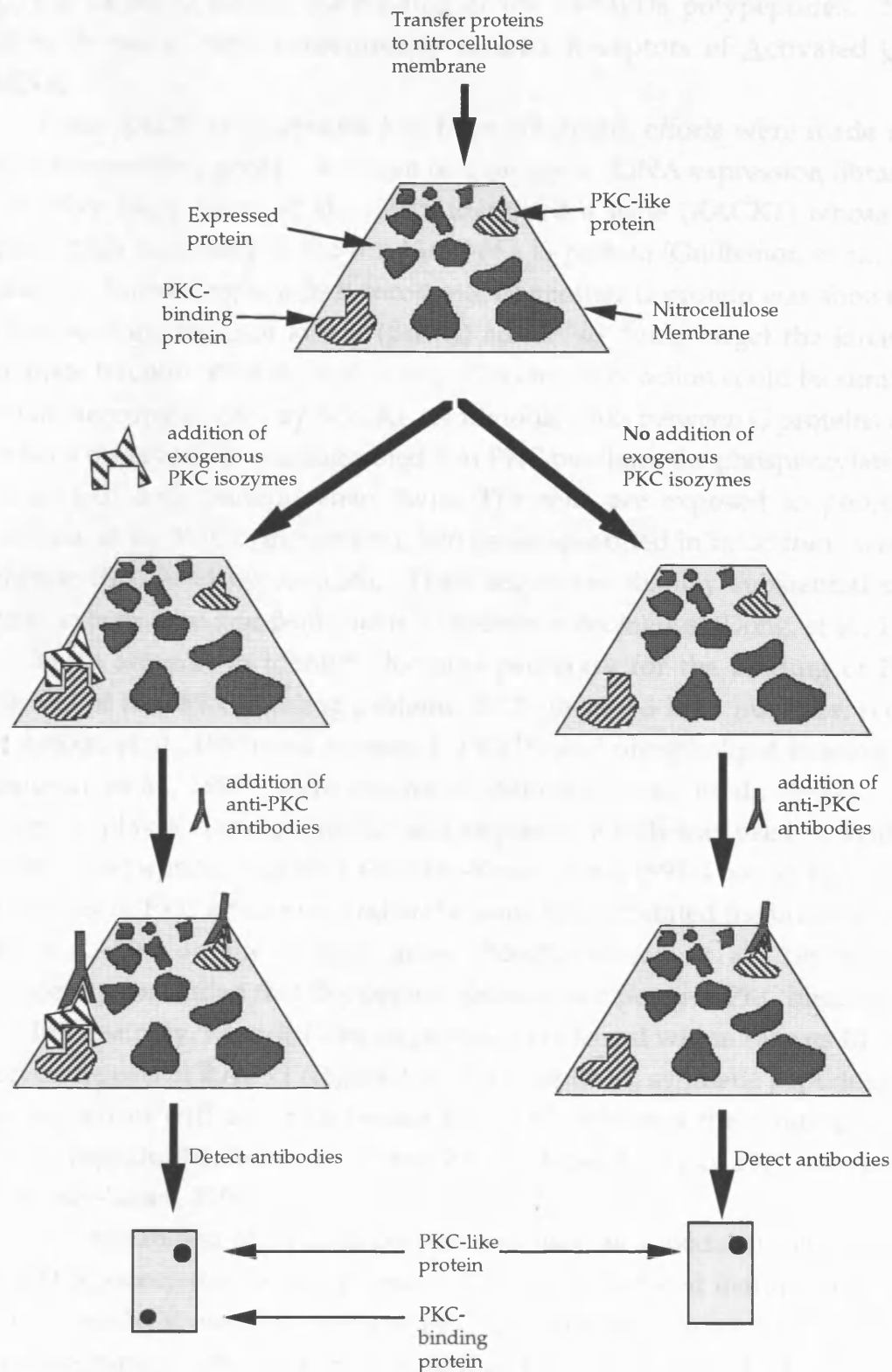


Figure 1.5

Overlay assay for the detection of PKC-binding proteins. Proteins of interest, derived from SDS-PAGE or a library screen for examples, are transferred to nitrocellulose. The membrane is subsequently washed in the presence or absence of exogenous PKC isozymes. Unbound PKC is then washed off. Bound PKC is detected with anti-PKC antibodies. PKC-like proteins bound to the membrane are recognised by these antibodies irrespective of the addition of exogenous PKC. Conversely, PKC-binding proteins bound to the membrane, should only be detected after the addition of exogenous PKC (see text for details).

PKC, but failed to inhibit the binding of the 30-33kDa polypeptides. The PKC-binding proteins were consequently termed Receptors of Activated C Kinase (RACKs).

Once RACK polypeptides had been identified, efforts were made to isolate their corresponding genes. A screen of a rat brain cDNA expression library, using the overlay assay (Ron, et al., 1994), identified a gene (RACK1) whose product displays high homology to the β subunit of a G protein (Guillemot, et al., 1989; see chapter 3). Interestingly, a $\beta\gamma$ heterodimer of another G protein was shown to bind the β -adrenergic receptor kinase (β ARK) and in so doing target the kinase to the particulate fraction (Pitcher, et al., 1992). This mode of action could be similar to the possible targeting of PKC by RACK1. Additional links between G proteins and PKC have been observed. It was suggested that PKC mediates the phosphorylation of the γ subunit of a G protein when Swiss 3T3 cells are exposed to phorbol ester (Morishita, et al., 1995). In addition, two genes identified in rat kidney, were found to encode PKC-binding proteins. Their sequences display substantial sequence identity to human α - and β -adducins (G protein homologues) (Dong, et al., 1995)

In an attempt to identify domains necessary for the binding of PKC, the sequences of two PKC-binding proteins, KCIP (a 14-3-3 PKC inhibitor; see section 1.3ii; Aitken, et al., 1990) and annexin I (a Ca^{2+} - and phospholipid binding protein) (Crompton, et al., 1988), were compared (Mochly-Rosen, et al., 1991a). The two proteins display a common amino acid sequence which was used to synthesise a peptide. This peptide, Peptide I, (Mochly-Rosen, et al., 1991a) bound PKC *in vitro*, in the presence of PKC activators, and at the same time inhibited the binding of PKC to putative RACKs in the overlay assay (Mochly-Rosen, et al., 1991b). It was consequently concluded that this peptide represents a putative PKC binding domain.

Interestingly, Peptide I-like sequences were found within repeats III and VI of the seven repeats of RACK1 (Figure 1.6). Like Peptide I, synthetic peptides based on these sequences (rIII and rVI) bound PKC and inhibited the binding of PKC to RACKs. Peptide rVI, however, bound PKC independently of PKC activators (Ron and Mochly-Rosen, 1994).

The maturation of *Xenopus* oocytes was used as a model system to study the PKC-RACK association *in vivo* (figure 1.7). Insulin-induced maturation of oocytes was previously shown to mediate PKC translocation from the cytosol to the particulate fraction. Microinjection of purified RACK or Peptide I interfered with the normal translocation of endogenous PKC and delayed oocyte maturation (Ron and Mochly-Rosen, 1994). Interestingly, peptide rVI of RACK1 induced oocyte maturation in the absence of insulin (figure 1.7). These results indicated that Peptide I, as well as purified RACK, competed with the cellular RACK for PKC and thereby inhibited PKC translocation. In contrast, Peptide rVI promoted PKC translocation

A) RACK1 DNA Sequence

```

1   GGCACGAGGG GTCGCGGTGG CAGCCGTGCG GTGCTTGGCT CCCTAAGCTA
51  TCCGGTGCCA TCCTTGTCGC TCGGCGGACT CGCAACATCT GACGCCATGA
101 CCGAGCAAAT GACCCCTTCGT GGGACCCTCA AGGGCCATAA TGGATGGGTT
151 ACACAGATCG CCACCACTCC GCAGTTCCCG GACATGATCC TGTGCGCGTC
201 TCGAGACAAG ACCATCATCA TGTGGAAGCT GACCAGGGAT GAGACCAACT
251 ACGGCATACC ACAACGTGCT CTTCGAGGTC ACTCCCACTT TGTTAGCGAT
301 GTTGTTCATCT CCTCTGATGG CCAGTTTGCC CTCTCAGGCT CCTGGGATGG
351 AACCTTACGC CTCTGGGATC TCACAACGGG CACTACCACG AGACGATTTG
401 TCGGCCACAC CAAGGATGTG CTGAGCGTGG CTTTCTCCTC TGACAACCGG
451 CAGATTGTCT CTGGGTCCCG AGACAAGACC ATTAAGTTAT GGAATACTCT
501 GGGTGTCTGC AAGTACACTG TCCAGGATGA GAGTCATTCA GAATGGGTGT
551 CTTGTGTCCG CTTCTCCCG AACAGCAGCA ACCCTATCAT CGTCTCCTGC
601 GGATGGGACA AGCTGGTCAA GGTGTGGAAT CTGGCTAACT GCAAGCTAAA
651 GACCAACCAC ATTGGCCACA CTGGCTATCT GAACACAGTG ACTGTCTCTC
701 CAGCTGGATC CCTCTGTGCT TCTGGAGGCA AGGATGGCCA GGCTATGCTG
751 TGGGATCTCA ATGAAGGCAA GCACCTTTAC ACATTAGATG GTGGAGACAT
801 CATCAATGCC TTGTGCTTCA GCCCCAACCG CTACTGGCTC TGTGCTGCCA
851 CTGGCCCCAG TATCAAGATC TGGGACTTGG AGGGCAAGAT CATGGTAGAT
901 GAACTGAAGC AAGAAGTTAT CAGCACCAGC AGCAAGGCAG AGCCACCCCA
951 GTGTACCTCT TTGGCTTGGT CTGCTGATGG CCAGACTCTG TTTGCTGGCT
1001 ATACCGACAA CTTGGTTCGT GTATGGCAGG TGACTATTGG TACCCGCTTAA
1051 AAGTTTATGA CAGACTCTTA GAAATAAACT GGCTTTCTG

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B) Complete RACK1 Protein Sequence

Repeat I	MTEQMTLRGTLKGHNWVQ	IATTPQFPDMILSASRDKTIIMWKLTRDET
Repeat II	YGIPQRALRGHSHFVS	DVVISSDGQFALSGSWDGLRLWDLT
Repeat III	TGTTTTRRFVGHTK <u>DVL</u>	<u>SVAF</u> SSDNRQIVSGSRDKTIKLWNTLG
Repeat IV	VCKYTVQDESHSEWVSCVRFSPNSSNP	IIVSCGWDKLVKVNLA
Repeat V	NCKLKTNHIGHTGYLN	TVTVSPDGS LCASGGKDGQAMLWDL
Repeat VI	NEGKHLYTLDG <u>DI</u>	<u>NALCF</u> SPNRYWLCAATGPSIKIWDLEGKIMVDE
Repeat VII	LKQEVISTSSKAEP	PQCTSLAWSADGQTLFAGYTDNLVRVWQVTIGTR

Consensus	GHS	V	V	SSD	ILSG	D	TIKLW	L
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C) Synthetic Peptides

PEPTIDE rIII	DVLSVAF
PEPTIDE rVI	DIINALCF

Figure 1.6

RACK1 sequence data. A) Sequence analysis of the RACK1 DNA revealed an open reading frame of 951bp. The putative start codon (ATG at 97) and stop codon (TAA at 1048) are underlined. B) The complete predicted amino acid sequence (317 residues) indicated the presence of seven repeats, as aligned in the figure (see text for details). C) Synthetic peptides, based on sequences from two of the repeat sequences, were obtained; rIII and rVI. These peptides were used for further analysis of RACK1 function (see text for details). (Data based on Ron *et al.* 1994).

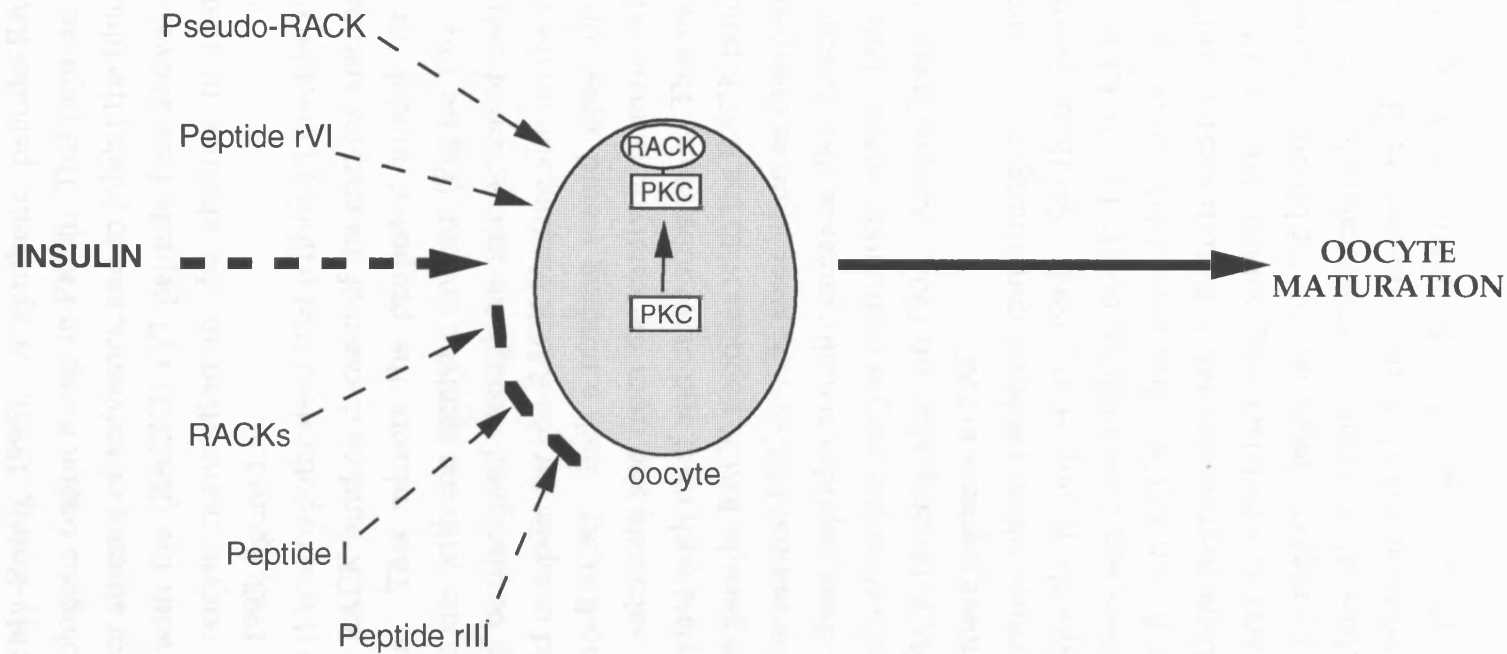


Figure 1.7

Analysis of domains, thought to participate in the PKC-RACK association, using the maturation of oocytes as an *in vivo* model system.

Insulin-induced maturation of *Xenopus* oocytes mediates the translocation of PKC from the cytosol to the particulate fraction. Oocyte maturation is inhibited when purified RACK proteins, Peptide I (based on homology found in two PKC binding proteins) and Peptide rIII (based on sequence found in RACK1) are microinjected. It is thought that the addition of these factors to the oocyte prevents the normal cellular PKC-RACK association. Conversely, the microinjection of Peptide rVI (based on sequence found in RACK1) and Pseudo-RACK (based on sequence found in PKC β displaying homology to RACK1) stimulated oocyte maturation in the absence of insulin. It is thought that these two peptides promote the 'open' active conformation of PKC, and its subsequent translocation (see text for details).

and kinase activation. These observations formed the basis for a model explaining RACK-PKC interaction (see below).

Another observation which contributed to the model was the interesting finding of short sequences in RACK1 and PKC α , β , γ , δ , ϵ , θ , ζ and η which display moderate identity (Ron and Mochly-Rosen, 1995). A synthetic 'pseudo-RACK' peptide, was designed to the homologous region found in PKC β . This peptide was found to bind PKC in the presence or absence of activators, and to inhibit the binding of PKC β to RACK1 *in vitro*. As with the (RACK1) rVI peptide (see above), the pseudo-RACK peptide induced oocyte maturation in the absence of insulin induction (Ron and Mochly-Rosen, 1995; figure 1.8).

Based on these observations it was hypothesised that within an inactive PKC, the pseudo-RACK site binds to the RACK-binding domain of the enzyme stabilising its inactive 'closed' conformation. This mirrors the proposed model for the interaction of the pseudo substrate site with the catalytic domain (Figure 1.8). The inactive, folded conformation could be disrupted through the interaction of pseudo-RACK or rVI peptides which would compete for the RACK binding site on the PKC molecule (see figure 1.8 for pseudo-RACK). Such a binding would 'open' up the folded PKC releasing the pseudo substrate site from the catalytic domain which would in turn be free to phosphorylate both itself and other substrates. This model implies that the interaction of rVI or pseudo-RACK peptides with the RACK binding site of PKC would interfere with the normal PKC-RACK association as observed *in vitro* (see below). The finding that these peptides actually enhance PKC function *in vivo*, and therefore its cellular translocation (see oocyte maturation above), indicates the possible existence of other RACK binding sites on PKC. Alternatively, they could reflect a transient binding of these peptides to PKC.

In addition to cPKC isozymes, other proteins containing a C₂ domain (including PLC γ) bind RACK1 (Mochly-Rosen, et al., 1992). Synthetic peptides reflecting the sequence homology between these proteins bound to RACK1 *in vitro* and inhibited the association of PKC β with RACK1 (Ron, et al., 1995). *In vivo* studies demonstrated that the presence of these peptides inhibited the translocation of PKC β but that the translocation of PKC δ and ϵ , which lack a C₂ domain, were unaffected. It is conceivable, therefore, that the C₂ region is important for the binding of classical isozymes to RACK1. As each classical isozyme is translocated to a different intracellular compartment upon activation it is likely that non-conserved regions of PKC will also exert some effect on the binding to RACKs (James and Olsen, 1992, Walker, et al., 1995).

In rat hepatocytes, two distinct groups of RACK-like proteins were detected (Robles-Flores and Garcia-Sainz, 1993). In the presence of PS, PKC was found to bind to a group of 12-14kDa proteins in a Ca²⁺-independent manner, and to a second group of 28-36kDa proteins in a Ca²⁺-dependent manner. It was suggested that the

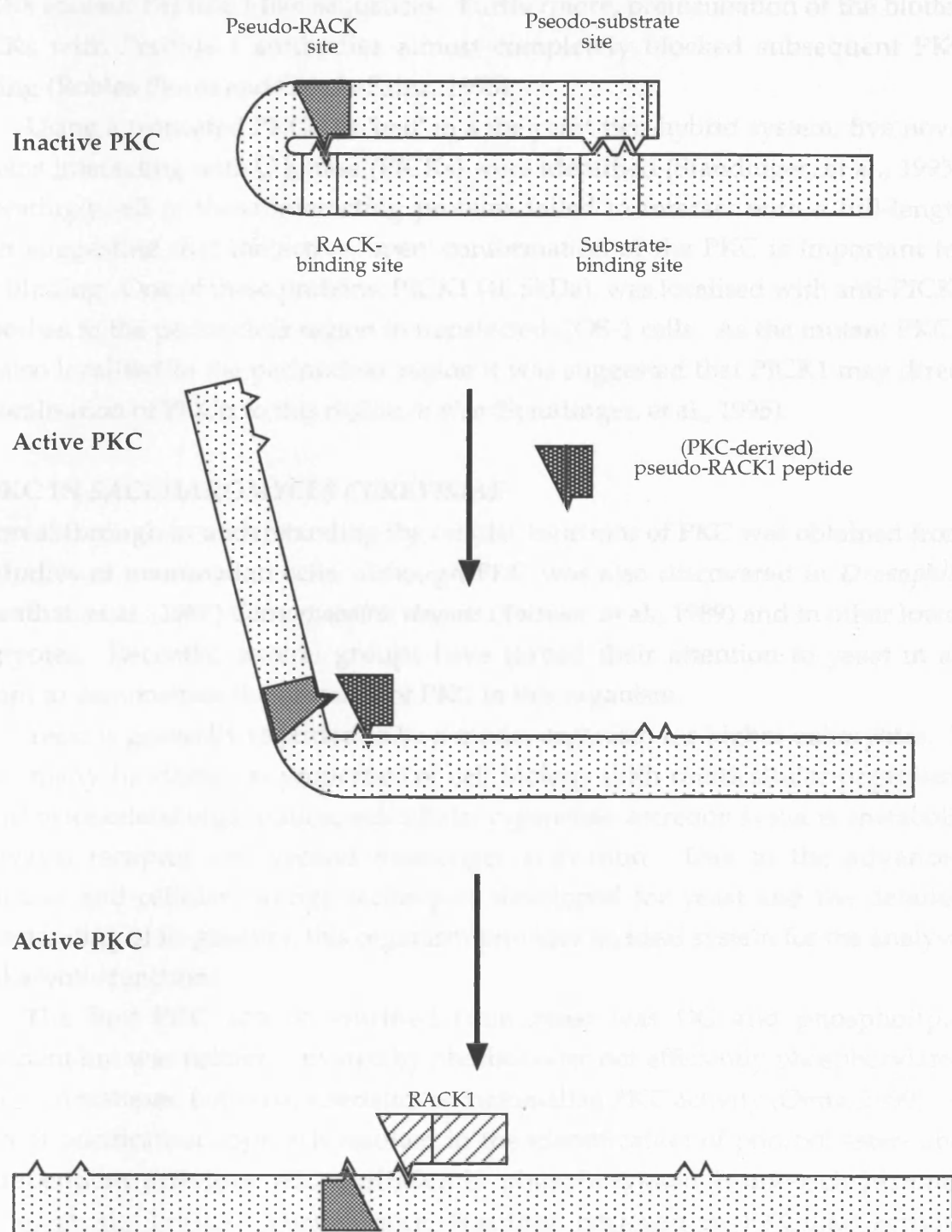


Figure 1.8

Model of the pseudo-RACK1 site in the inactive and active forms of PKC. The inactive PKC enzyme is maintained in a 'closed' conformation by the intramolecular associations of the pseudo-substrate site with the substrate-binding site and a pseudo-RACK1 site with the RACK1-binding site. An 'open' conformation of PKC is promoted by its association with PKC activators or a synthetic peptide homologous to the pseudo-RACK1 site. An active 'open' conformation of PKC is maintained by continued association with PKC activators, and/or with RACK1. (Based on Ron and Mochly-Rosen, 1995).

two groups of putative RACKs have varying affinities for different PKC isozymes. Western blot analysis with anti-Peptide I antibodies suggested that these putative RACKs contain Peptide I-like sequences. Furthermore, preincubation of the blotted RACKs with Peptide I antibodies almost completely blocked subsequent PKC binding (Robles-Flores and Garcia-Sainz, 1993).

Using a truncated PKC α as 'bait' in a the yeast two-hybrid system, five novel proteins interacting with C kinase (PICKs) were identified (Staudinger, et al., 1995). Interestingly, all of these interacting proteins failed to interact with a full-length PKC α suggesting that the active 'open' conformation of the PKC is important for their binding. One of these proteins, PICK1 (46.5kDa), was localised with anti-PICK1 antibodies to the perinuclear region in transfected COS-1 cells. As the mutant PKC α was also localised to the perinuclear region it was suggested that PICK1 may direct the localisation of PKC α to this region *in vivo* (Staudinger, et al., 1995).

1.5 PKC IN *SACCHAROMYCES CEREVISIAE*

The breakthrough in understanding the cellular functions of PKC was obtained from the studies of mammalian cells, although PKC was also discovered in *Drosophila* (Rosenthal, et al., 1987) *Caenorhabditis elegans*. (Tabuse, et al., 1989) and in other lower eukaryotes. Recently, several groups have turned their attention to yeast in an attempt to demonstrate the presence of PKC in this organism.

Yeast is generally regarded to be a model organism for higher eukaryotes. It shares many fundamental properties of cell biology with multicellular organisms such as cytoskeletal organisation, subcellular organelles, secretion systems, metabolic pathways, receptor and second messenger activation. Due to the advanced molecular and cellular biology techniques developed for yeast and the detailed understanding of its genetics, this organism provides an ideal system for the analysis of eukaryotic functions.

The first PKC activity purified from yeast was DG and phospholipid dependent but was neither activated by phorbol ester nor efficiently phosphorylated lysine-rich histones, both characteristics of mammalian PKC activity (Ogita, 1990). A different purification approach resulted in the identification of phorbol ester- and DG-dependent activities which efficiently phosphorylated lysine-rich histones (Simon, et al., 1991b). Furthermore, fractions enriched in PKC-like activity specifically bound TPA and contained an 85kDa protein recognised by monoclonal antibodies raised against rat brain PKC. Interestingly, as in mammalian cells, three peaks of PKC activity were purified, suggesting the presence of isozymes.

An isolated PKC-like gene from *Saccharomyces cerevisiae*, *PKC1*, was found to be essential for cell growth (Levin, et al., 1990). The predicted size of the Pkc1p protein, 131kDa, was much larger than that of most mammalian isozymes and larger than the 85kDa protein(s) described biochemically (Ogita, 1990; Simon, et al., 1991b).

Although Pkc1p showed approximately 50% amino acid similarity to the catalytic region of conserved rat PKC isozymes, its sequence similarity to the regulatory domain was significantly lower. The putative Ca^{2+} -binding region of Pkc1p, for example, displayed only 17% sequence identity to the mammalian PKC. The limited sequence homology of the Pkc1p regulatory domain to that of mammalian isozymes was reflected in its activator requirements. Recombinant Pkc1p is not activated by PS, Ca^{2+} and DG indicating that this enzyme shows most similarity to the novel mammalian PKC isozymes and possibly requires additional cofactors for its complete activation (Antonsson, et al., 1994, Watanabe, et al., 1994). The substrate preferences of Pkc1p *in vitro* includes a pseudo substrate peptide, myelin basic protein and some histones, similar to the preferences of mammalian isozymes, and in particular, to PKC δ and ϵ (Antonsson, et al., 1994). Furthermore, the residues surrounding the target phosphorylation site in the substrates of Pkc1p showed similarity to the consensus sequence of mammalian substrates (Watanabe, et al., 1994).

Mutations in *PKC1* arrest yeast growth and cell division and are accompanied by cessation of protein synthesis, rapid loss of viability, and release of cellular material into the medium (Levin and Bartlett-Heubusch, 1992). A $\Delta pkc1$ mutant was nevertheless capable of proliferation in osmotically stabilising medium, but underwent rapid cell lysis when shifted to hypo-osmotic medium.

The osmotic sensitivity of Pkc1p is underlined by the observation that the *PKC1* mutant *hpo2* is involved in the accumulation of β -1,3-glucan necessary for cell wall rigidity (Shimizu, et al., 1994). *PKC1* is thought to function, in this case, as a negative regulator of the gene *BGL2*, which expresses a β -1,3-endoglucanase. It is plausible that Bgl2p, which has affinity for chitin, plays a role in decomposing glucan at the bud emergence site to allow extension of the cell wall. *PKC1* mutation and/or overproduction of Bgl2p would be expected to increase cell wall digestion, prevent bud enlargement, and arrest cell growth. All these phenotypes were indeed described for *PKC1* mutants. *PKC1* is therefore believed to have a role in synchronising cell wall metabolism with cell growth (Shimizu, et al., 1994). The role(s) of Pkc1p in cell wall metabolism is further illustrated by the findings that mutations in other genes, *KRE6* (K1 killer toxin resistant) and its functional homologue *SKN1*, are independently involved in the production of (1-6)- β -glucan. *KRE6* mutants lead to synthetic lethality with several members of the *PKC1*-mediated MAP kinase pathway (see below), including *PKC1* (Roemer, et al., 1994). As yet, it is unclear whether *KRE6* is a member of the *PKC1* pathway or participates in a parallel pathway affecting cell wall biosynthesis.

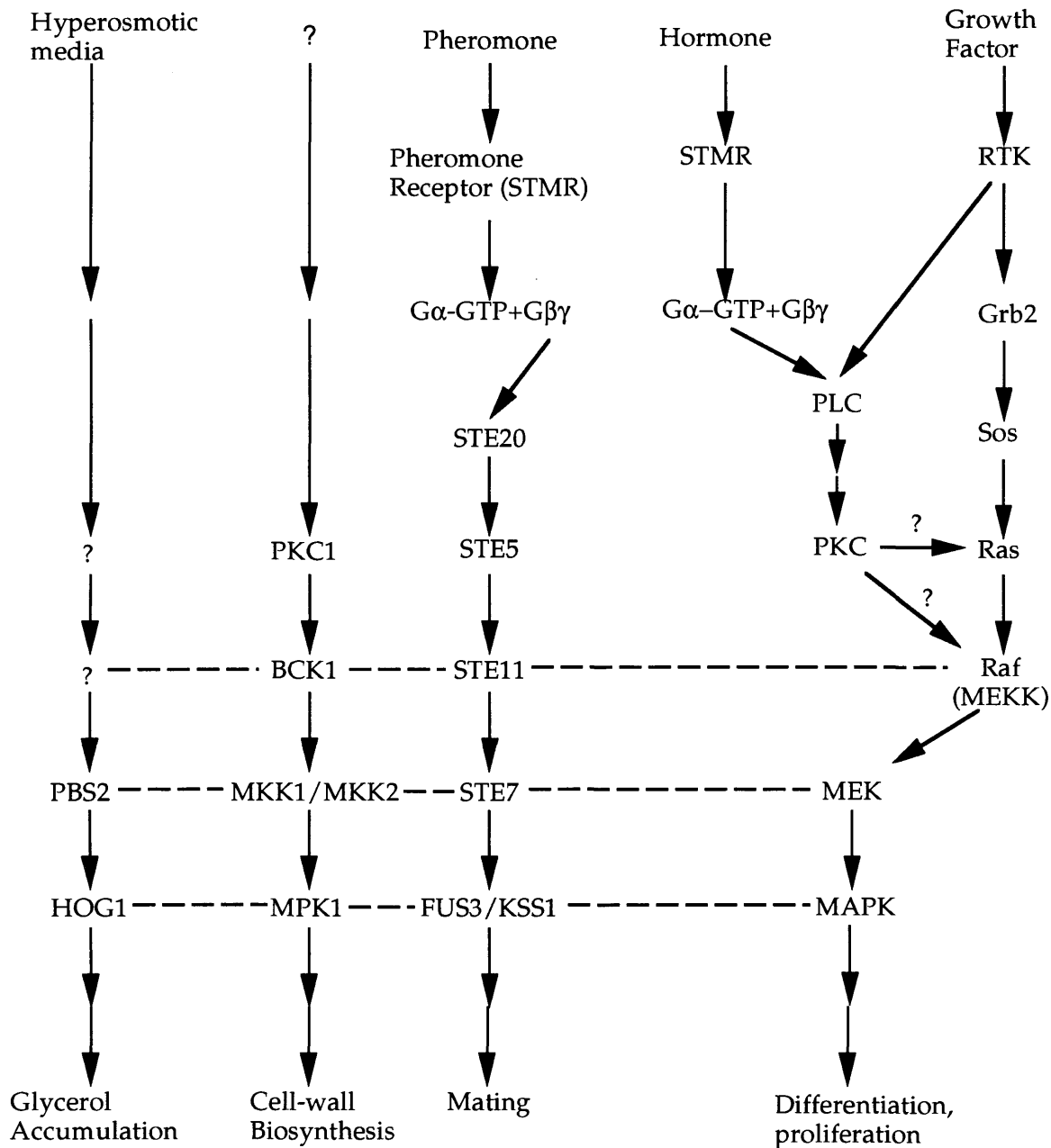
The involvement of Pkc1p in cell wall metabolism was also shown by genetic analysis of staurosporine sensitive mutants. Staurosporine, is one of the most specific PKC inhibitors available and is thought to inhibit PKC by binding to the

enzyme directly. Mutants in *Saccharomyces cerevisiae*, designated *stt* for staurosporine and temperature sensitivity, were isolated to investigate the physiological role of PKC in yeast (Yoshida, et al., 1992). One such mutant, *Stt1*, was found to be allelic of *PKC1*. Other mutant analysis revealed *Stt3p*, which is involved in protein glycosylation, *Stt10p*, involved in the sorting of vacuolar proteins (Yoshida, et al., 1995) and *Stt4p*, which shows sequence identity to mammalian PI-3 kinase but is actually required for PI-4P production (Yoshida, et al., 1994).

The signal transduction pathway which leads to cell wall biosynthesis was elucidated through suppressor analysis of *pkc1* mutants and the yeast two-hybrid system. Extragenic suppressors of a *pkc1* deletion mutant were all assigned to a single locus designated *BCK1* (for bypass of C kinase) (Lee and Levin, 1992). *BCK1* displays homology to the MEKKs of mammalian cells (Raf) indicating that Pkc1p functions upstream of a MAP kinase module (figure 1.9). This finding was an exciting result as it suggested that evolutionally diverse organisms contain common signal transduction pathways (Blumer, 1995 and references therein). Genetic evidence indicates that Bck1p connects Pkc1p with a MAP kinase cascade which includes Mkk1p, Mkk2p, and Mpk1p (figure 1.9). The participation of these proteins is underlined by the fact that multiple copies of either *MKK1* or *MKK2*, a pair of MAP kinase kinase homologues, suppress the lysis of *pkc1* mutants (Blumer, 1995). *MPK1*, the MAP kinase at the base of this yeast MAP kinase module (figure 1.9), is thought to phosphorylate a variety of substrates, including transcription factors.

The yeast two-hybrid system was utilised to confirm the genetical findings and to analyse the *in vivo* interactions between Pkc1p and proteins of the MAP kinase cascade (Paravicini and Friedli, 1996). As predicted from the genetical studies (figure 1.9), Bck1p was found to interact with the C-terminal catalytic domains of Mkk1p and Mkk2p, while Mkk2p was found to interact with Mpk1p. However, contrary to the genetical evidence based on mutant analysis, no interaction was detected between Pkc1p with Bck1p, perhaps indicating that they only transiently interact. Interestingly, Pkc1p interacted with activated forms of Mkk1p but not Mkk2p. It is possible that Pkc1p interaction with downstream members of the MAP kinase pathway provides an additional mechanism of regulating the MAP kinase pathway (Paravicini and Friedli, 1996).

Saccharomyces cerevisiae possesses at least another two signalling pathways that include MAPK homologues (figure 1.9). One of these pathways responds to mating pheromones and the other to osmotic stress (Herskowitz, 1995). Expression of mammalian MEKK suppresses the effects of a $\Delta bck1$ mutation in *Saccharomyces cerevisiae* but not those of a $\Delta mpk1$ mutation (figure 1.9), indicating that MEKK replaces its yeast homologue rather than bypassing the mutation. The mammalian MEKK also fails to suppress yeast mutations in the osmotic stress or mating pheromone pathways, indicating that these pathways (figure 1.9) are distinct and

**Figure 1.9**

MAP kinase pathways in yeast and higher eukaryotes. The analysis of signal transduction pathways has revealed a series of kinases which are highly conserved throughout evolution. These kinases make up a module referred to as the MAP kinase cascade. Several MAP kinase cascades have been observed in yeast, each responding to a different stimulus. Although these cascades are highly homologous, they are almost entirely independent of one another.

independent of each other (Blumer, et al., 1995). Furthermore, overexpression of *NPK1* in yeast, a tobacco gene with a catalytic domain that is homologous to Bck1p and Ste11p of the pheromone response pathway (figure 1.9), complements *pkc1* and *bck1* mutants but not *ste11* mutants (Banno, et al., 1993). These data show that components of MAP kinase pathways are evolutionary well conserved, but while an organism may have several functional MAP kinase pathways, operating in parallel, they are essentially independent of each other (figure 1.9).

It is evident from the analysis described above that Pkc1p has a prominent role in the signal transduction pathways leading to cell wall biosynthesis. Nevertheless, analysis of mutants with defective Pkc1p activity suggested that it is involved in other cellular processes. A possible protein interacting with Pkc1p, Rho1p, has been identified (Nonaka, et al., 1995). Rho1p is a small GTP-binding protein essential for bud formation. Studies using indirect immunofluorescence microscopy indicated that Rho1p is localised to the cortical actin patches and the presumptive bud site, suggesting that it associates with the actin cytoskeleton as observed in mammalian cells (Nonaka, et al., 1995). A dominant suppressor mutation of a $\Delta\rho1$ strain was found to be in the pseudo substrate domain of *PKC1* (Nonaka, et al., 1995). Yeast two-hybrid studies indicated the interaction of the C₁ domain of Pkc1p with Rho1p-GTP (but not with Rho1p-GDP). The data suggests that Rho1p could promote the activation of Pkc1p by dissociating its pseudo substrate site from the catalytic site. Alternatively, Rho1p may localise Pkc1p to the actin cytoskeleton in a manner proposed for RACK proteins (Nonaka, et al., 1995).

Pkc1p was also implicated in the metabolism of DNA as a mutant allele of *pkc1* (*pkc1-4*) was found to confer a hyper-recombination phenotype (Huang and Symington, 1994). In addition, *PKC1* mediates phosphorylation of the yeast CTP synthetase leading to a 3-fold increase in the activity of this enzyme (Yang, et al., 1996).

The entry of *Saccharomyces cerevisiae* into the mitotic cell cycle is regulated late in the G₁ stage at a point defined as START and requires *CDC28*, in association with the cyclins, *CLN1*, *CLN2*, and *CLN3* (figure 1.10). Phosphorylations by the Cln/Cdc28 complexes are responsible for triggering the DNA replication, spindle pole body duplication, and bud emergence associated with passage through START (Marini, et al., 1996 and references therein). Mutant analysis placed the activities of both Pkc1p and Mpk1p downstream of the cyclin-dependent kinase, Cdc28p (Marini, et al., 1996). A post-START Cdc28-dependent stimulation of DG production was presumed to be responsible for the activation of the *PKC1*/MAP kinase cascade although Pkc1p activation by DG could not be demonstrated (Antonsson, et al., 1994, Watanabe, et al., 1994).

The completion of the yeast genome sequencing project made the search for PKC homologues possible. The genome sequence data suggests that *PKC1* is the

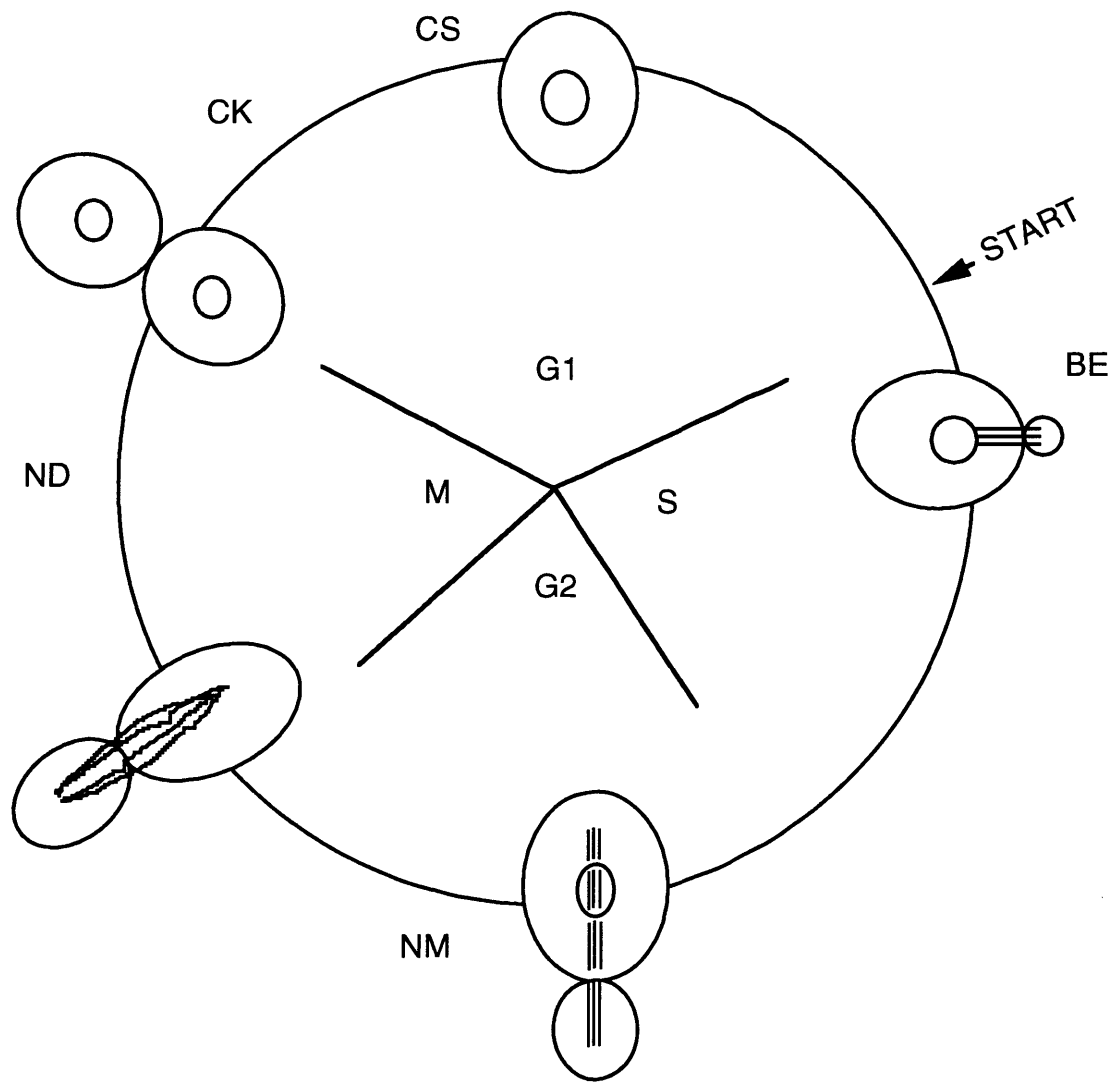


Figure 1.10

The cell cycle of *Saccharomyces cerevisiae*. The yeast cell cycle is divided into a G1 phase, which precedes the initiation of chromosomal DNA replication; an S phase, in which chromosomal DNA is replicated; a G2 phase and an M phase, during which mitosis and nuclear division occur. BE, bud emergence; NM, nuclear migration; ND, nuclear division; CK, cytokinesis; CS, cell separation. (Based on Pringle and Hartwell, 1981)

only yeast gene showing significant sequence homology to mammalian PKC. This is surprising considering the previously obtained biochemical data (Simon, et al., 1991a). The yeast genome sequencing project did reveal, however, genes sharing high sequence homology to particular domains of mammalian PKC and it is therefore plausible that functional homologues of mammalian PKC exist.

1.6 AIMS OF THE PROJECT

As more components of signal transduction pathways are revealed, the emphasis on understanding the regulation and control of these pathways increase. PKC appears to be a pivotal protein that has a role in many of the signal transduction pathways characterised in higher eukaryotic cells. The regulation of PKC activity is therefore envisioned to be a major point of signal transduction control. One mode of controlling PKC activity has been the proposed action of PKC receptors that compartmentalise individual PKC isozymes. This localisation of PKC is believed to direct the kinase to particular substrates that ultimately influence the final cellular response.

Many components of mammalian signal transduction pathways are found to be conserved in yeast. The purification of enzymatic activities from yeast showing properties characteristic of mammalian PKC initiated the cloning and characterisation of the yeast gene, *PKC1*. The indication that PKC-dependent signal transduction pathways existed in yeast suggested the possibility that PKC receptors would also be present.

Using an immunological screen of expression libraries we aimed to identify, isolate and characterise putative receptors of PKC from both mammalian and yeast cells. The cloning of putative RACKs from both organisms would demonstrate a universal requirement for these proteins, and contribute to the understanding of the regulation of PKC.

CHAPTER 2.

MATERIALS AND METHODS

2.1 Yeast Culture Media

Yeast were routinely grown in YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose). For solid medium 2% (w/v) agar was added to the liquid medium.

Synthetic minimal medium (SD) contained 0.67% (w/v) Bacto-yeast nitrogen base, 2% (w/v) glucose; 2% (w/v) agar and amino acid supplements were added as and when appropriate.

Completely defined yeast medium [(M63), Clifton et al, 1978] was used in phenotypic analysis and contained 100mM KH_2PO_4 , 15mM $(\text{NH}_4)_2\text{SO}_4$, 0.8mM MgSO_4 and 2 μM $\text{Fe}_2(\text{SO}_4)_3$, the pH was adjusted to 6.5 with KOH. This was supplemented with the vitamins thiamine HCl (0.4mg/l), pyridxine (0.4mg/l), pantothenate (0.4mg/l), biotin (0.02mg/l) and myo-inositol (2mg/l), a carbon source (2% final) and any auxotrophic requirements.

Diploid yeast strains were induced to sporulate by plating out on sporulating medium (1% potassium acetate, 0.1% (w/v) yeast extract, 0.05% (w/v) glucose, 2% (w/v) agar); nutritional supplements were added as required. Growth of strains on pre-sporulation media was often carried out for a more dramatic starvation response (YPD including 10% glucose).

2.2 Bacterial Culture Media

Luria - Bertani Medium contained 1% Bacto-tryptone, 0.5% bacto-yeast extract, and 1% NaCl, the pH was adjusted to 7.5 with NaOH. Ampicillin was added at 100 $\mu\text{g}/\text{ml}$ (final) when growing or selecting for strains carrying plasmids encoding β -lactamase.

M9 minimal medium contained 10% of M9 salts (15.1% Na_2HPO_4 , 3% KH_2PO_4 , 0.5% NaCl, and 1% NH_4Cl), 0.4% glucose, and 1% CM (0.01M CaCl_2 , 0.1M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$).

Phage were plated out in soft overlay agar (BTL, which consisted of 1% trypticase, 0.5% NaCl, and 0.7% Difco agar) onto bottom layer agar (BLA, as BTL except with 1.5% agar).

Electroporated bacteria were recovered in SOC media; 2% Bacto-tryptone, 0.5% bacto-yeast extract, 0.05% NaCl, 2.5mM KCl, 5mM MgCl_2 , 5mM MgSO_4 and 20mM glucose (added after autoclaving).

2.3 Strains, Phage, Plasmids, Oligonucleotides And Primers Used During This Study

Table 2.1

Yeast strains	Genotype	Source
842	<i>Mat(a/α ade2, trp1, leu2, his3, ura3, can^{R1}-100</i>	K. Nasmyth
W303 (a or α) [His ⁺]	<i>Mat(a or α) ade2-1, trp1-1, leu2-3-112, ura3-1,</i>	P. Meacock
W303 (a or α) [His ⁻]	<i>Mat(a or α) ade2-1, trp1-1, leu2-3-112, his3-11-15, ura3-1,</i>	P. Meacock
HDs (a or α) [H ⁻]	<i>Mat(a or α) ade2-1, trp1-1, leu2-3-112, his3-11-15, ura3-1, rck2::URA3</i>	This study
HDl [H ⁺] α	<i>Matα ade2-1, trp1-1, leu2-3-112, ura3-1, Δrck2::URA3</i>	This study
HDl (a or α) [H ⁻]	<i>Mat(a or α) ade2-1, trp1-1, leu2-3-112, his3-11-15, ura3-1, Δrck2::URA3</i>	This study
HDs α [H ⁺] ρ ⁻	<i>Matα ade2-1, trp1-1, leu2-3-112, ura3-1, rck2::URA3 ρ⁻</i>	This study
HDl α [H ⁺] ρ ⁻	<i>Matα ade2-1, trp1-1, leu2-3-112, ura3-1, Δrck2::URA3, ρ⁻</i>	This study
DDs	<i>Mat(a/α ade2-1/ade2-1, trp1-1/trp1-1, leu2-3-112/leu2-3-112, his3-11-15/HIS3, ura3-1/ura3-1, rck2::URA3/RCK2</i>	This study
DDl	<i>Mat(a/α ade2-1/ade2-1, trp1-1/trp1-1, leu2-3-112/leu2-3-112, his3-11-15/HIS3, ura3-1/ura3-1, Δrck2::URA3/Δrck2::URA3</i>	This study

Table 2.2

Bacterial strains	Genotype	Source
<i>E. coli</i> Y1090	F ⁻ $\Delta(lac)U169 lon^{-} 100 araD139 rpsL(Str^r)$ <i>supF mcrA trpC22::Tn10</i> (pMC9; Tet ^r Amp ^r)	Huynh, <i>et al.</i> (1985)
<i>E.coli</i> DH5 α	<i>supE44, $\Delta lacZ$ U169 ($\Phi 80 lacZ \Delta M15$), endA1, recA1, hsdR17, thi-1, gyrA96, relA1</i>	Hanahan (1983)
<i>E.coli</i> BL21(DE3)	<i>hsdS, gal(lclts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)</i> with DE3, a λ prophage carrying the T7 RNA polymerase gene	Studier and Moffat (1986)
<i>E.coli</i> TB1	F ⁻ <i>ara $\Delta(lac-proAB) rpsL \phi 80 dlacZ \Delta M15 hsdR17$</i> (r _k ⁻ m _k ⁺)	D. Mochly-Rosen

Table 2.3

Phage	Description	Source
λ gt11	Phage used for expression library construction	Young and Davis (1985)
λ EMBL3	Phage used for genomic library construction	P. Meacock
λ 3.1/3.3/7.1/1.2/7.2	λ gt11 phage clones isolated as putative yeast RACKs	This study

Table 2.4

Plasmids (Genetic marker)	Description	Source
pUC18/19 (amp)	Plasmid used for cloning	This laboratory
pIC19R/20H (amp)	Plasmid used for cloning based on the pUC vectors	This laboratory
yCP50 (URA3, amp, tet)	Yeast centromeric shuttle vector	Rose <i>et al.</i> , (1987)
pEMBLyex4 (amp, URA)	Shuttle vector allowing gene expression in yeast	Baldari and Cesarini (1985)
pYRG19- Δ BamHI (amp)	Plasmid source for <i>Sma</i> I fragment containing URA3	M. Murray (this laboratory)

Plasmids (Genetic marker)	Description	Source
pET3a (amp)	Plasmid expression vector	A. H. Rosenberg (1987)
pMAL (amp)	Plasmid expression vector producing MBP fusions	New England Biolabs
pGEX-2T/pGEX-4T (amp)	Plasmid expression vectors producing GST fusions	Pharmacia
p3.1/3.3/7.1/1.2/7.2 (amp)	Recombinant plasmids containing inserts derived from λ gt11 phage clones	This study
pEH400 (amp)	pUC19-based recombinant plasmid containing <i>EcoRI-HindIII</i> fragment (~400bp) from p7.2	This study
pEH900 (amp)	pUC19-based recombinant plasmid containing <i>EcoRI-HindIII</i> fragment (~900bp) from p7.2	This study
pRCK2 (amp)	pUC19-based recombinant plasmid with <i>HindIII</i> fragment (~3.5kb) containing <i>RCK2</i> gene	This study
pPP1400 (amp)	pUC19-based recombinant plasmid containing <i>PstI</i> fragment (~1400bp) derived from pRCK2	This study
pEE1100 (amp)	pUC19-based recombinant plasmid containing <i>EcoRI</i> fragment (~1100bp) derived from pRCK2	This study
pEE1800 (amp)	pUC19-based recombinant plasmid containing <i>EcoRI</i> fragment (~1800bp) derived from pRCK2	This study
pRCK2-P (amp)	Recombinant plasmid of pRCK2 with ~1.4kb <i>PstI</i> fragment removed	This study
pLJBAM (amp)	pUC19-based recombinant plasmid containing oLJBAM-mediated PCR product	This study
pDMR32 (amp)	Plasmid based on pMAL which includes FLAG antigenic tag	D. Mochly-Rosen
pRCK2m (amp)	Plasmid pDMR32 containing <i>RCK2</i> fragment (MBP fusion)	This study

Plasmids (Genetic marker)	Description	Source
pRCK2g (amp)	Plasmid pGEX-2T containing <i>RCK2</i> fragment (GST fusion)	This study
pRCK2p (amp)	Plasmid pET-3a containing <i>RCK2</i> fragment	This study
pRCK2Δ (amp)	Plasmid containing deleted <i>RCK2</i>	This study
pRCK2δ (amp)	Plasmid containing disrupted <i>RCK2</i>	This study
pRCK2yex (amp, <i>URA</i>)	Plasmid pEMBLyex4 containing <i>RCK2</i> fragment	This study

Table 2.5

Oligonucleotides	Description	Source
oLJBAM	Synthetic oligonucleotide primer (30-mer) containing <i>Bam</i> HI site (5'-AACAAAATGGATCCTTTGAGAATATGTCCG-3')	This study
oLJNCOA	Synthetic oligonucleotide primer containing <i>Nco</i> I and <i>Eco</i> RI sites (5'-GGTATCGTACAGGCCATGGAATCAAGAAG-3')	This study
oLJNCO1	Synthetic oligonucleotide primer containing <i>Nco</i> I site (5'-GCTGTCATTGAAAGACCATGGAATATTCTGG-3')	This study

Table 2.6

Primer	Description	Source
λ gt11 forward	Located 16bp upstream of the <i>Eco</i> RI cloning site of λ gt11 (5'-GGTGGCGACGACTCCTGGAGCC-3')	John Kyte
λ gt11 reverse	Located 22bp downstream of the <i>Eco</i> RI cloning site of λ gt11 (5'-GACACCAGACCAACTGGTAATG-3')	John Kyte
pUC forward (#1212)	Located 40bp upstream of the multiple cloning site pUC plasmids (5'-GTTTCCCAGTCACGAC-3')	John Kyte
pUC reverse (#1233)	Located 48bp upstream of the multiple cloning site pUC plasmids (5'-AGCGGATAACAATTTCACACAGGA-3')	John Kyte

2.4 General Procedures

2.4.1 Centrifugation

Centrifugation of solutions up to 1.5ml was performed in microcentrifuge tubes in a MSE Microcentaur. Larger volumes were centrifuged using either a Sorvall RC5B Refrigerated Superspeed or an Omnispin Sorvall centrifuge. Ultracentrifugations were carried out in either a Beckman TL-100, Beckman L5-65B or a Sorvall OTD65B ultracentrifuge.

2.4.2 Isolation and purification of plasmid DNA

a. Large scale alkaline lysis

Plasmid DNA was prepared (Birnboim and Doly, 1979) as described on pages 90-91 in Maniatis et al. (1982). Plasmids were purified by banding on isopycnic CsCl density gradients, essentially as described on pages 93-94 in Maniatis et al., (1982).

b. Minipreparations of plasmid DNA

All "minipreps" of plasmid DNA were carried out essentially as described by Birnboim and Doly (1979).

2.4.3 Phenol/chloroform extraction

Aqueous solutions of DNA were extracted with phenol/chloroform (1:1) in order to remove any contaminating proteinaceous material. An equal volume of phenol/chloroform was added to the DNA solution, the mixture was vortexed to

form an emulsion and centrifuged. The upper aqueous phase contains the DNA and any proteinaceous debris is located at the interphase between the aqueous and phenolic phases. This step was followed by extraction with an equal volume of chloroform to remove phenol traces, followed by ethanol precipitation.

2.4.4 Ethanol precipitation of DNA

DNA was precipitated with 2 volumes of 100% ethanol in the presence of 0.3M Na acetate, pH 5.5. Precipitation was carried out at -20°C for greater than 30 mins. After centrifugation the pellet was washed with 80% ethanol, allowed to dry partially before being resuspended in TE (10mM Tris pH 8.0, 1mM EDTA) or sterile distilled water.

2.4.5 Restriction endonuclease digestion of DNA

DNA was digested using restriction endonucleases with in the appropriate REact™ buffer (Bethesda Research Laboratories) as supplied. Restriction digests were performed as per manufacturer's recommendations.

2.4.6 Agarose gel electrophoresis

All DNA molecules with the exception of oligonucleotides were analysed by agarose gel electrophoresis. The percentage agarose (w/v) in the gel was selected to suit the requirements of separation and resolution of each particular run. Agarose dissolved in TAE (40mM Tris Acetate, 1mM EDTA) was poured molten into the gel mould and polymerised at room temperature. DNA samples were loaded in sample buffer (TE pH 7.5 containing 20% glycerol and 0.025% bromophenol blue) and electrophoresis was performed in TAE, pH 8.0, voltage and time course as required.

2.4.7 Visualisation of agarose gels

DNA was visualised by staining with 0.5µg/ml ethidium bromide. Gels stained with ethidium bromide were photographed on a UV transilluminator using a Polaroid MP-4 Land Camera and Kodak T max 100 panachrome professional film, which was processed using LX 24 X-ray developer and Kodak 3000 film fixer and hardener.

2.4.8 Size Determination of DNA fragments

The length of DNA fragments was deduced by plotting the distance migrated by respective fragments in agarose against distance migrated by standards such as λ /HindIII or Φ X174/HaeIII under identical conditions.

2.4.9 Isolation of DNA fragments from agarose

A number of methods were employed in purifying DNA fragments from agarose gels: the glass milk method of Vogelstein and Gillespie (1979), the "deathwish" technique of electroelution onto dialysis membrane and also the isolation of DNA from an agarose block by spinning it through polyallomer wool and recovering the aqueous solution containing the DNA.

2.4.10 Dephosphorylation of plasmid DNA

Vector DNA was dephosphorylated as described in Maniatis *et al.*, (1982) pp 133-134 using calf intestinal phosphatase (BCC). Alternatively, shrimp alkaline phosphatase was used. This enzyme, purified from Arctic shrimp (*Pandalus borealis*) has approximately the same specific activity as calf intestinal phosphatase but is completely and irreversibly inactivated after heating at 65°C for 15 mins. In addition, it functions efficiently in standard REact buffers used for restriction endonuclease digestion. Shrimp alkaline phosphatase was used according to the manufacturers recommendations (USB).

2.4.11 T4 Polymerase blunting of DNA termini

T4 DNA Polymerase (Gibco BRL) has a 3'-5' exodeoxyribonuclease activity, but lacks the 5'-3' exodeoxyribonuclease activity. The enzyme can therefore produce blunt-ended DNA by digesting 3' overhangs, and complementing 5' overhangs. The DNA (up to 1µg) was added to 2 µl of T4 Polymerase buffer (10X) [0.33M Tris acetate (pH 7.9), 0.66M potassium acetate, 0.1M magnesium acetate, 5mM DTT, 1mg/ml BSA] and the volume then made up to 20µl with H₂O. 1µl of each dNTP (2mM) and ~2.5 units of T4 DNA Polymerase were added to the 20µl. The DNA was incubated at 37°C for 15 minutes after which the reaction is terminated by a 5 minute incubation at 70°C.

2.4.12 Ligation reaction

Vector DNA was digested with the appropriate restriction enzyme(s) and the reaction was stopped by incubation at 65°C for 10 mins. The DNA was extracted with phenol/chloroform followed by chloroform extraction, and if necessary, dephosphorylated as described above.

Ligation reactions were set up with the appropriate vector DNA to insert ratios in reactions containing 5x ligation buffer (GIBCO BRL) and 1 unit T4 DNA ligase (GIBCO BRL). Ligation reactions of "sticky-ended" fragments were incubated at 15°C overnight, whereas reactions of "blunt-ended" fragments were incubated at 37°C overnight.

2.4.13 Chemical transformation of *Escherichia coli*

Exponentially growing cells in 50ml luria broth ($OD_{600} \approx 0.5$) were spun down in sterile tubes, gently resuspended in 5ml of MR solution (10mM MOPS, pH 7.0 and 10mM RbCl) and spun down immediately. The pellet was resuspended in 5ml MRC (100mM MOPS, pH 6.5, 10mM RbCl and 50mM $CaCl_2$) and left on ice for a minimum period of 60 mins. The cells were pelleted and finally resuspended in 2ml of ice cold MRC.

150 μ l-200 μ l of competent cells were aliquoted into microcentrifuge tubes and the appropriate DNA was added to each tube and left on ice for 60 mins. Cells were heat-shocked by placing them at 50°C for exactly 35 secs and then cooled on ice for 1 minute. 1ml Luria broth was added to each tube, which were incubated at 37°C without shaking for 60 mins. The cultures were spun down and the pellets were gently resuspended in Luria broth and plated onto Luria agar containing the appropriate antibiotics. In case of blue-white selection, 25 μ g/ml X-gal and 25 μ g/ml IPTG were added to the plate. The plates were incubated at 37°C overnight.

2.4.14 Electroporation of *E. coli*

Log-phase cells ($OD_{600} \approx 0.6$) were washed extensively in ice-cold distilled water. 100mls of initial culture were finally reduced to a volume of 200 μ l, and 40 μ l of competent cells were used for each transformation. Electroporation was done following the method of Dower *et al.*, (1988) using a Bio-Rad Gene Pulser using the settings of: 1.5kV, 25 μ FD, 1000 Ω). Transformed cells were recovered in pre-warmed SOC broth, incubated at 37°C for ~60 mins, and plated onto Luria agar medium containing the appropriate antibiotics.

2.4.15 Extraction of Genomic DNA from Yeast

Exponentially growing cells were pelleted and washed in a solution of 1.2M Sorbitol, 50mM Tris pH 7.5, 50mM KCl, 20mM EDTA and 1% β -Mercaptoethanol. After another centrifugation (5mins, 3000g) they were resuspended in the same buffer containing 1mg/ml Zymolyase 100T and incubated at 37°C until spheroplasts formed (approximately 15 mins). Spheroplasts were lysed in 100mM NaCl, 50mM Tris pH 7.5, 100mM EDTA, 1% SDS, 100 μ g/ml Proteinase K, and incubated at 65°C for 15 mins followed by an incubation at 55°C for 45 mins.

The lysate was extracted 3x with an equal volume of phenol-chloroform and once with chloroform prior to ethanol precipitation. The pelleted nucleic acid was washed with 80% ethanol, dried and resuspended in 10mM Tris pH 7.5 containing 10 μ g/ml RNase A and incubated at 65°C for 15 mins.

The DNA was recovered by adjusting the solution to 0.3M sodium acetate and precipitating with 2 volumes of ethanol. The pellet was then washed with 80% ethanol, dried and resuspended in sterile double distilled water.

2.4.16 Transformation into yeast

a) electroporation

50ml culture of exponentially growing cells ($OD_{600} \approx 0.5-0.6$) was spun down at 3,000rpm in an Omnispin centrifuge. The pellet was washed 5-6 times with sterile distilled water. The cells were resuspended in 1 ml sterile distilled water for electroporation.

Electroporation was done following the method of Dower *et al.* (1988) using a Bio-Rad Gene Pulser using the settings of: 5kV/cm, 25 μ FD capacitance, 1000 Ω resistance. Transformed cells were recovered in pre-warmed SOC broth, incubated at 37°C for ~60 mins, and plated onto agar medium containing appropriate antibiotics.

b) chemical transformation

50ml culture of exponentially growing cells ($OD_{600} \sim 0.4$) was pelleted and washed 4 times in sterile distilled water. The cells were then washed twice in 1xTE, 100mM LiAc and finally resuspended in 1ml of TE/LiAc ($\sim 2 \times 10^9$ cells/ml). 50 μ l of cells were added to 1-10 μ g of DNA and vortexed briefly. 300 μ l of 40% PEG₃₃₅₀, 1xTE, 100mM LiAc then added to the cells which were then vortexed briefly and incubated at 30°C for 30 mins. Cells were heat shocked at 42°C for 20 mins, after which they were washed twice in sterile distilled water and plated onto suitable media.

General reaction conditions: 1-10ng DNA template, 50mM KCl, 10mM Tris-HCl (pH 8.8), 1.5mM MgCl₂, 0.1% (w/v) gelatin, 200 μ M each dNTP, 1 μ M of each primer and 2-5 units *Taq* DNA polymerase (Stratagene). Conditions for the PCR of each fragment were set up according to the length of the fragment to be amplified and the GC content of the primers used. The PCR steps included: denaturation at 95°C, annealing and an elongation step (usually 30 cycles). *Pfu* DNA polymerase used instead of *Taq* polymerase when high-fidelity amplification is required due to its proof-reading ability.

2.4.18 Yeast sporulation, tetrad dissection and matings

A diploid yeast strain was induced to sporulate by starvation (see sporulation media, section 2.1) at 30°C. Sporulating diploid cells produce asci containing 4 haploid spores (tetrads). The wall of the ascus was digested with 5% β -glucuronidase and the individual spores were separated using a Singer tetrad dissector. Individual spores were germinated on YPD agar plates at 30°C.

To test the ability to mate, haploid yeast strains were streaked onto a single YPD plate, one strain streaked in horizontal rows, the other strain in vertical rows.

After incubation of the plate at 30°C for several days, yeast cells growing at the points of cross-over were tested for the presence of diploid cells.

2.4.19 Phenotype Analysis Of Mutant Yeast

a) Defined media:

Cells to be analysed were grown overnight in 5mls of defined media at 30°C. 200µl of each overnight culture were placed into wells of a sterile 95-well microtitre plate in an organised array. If space allowed, the array was repeated to give results in duplicate. Using a sterilised 'hedgehog' (metallic apparatus from which prongs protrude, spatially mirroring the wells of the microtitre plate) cells were transferred from the microtitre plate to a petri dish containing pre-defined solid medium. The cell suspension was allowed to dry into the medium before the plate was incubated at a suitable temperature. The extent of growth was assessed after 24-48 hours.

b) Spot tests:

Yeast cells were grown to OD₆₀₀≈0.7. The optical densities of cultures tested were measured accurately to ensure that the number of cells were equivalent. YPD media containing 0.7% agarose was pre-warmed to 50°C. 400µl of yeast culture was added to 4mls of YPD/agarose, mixed quickly, and poured onto pre-warmed (30°C) YPA plates. The plates were then left to solidify. Drops of appropriate solutions were subsequently added to the plates and left to diffuse into the plate. Plates were incubated at 30°C for 24-48 hours. Assuming the substance added to the plate is detrimental to the growth of yeast, a halo of no growth will appear around the site of addition. The radius of the halo corresponds to the sensitivity of the yeast to the substance.

2.4.20 Chromosome assigning of genes

Two hybridisation membranes were obtained from American Type Culture Collection (ATCC) which contained blotted DNA from mapped fragments obtained from the genome of *Saccharomyces cerevisiae* AB972 (ATCC 76269). One membrane contained recombinant bacteriophage lambda while the other recombinant cosmids. Gene sequence was radioactively labelled and used as a probe to hybridise against the two membranes. The hybridising genomic clones were subsequently correlated to a yeast chromosome and approximate chromosomal position by using a supplied computer programme.

2.4.21 Colony screening

Colony hybridisations were carried out as described (Buluwela, et al., 1989). Hybond N (Amersham International) nylon filters and plates were pierced with a

needle for orientation purposes to allow subsequent alignment of positive signal with corresponding colonies.

2.4.22 Plating out phage library

Serial dilutions of bacteriophage were made in lambda buffer (50mM Tris, pH 7.5, 10mM MgCl₂, 100mM NaCl) and 0.1ml of each dilution was added to 0.2ml of an overnight culture of *E.coli* (C600) grown in Luria Broth supplemented with 10mM MgSO₄ and 0.2% maltose. The mixtures were incubated at 37°C for 20 mins, were added to 3ml of molten (50°C) top layer agar (TLA) which was then poured onto pre-warmed BLA plates. The plates were incubated at 37°C for approximately 16 hrs.

2.4.23 Plaque screening

Plaque lifts were carried out according to the method of Benton and Davis (1977) as described in Maniatis *et al* (1982) pp320-321, using Schleicher and Schuell nitrocellulose discs. Filters and plates were pierced with a needle for orientation purposes to allow subsequent alignment of positive result with corresponding plaque.

2.4.24 Detection and isolation of positive phage/colonies

The filters from the plaque/colony lifts were hybridised following protocols described for Western or Southern blots depending on the type of screen. Positive plaques/colonies were identified by aligning the plates from which the lifts were taken with the developed filter. The plaques were picked ('plugged') from the plates using a pasteur pipette and stored in lambda buffer (50mM Tris, pH 7.5, 10mM MgCl₂, 100mM NaCl). Colonies were picked with a toothpick and streaked onto fresh media. The plaques/colonies were purified to homogeneity by several rounds of screening before isolating the corresponding DNA.

2.4.25 Isolation and purification of phage DNA

In this method (Blattner *et al*, 1977) 4 or 5 'plugs' of agar each containing a plaque with the surrounding bacteria were picked from a fresh plate using the large end of a pasteur pipette and drop into a 2l flask containing 200ml of Luria broth containing 10mM MgSO₄ and 0.2% maltose. The cultures were incubated overnight at 37°C. The presence of cell debris in the culture indicates bacterial lysis.

Lysates were incubated with 5% chloroform and agitated at 37°C for 15 mins prior to the addition of DNase I and RNase A at 100µg/ml and 50µg/ml respectively. NaCl was added to a final concentration of 1M and the lysates were left on ice for 30 mins before the cell debris was pelleted by centrifugation (Sorval RC5C) at 8000rpm for 10 mins. Solid PEG 6000 was added to the supernatant to give a final

concentration of 10% and dissolved with gentle stirring at room temperature. The solution was then incubated on ice for 1hr in order to precipitate the phage which were harvested by centrifugation (Sorval RC5C) at 8000rpm for 10 mins at 4°C. The phage pellet was resuspended in 3ml of lambda buffer at 4°C, the solution was centrifuged (Sorval RC5C) for 2 mins at 4°C at 8000rpm and the supernatant collected. The pellet was resuspended in 1.5ml of lambda buffer and spun as described. The supernatants (phage suspensions) were pooled before being layered onto a CsCl step gradient made up in lambda buffer. Solutions of CsCl of densities 1.7g/ml, 1.5g/ml and 1.3g/ml were layered into a disposable Ultra-Clear™ centrifuge tube in the ratio; 2ml:3ml:2ml respectively and the phage suspension was layered on top. The tube was placed in SW40 swingout rotor and centrifuged at 35000rpm for 1hr at 20°C to band the phage particles at their buoyant density. The phage were recovered from the gradient by puncturing the side of the tube with an 18G needle. The phage suspension was dialysed for several hours against lambda buffer to remove CsCl before extracting DNA from the particles.

2.4.26 DNA extraction

The phage DNA was isolated by the addition of SDS to a final concentration of 1% and extraction with 50% volume of phenol/chloroform. The aqueous phase was re-extracted three times before the DNA was precipitated with 100% ethanol. The resultant pellet was rinsed with 80% ethanol and dissolved in 10mM Tris pH 7.5 containing 50µg/ml RNase A and incubated at 65°C for 10 mins. Phenol extraction of the solution was followed by three ethanol precipitations and a 70% ethanol wash before the DNA was suspended in sterile distilled water.

2.4.27 Double-stranded sequencing of plasmid DNA:

a) T7 Polymerase

The concentration of template DNA was adjusted so that 8µl of the solution contained 1.5-2µg of double-stranded plasmid DNA. The 8µl solution of double-stranded plasmid DNA was then denatured by adding 2µl of 2M NaOH and incubated at room temperature for 10 mins. The mixture was neutralized with 3µl 3M Na acetate pH 4.8 and 7µl distilled water. The single-stranded plasmid DNA was ethanol-precipitated with 60µl of 100% ethanol and left on dry ice/methanol for 15 mins. The pellet was collected by centrifuging for 10 mins, washed with ice-cold 80% ethanol, re-centrifuged for 10 mins, dried briefly and resuspended in 10µl of distilled water.

The annealing reaction was set up as follows: in a microcentrifuge tube, 10µl of denatured template DNA from above was mixed with 2µl of annealing buffer (280mM Tris-HCl, pH 7.5, 100mM MgCl₂ and 350mM NaCl) and 2µl of primer

solution (5ng/ μ l). The mixture was incubated at 37°C for 20 mins and then left at room temperature for at least 10 mins.

Termination mixes, 'G' mix {N solution [150 μ M (dATP, dCTP, dTTP and dGTP), 10mM MgCl₂, 40mM Tris-HCl/pH 7.5 and 50mM NaCl] and 15 μ M ddGTP}, 'A' mix {N solution and 15 μ M ddATP}, 'T' mix {N solution and 15 μ M ddTTP}, and 'C' mix {N solution and 15 μ M ddCTP} were pipetted, respectively, into the corresponding tubes.

Using ice-cold enzyme dilution buffer (10mM Tris-HCl, pH 8.0 and 1mM EDTA), the stock of T7 DNA polymerase was diluted to a concentration of 1.5 units μ l⁻¹. The labelling reaction was performed as follows: in a microcentrifuge tube, the annealed template and primer from the above reaction were mixed with 2 μ l of labelling mixture (2 μ M dGTP, 2 μ M dCTP and 2 μ M dTTP), 10 μ Ci [α -³⁵S]dATP α S, and 2 μ l of 1.5 units μ l⁻¹ T7 DNA polymerase. The mixture was incubated at room temperature for 5 mins. 4.5 μ l of this reaction mixture was transferred into each sequencing mixture, which had been held for at least 1 minute at 37°C. The reaction was continued for a further 5 mins and stopped by adding 5 μ l of stop solution (10mM EDTA, pH 8.0, 0.025% xylene cyanol and 0.025% bromophenol blue in 95% deionised formamide). The mixtures were incubated immediately at 80°C for 2 mins; if the sequencing was not performed in the same day, they could be stored at -20°C for up to one week.

b) Cyclist™ ExoPfu

Cycle sequencing was carried out as described in the manufacturer's (Stratagene) protocol. Briefly, 200fmol (10⁻¹⁵mol) of template DNA, 1pmol of primer, 10x sequencing buffer (Stratagene) containing 50 μ M dCTP/TTP/GTP and 20 μ M dATP, 10 μ Ci of [α -³⁵S]dATP, and 2.5U *Pfu* polymerase were divided into four termination mixes containing 15 μ M ddNTPs. The cycle sequencing reaction was carried out as follows: initial delay (95°C)-4 mins; denaturation (95°C)-1 min; annealing (55°C)-1 min; extension (70°C)-2 mins; 30 cycles. Conditions were altered according to the template used, and the annealing temperature of primer.

2.4.28 Electrophoresis of DNA fragment from sequencing reaction

Sequencing gels were cast in a 20x50 cm mould comprising two thoroughly cleaned glass plates. The smaller plate was siliconised using 3ml of 2% dimethyldichlorosilane dissolved in 1,1,1,-trichloroethane. The 6 % gradient gel was prepared from the following stock solutions:

- 40% acrylamide stock (380g acrylamide and 20g bisacrylamide per litre double-distilled water) was filtered through Whatman no.1 paper and stored at 4°C in the dark;

- 10x TBE pH 8.3 (109g Tris, 55g boric acid and 9.3g EDTA) was filtered as above and stored at room temperature.

Working solutions were as follows:

- 1x stock solution (6% acrylamide, 1x TBE, and 50% urea) was filtered and stored at 4°C in the dark.

- 2.5x stock solution (6% acrylamide, 2.5x TBE, 50% urea, 5% sucrose and 0.025% bromophenol blue) was filtered and stored at 4°C in the dark.

The gel was cast following the addition of 0.03% APS and 0.03% TEMED to 12.5ml and 75ml of the 2.5x and 1x stock solutions, respectively. 8ml of the 1x stock was mixed in a 25ml pipette with 12ml of 2.5x stock by introducing an air bubble. The gradient was poured down one edge of the mould and levelled before the rest of the 1x stock solution was added, avoiding the formation of any air bubbles in the gel. Inverted sharktooth combs were placed at the top of the gel to ensure a level air-gel interphase and were removed following polymerisation and inverted in such a way that they barely deformed the surface of the gel. The top reservoir of a BRL (model S2) tank was filled with 1x TBE, whereas the bottom was filled with 2.5x TBE. The samples were loaded following 2 minute incubation at 75-80°C. Electrophoresis was performed at 50°C (50-60W) for 3-4 hrs. The gel, supported on a larger plate, was then fixed with 10% glacial acetic acid and 10% methanol for 15 mins at room temperature, dried under vacuum at 80°C for 2 hrs and exposed to Fuji-XR X-ray film overnight at room temperature before being developed.

2.4.29 Analysis of sequencing data

All DNA sequences and peptide manipulations were carried out using the suite of computer programmes written by the University of Wisconsin Genetics Computer Group run on the Vaxcluster computer system or on a Macintosh using the MacVector programme.

2.4.30 Oligonucleotide purification

In order to complete the sequencing of clones or to carry out PCR amplification it was often necessary to synthesise a number of specific oligonucleotide primers. Primers were obtained from J. Kyte and D. Langton, Department of Biochemistry, Leicester. Purification of the primers was carried out by precipitation with 3 volumes of ethanol in the presence of 0.3M Na acetate, pH 5.5. The primers were washed with 80% ethanol and then checked using a spectrophotometer (Hitachi U-2000) for concentration (OD₂₆₀) and purity. The primers were diluted to a working concentration of 50µg/µl for PCR amplification and 5µg/µl for sequencing.

2.4.31 RNA extraction

A pellet of yeast cells from a mid-log culture was frozen in denaturing buffer (4M guanidine thiocyanate, 25mM Na citrate, 0.5% Na sarcosyl, 0.1M β -mercaptoethanol) prior to their disintegration using a Hughes press apparatus. The Hughes press is comprised of a loading chamber and a collection chamber separated by a fine aperture. Cell breakage is achieved when a tightly fitting plunger is hydraulically pushed into the loading chamber thus forcing the frozen yeast cells through the narrow aperture into the collection chamber shearing single cells in the process. To the resultant lysate 1/10 volume of 3M Na acetate pH 4.0 was added and then the solution was extracted with an equal volume of phenol (water saturated) and 1/5 volume of chloroform/isoamyl alcohol (24:1) following a 10 minute incubation at 60°C. This extraction was repeated before precipitating the RNA with an equal volume of isopropanol on ice for 30 mins. The RNA was pelleted by centrifugation at 10,000g for 10 mins, washed with 80% ethanol, allowed to dry and resuspended in Diethylpyrocarbonate (DEPC) treated water.

2.4.32 Electrophoresis of RNA on agarose gels

RNA was denatured prior to electrophoresis by incubation with 1-3 volumes of GFM buffer (1.1M glyoxal pH 6.9, 78% formamide, 10mM MOPS pH 7.0) at 55°C for 15 mins. Denatured RNA was loaded onto a neutral 1x MOPS gel (1.5% agarose dissolved in 20mM MOPS, pH 7.0, 5mM Na acetate, 0.1mM EDTA) in 1/10 volumes of RNA-sample buffer (40% deionised formamide, 50% glycerol, 1x MOPS, pH 7.0, 0.025% xylene cyanol, 0.025% bromophenol blue) and electrophoresed for 2-3 hrs in 1x MOPS, pH 7.0 at 60mA. If the RNA was to be directly visualised on the gel, for quantitation and qualitative purposes , 1 μ l of 0.2mg/ml ethidium bromide solution was added to the sample prior to denaturation.

2.4.33 Southern blotting

The Southern blotting procedure used was essentially that described on pp 382-386 in Maniatis et al (1982).

2.4.34 Radiolabelling of probe DNA by random priming

This method was first described by Feinberg and Vogelstein (1983). 10-30ng of probe DNA was denatured by boiling for 3-5 mins prior to the addition of the labelling reaction mix. This reaction mix contained 30% OLB (100mM dATP, 100mM dTTP, 100mM dGTP, 250mM Tris-HCl pH 8.0, 25mM MgCl₂, 52mM β -mercaptoethanol, 1mM HEPES pH 6.6, 540 μ g/ml hexadeoxyribonucleotides [Pharmacia]), 0.5 μ g/ml DNase free BSA, 10 μ Ci [³²P] α -dCTP and 0.5 units of Klenow fragment of DNA

polymerase I. The reaction was incubated at room temperature for 5-16 hrs before being denatured by boiling prior to its addition to the hybridisation solution.

2.4.35 DNA hybridisation

This procedure was based on that described by Church and Gilbert (1984). Prehybridisation and hybridisation were carried out in a solution of 0.5M Na Phosphate pH 7.2 (see below), 7% SDS and 1mM EDTA at 65°C when using homologous DNA probes; or 5x SSPE (see below), 5x Denhardtts (see below), 0.5% SDS and 5µg/ml sheared, denatured Herring sperm DNA at 55°C for heterologous probes.

1M Na Phosphate- 134g of Na₂HPO₄ made up to 1045ml to give 1M of PO₄ (pH adjusted to 7.2 with orthophosphoric acid)

20x SSPE- 174g NaCl, 27.6g NaH₂PO₄H₂O and 7.4g EDTA. pH to 7.4 with 10N NaOH per litre.

50x Denhardtts- 5g Ficoll, 5g Polyvinylpyrrolidone, 5g BSA (Fraction V) made up to 500ml with water and filtered.

2.4.36 Washing of the filters

After hybridisation the blots were washed in suitable solution concentrations to provided the required stringency. For hybridisations using Denhardtts (see section 2.4.35), concentrations ranged from 3x SSPE (see section 2.4.35), 0.5% SDS for low stringency to 0.1x SSPE, 0.1% SDS for maximum stringency. For Na Phosphate hybridisations (see section 2.4.35), 0.5M PO₄ pH 7.2, 1% SDS was used for low stringency and 40mM PO₄, 1% SDS for high stringency.

2.4.37 Northern blot

RNA was transferred from the gel to Hybond-N filters following electrophoresis (Northern blot). The transfer was performed exactly as for a Southern blot. The filter was rinsed briefly in 5x SSPE, pH 7.4, and dried at room temperature before the RNA was fixed onto the filter by exposure to UV light for 5 mins. The RNA was deglyoxylated by baking the blot at 80°C for 2 hrs before hybridisation.

2.4.38 RNA hybridisation

Hybridisation was carried out as previously described for Southern blots.

2.4.39 Autoradiography of samples labelled with ³²P-dCTP

A sheet of Fuji X-ray film was placed over the filter to be autoradiographed in a tightly-closing cassette containing an intensifying screen (Genetic Research Instrumentation Ltd., Dunmow, Essex) and the exposure carried out at -70°C for the length of time required to give suitable band intensity. The use of an intensifying

screen amplifies the signal but also reduces the definition of bands. Therefore, if maximum band sharpness was required, exposure was carried out at room temperature.

The X-ray film was processed using LX 24 X-ray developer, FX-40 X-ray liquid fixer and HX-40 X-ray liquid hardener (all from Eastman Kodak Company, Rochester, New York, USA).

2.4.40 Mini polyacrylamide gel electrophoresis

Proteins were resolved by SDS-PAGE using a discontinuous buffer system according to the method of Laemmli (1970). Two clean glass plates were used to make a cassette by aligning spacers on three sides of the plates. Bulldog clips were used to clamp the mould. The mould was then sealed by pouring molten 1% agarose down three sides of the cassette. The separating gel was cast in the mould: 12.5% acrylamide (3% acrylamide [w/v], 0.8% [w/v] bisacrylamide), 375mM Tris-HCl, pH 8.8, 0.1% SDS, 0.025% APS and 0.002% TEMED. Butan-2-ol was pipetted onto the surface of the gel before it polymerised to ensure a level interface. The butan-2-ol was removed after the separating gel had polymerised. The stacking gel (5% acrylamide, 125mM Tris-HCl, pH 6.8, 0.1% SDS, 0.3% APS and 0.002% TEMED) was cast, the comb inserted and left for 15 mins until polymerisation was complete.

The mould was placed in the electrophoresis tank containing running buffer (250mM glycine, 25mM Tris and 0.15% SDS, pH 8.8). The comb was removed before the samples were loaded into the wells and electrophoresis was carried out at 275V for 45 mins. The gel was stained in 0.25% (w/v) Coomassie Blue R, 50% methanol and 10% glacial acetic acid for 10-15 mins followed by destaining using a solution of 10% glacial acetic acid and 20% methanol, until the background staining was removed.

2.4.41 Western blotting

a) Electroblothing

After separation of proteins by SDS-PAGE, a piece of nitrocellulose membrane was cut to the dimensions of the gel. The membrane was carefully laid on top of the gel, taking care to eliminate any air bubbles. The gel and membrane were sandwiched between 3MM paper and foam pads that had been pre-soaked in transfer buffer (250mM glycine, 25mM Tris, pH 8.8). The sandwich was placed in an electroblothing tank with the membrane oriented towards the cathode and transfer was carried out at 300mA for 45 minutes at 4°C. After electroblothing, the proteins were visualised and fixed to the membrane by staining with Ponceau S (supplied by BDH).

b) Semi-dry blotting

A Semi-dry Electroblotter (AutoGen Instruments UK Ltd) was a more effective method of blotting and used extensively. The acrylamide gel and nitrocellulose membrane were sandwiched between 3MM paper soaked in appropriate buffers. From anode to cathode: 2 layers of 3MM soaked in 300mM Tris, 20% methanol pH10.4; 1 layer soaked in 25mM Tris, 20% methanol pH10.4; nitrocellulose; gel; 3 layers soaked in 25mM Tris, 40mM 6-amino hexanoic acid, 20% methanol pH9.4. Blotting was carried out at 0.8-2mA constant current per cm² of gel area, for 45-90 mins. After electroblotting, the proteins were visualised and fixed to the membrane by staining with Ponceau S.

2.4.42 Immunodetection of proteins

Non-specific binding of IgG was reduced by incubating the blot in a blocking solution (TBS [0.15M NaCl, 0.01M Tris pH 7.4] containing 3% BSA and 1% caesin or 5% baby milk [Cow & Gate]) overnight at 4°C. After blocking the membrane was washed three times in TBS and then incubated for 3 hrs at room temperature, or overnight at 4°C, with appropriate primary antibody diluted in TBS. The blot was again washed several times with TBS followed by incubation for 1 hour with appropriate secondary antibody.

2.4.43 Developing reaction

The blot was washed several times for 5 minutes with TBS prior to being placed in developing solution. Western blots were developed using iLUmin8, which is based on the ECL system of Pharmacia. iLUmin8 was developed by Dr M. Murray, Leicester University.

2.4.44 Preparation of yeast cell extracts

a) Enzymatic breakage

20mls of yeast cells (OD₆₀₀~0.6) were harvested by centrifugation and resuspended in 1ml SCE (1.2M sorbitol, 0.1M sodium citrate pH 5.8, 0.01M EDTA) containing 0.01% β-Mercaptoethanol, 100µg/ml zymolyase. The cell suspension was incubated at 30°C for 30-60 mins in order to produce spheroplasts. The spheroplasts were washed several times with SCE, resuspended in SDS-PAGE loading buffer and boiled for 5 mins prior to running on a gel for subsequent protein analysis.

b) Physical breakage

Yeast cells were harvested by centrifugation and resuspended in SCE (1.2M sorbitol, 0.1M sodium citrate pH 5.8, 0.01M EDTA). The cells suspension was frozen at -20°C and the yeast 'ice' inserted into the loading chamber of a pre-chilled Hughes press

(see 2.4.31). After mechanically pressing the Hughes press was opened and the broken yeast cells removed from the collecting chamber.

2.4.45 Overlay assay

Nitrocellulose filters resulting from Western blotting or plaque/colony library screening were incubated as follows:

a) **blocking overlay solution** for greater than 1 hour at room temperature (RT).

b) wash 4 times with TBS for 5 minutes each at RT.

c) purified PKC isozymes ($\alpha, \beta, \gamma, \delta, \epsilon, \zeta$) and activators;

750 μ l	overlay buffer
100 μ l	PKC isozymes (1.5 μ M)
50 μ l	PS (5-20 μ g/ml), DG (0.8 μ g/ml)
100 μ l	Ca ²⁺ (1mM)

incubate at RT for 30-60 minutes

d) wash 4 times with overlay wash for 5 minutes each at RT

e) dilute primary antibodies (α, β, γ monoclonals diluted 1:1000, δ, ϵ, ζ polyclonals diluted 1:300) in TBS and 0.1% PEG, and incubate at RT for a minimum of 3 hours (antibodies supplied by Seikagaku Kogyo, Tokyo).

f) wash 4 times with TBS for 5 minutes each at RT.

g) incubate with alkaline phosphatase-conjugated antibodies in TBS and 0.1% PEG at RT for 60 minutes.

h) wash 4 times with TBS for 5 minutes each at RT.

i) incubate with **alkaline phosphatase substrate solution** to visualise alkaline phosphatase labelled proteins.

NB Visualisation of antibodies can be achieved with horseradish peroxidase linked secondaries in association with an ECL system, as well as other alternatives.

blocking overlay solution

3%	BSA
0.1%	PEG
0.2M	NaCl
50mM	Tris pH7.5

overlay buffer

0.1%	PEG
0.2M	NaCl
0.1mM	CaCl ₂
12mM	2-mercaptoethanol
50mM	Tris pH7.5

overlay wash

overlay buffer including:

0.1%	BSA
5mg/ml	leupeptin
10mg/ml	soybean trypsin inhibitor

alkaline phosphatase

substrate solution

7.5ml **AP buffer**
50µl NBT stock
50µl BCIP stock (25mg/ml)

AP buffer

100mM Tris-HCl pH9.5
100mM NaCl
5mM MgCl₂

NBT Nitro-Blue Tetrazolium

BCIP 5-Bromo-4-Chloro-3-Indolyl Phosphate

10X PBS:

NaCl 80g
KCl 2g
Na₂HPO₄ 14.4g
KH₂PO₄ 2.4g

10X TBS:

Tris-HCl pH 7.5 30.285g
NaCl 43.83
pH 7.2

quantities per litre

2.4.46 Overexpression of pDMR32/pGEX fusion

The protocols used for the overexpression and purification of the MBP and GST fusion proteins were essentially that recommended by the manufacturer (New England Biolabs and Pharmacia respectively). 400mls of culture were grown to late log phase (OD₆₀₀~0.7) and then induced for ~3 hrs with 1mM IPTG. Induced cells were pelleted at 5,000g and resuspended in ~15mls column buffer. Cells were frozen at -20°C overnight and then thawed at RT. Bacterial cells were broken by sonication (3-4 cycles of 30secs sonication and 30secs on ice). Sonicated cells were spun at 9,000g to pellet cell debris and the supernatant (pre-column) taken.

2.4.47 Purification of pDMR32/pGEX fusion

Amylose resin/glutathione sepharose was added to a 1cm x 10cm column to give a 2cm³ bed volume. The matrix was washed with at least 8 volumes of column buffer before the pre-column fusion-protein extract was added. The column had a flow rate of ~0.5mls/min. The column was washed again with at least 8 volumes of column buffer to remove any unbound material. Fusion proteins were eluted with 10mM maltose in column buffer (MBP fusions) or 10mM Glutathione, 50 mM Tris-HCl pH8.0 (GST fusion). Fractions of 300µl were collected and 10µl of each analysed by SDS-PAGE.

2.4.48 Overexpression of Rck2p in yeast (pEMBLyex4)

Expression of a recombinant protein from the vector pEMBLyex4 is induced by galactose-containing media and inhibited in glucose-containing media. Transformed

yeast are incubated at 30°C in glucose-containing media (2%) until log phase growth is achieved. Exact conditions are dependent on the construct used and should be assessed in a trial experiment first. Yeast cells are subsequently pelleted and washed in non-glucose media and then resuspended in galactose-containing media (2%). Galactose induction at 30°C occurs over an appropriate period of time depending on the construct used (3-12 hrs). Initial growth can be carried out in raffinose-containing media (2%), if glucose inhibition is unnecessary, in which case galactose can be added directly to the culture without washing of the cells.

2.4.49 Generation of monoclonal antibodies

a) Immunisation

Three female BALB/c mice were required for each fusion. Injection of the antigen (approximately 30-50µl per foot) into the footpads of the hindlegs occurs on days -17 (in complete Freud's adjuvant, FA), -14 (in incomplete FA), -10 (in PBS), -7, -4, and before the fusion (=day 0). Removal of the popliteal lymph nodes (under the 'knee') of all three mice is subsequently carried out.

b) Fusion

The cells from the lymph nodes are suspended in Hepes-buffered DMEM-medium by grating them between the frosted ends of microscope slides. Bigger pieces are removed and the cells pelleted and washed with Hepes-medium.

Myeloma cells, X63Ag8.658 (Kearney, et al., 1979), from 10-12 petri dishes were pelleted and washed in Hepes-medium (pellet should be larger than that of the lymphocytes). Both pellets were resuspended, mixed and pelleted together. 1ml PEG 4000 (1g/ml in Hepes-medium, adjust to pH7.4 with NaOH and sterile filter) was slowly (over about 1 minute) added to the pellet while the tube was slightly shaken. After incubation in a 37°C waterbath for about 1 minute, 20 ml of hepes-medium was added to dilute the PEG (1ml in 30 secs, 3ml in 30 secs, 16ml in 60 secs). The tube was left in the warm water bath for 5 mins, cells were pelleted, resuspended in selective medium (Optimem+AH [azaserine/hypoxanthine]; GibcoBRL) and plated onto fifteen 24-well plates (per fusion) with macrophages as feeder cells.

c) Selection

Even after an efficient fusion only approximately 1% of the starting cells are fused. Although the cells from the lymph nodes do not grow in tissue culture, the unfused myeloma cells must be eliminated. Consequently, the myeloma cells used have a mutation in one of the enzymes required for the salvage pathway of purine biosynthesis. For the X63-Ag8.653 cell line the mutation is in the hypoxanthine-guanine phosphoribosyl transferase gene (HPRT). As the salvage pathway for

purine biosynthesis is therefore blocked, myeloma cells must rely on the *de novo* pathway for synthesis. After the fusion of myeloma and lymph cells the culture medium is supplemented with azaserine which blocks *de novo* synthesis of purines by covalently binding to glutamine phosphoribosyl amidotransferase and phosphoribosyl glycinamidine synthase. As both the pathways necessary for purine synthesis are now blocked, unfused myeloma cells die. Fused myeloma cells, however, possessing a functional HPRT gene from the lymph cells, can survive using the salvage pathway providing a substrate for purine synthesis is provided (hypoxanthine).

d) Harvesting

After the fusion cells have been cultured the hybridomas were examined every 1-2 days to check on the progress of their growth. The cells grew faster on the periphery of the wells so the whole plate had to be examined. If the cells were large enough then 1ml of supernatant from each well was taken under sterile conditions and tested by ELISA and Western blot. Positive clones were then harvested and frozen. Hybridoma cells were pipetted into a 10ml sterilin; the last 3-4mls were used to resuspend the cells which adhere to the growth plate. Care was taken to avoid pipetting bubbles into the sterilin. A few cells were left on the plate and 5mls of fresh growth media added to allow continued growth.

e) Subcloning

The day prior to subcloning two or three 96-well microtitre plates were prepared for each clone of interest. Macrophage cells recovered from one mouse were taken up in a small volume (about 0.5ml) of OPTIMEM (GibcoBRL) cooled to 4°C, and then resuspended in 50mls of cold media. 100µl was pipetted into each well of the three ELISA plates and the macrophage cells were incubated overnight at 37°C. The following day the microtitre plates were checked microscopically for contamination.

The plates of clones that were harvested the day before were also checked for contamination. The plates should be viewed through a microscope when checking for contamination and it is also essential that a high proportion of cells are viable. If a sufficiently high proportion of cells were viable then the cells were resuspended and transferred into a 10ml sterilin. As before a few cells were left behind and 5ml AH supplemented growth media was added and the plate returned to the incubator. The harvested cells were serially diluted before being pipetted onto the microtitre plates. The number of cells per ml was established microscopically by counting them in a counting chamber. 100µl of appropriate dilutions are added to each well. If two ELISA plates were used for each dilution, 100, 20 and 5 cells/well dilutions (4 rows of each) occupied one plate, while 6 rows of 1 and 0.5 cells/well dilutions occupied the second ELISA plate. The cells were checked for contamination every 1-

2 days and wells containing single colonies noted. After about 1 week the supernatants can be tested by ELISA. This was done in duplicate with 50µl of supernatant from each well. If a well showed a positive response in the ELISA the clone was picked and grown up on 24-well plates-prepared the day before with macrophage. After a few days the supernatants were tested by Western blot.

CHAPTER 3.

CLONING AND ANALYSIS OF RACK1 [work carried out in collaboration with Dr D. Mochly-Rosen and published (Ron, et al., 1994)]

3.1 Introduction

Previous research by Dr Mochly-Rosen's group identified and purified proteins of ~30kDa which bound activated PKC (see introduction). These findings initiated the attempt to clone and characterise the respective genes. As antibodies to PKC-binding proteins were unavailable, the direct screening of an expression library was not possible. Instead, an indirect method of detecting these proteins was employed, namely; the overlay assay (see section 2.4.45). The rationale behind this assay was to identify clones from an expression library which expressed polypeptides that could bind exogenous PKC in the presence of PKC activators (see figure 1.5).

3.2 Isolation Of cDNA Encoding A PKC-Binding Protein

An expression library of rat brain cDNA was constructed in the λ phage cloning vector Uni-Zap XR (Stratagene). Approximately 2.4×10^6 recombinants were screened using an overlay assay based on that described by Wolf (Wolf and Sahyoun, 1986; see figure 1.5). The expression of recombinant phage proteins was induced by IPTG and the proteins transferred to nitrocellulose filters. The filters were processed with suitable buffers (see section 2.4.45) prior to their incubation with a mixture of PKC isozymes (including α , β , γ , δ , ϵ , and ζ) in the presence of phosphatidylserine, diacylglycerol, and calcium. After subsequent washing, plaques containing proteins bound to PKC were detected using anti-PKC antibodies. From 35 initial recombinant proteins, only one required exogenous PKC for its detection; the remainder were detected with anti-PKC antibodies without the presence of PKC. The corresponding 34 recombinant phage were presumed to express PKC polypeptides, while the remaining single phage was thought to express a putative RACK. The phage carrying the putative RACK encoding sequence was purified and its cDNA insert isolated as phagemids by *in vivo* excision of the cloning vector (Stratagene).

3.3 Analysis Of The Putative RACK1 Gene

The putative RACK1 encoding DNA was sequenced entirely by D. Ron using 11 synthetic oligonucleotides. Analysis of the sequence revealed a large open reading frame (951bp) which was compared to sequence databases (see figure 1.6). It was found that the predicted polypeptide sequence was 100% identical to the chicken (C12.3) and human (H12.3) genes, previously identified as homologues of the β subunit of G proteins (Ron, et al., 1994).

In order to analyse the biochemical properties of RACK1, the open reading frame of the gene was cloned into the pMAL expression vector (New England Biolabs; see table 2.4). This expression vector produces a RACK1 polypeptide which is fused at its amino terminus to the maltose-binding protein (for details see section 6.3). This fusion was subsequently expressed in an *E. coli* host and purified from the bacterial extract using affinity chromatography on amylose resin (see section 2.4.47). Protease digestion of the fusion protein with Factor Xa (Stratagene) released the RACK1 polypeptide from the maltose-binding moiety, and made the subsequent investigation of the biochemical properties of RACK1 possible.

The recombinant RACK1 bound PKC in the overlay assay and its binding was maximal in the presence of PKC activators. Of all the PKC isozymes tested, RACK1 bound PKC β preferentially and this binding was found to be concentration-dependent. RACK1 was a poor substrate for PKC phosphorylation indicating that the RACK1-PKC interaction was not simply that of an enzyme and its substrate. Furthermore, peptides homologous to the pseudosubstrate site of PKC, or to a substrate consensus sequence, failed to inhibit the interaction of PKC with RACK1. Thus, the regions of PKC involved in RACK1 interaction are evidently distinct from those required for binding a substrate. Finally, it was shown that RACK1 interacts specifically with PKC, and shows no binding affinity to either the calcium/calmodulin-dependent kinase or the RII α subunit of cAMP-dependent protein kinase. These results correlated well with the data obtained previously for biochemically purified RACKs (Mochly-Rosen, et al., 1991a). As the RACK1 protein met all the criteria defined for a PKC receptor, its respective gene was considered to be the first encoding a genuine receptor of activated C kinase (Ron, et al., 1994).

Further experimentation identified sequences in RACK1 which were necessary for binding to PKC. Peptides designed against these sequences interfere with the normal RACK1 association with PKC both *in vitro* and *in vivo* (see introduction). Furthermore, sequences found in RACK1 are also present in PKC isozymes. These pseudo-RACK sequences are thought to maintain the PKC enzyme in a 'closed', inactive conformation similar to the mechanism of regulation described for the pseudosubstrate domain (see figure 1.8).

3.4 RACK1 Hybridisation To Yeast Genomic DNA

The identification and cloning of RACK1 from rat brain initiated the investigation for homologous sequences in yeast. This was particularly interesting due to the previously characterised *PKC1* gene isolated in *Saccharomyces cerevisiae* (see introduction). The observation that RACK1 showed greater than 90% identity to the 12.3 chicken and human genes, suggested that, at least in higher eukaryotes, the sequence was very well conserved (Ron, et al., 1994). The complete RACK1 DNA was radioactively labelled and used as a probe in Southern blot analysis against

Saccharomyces cerevisiae genomic DNA digested with several restriction enzymes. Low stringency hybridisation conditions (see section 2.4.36) were used to accommodate sequence differences between organisms due to evolution and codon usage. The RACK1 probe weakly hybridised to rDNA fragments, due to high localised DNA concentrations, but did not hybridise to other genomic DNA (data not shown). Attempts to screen a yeast genomic library with the probe also failed to detect positively reacting sequences. These results suggested that a homologue of mammalian RACK1 did not exist in yeast.

3.5 IDENTIFICATION AND ANALYSIS OF *y*RACK1 (CDYS:M_F190)

The completion of the Yeast Genome Sequencing Project provided a unique opportunity for finding yeast homologues of previously characterised genes. Comparing the sequence of the RACK1 protein with the completed yeast genome data identified significant identity (67% similarity) to the putative product of the ORF designated CDYS:M_F190 (figure 3.1). This high degree of similarity contrasts with the lack of hybridisation during the Southern analysis described above. The DNA sequence displays an overall identity of 52% which includes a short region of ~30bp, around the middle of the gene, with 90% identity. It is intriguing, therefore, that this yeast sequence did not hybridise to the radioactively labelled RACK1 DNA. (Further analysis of *y*RACK1 is described in chapter 6).

3.6 Discussion

The cloning and analysis of the mammalian RACK1 gene completed the preliminary research carried out on purified proteins, and proved that PKC receptors do exist. This data confirmed the speculation surrounding the translocation of PKC and provided indications for the mechanism(s) of PKC regulation. The cloning of the RACK1 gene provided a powerful tool for further research which has since uncovered some interesting findings (see introduction).

The use of RACK1 as a probe against yeast genomic DNA was an obvious method for obtaining a yeast homologue of the mammalian gene. Unfortunately, the RACK1 gene failed to hybridise to any yeast genomic DNA with significant specificity suggesting the absence of a yeast homologue to this gene. It did not preclude the possibility that a functional homologue existed, however.

The completed Yeast Genome Sequencing Project identified a sequence which displays high homology to RACK1 at both the DNA and protein levels. The function of this gene is unknown and no information regarding its product has been published. Since this gene could be the yeast RACK1 homologue, a preliminary characterisation of its product was initiated (see chapter 6).

rack1 x yrack (nucleotide sequence)

Gap Weight:	5.000	Average Match:	1.000
Length Weight:	0.300	Average Mismatch:	0.000
Quality:	274.6	Length:	972
Ratio:	0.479	Gaps:	2
Percent Similarity:	52.252	Percent Identity:	52.252

```

1 .....atgaccgagcaaatgacccttcgtgggaccctcaagggccataa 44
1 ATGGCATCTAACGAAGTTTGTAGTTTGTAGAGGTACCTTGGAAAGGTCACAA 50
45 tggatgggttacacagatcgccaccactccgcagttcccgacatgatcc 94
51 CGGTTGGGTCACATCTTGGCTACTTCTGCTGGTCAACCAACCTATTGT 100
95 tgtcggcgctctcgagacaagaccatcatcatgtggaagctgaccagggat 144
101 TGTCCGCTTCCCGTGATAAGACTTTGATCTCCTGGAAGTTGACTGGTGAC 150
145 gagaccaactacggcataccacaacgtgctcttcgaggtcactcccactt 194
151 GACCAAAAGTTTGGTGTCCAGTTAGATCTTCAAGGGTCACAGTCACAT 200
195 tgtagcgatgttgatcatctcctctgatggccagtttgccctctcaggct 244
201 TGTCCAAGACTGTACTTTGACTGCTGACGGTGCCTTACGCTTGTCTGCTT 250
245 cctgggatggaaccctacgcctctgggatctcacaacgggcactaccacg 294
251 CTGGGACAAGACCTTGAGATTATGGGATGTTGCCACCGGTGAAACCTAC 300
295 agacgatttgctcgccacaccaaaggatgtgctgagcgtggctttctctc 344
301 CAAAGATTTCGTCGGTCACAAGTCCGATGTTATGTCCGTTGACATTGACAA 350
345 tgacaaccggcagattgtctctgggtcccgagacaagaccattaagttat 394
351 GAAGGCTTCATGATTATCTCTGGTTCCCGTGACAAGACCATCAAGGTCT 400

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395 ggaatactctgggtgtctgcaagtacactgtccaggatgagagtcattca 444
401 GGACCATCAAAGGTCAATGTTTGGCCACTTTGTTGG.....GTCACAAT 444
445 gaatgggtgtcttgtgtccgcttctccccgaacagca.....g 482
445 GACTGGGTTTCCCAAGTCAGAGTTGTTCCAAACGAAAAAGCTGATGATGA 494
483 caaccctatcatcgctctcctgcggatgggacaagctgggtcaaggtgtgga 532
495 CTCTGTCAACCATCATTTCTGCCGGTAACGACAAAATGGTTAAGGTATGTG 544
533 atctggctaactgcaagctaagaccaaccacattggccacactggctat 582
545 ATATATTTTCTTCCATGATAGAATATGA..... 573

```

rack1 x yrack (amino acid sequence)

Gap Weight:	3.000	Average Match:	0.540
Length Weight:	0.100	Average Mismatch:	-0.396
Quality:	164.6	Length:	323
Ratio:	0.862	Gaps:	2
Percent Similarity:	67.027	Percent Identity:	51.351

```

1 ..mteqmtlrgtlkghngwvtqiattppqfpdmilsasrdktiimwkltrd 48
1 MASNEVLVLRGTLEGHNGWVTSLATSAAGQPNLLLSASRDKTLISWKLTD 50
49 etnygipqralrghshfvsdvvisdggqfalsgswdgtlrlwdlttggtt 98
51 DQKFGVPVRSFKGHSHIVQDCTLTADGAYALSASWDKTLRLWDVATGETY 100
99 rrfvghtkdvlsavfssdnrqivsgsrdktiklwntlgvckytvgdeshs 148
101 QRFVGHKSDVMSVDIDKKASMIISGSRDKTIKVWTIKGQCLATLLG..HN 148
149 ewvscvrfspn....ssnpiivscgwdklvkvnlancklktnhightgy 194
149 DWVSQVRVVPNEKADDSVTIISAGNDKMKVCDIFSFDRI*..... 191

```

Figure 3.1
Sequence homology between *RACK1* and *yRACK*. Both the nucleotide and amino acid sequences were compared using the Gap programme of the GCG computer package. *yRACK* (uppercase) showed 52% and 67% homology to *RACK1* (lowercase) nucleotide and amino sequences respectively.

CHAPTER 4.

ISOLATION OF GENES ENCODING PUTATIVE YEAST RACKs

4.1 Introduction

Genetical and biochemical evidence suggested that yeast has a gene(s) which is significantly homologous to mammalian PKC genes (Antonsson, et al., 1994, Watanabe, et al., 1994). Furthermore, yeast exhibits PKC activity that is typical of several distinct PKC isozymes (Ogita, et al., 1990, Simon, et al., 1991a). As yeast cells display homology to mammalian cells with regard to PKC, it was reasonable to hypothesise that the homology could extend to RACKs. Initial analysis of *Saccharomyces cerevisiae* cell extract using the overlay assay indeed suggested the existence of yeast proteins which bound activated PKC isozymes (E. Orr and D. Mochly-Rosen, unpublished data). This observation called for an investigation into the properties of the putative yeast RACKs. Although the attempt to obtain a yeast RACK1 homologue, through DNA hybridisation with genomic DNA, was unsuccessful (see chapter 3), the identification of functional RACK1 homologues, using the overlay assay, was attempted.

4.2 Screening A Commercial Yeast Expression Library

The yeast genome is made up of 16 linear chromosomes containing approximately 14Mb of DNA, of which approximately 12Mb is present as single-copy DNA and 1-2Mb is rDNA (Olson, 1991). Extrachromosomal DNA is represented by 50-100 copies of the 6.3kb 2 μ plasmid and 20-40 copies of the circular 75-80kb mitochondrial DNA. As the yeast genome is small with very little 'spacer' DNA between genes, it is an attractive organism from which to engineer a genomic library.

A commercial expression library (Clontec) was used in the screening for putative RACK-expressing clones. This library was prepared by shearing yeast genomic DNA and ligating the fragments (average size of ~1.8kb) into a λ gt11 phage vector using *Eco*RI linkers (figure 4.1). This strategy should produce a selection of random genomic fragments allowing expression of all possible open reading frames.

The library was screened using the overlay assay modified from that developed by Wolf (Wolf, et al., 1985). This assay was based on the identification of putative RACK-encoding sequences due to the binding of their products to exogenous PKC in the presence of PKC activators (see figure 1.5). In the course of this screening, additional clones expressing putative PKC were also isolated. These clones, unlike those expressing RACKs, were recognised by anti-PKC antibodies without the addition of exogenous PKC (see figure 1.5).

Approximately 1×10^5 plaques, representing sequences equivalent to 10 yeast genomes, were screened. The screen identified 5 putative yeast RACK clones and 16

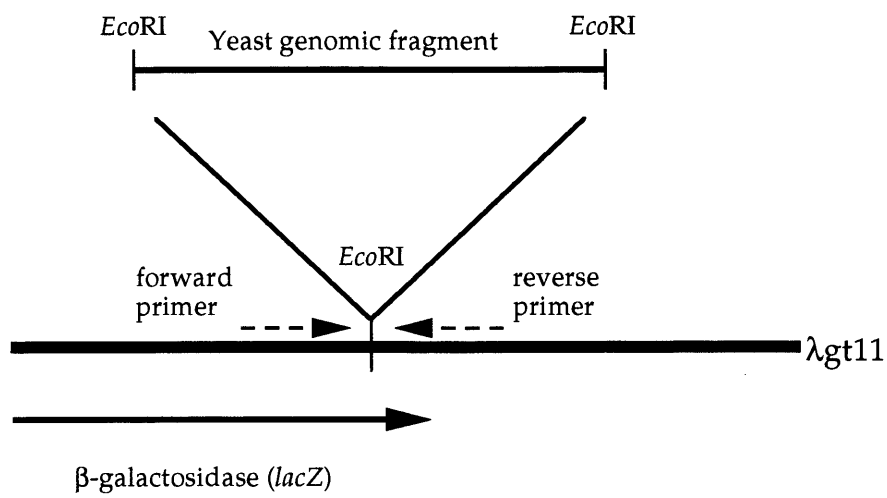


Figure 4.1

λ gt11 phage yeast genomic library (43,700bp). Sheared fragment of *Saccharomyces cerevisiae* were ligated into the unique *EcoRI* site of λ gt11 using *EcoRI* linkers. The average fragment size was 1.8kb, ranging in size between 0.8-5kb (Clontec). IPTG induction allowed translation of an ORF present within the inserted fragment as a fusion with β -galactosidase. λ gt11 primers were available for PCR amplification of the insert (see text for details).

putative PKC clones (figure 4.2). Interesting clones were rescreened several times to confirm their authenticity and to obtain pure stocks of recombinant phage .

4.3 IPTG Dependency Of Putative Rack Expression

The λ gt11 expression library is based on the ability of an inserted DNA sequence to be transcribed and translated as part of the *lacZ* gene, present in the phage (Young and Davis, 1985). This fusion protein is overexpressed by the addition of IPTG which induces the *lacZ*-directed expression. A screen to examine the requirement of IPTG for the expression of the 5 RACK recombinant proteins was subsequently carried out. Although the results from this experiment were not clear, it appeared that 3 phage, λ 3.3, λ 3.1, and λ 7.1 (see table 2.3), continued to exert their RACK activity in the absence of IPTG (data not shown). This IPTG-independent transcription/translation suggested the presence of sequences within the cloned fragments able to act as a yeast promoter within the bacterial system. The remaining 2 recombinant phage, λ 1.2 and λ 7.2 (see table 2.3), appeared to lose their ability to bind activated PKC in the absence of *lacZ* induction (see later).

4.4 Specificity Of The Recombinant Yeast RACK For PKC Isozymes

Proteins expressed by λ 3.3, λ 3.1, λ 1.2 and λ 7.2 were analysed for their binding preferences toward individual mammalian PKC isozymes. In the original screen, a mixture of PKC isozymes was added to the nitrocellulose filters containing the expressed proteins. The results of this screen showed that all four putative RACKs behaved identically. They appeared to bind δ -PKC very strongly and to a lesser extent the β - and ϵ -isozymes. α - γ - and ζ -PKCs did not appear to bind at all (data not shown).

4.5 The Five Recombinant Phage Inserts Represent Two Genomic Fragments

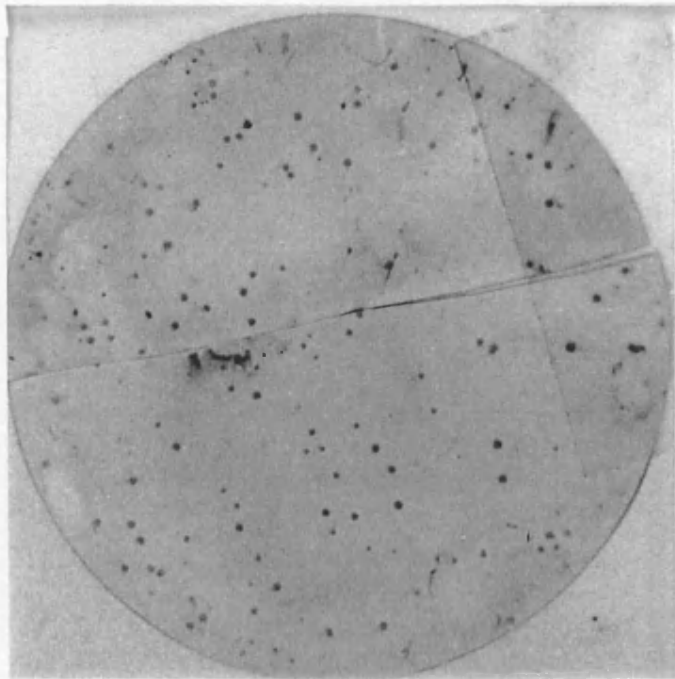
Inserts from two of the five putative RACK recombinant phage, λ 3.3 and λ 1.2, were radioactively labelled with ^{32}P and used in hybridisation studies. They were used to probe the DNAs of the all 5 putative phage transferred to nitrocellulose membranes. The labelled 3.3 probe strongly hybridised to DNA from phage λ 3.3, as anticipated, as well as to λ 3.1 and λ 7.1. The 1.2 probe hybridised strongly only to DNA prepared from phage λ 1.2 and λ 7.2.

PCR analysis of all 5 inserts, using primers designed to λ gt11 sequences flanking the inserts (see table 2.6), suggested that phage λ 3.1, λ 3.3, and λ 7.1 contain inserts of approximately 800bp. PCR analysis also indicated that the inserts of both λ 1.2 and λ 7.2 were approximately 1.3kb in size (figure 4.3).

The data obtained from both hybridisations and PCR analyses strongly suggested that the inserts present in phage λ 3.1, λ 3.3, and λ 7.1 were identical, as were those present in phage λ 1.2 and λ 7.2.

Figure 4.2

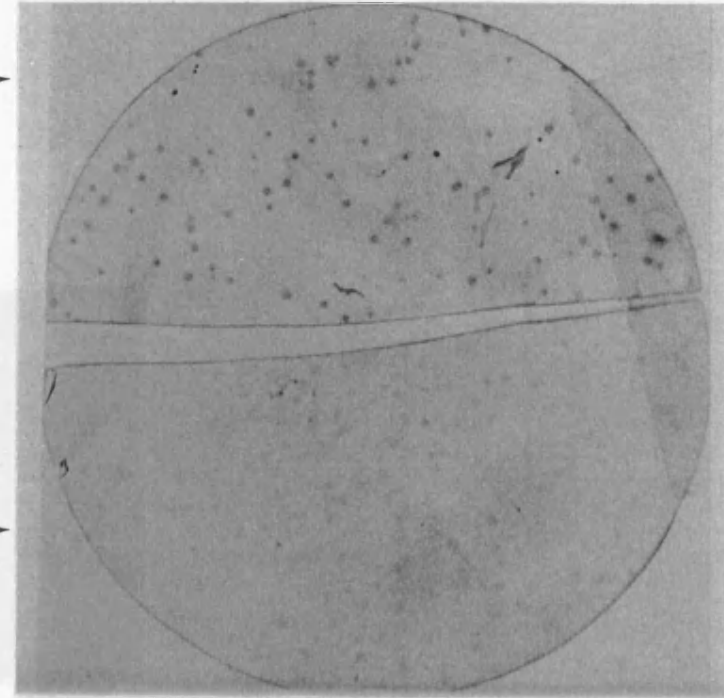
Nitrocellulose filters resulting from an overlay assay of a λ gt11 expression library. Nitrocellulose filters soaked in IPTG (to induce protein expression) were placed on plates containing plaques of λ gt11 clones. Incubation of the filters allowed expressed polypeptides to be transferred from the plaques onto the nitrocellulose. Filters were subsequently removed and treated with appropriate overlay assay buffers (see materials and methods). Filters were halved and each half treated separately. One half was incubated with exogenous PKC and PKC activators, while the other half was untreated. The filters were then washed thoroughly, and PKC present on the filters was detected with anti-PKC antibodies. Recombinant proteins recognised by the anti-PKC antibodies in the **absence** of exogenous PKC were indicative of PKC-like proteins. Recombinant proteins recognised by the anti-PKC antibodies only in the **presence** of exogenous PKC were indicative of RACK proteins. (See text for details)



Nitrocellulose filter resulting from the overlay assay of a putative PKC-expressing clone.

Half of the filter was treated with exogenous PKC in the presence of PKC activators

Half of the filter was untreated with exogenous PKC



Nitrocellulose filter resulting from the overlay assay of a putative RACK-expressing clone.

4.6 Sequence Analysis Of Plasmid p3.3

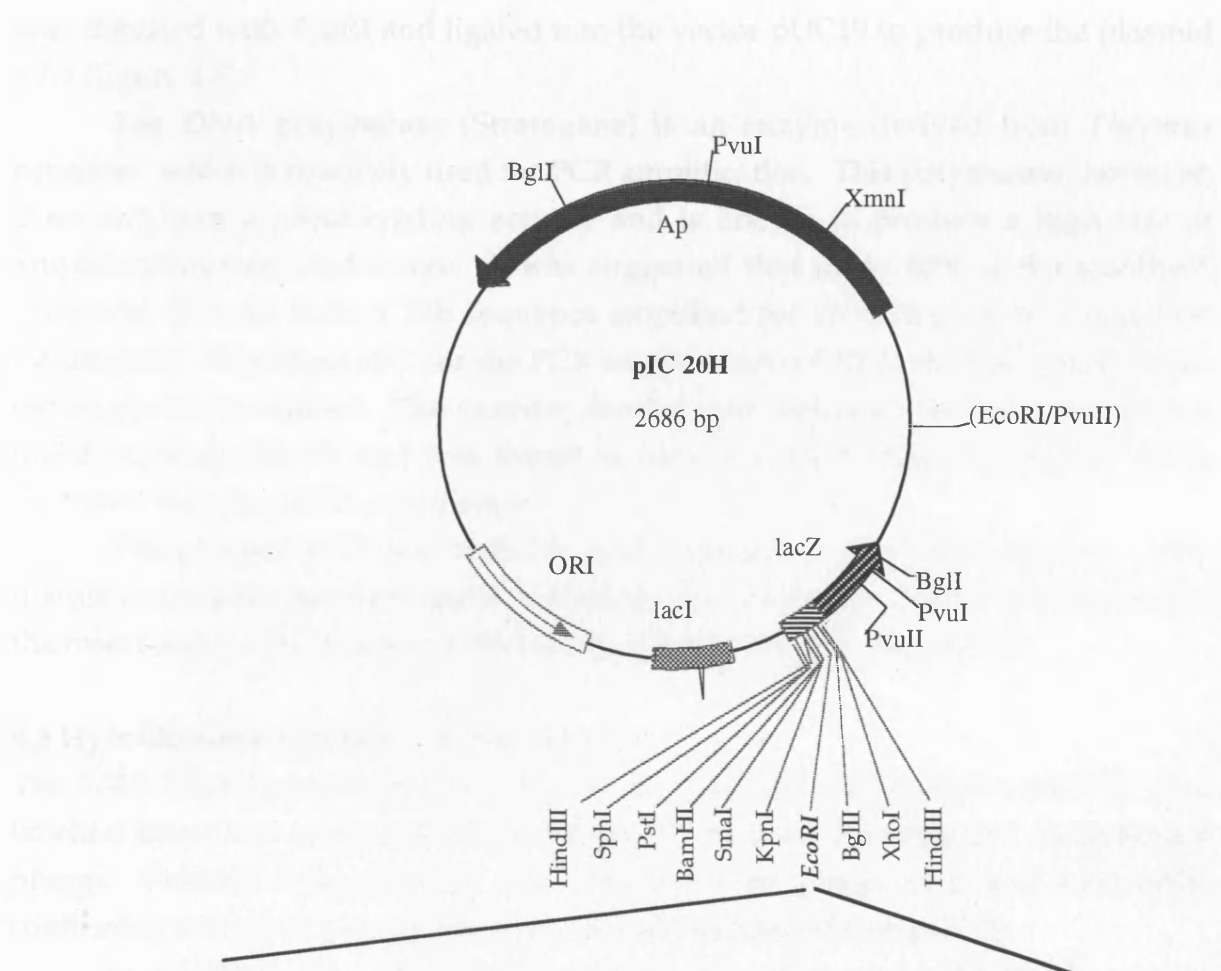
The insert from one of the 5 putative recombinant RACK phage, λ 3.3, was purified and subcloned into the vector pIC 20-H (see table 2.4) using the *EcoRI* sites (see figure 4.4) to produce the plasmid p3.3 (see table 2.4). *EcoRI* digestion of λ 3.1 DNA was used to subclone its insert into the vector pUC18 (see table 2.4) to produce the plasmid p3.1 (see table 2.4). The termini of the insert 3.1 were sequenced using pUC-based primers (see table 2.6). This partial DNA sequence was compared to the complete sequence of insert 3.3 (see paragraph below) and confirmed that they were identical. Sequence analysis of 7.1 was not considered to be necessary to confirm its identity to 3.1 and 3.3 fragments.

The entire 3.3 insert was sequenced using pUC-based primers (see table 2.6) which anneal to sequences flanking the polylinker of the vector. Initial sequencing of the insert termini identified a unique *XhoI* site 218bp downstream of the 5' end of the fragment (figure 4.4). Using this site, and the unique *XhoI* site in the polylinker of p3.3 (figure 4.4), the 5' portion of the previously sequenced DNA could be removed to allow the remaining fragment to be sequenced accurately without the requirement of additional primers.

Sequencing data demonstrated that the fragment was 736bp in length (figure 4.4). Database comparison showed that 362bp of this sequence represents a gene encoding the transfer RNA for isoleucine (tRNA_{ile}) (Ogden, et al., 1984). The remaining sequence displays an open reading frame of 78 amino acids beginning at an ATG (502bp) and continuing towards the 3' end of the fragment. 'TATA' and 'CAAT' boxes, commonly recognised in yeast promoters (Guarente, 1992), were identified 107bp and 140bp upstream of the ATG, respectively (figure 4.4). An adenine residue was found 3bp upstream of the initiation codon in common with other yeast promoters (Guarente, 1992). This open reading frame was predicted to encode a polypeptide of approximately 7kDa and was presumed to be responsible for the observed RACK activity. The protein sequence, when compared to the computer database, was later found to be identical to Gcd6p which was characterised as a gene encoding a protein necessary for the nitrogen starvation response (Bushman, et al., 1993). (The full characterisation of the gene was carried out in the laboratory by W. Richards).

4.7 Restriction Analysis Of Plasmid p7.2

A common problem associated with λ gt11 phage is the inability to digest efficiently its DNA following standard purification protocols (Murray, et al., 1993). Such difficulties were encountered when attempting to subclone the 1.3kb *EcoRI* insert of λ 7.2. So as to circumvent this complication, the 1.3kb insert was amplified using PCR with commercially available λ gt11 primers (see table 2.6). The PCR product



<u>EcoRI</u>					
1	gaattctcag	aaagagctat	tttttttttt	aataaaaatta	ttagtactaa
51	ttccagtata	aataacaatg	ttattttacgt	ctcttttcgaa	aatgctcgtg
101	tagctcagtg	gttagagctt	cgtgcttata	gcaacattcg	gtttccgaag
151	tttctgtgcc	aaagaccttt	caaacaggcc	tttaaaagca	acgcgaccgt
201	cgtgggttca	aacccacCT	CGAGcacttt	ctcttttttt	ttcacacgcg
<u>XhoI</u>					
251	tttgcaacat	gcccgattta	catgagctta	tttgacttta	gatggttttc
301	agttcttttt	tttttttttt	tttttttcag	tagattaatg	cgaagagtta
351	aatca caat	tcaaagatgg	cagatggacc	accttggtga	taag tataaa
401	aataactaaa	agtgcgtctt	gtgttaaaaa	tagaatagaa	accactcata
451	aggcaaaaga	tggctggaaa	aaagggacaa	aagaaagtgg	actaggcaac
501	cATGgaaaga	actctgatat	ggatgttgaa	gatcgtctcc	aggccgttgt
551	cttgacagac	tcttatgaaa	ctaggtttat	gccactgaca	gctgtcaagc
601	caagggtgtt	gctgccactg	gctaacgtac	ctctcattga	atacacctta
651	gaatttttgg	ctaaggctgg	cgtacatgaa	gttttcttaa	tttgctcttc
701	tcattgccaac	caaattaatg	actatattga	gaattc	736
<u>EcoRI</u>					

Figure 4.4

Construction and sequence of p3.3. Using universal pUC primers (table 2.6), the subcloned insert of clone λ 3.3 was sequenced directly from the pIC 20H vector. The 736bp insert has two open reading frames. The underlined sequence shows the tRNA isoleucine precursor gene (42bp-233bp). The predicted RACK-encoding open reading frame is also shown (502bp-736bp). Promoter elements are also shown; **caat** (356bp) and **tataa** (395bp).

was digested with *EcoRI* and ligated into the vector pUC19 to produce the plasmid p7.2 (figure 4.5).

Taq DNA polymerase (Stratagene) is an enzyme derived from *Thermus aquaticus* which is routinely used for PCR amplification. This polymerase, however, does not have a proof-reading activity and is known to produce a high rate of amplification-mediated errors. It was suggested that up to 40% of the amplified products, derived from a 1kb sequence amplified for 20 cycles, carry a mutation (Stratagene). Consequently, for the PCR amplification of λ 7.2, *Pfu* DNA polymerase (Stratagene) was utilised. This enzyme, derived from *Pyrococcus furiosus*, does have a proof-reading activity and was found to have a 12-fold higher fidelity of DNA synthesis than *Taq* DNA polymerase.

The plasmid p7.2 (see table 2.4) was subjected to restriction analysis. Two unique restriction sites were found: a *KpnI* site, approximately 200bp from one end of the insert and a *HindIII* site a further 200bp downstream (see figure 4.5).

4.8 Hybridisation Analysis Of Plasmid p7.2

The 1.3kb insert present in plasmid p7.2 was radioactively labelled with ^{32}P . The labelled insert was used to hybridise phage DNAs from the original 5 recombinant phage. Positive hybridisation was detected with phage λ 1.2 and λ 7.2 only, confirming that the 7.2 insert had been correctly subcloned into pUC19.

In addition, the radioactively labelled 7.2 insert was hybridised to yeast genomic DNA digested with several restriction enzymes (figure 4.6). The probe hybridised to two *KpnI* fragments (~7kb and ~10kb), two *StyI* fragments (~0.6kb and ~2kb) and two *HindIII* fragments (~4.0kb and ~6.5kb), suggesting the presence of these sites within the 1.3kb insert (figure 4.7b). The single genomic *EcoRI* fragment of approximately 1.3kb was also recognised by the probe confirming the genomic origin of this insert.

4.9 Sequence Analysis Of Plasmid p7.2

Two subclones, originating from insert 7.2, were constructed, making use of the *HindIII* site present approximately 400bp from one end of the insert (figure 4.7a). The plasmid p7.2 was simultaneously digested with *EcoRI* and *HindIII* to obtain two fragments of approximately 400bp and 900bp. Each of the fragments were ligated into the vector pUC19 (see table 2.4) to yield the subclones pEH400 and pEH900 (see table 2.4), respectively (figure 4.7c).

Both inserts were initially sequenced with universal pUC primers (see table 2.6). Synthetic primers were later designed, using the derived sequence, to complete the analysis. Sequence data demonstrated that the ~1.3kb *EcoRI* fragment is in fact 1166bp in length and that the *HindIII* digest generated 380bp (pEH400) and 786bp (pEH900) fragments (figure 4.7c). DNA sequence analysis identified a large open

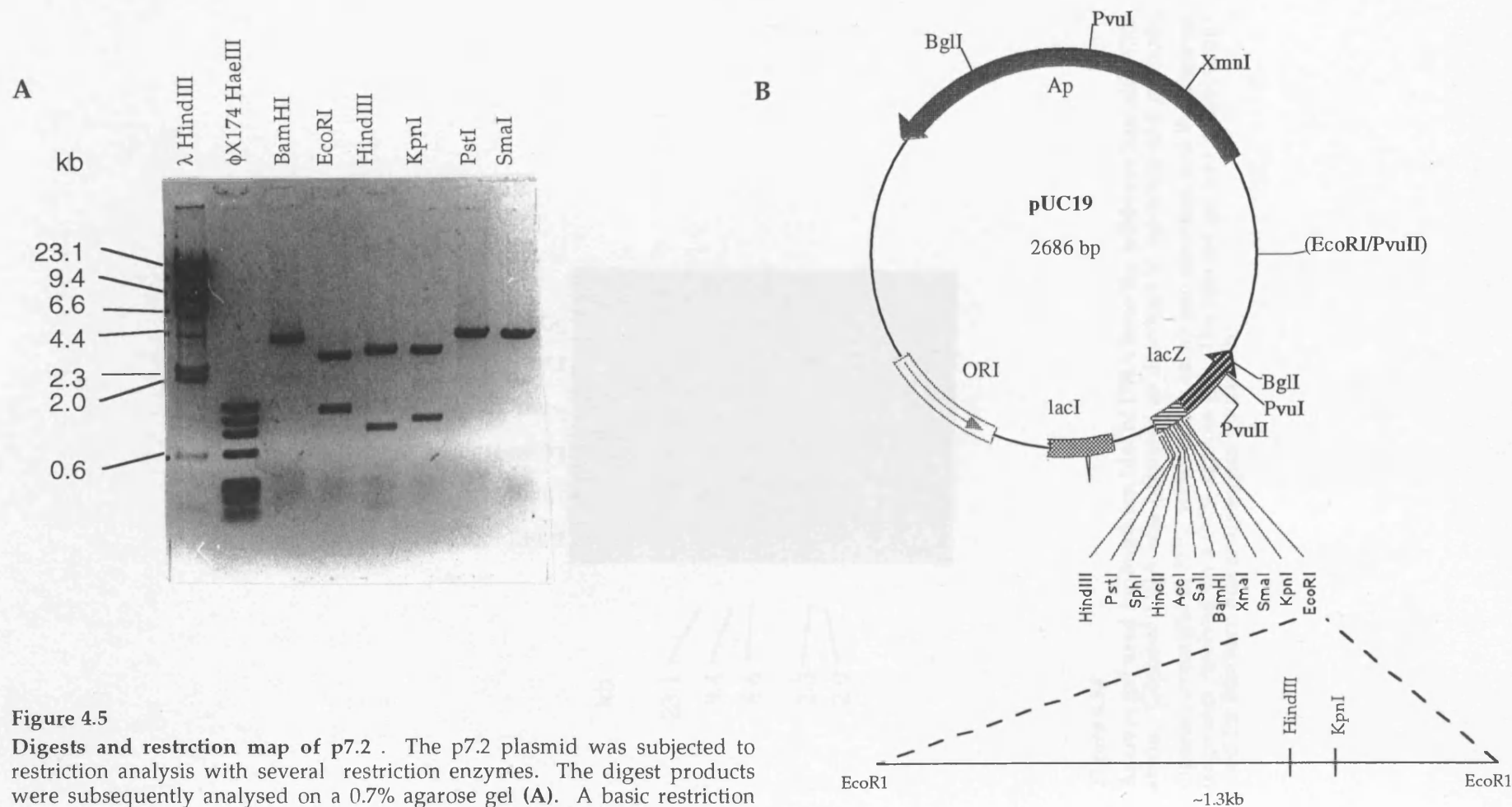


Figure 4.5

Digests and restriction map of p7.2. The p7.2 plasmid was subjected to restriction analysis with several restriction enzymes. The digest products were subsequently analysed on a 0.7% agarose gel (**A**). A basic restriction map was derived from the digestion analysis (**B**). The ~1.3kb insert was found to contain single sites for both *Hind*III and *Kpn*I restriction enzymes.

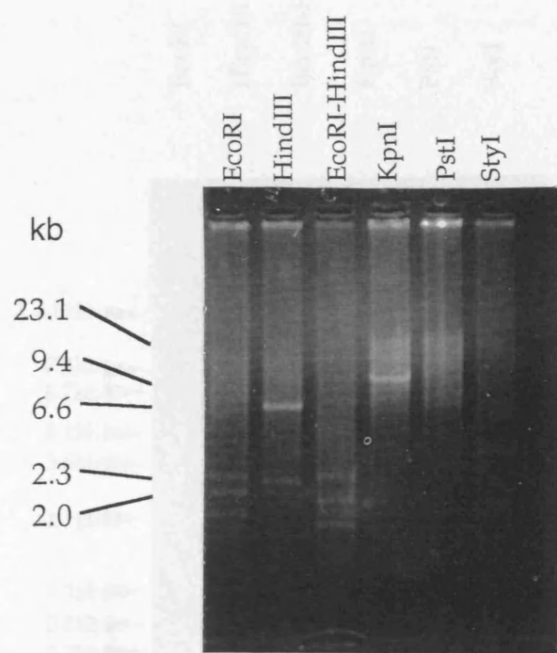


Figure 4.6a

Agarose gel used for Southern blot of DNA from the wild-type haploid W303 strain. Genomic DNA was prepared as described in materials and methods, digested overnight at 37°C with various restriction enzymes, and the digested fragments resolved on a 0.7% agarose gel. The agarose gel was subsequently used for Southern blot analysis (see figure 4.6b)

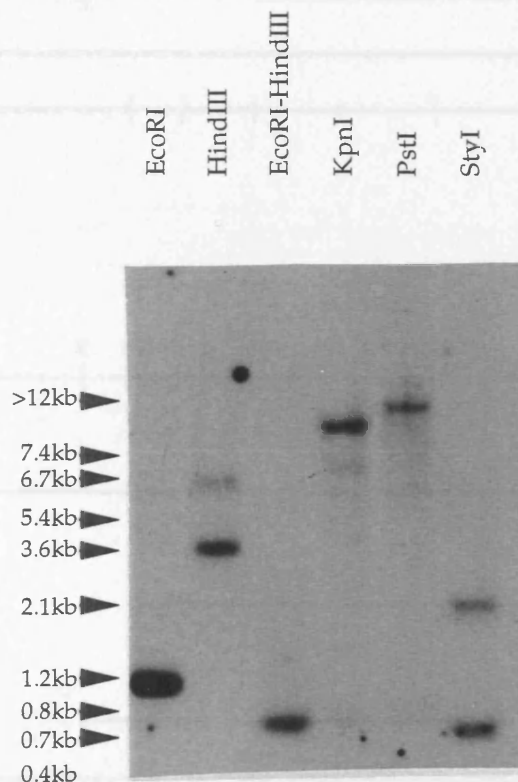


Figure 4.6b

Autoradiograph of a Southern blot of DNA extracted from the wild-type haploid W303 strain. Genomic DNA was prepared, digested with various restriction enzymes, and used for Southern blot analysis (see figure 4.6a). The labelled probe used for hybridisation was the original 1.3kb *EcoRI* fragment isolated from λ gt11. High stringency hybridisation and washing was carried out (65°C , 0.1M NaHPO_4), and exposure was for 4 weeks. (For explanation of Southern blot, see figure 4.6c).

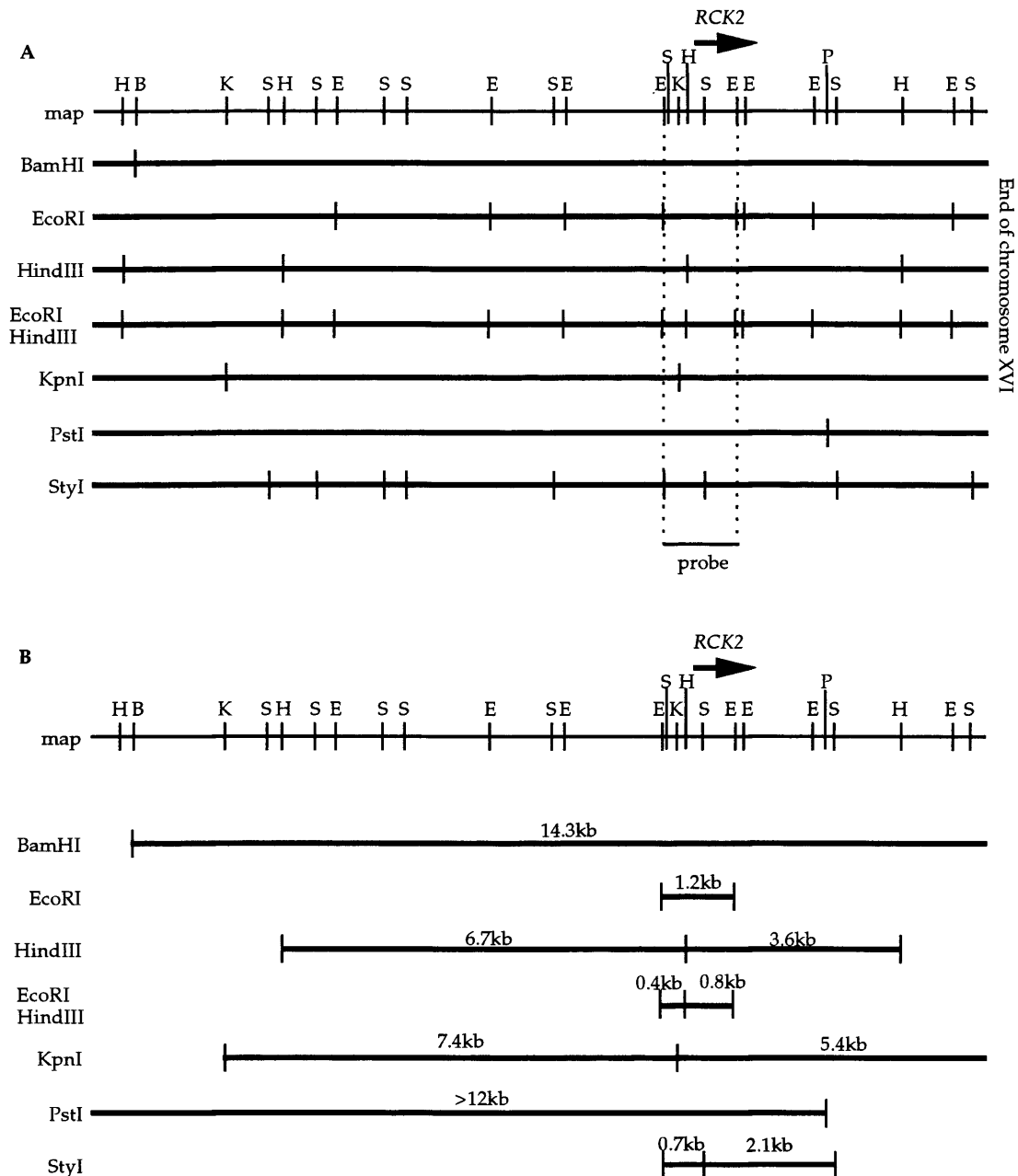


Figure 4.6c

Explanation of yeast genomic Southern blot using the 1.3kb *EcoRI* fragment from phage λ 7.2 as a probe. A) Restriction map of yeast genomic DNA according to the yeast genomic sequencing project. Restriction sites of individual restriction enzymes shown also. B) The restriction fragments predicted to hybridise to the probe are described along with the predicted fragment sizes. The fragments correspond to the bands observed on the autoradiogram in figure 4.6b. (The data suggests that the *KpnI* digest was incomplete)

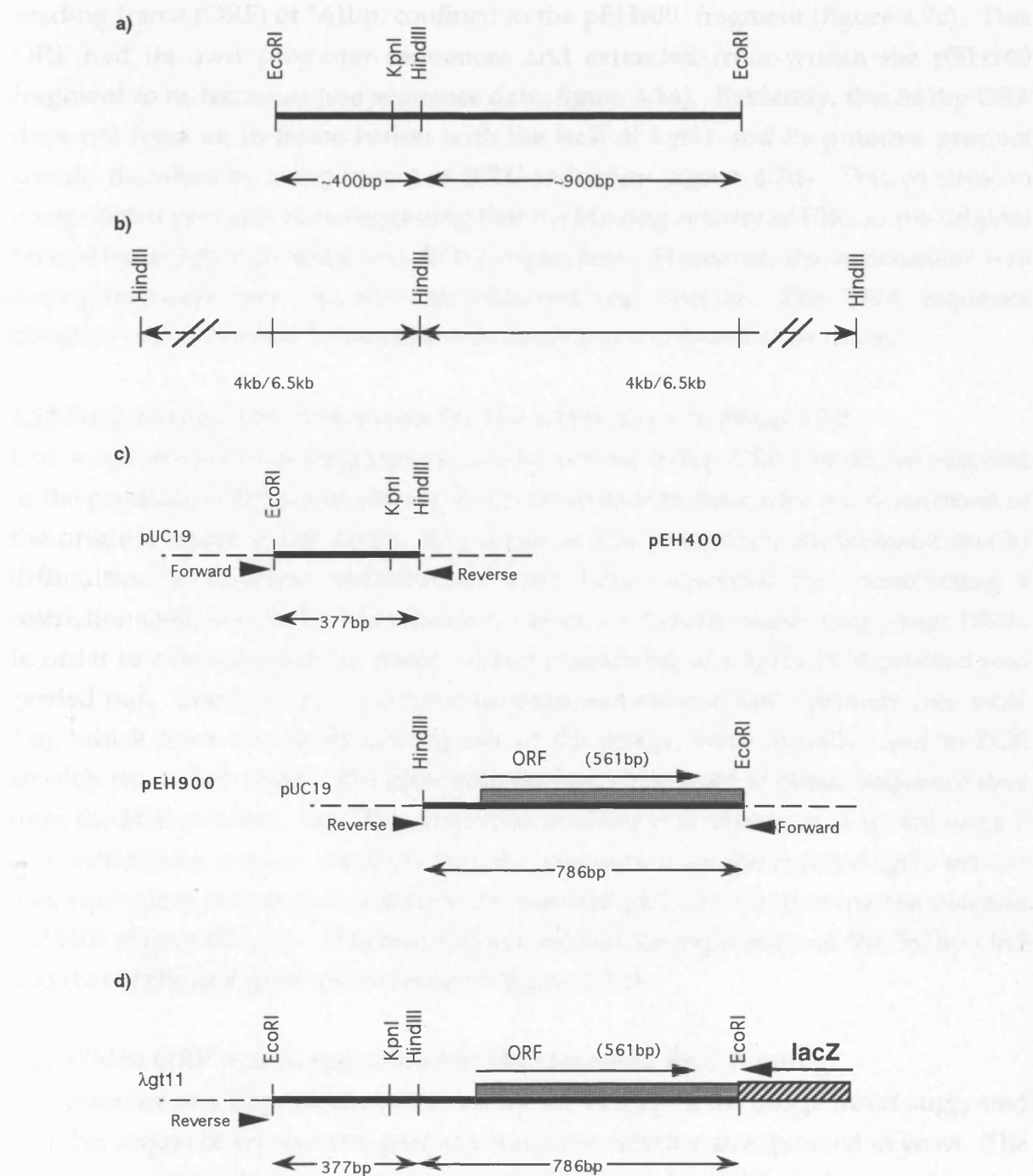


Figure 4.7

Manipulation of the ~1.3kb insert from λ 7.2. **a)** The ~1.3kb *EcoRI* fragment contained unique restriction sites for *HindIII* and *KpnI*. **b)** Southern hybridisation analysis of genomic DNA using the *EcoRI* fragment as a probe indicated that this fragment spans *HindIII* fragments of ~6.5kb and ~4.0kb. **c)** Utilising the unique *HindIII* restriction site, the *EcoRI* ~1.3kb fragment was digested into two *HindIII*-*EcoRI* fragments of ~400bp and ~900bp. These fragments were subcloned into pUC19 vector to produce pEH400 and pEH900 subclones. Sequence analysis of these two subclones revealed that the insert of pEH400 was 377bp in size, and that of pEH900 was 786bp in size. A large open reading frame (561bp) was identified within the pEH900 fragment. **d)** Comparing the sequence obtained from the reverse *λgt11* primer and the reverse pUC19 primer (see text for details) helped to deduce the orientation of the ~1.3kb *EcoRI* fragment within the *λgt11*. It was found that the 561bp ORF of pEH900 was expressed in the opposite direction to that of β -galactosidase in *λgt11* (see text for details).

reading frame (ORF) of 561bp, confined to the pEH900 fragment (figure 4.7c). This ORF had its own promoter sequences and extended from within the pEH900 fragment to its terminus (see sequence data, figure 4.14). Evidently, this 561bp ORF does not form an in-frame fusion with the *lacZ* of λ gt11 and its putative product should therefore be independent of IPTG induction (figure 4.7d). This conclusion contradicted previous data suggesting that the binding activity of PKC to the original recombinant λ gt11 proteins was IPTG-dependent. However, the experiment was only performed once and the data obtained was unclear. The DNA sequence obtained was compared to the computer databases and found to be novel.

4.10 Determining The Orientation Of The 1.3kb Insert In Phage λ 7.2

Due to the results indicating that the product of the 561bp ORF should not respond to the presence of IPTG (see above), it was important to determine the orientation of the original insert in the λ gt11. This analysis was potentially problematic due to difficulties in digesting recombinant λ gt11 DNA, essential for constructing a restriction map, and the lack of reliable methods for directly sequencing phage DNA. In order to overcome both problems, direct sequencing of a λ gt11 PCR product was carried out. Commercially available forward and reverse λ gt11 primers (see table 2.6), which flank the *EcoRI* cloning site of the phage, were initially used to PCR amplify the ~1.3kb insert. The same primers were then used to obtain sequence data from the PCR product. The DNA sequence obtained was of poor quality, although it was sufficiently clear to conclude that the sequence from the reverse λ gt11 primer was equivalent to that obtained from the forward pUC19 primer using the plasmid pEH400 (figure 4.7c/d). This result indicated that the expression of the 561bp ORF and that of the *lacZ* gene are convergent (figure 4.7d).

4.11 Which ORF Was Responsible For The Observed PKC Binding?

The presence of a large (561bp) ORF within the 1166bp of the phage insert suggested that this sequence represented part of a true gene which was expressed in yeast. The ORF was also linked to promoter sequences which could mediate expression independently of the *lacZ* fusion. Independent polypeptide expression of this type was observed relatively frequently (section 4.6, and personal communication of laboratory members). It was plausible, therefore, that this ORF was responsible for the PKC binding activity. However, preliminary data, although not conclusive, suggested that expression of the PKC binding activity was IPTG-dependent and hence required the expression of the λ gt11 promoter (section 4.3). This experiment was carried out once only in the laboratory of D. Mochly-Rosen. It was therefore impossible to repeat and confirm the data.

A thorough sequence analysis was carried out on the ORF fused to the β -galactosidase in the recombinant phage. The ORF fused in frame to β -galactosidase

was found to be 17 amino acids in length (figure 4.8). Although it was assumed that this ORF was not part of a *bona fide* gene (confirmed later by the Yeast Genome Sequencing Project), it could still encode a polypeptide which interacts with PKC. This short polypeptide was compared with sequences in the Swissprot computer database (figure 4.9). The 50 sequences obtained from the database which showed the greatest homology to the 17 amino acids, failed to provide any clue as to the possible binding activity of this short polypeptide to PKC. The 50 proteins had no apparent functional similarities which would suggest that the 17 amino acid polypeptide represented a functional domain of some kind. Very few proteins related to signal transduction were represented amongst the 50 proteins. The two sequences from the database which displayed the highest homology to the 17 amino acid polypeptide showed 59% and 47% identity respectively, but was mainly due to arginine residues (R), and failed to suggest a correlation of this polypeptide with PKC binding (figure 4.9a). Comparing the 17 amino acid polypeptide to RACK1, which contains regions that mediate PKC-association (see introduction), revealed very poor homology (less than 24% identity, figure 4.9b). Taken together, the data did not provide a convincing argument for the PKC binding activity of the 17 amino acid polypeptide, when expressed as a fusion with β -galactosidase. The sequence obtained from pEH900 (figure 4.7c) indicated that the 17 amino acid residues lie within the 561bp ORF. Overlapping genes are rare in yeast, especially those which are convergently expressed. Consequently, assuming the 561bp ORF was part of a truncated gene, it was unlikely that a polypeptide expressed as a fusion with β -galactosidase represented a true gene product. It was more plausible that the large 561bp ORF encodes the polypeptide responsible for the observed ability of the λ 7.2 expressed proteins to bind PKC.

4.12 Screening A λ EMBL3 Yeast Genomic Library

A λ EMBL3 yeast genomic library was prepared in our laboratory for routine screening (see table 2.3). The library was constructed through the ligation of *Bam*HI-digested λ EMBL3 with yeast genomic DNA, partially digested with *Sau*3A. The average size of inserts was approximately 10-15kb. It was hoped that by using this library a single genomic fragment containing the complete gene including the 561bp ORF would be obtained.

Approximately 1×10^4 phage were hybridised to the 1166bp insert of p7.2 (see table 2.4) which was radioactively labelled with ^{32}P . After several rounds of screening and re-screening, four positively hybridising phage were identified and isolated. All four hybridised to both fragments (380bp and 786bp) of clone p7.2 (see table 2.4). One of the four recombinant λ EMBL3 phage was chosen for further analysis.

EcoRI phage/insert
junction

---//---

S A **E F** r l c r q r r r v g r e r s *

TCG.GCG.**gaa.ttc**.cgt.ttg.tgc.aga.cag.agg.agg.aga.cgt.gta.ggt.aga.gaa.aga.agc.tga

----->>//----->

lacZ expression 17 amino acid open reading frame

Figure 4.8

Sequence of the junction between the λ gt11 β -galactosidase and a 17 amino acid polypeptide. An insert of ~1.3kb was isolated from a λ gt11 expression library (λ 7.2) and completely sequenced. The sequence data identified a large incomplete open reading frame (181 amino acids) within the insert which was thought to be responsible for the observed PKC binding (see text for details). This large open reading frame was associated with upstream regulatory sequences proposed to promote gene expression. The λ gt11 expression library, however, was constructed so that expression of inserts would be mediated by the lacZ gene cloned within the phage. Phage expression produces β -galactosidase fused to a polypeptide encoded by the insert sequence. Consequently, the possibility existed that a polypeptide fused to the β -galactosidase was responsible for the PKC binding rather than the large open reading frame. Analysis of the sequence suggested that a 17 amino acid polypeptide would be fused in frame to the λ gt11 β -galactosidase.

A)

(i) SPERM PROTAMINE (CYSTEINE-RICH PROTAMINE). . . .

```

              10
17aa          EFRLCRQRRRVGRERS
              | ||:|||| || |:
hsp1_s ARYRCCLTHSRSRCRRRRRRRCRRRRRRFGRRRRRRVCCRRYTTVVRCTRQ
              10      20      30      40      50

```

(ii) PTST PROTEIN. . . .

```

              10
17aa          EFRLCRQRRRVGRERS
              | ||:||||
ptst MKAWRRLRSPRFFRFALCQQRRRRFIAIPLPDRKRKADSDNFRHLVNNVQIRIRKHAHIS
              10      20      30      40      50      60

```

B)

Gap Weight:	3.000	Average Match:	0.540
Length Weight:	0.100	Average Mismatch:	-0.396
Quality:	9.1	Length:	317
Ratio:	0.535	Gaps:	0
Percent Similarity:	47.059	Percent Identity:	23.529

```

17aa  1 .....EFRLCRQRRR 10
              :: . ||
RACK1 51 NYGIPQRALRGHSHFVSDVVISSDGQFALSGSWDGTLLRLWDLTTGTTTRR 100
              . . . . .
11 RVGRERS..... 17
   ||:::
101 FVGHTKDVLSVAFSSDNRQIVSGSRDKTIKLWNTLGVCKYTVQDESHSEW 150
       .
       .
       .

```

Figure 4.9

Sequence comparisons with the 17 amino acid ORF. A) The 17 amino acid sequence was compared against the whole Swissprot protein database. The best two homologies found are shown. Analysis of the proteins showing homology to the 17 amino acid sequence failed to suggest a correlation of this polypeptide to a PKC binding ability. B) Comparison of the 17 amino acid sequence with RACK1 showed very poor homology and failed to indicate possible PKC binding domains in this polypeptide.

4.13 Isolating A Genomic Fragment Containing The Entire Gene

The DNA sequence of pEH900 indicated that the ORF, presumed to be responsible for the PKC binding activity, resided entirely within a 786bp *EcoRI-HindIII* fragment (see sequencing data, figure 4.14). In addition, Southern blot analysis of yeast genomic DNA, digested with *HindIII*, showed that the original 1166bp phage insert hybridised to genomic fragments of approximately 4- and 6.5kb (figure 4.6). Together, these results suggested that a single *HindIII* genomic fragment (4kb or 6.5kb) would contain the entire gene (presuming the gene was smaller than 4kb).

The recombinant λ EMBL3 was digested with *HindIII* and the digested DNA resolved on an agarose gel. Several fragments, varying in size between 3kb and 6kb, could be detected (figure 4.10). Considering the Southern blot data, this restriction pattern suggested that the gene would reside within a 4kb *HindIII* fragment (figure 4.6).

The λ EMBL3 DNA, digested with *HindIII*, was hybridised to the radioactively labelled 786bp insert of pEH900. The results confirmed that the gene resides within a fragment of ~4kb, although determining which of the several fragments hybridised to the probe proved difficult (data not shown). So as to overcome this problem, a 'shot-gun' cloning strategy was carried out. All the *HindIII* fragments produced from the digestion of the recombinant λ EMBL3 DNA were used in a ligation reaction with *HindIII*-digested pUC19 vector. *E. coli* transformants containing recombinant plasmid with the fragment of interest were identified by colony hybridisation (see section 2.4.21), using the above radioactively labelled 786bp insert as a probe. Isolated transformants were found to contain a recombinant pUC19 plasmid with a *HindIII* insert of approximately 3.5kb, which compared favourably with the 4kb fragment predicted from the Southern analysis. These recombinant plasmids were designated pRCK2 (see table 2.4).

4.14 Characterisation Of The 3.5kb λ EMBL3 Fragment

Plasmid pRCK2 (see table 2.4) was digested with several restriction enzymes to deduce a basic restriction map (figure 4.11). One end of the fragment includes both *StyI* and *EcoRI* sites at positions which compared favourably with data obtained from the sequencing analysis of pEH900 (see sequencing data, figure 4.14). Two additional *EcoRI* sites, as well as a unique *PstI* site, were also identified from the restriction data (figure 4.11).

A ~2.4kb *HindIII-PstI* fragment, containing the RCK2 ORF (figure 4.11), was radioactively labelled and used to hybridise yeast genomic DNA digested with various enzymes (figure 4.12). This Southern data, in conjunction with the previous hybridisation data obtained with a different probe (figure 4.6) complemented the restriction map analysis. The collective results were later confirmed by the Yeast Genome Sequencing Project.

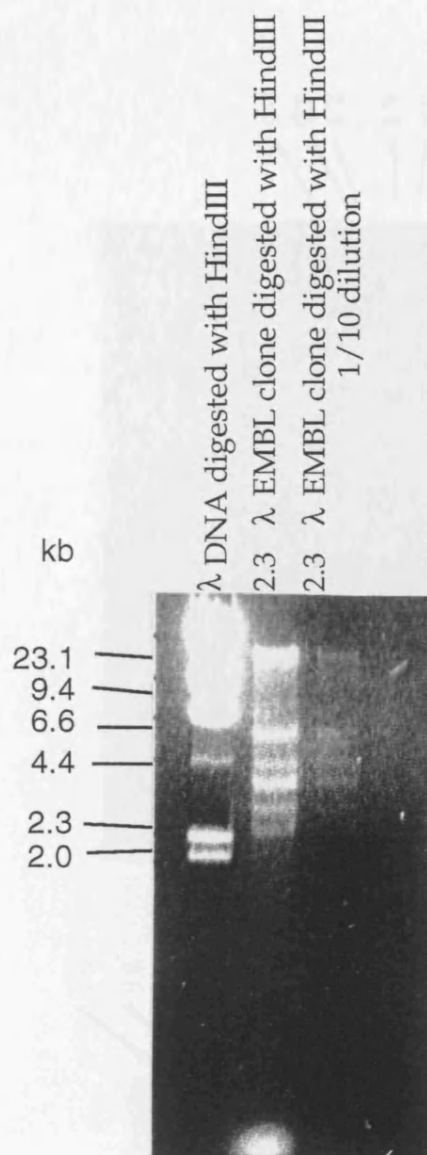


Figure 4.10

Agarose gel showing λ EMBL3 DNA digested with *Hind*III. Previous data had suggested that the complete yeast *RCK2* gene resided on a *Hind*III genomic fragment (see text for details). Subsequent screening of a λEMBL3 phage library resulted in the isolation of clone 2.3. This phage clone was digested with *Hind*III and the digestion products resolved on a 0.7% agarose gel.

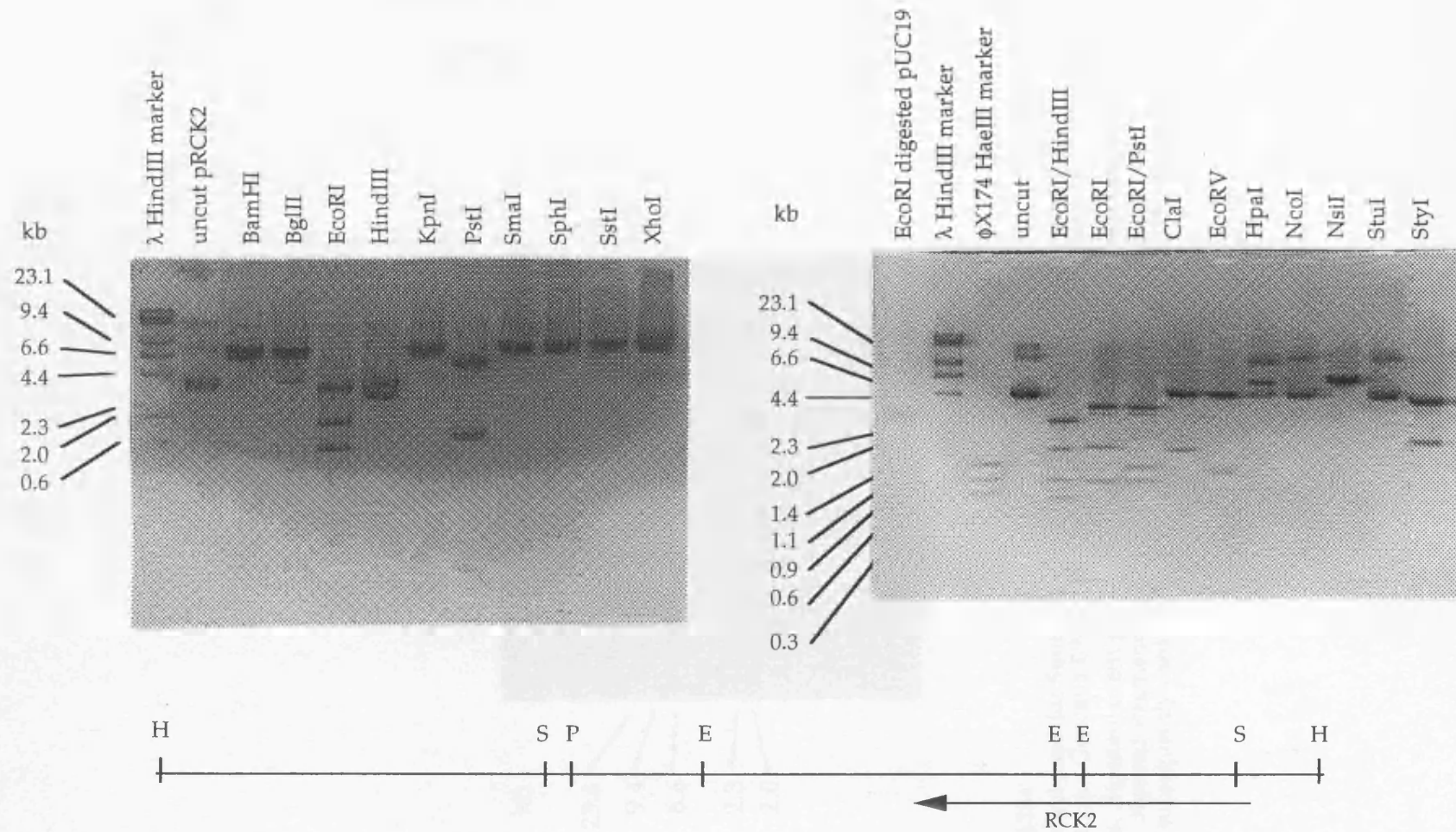


Figure 4.11

Agarose gels showing pRCK2 restriction digests and the subsequently derived restriction map. A ~3.7kb *HindIII* fragment from a λEMBL3 genomic library containing the *RCK2* gene was subcloned into pUC19. This subclone was digested with several common restriction enzymes and a basic restriction map of the insert was deduced. H=*HindIII*, S=*StyI*, P=*PstI*, E=*EcoRI*.

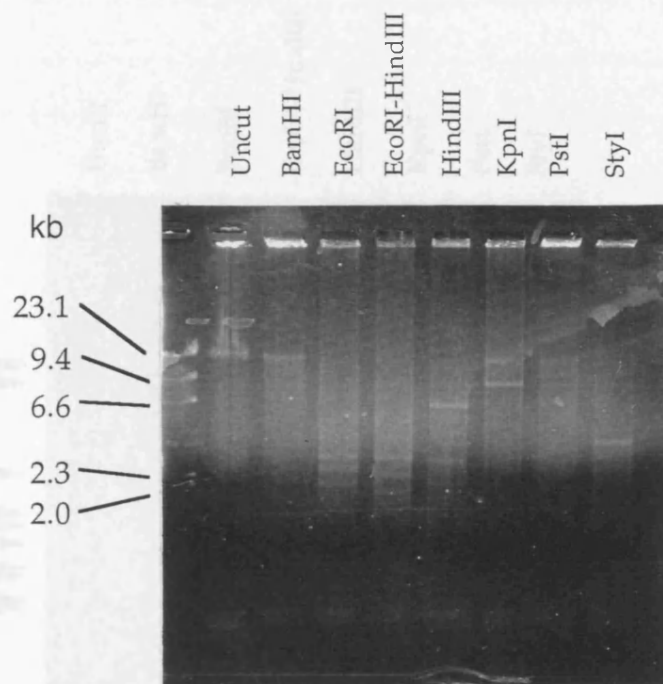


Figure 4.12a

Agarose gel used for Southern blot of DNA from the wild-type haploid W303 strain. Genomic DNA was prepared as described in materials and methods, digested overnight at 37°C with various restriction enzymes, and the digested fragments resolved on a 0.7% agarose gel. The agarose gel was subsequently used for Southern blot analysis (see figure 4.12b)

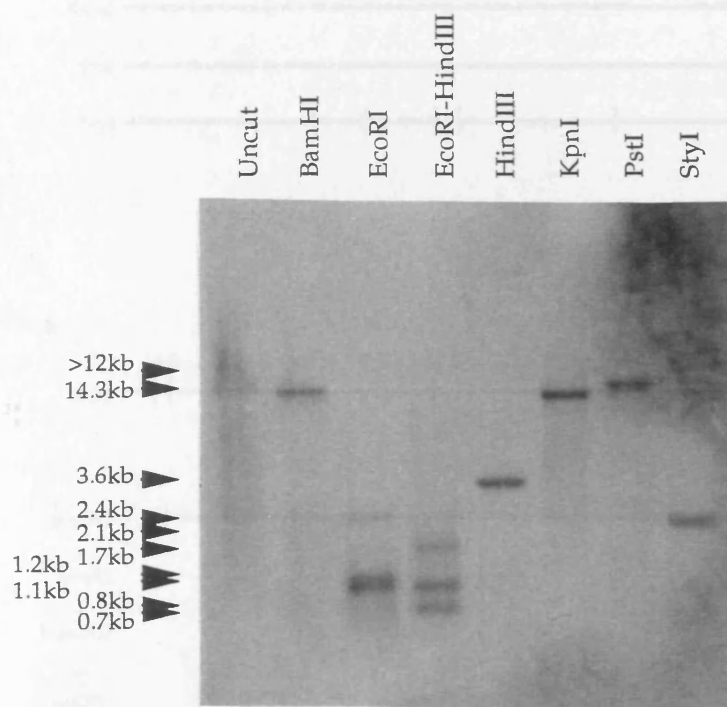


Figure 4.12b

Autoradiograph of Southern blot of DNA extracted from the wild-type haploid W303 strain. Genomic DNA was prepared, digested with various restriction enzymes, and used for Southern blot analysis (see figure 3.2.6b). The labelled probe used for hybridisation was a 2.4 kb *HindIII*-*PstI* fragment containing RCK2. High stringency hybridisation and washing was carried out (65°C , 0.1M NaHPO_4), and exposure was for 1 week.

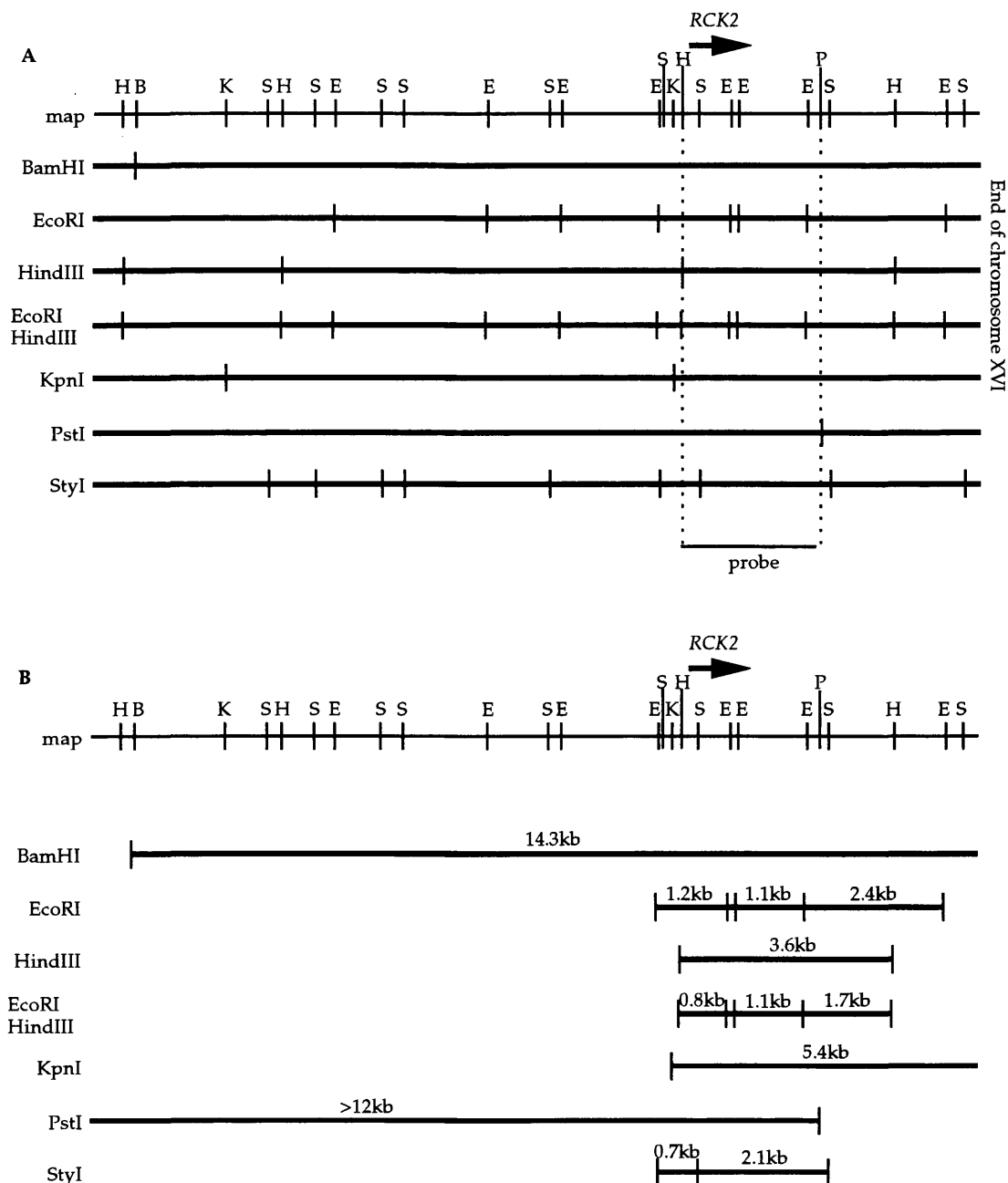


Figure 4.12c

Explanation of yeast genomic Southern blot using a *HindIII*-*PstI* fragment containing *RCK2* as a probe. A) Restriction map of yeast genomic DNA according to the yeast genomic sequencing project. Restriction sites of individual restriction enzymes shown also. B) The restriction fragments predicted to hybridise to the probe are described along with the predicted fragment sizes. The fragments correspond to the bands observed on the autoradiogram in figure 4.12b. (The data suggests that the *KpnI* digest was incomplete)

The 3.5kb fragment was initially sequenced using commercially available forward and reverse pUC primers (see table 2.6). As anticipated, the DNA sequence obtained from one end of the fragment (using the reverse primer) was identical to that obtained from pEH900, confirming the authenticity of the λ EMBL3 insert. Sequence from the forward primer displayed no similarity to sequences in the computer database.

The 3.5kb insert was subcloned into smaller fragments (figure 4.13). Using the unique *Pst*I site within the insert and the *Pst*I site within the polylinker of the pRCK2, a 1.4kb *Pst*I fragment was subcloned into the *Pst*I site of pUC19. This clone was designated pPP1400 (see table 2.4). Using one of the three *Eco*RI sites within the insert and the *Eco*RI site in the polylinker of pRCK2, a ~1.8kb *Eco*RI fragment was subcloned and designated pEE1800 (see table 2.4). Finally, using two *Eco*RI sites within the insert, a 1.1kb *Eco*RI fragment was similarly subcloned and designated pEE1100 (see table 2.4). These subclones provided the basis for the sequencing strategy used to characterise the complete gene containing the 561bp ORF (figure 4.13).

4.15 Sequencing Data Of RCK2 (Accession number; Genbank X86735 [LEE1])

Using universal pUC-based primers (see table 2.6) and designed synthetic primers, the DNA sequence of the subclones described above was determined so that a contiguous sequence of approximately 1.5kb could be obtained. This 1.5kb included the whole 561bp ORF and flanking sequences. Both DNA strands were sequenced at least twice. The sequencing data identified an ORF of 903bp which included the 561bp previously characterised in pEH900 (figure 4.14). This ORF was designated *RCK2* (*R*eceptor of *C* *K*inase) and was predicted to encode a protein of 301 residues (~33kDa).

Just as in higher eukaryotes, TATA boxes are an essential element of yeast promoters. In yeast, however, initiation is not confined to a site 30 base pairs downstream of the TATA box and can occur within a window of 30-120 nucleotides (Guarente, 1992). In the case of *RCK2* there are several upstream sequences of TATA: -34, -136, -203, plus many sequences of TATTA, between -19 and -149 (figure 4.14). Without any experimental evidence, the importance of any of these sequences is unknown.

Several regulatory elements are found upstream of *RCK2*. Pheromone-stimulated transcription is mediated by a *cis*-acting pheromone response element (PRE) displaying the consensus sequence: 5'-TGTTTCT-3' (Sprague Jr and Thorner, 1992). One conserved PRE and one PRE containing the first 5 residues of the 7 nucleotide consensus sequence (PRE') are found upstream of the initiation ATG codon of *RCK2* (figure 4.14). These two elements are in opposite orientations, although orientation is often not important for upstream activator sequences (UAS).

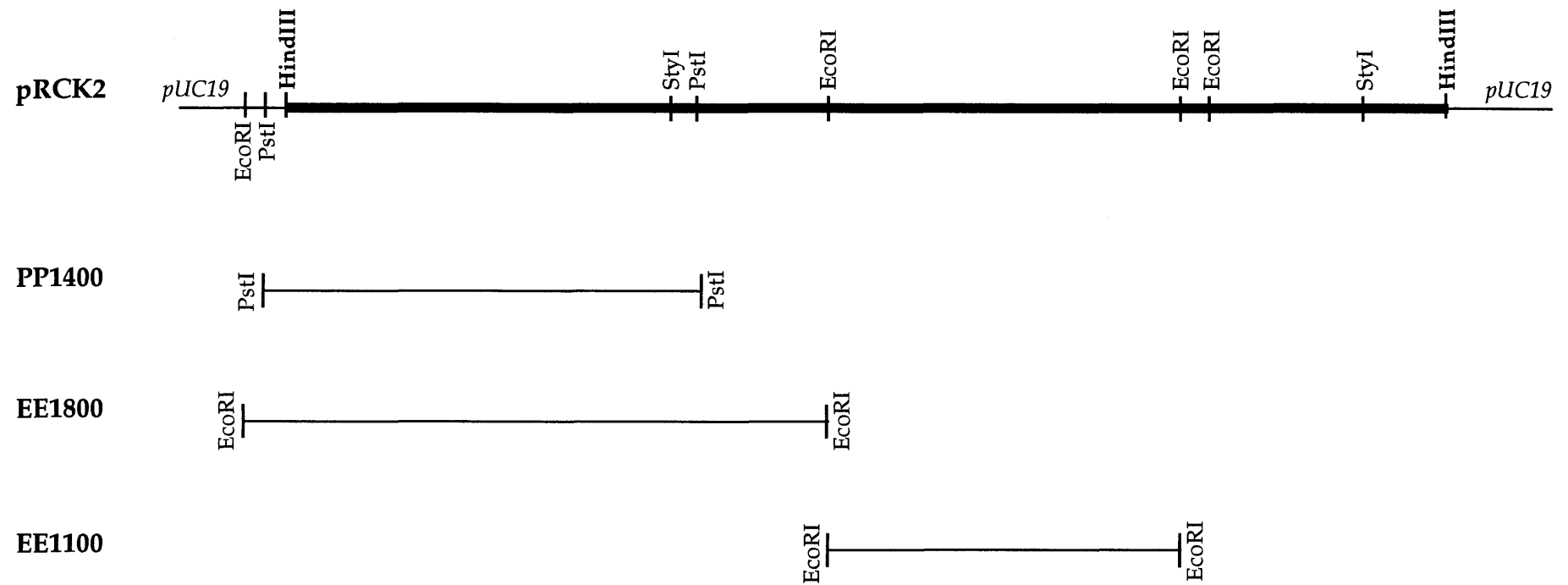


Figure 4.13

Restriction map of pRCK2 showing the subclones used for sequence analysis. Restriction analysis of pRCK2 allowed a simple restriction map to be derived. Suitable restriction sites were observed which, along with restriction sites present within the pUC19 polylinker, allowed three subclones to be made (see text for details). All derived constructs were subsequently used for sequence analysis.

EcoRI StyI
1 gaattcccccg ccactcttgc cccgccccctt aaaacgcca ggtgaaaggg agctctacta
61 caggccccgcc acagtatgat gtgggataaa aaagatgata ggaagaaaag gtgcagacaa
121 catcctgtat ggggtgcacg gtgtggcagt gctagtaata aaatgatgtg gagattaaca
181 tgttgtggta cgggtattctt atcttcaagg KpnI taccacagta taattcatgg cctttatcat
241 tttttatttg tattatgtat SCB tttcgtgctt atcttgtaat ttttaagttt tggatttcgg
301 tgcttcgtgt agttcttccc ctatatctca atctaaagta aattattaca aaatatgggtg
361 gtgatctttt aatctcaaag ctctcttttac tccgggcatt ataaaaacag aatctcttag
421 cagttactct tttccctttt ctcttggtgcc gtcataattag agtaattata atttcttctt
481 tttcggcttc cctt PRE tgtttc tcaaaaatct cgattttatt atctaact atcataacta
541 ctactattac tattactatt attattatta taaatcttaa aactattatt tgc PRE' taaaaa
601 aa ATGGATGC TTTTGAGAAT ATGTCCGTAT CAAATCATCC GGGCGGTAAC GCAAGAAGAA
661 ACTCCCAATC AGCAAACGAA ATGCTTGCAT CGCAAATTCA GGATTTCAG AATATTCCAA StyI
721 GGTCTTTCAA TGACAGCAAT GCAAATGTAA ATTTGTCAA AAATTGTACT GTCGGCAATC
781 AAC TGCCTTT CTCATCACGT CAACAAAAA TTATCATGGA ACATTTACTG ATAACGAAGA
841 ACAATTCTCA ACAGCAGAAG GACTATTAC ACGTGCCATG CAAATTTTTT AAGATGGGCA
901 ATTGCCAAGC TGGCTCGTCT TGTCCCTTTT CCCATTCCCC AGATATTATC AGTTCTGCGA
961 ATAATTTACC TTGCAAGTAT TTCGCAAAGG GTAACGTAA ATTCGGTAAC AAGTGTGTTA
1021 ATGCTCATGT GCTGCCAAAT GGGTTCAAGA TGAACAGTAA AGAACCTATT GACATCACTC
1081 CCCCTTCACA AAACAAC TAC TTATCCCATG CTAGATCAGC TTCTTTCTCT ACCTACACGT
1141 CTCCTCCTCT GTCTGCACAA ACGGAATTCT CTCACCTGTC TTCGAATGCA AACTTCTTTT EcoRI
1201 CCTCGCAATA CCTAATGTAT TCGCCTCAGA AAAGTCCAGA GGCCCTATAT ACTGAATTCT EcoRI
1261 TTTCTCCCCC TTCGTCTTCT TCTTCGTACA TCAACTATAG CTATAATAAC TCCAACATTA
1321 ATGCCTATTC TCCAGTCTCT TCGTCTTCAT CTAACATTTG GCAAGAGCAA GGCCAAACTA
1381 CGTTATCGAA TCCGAGTGTG AATCAGAATT TAAGGTATCG TACAGGCCCT GCAATTCAAG
1441 AAGAATCAGA TAATGAAATT GAAGATTTAC TTATTCACAA TTTCAATTCC AGATACTGTC
1501 ATGAATGA ag agccgcccc tccatagttt ttcctagttc aagcattaat ataccggacc
1561 actctacatc ttccgaaagc cggaaaataa gaacaaaaaa

Figure 4.14

Sequence of RCK2 and flanking regions. The RCK2 ORF (uppercase) begins from an ATG (603) and ends with TGA (1508). A consensus SWI4/SWI6 cell cycle box (SCB) is found 5' to RCK2 in addition to consensus pheromone response elements (PRE and PRE'). Sequence indicative of a polyadenylation site is also underlined (lowercase). Restriction sites are indicated by overline.

In addition, the consensus sequence CACGA₄ of the *SWI4*/*SWI6*-dependent cell cycle box (SCB) is also present upstream of *RCK2* (figure 4.14). For example, SCB sequences are found repeated ten times upstream of the *HO* gene whose expression initiates the mating-type switch (Breedon and Nasmyth, 1987).

Unlike the step-wise processing of the 3' termini of mRNA in higher eukaryotes, the processing in yeast appears less ordered. It was suggested that transcriptional termination is directly coupled to mRNA polyadenylation, as all transcripts contain poly(A) additions. Many yeast genes, including *RCK2*, contain the tripartite sequence TAG...TAGT...TTT in the 3' non-translated region. This sequence is thought to be necessary for polyadenylation and the correct processing of the mRNA (figure 4.14); (Henikoff and Cohen, 1984, Irniger, et al., 1991). *RCK2* also contains at least one hexanucleotide sequence of AAUAAA commonly associated with RNA processing (polyadenylation) in higher eukaryotes.

4.16 RCK2 Gene And Protein Sequence Analysis

The sequences of *RCK2* and its predicted protein were compared to sequences present in the various computer databases. Both sequences showed no significant homology to any previously characterised sequences. In addition, comparison of *RCK2* and Rck2p to *RACK1*, *GCD6*, and other genes/proteins found to have PKC binding activity failed to pick up any significant homologies. Interestingly, however, when Rck2p was compared with itself two repeat sequences were detected. A sequence of PCKXFFXGXGXC was found twice in the 5' half of the protein, while the sequence SPXSSSSSXI was found twice in the 3' half of the protein (figure 4.15). These motifs were compared to the computer databases but failed to match any known sequences. Furthermore, no significant matches were obtained when Rck2p was compared with the 'motifs' programme of the GCG computer package (Devereux, et al., 1984). The sequences alone, therefore, provided little information concerning the function of *RCK2*. (The *RCK2* sequence was compared to the whole yeast genome after the completion of the genome sequencing project but still did not detect any significant homology to other sequences except to itself).

The secondary structure of Rck2p (figure 4.15) is predicted to be an irregular, globular protein (MIPS Yeast Genome Sequencing Project). Further computer analysis of the predicted secondary structure (figure 4.16) neither provided clues as to the possible function of Rck2p, nor showed any peculiarities which could be assessed (Chou and Fasman, 1978, Devereux, et al., 1984, Garnier, et al., 1978, Kyte and Doolittle, 1982).

4.17 Assigning RCK2 To A Yeast Chromosome

The 1166bp fragment from pEH900 was isolated and radioactively labelled with ³²P. This probe was subsequently used to hybridise American Type Culture Collection

1	MDAFENMSVSNHPGGNARRNSQSANEMLASQIQDFQNIPRSFNDSNANVN	50
	ccccccccccccccccccccccccchhhhhhhhhhhhhhhcccccccccccccc	
51	LSKNCTVGNQLPFSSRQQKIIMEHLLITKNNSQQQKDYSHV <u>PCKFFKMGN</u>	100
	ccccccccccccccccchhhhhhhhhhhheeeccccccccccccccccceeecccc	
101	<u>CQAGSSCPFSHSPDI</u> ISSANNL <u>PCKYFAKGN</u> CKFGNKCVNAHVLPNGFKM	150
	ccccccccccccccccccccccccceeeccccccccccccceeecccccccc	
151	NSKEPIDITPPSQNNYLSHARSASFSTYTSPPLSAQTEFSHSASNANYFS	200
	cc	
201	SQYLMYSPQKSPEALYTEFF <u>SPPSSSSSY</u> INYSYNNSNINAY <u>SPVSSSSS</u>	250
	ceeeccccccccchhhhhccccccccccccceeecccccccccccccccccc	
251	<u>NIWQEQGQT</u> TLNPSVNQNLRRTGPAIQEESDNEIEDLLIHNFNSTRYCH	300
	ceeeccccccccccccccccccccccccchhhhhccchhhhhhhcccccccccc	
301	E 301	
	C	

h=alpha-helix

c=coil

e=extended β -sheet

(Lipman and Pearson, 1985)

Figure 4.15
The predicted secondary structure of Rck2p (data obtained from the MIPS Yeast Genome Project). The predicted secondary structure of Rck2p suggests this protein is an irregular, globular protein (Lipman and Pearson, 1985). Rck2p showed no significant homology to protein sequences present in the computer databases. Interestingly, however, when Rck2p was compared to itself, two repeat sequences were revealed. A sequence of PCKXFFXGXC was found twice in the 5' half of the protein while a sequence of SPXSSSSXI was found twice in the 3' half of the protein. These motifs were also compared against the computer databases without detecting any significant matches. Furthermore, no significant matches were obtained when Rck2p was compared to the 'motifs' databases of the GCG computer package.

Figure 4.16

Analysis of Rck2p using the PeptideStructure and PlotStructure programmes of the GCG package (Devereux *et al.*, 1984).

KD hydrophilicity plot: graphs the local hydrophilicity of the protein along its amino acid sequence using the Kyte-Doolittle scale (Kyte and Doolittle, 1982). When the line is in the upper half of the frame, it indicates a hydrophobic region and when it is in the lower half, a hydrophilic region.

Surface probability plot: shows which regions of the protein are likely to lie on the surface of the protein. Values above the horizontal line indicate an increased probability of being found on the protein surface.

Flexibility plot: reflects regional flexibility of the polypeptide chain.

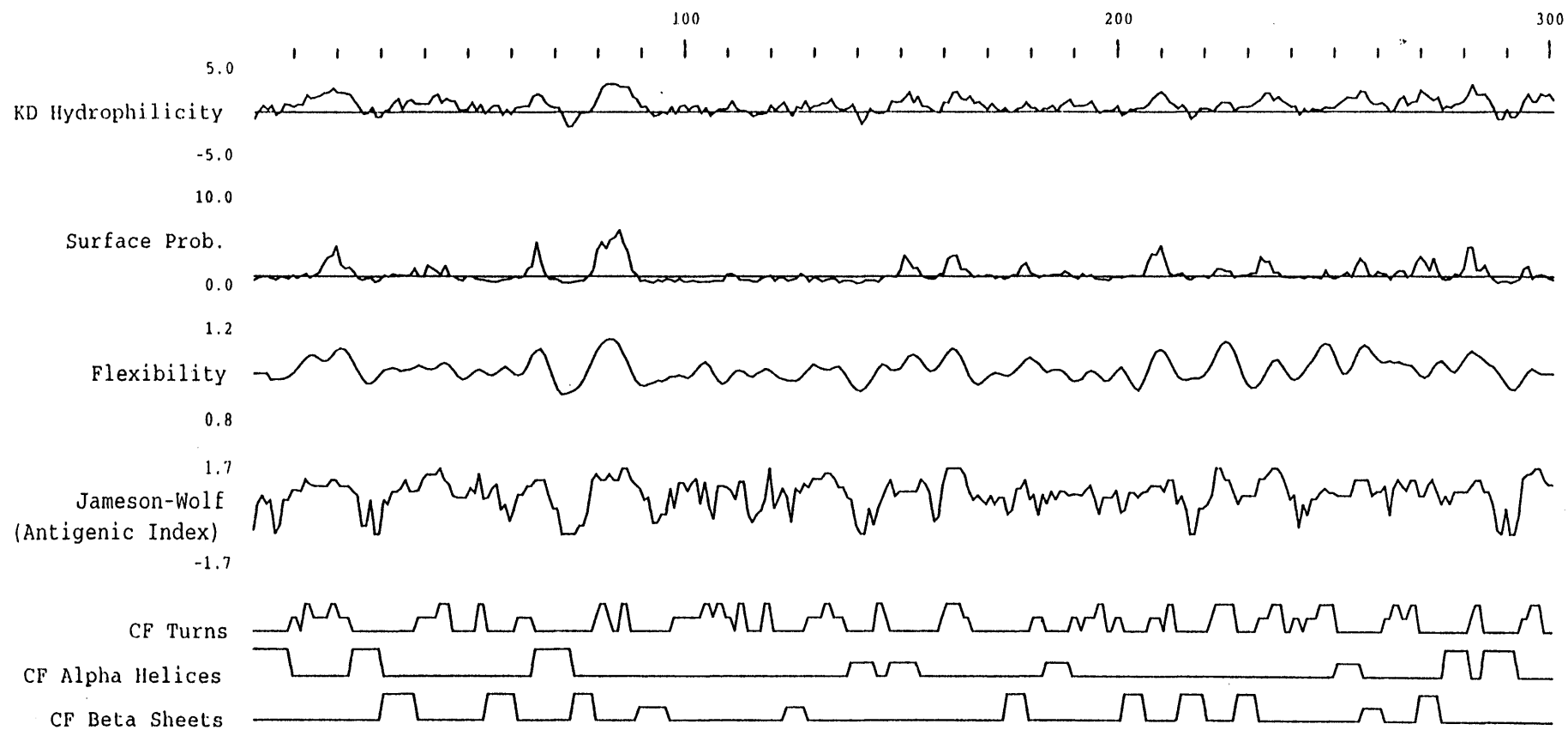
Antigenicity plot: indicates possible exposed surface peaks of the protein which may be antigenic sites. Analysis combines information from hydrophilicity, surface probability and backbone flexibility predictions along with the secondary structure predictions of Chou-Fasman (Chou and Fasman, 1978) and Robson-Garnier (Garnier *et al.* 1978) in order to produce a composite prediction of the surface contour of a protein.

Secondary structure plots: indicates regions of α -helix, β -sheet and reverse turn or coil. Predictions have been made using the Chou-Fasman method (CF).

PLOTSTRUCTURE of: rck2.p2s February 25, 1997 13:16

PEPTIDESTRUCTURE of: rck2.pep Ck: 8803, 1 to: 301

TRANSLATE of: rck2.seq check: 9287 from: 1 to: 905



(ATCC) membranes containing *Saccharomyces cerevisiae* genomic DNA derived from ordered recombinant lambda phage and cosmids (see section 2.4.20). The results suggested that *RCK2* is located on the right arm of chromosome XVI. This result was later confirmed by the Yeast Genome Sequencing Project.

4.18 Northern Blot Analysis

Northern blot analysis was carried to assess whether the *RCK2* gene is expressed *in vivo* and to determine the transcript size. RNA was purified from several yeast strains including wild-type, strains containing both *RCK2* disruptions and deletions (see chapter 5), and a strain in which *RCK2* transcription was induced (see section 5.8). Several attempts to identify an *RCK2* transcript failed. A radioactively labelled *RCK2*-containing probe, that was successfully used in Southern analysis, also failed to identify a transcript on an RNA blot previously used successfully to identify an unrelated transcript (*VRP1*; Donnelly, personal communication). It is possible that *RCK2* RNA is very unstable and rapidly turned over, or that transcription is tightly controlled by additional factors, or that *RCK2* transcription is limited to a short window of time during the cell cycle, or a combination of these factors.

4.19 Discussion

The sequence data of *RCK2* indicate that this gene is novel and unique, showing no significant homologies with previously characterised genes. This may indicate that Rck2p provides a specialised function in yeast. The predicted protein sequence displays no motifs or domains which could be attributed to a possible function. Although particular sequences present in RACK1 (see introduction) have been attributed to the binding of PKC, a PKC-binding domain common to all PKC-binding proteins has not been found. In the absence of reliable methods for predicting the tertiary structure of proteins, it would be unwise to dismiss the possibility that Rck2p may display conformational homologies with PKC-binding proteins.

Sequence homologies between proteins predicted to bind PKC is an interesting topic. Firstly, as discussed above, a common PKC-binding domain has not universally been identified suggesting the tertiary structures of PKC-binding proteins may be important for such an activity. Secondly, the PKC isozyme specificity of PKC-binding proteins may be determined by the latter. This means that PKC-interacting proteins would display sequence divergence depending on the PKC isozymes they associate with. Finally, the binding specificity of the PKC isozymes to other proteins could be determined by specific sequences in the various isozymes. For example, proteins proposed to bind PKC (*e.g.* RACK1, KCIP, lamin B, and Ras) were associated previously with functions unrelated to PKC interaction. Could it be possible, therefore, that PKC isozymes simply bind to a protein already

positioned at a particular intracellular location *via* its specific domain? For instance, an isozyme which attained an affinity for G proteins (RACK1) would become associated with the plasma membrane, while an isozyme attaining an affinity for nuclear proteins (lamin B) would become associated with the nucleus. In this hypothesis, the G protein and nuclear protein involved would not necessarily be expected to have sequence homology attributed to PKC-binding.

There are many yeast genes which are stimulated in response to the mating pheromones (Sprague Jr and Thorner, 1992). These include genes responsible for pheromone production, cell pairing, cell cycle arrest, and mating-related morphological changes, as well as genes involved in the recovery after such events. The magnitude of induction varies considerably depending on the genes involved. Pheromone-stimulated transcription is mediated by a *cis*-acting pheromone response element (PRE). The transcriptional activator, Ste12p, binds poorly to a single PRE but binds cooperatively if two or more elements are present in tandem (Sprague Jr and Thorner, 1992). Although two PRE elements are present upstream of *RCK2*, they are in opposite directions and at a distance longer than previously observed (~100bp apart). Many pheromone-responsive genes also contain a second *cis*-acting sequence, the 'P-box' that serves as the binding site for a general transcription factor, Mcm1p (Sprague Jr and Thorner, 1992). The Ste12p was shown to bind a single PRE if juxtaposed to a 'P-box'. There are no 'P-boxes' found upstream of *RCK2*, however.

Ten tandem repeats of the SCB sequence are found upstream of the *HO* gene, which is responsible for mating-type switching. Swi4p and Swi6p (Swi, required for mating-type switching) are present in a multisubunit complex called the cell cycle box factor (SCBF), which binds to SCB sequences. SCBF binding to SCB sequences is regulated by *CDC28*-dependent phosphorylation. Consequently, *HO* transcription is induced by the SCBF only during the G₁ phase of the cell cycle when the *CDC28* kinase is active. This SCBF-mediated regulation is also observed for other genes expressed in G₁, including the cyclins *CLN1* and *CLN2* (Herskowitz, et al., 1992). Although genes found to be regulated by the SCB factor have multiple SCB sequences, the presence of a single SCB sequence upstream of *RCK2*, in addition to two PRE sequences, suggests that *RCK2* could be cell cycle regulated and/or responsive to mating pheromones. The failure to identify an *RCK2* transcript in asynchronous yeast populations may reflect this tight regulation (see chapter 5).

CHAPTER 5.

RCK2 GENE DISRUPTION AND DELETION

5.1 Introduction

The ability to manipulate the genome is a powerful feature of yeast genetics. Such manipulation is possible due to the relatively high transformation efficiency of exogenous DNA into yeast cells. Consequently, plasmids containing autonomously replicating sequences (ARS) can be introduced and maintained within yeast cells, similar to plasmids routinely used in bacterial cells. In addition, linear DNA sequences can be targeted, through homologous recombination, into specific sites of the genome after their introduction into yeast cells. Manipulated DNA is therefore capable of replacing wild-type sequences. Thus, controlled and detectable genomic manipulations can routinely be carried out in yeast to assess the role of interesting DNA sequences *in vivo*.

Replacing a wild-type allele with a mutant one allows the study of a mutant phenotype possible. The most commonly used method for constructing genomic mutations is that of gene disruption, whereby a foreign fragment of DNA is introduced into the ORF of the gene of interest, disrupting its normal expression. The foreign fragment is usually an auxotrophic marker which allows the selection for transformed cells carrying the manipulated DNA.

5.2 RCK2 Gene Disruption

The plasmid pRCK2-P (see table 2.4) was the primary source of *RCK2* sequence used for genetic manipulation (figure 5.1). This plasmid was constructed by digesting pRCK2 (see table 2.4) with *Pst*I and removing an internal 1.4kb *Pst*I fragment (figure 5.1). A *Sty*I restriction site, approximately 110bp downstream of the ATG start codon of *RCK2*, is unique within pRCK2-P and was utilised for gene disruption (figure 5.1). A *Sty*I-linearised plasmid was treated with T4 DNA polymerase (see section 2.4.11) which converted its 5' overhanging termini into blunt ends. This linearised plasmid was then ligated with a 1.1kb *Sma*I fragment (derived from pYRG19- Δ *Bam*HI, see table 2.4) which contained a *URA3* gene as a selectable marker. Ligation mixtures were transformed into *E. coli* and plasmids isolated from the transformants were analysed by agarose gel electrophoresis (figure 5.2). A correct plasmid construct, pRCK2 δ (see table 2.4), was digested with *Hind*III and *Eco*RI and the resulting ~1.9kb *URA3*-containing fragment (figure 5.1) was used to transform the wild-type haploid strain, W303 (see table 2.1). The *URA3* sequence in this fragment is flanked on either side by approximately 300bp of sequence derived from the *RCK2* locus. This flanking DNA should promote homologous recombination and allows the subsequent introduction of the *URA3* allele into the wild-type *RCK2* locus in the

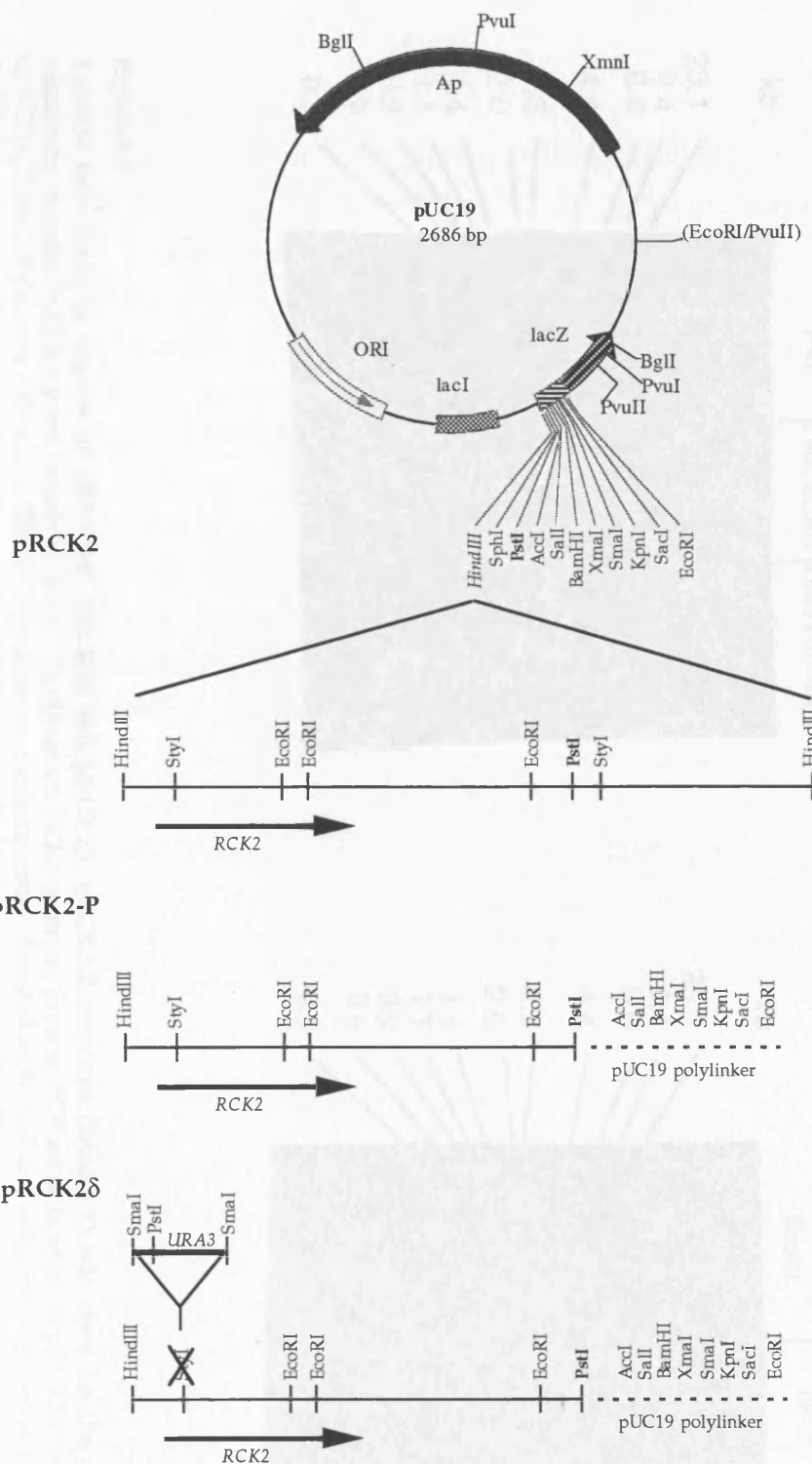


Figure 5.1

Construction of an RCK2 disruption. The RCK2-containing HindIII fragment of pRCK2 was reduced in size by removing a ~1.4kb PstI fragment (single PstI sites are present in both the fragment and the vector). This modified plasmid was called pRCK2-P. A 1.1kb SmaI fragment, containing URA3, was ligated into the blunt-ended StyI site of pRCK2-P to produce pRCK2 Δ . This last plasmid was used as a source of DNA for yeast transformations (see text for details).

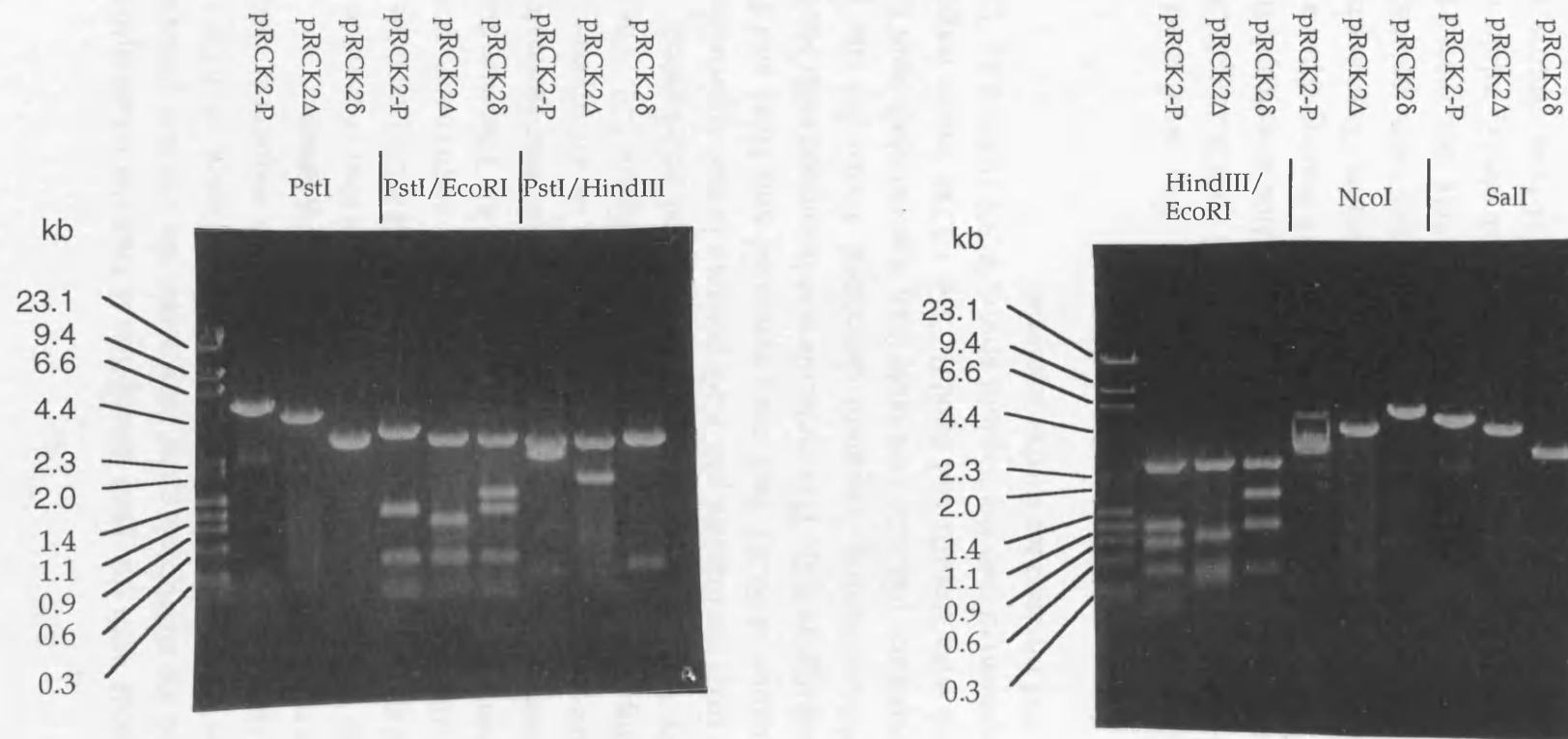


Figure 5.2

Agarose gels showing digests of pRCK2-P, pRCK2 δ and pRCK2 Δ . pRCK2-P construct (table 2.4) was used for the preparation of DNA containing mutated *rck2* for yeast transformation. To obtain an *RCK2* deletion, inverse PCR amplification of pRCK2-P was carried out using LJNCOA/LJNCOI primers (table 2.5). The PCR product was subsequently digested with *Nco*I and religated to generate pRCK2 Δ . To obtain a *RCK2* disruption, a *URA3* cassette was ligated into the *Sty*I site of RCK2-P to generate (pRCK2 δ).

yeast genome. Uracil prototrophs were selected after the transformations for further analysis.

5.3 *RCK2* Gene Deletion

A problem which may occur with the gene disruption is that the transcription of sequences downstream of the disrupted gene may affect the mutant phenotype. Consequently, a deletion of almost the entire coding sequence of *RCK2* was constructed. Care was taken, however, not to interfere with sequences flanking *RCK2* which could contain regulatory sequences for neighbouring genes.

The strategy used to construct an *RCK2* null mutant was that of inverse PCR (figure 5.3). Two synthetic oligonucleotides, oLJNCOA and oLJNCO1 (see table 2.5), incorporating *NcoI* restriction sites, were designed to sequences present approximately 100bp within each of the *RCK2* termini (figure 5.4). The introduction of the *NcoI* site in the primer oLJNCOA, through base-mismatch, also generated an adjacent *EcoRI* site (figure 5.4). These primers were used in an inverse PCR amplification reaction using pRCK2-P plasmid as a template (figure 5.3). The PCR product, of approximately 4.5kb, was digested with *NcoI* and self-ligated. The resulting plasmid differed from the original pRCK2-P plasmid in that approximately 800bp of the coding sequence of *RCK2* had been removed and *NcoI* and *EcoRI* restriction sites incorporated (figure 5.2). This molecule was linearised with *NcoI* and ligated to the 1.1kb *URA3*-containing fragment described above for the gene disruption. The correct construct, pRCK2 Δ (see table 2.4), was isolated from *E. coli* transformants and digested with *HindIII* and *BamHI*. The ~2.7kb linear fragment containing *URA3*-was transformed into the haploid strain, W303 (table 2.1). Uracil prototrophs were selected and retained for further analysis.

5.4 Yeast Transformants

The laboratory wild-type yeast strain W303 (see table 2.1) was used for transformation of the deletion/disruption constructs of *RCK2* (pRCK2 Δ /pRCK2 δ). Two haploid W303 strains were available (see table 2.1): histidine auxotroph (His⁻) and histidine prototroph (His⁺) strains. Haploid strains of both mating types α and a were transformed with the disrupted and deleted gene constructs. Transformants were selected on uracil-lacking media and several Ura⁺ colonies were isolated for further analysis. Strains containing a disrupted (*rck2*) allele were designated HDs (H=haploid; Ds=disruption) while those containing a deleted ($\Delta rck2$) allele were designated HD1 (D1=deletion) (see Table 2.1). Haploid wild-type strains were designated HWT (WT=wild-type).

Genomic DNA was prepared for Southern blot analysis from various transformants (see section 2.4.33). DNA was digested with *EcoRI* and hybridised to a radioactively labelled probe containing the *RCK2* sequence on a *HindIII*-*PstI*

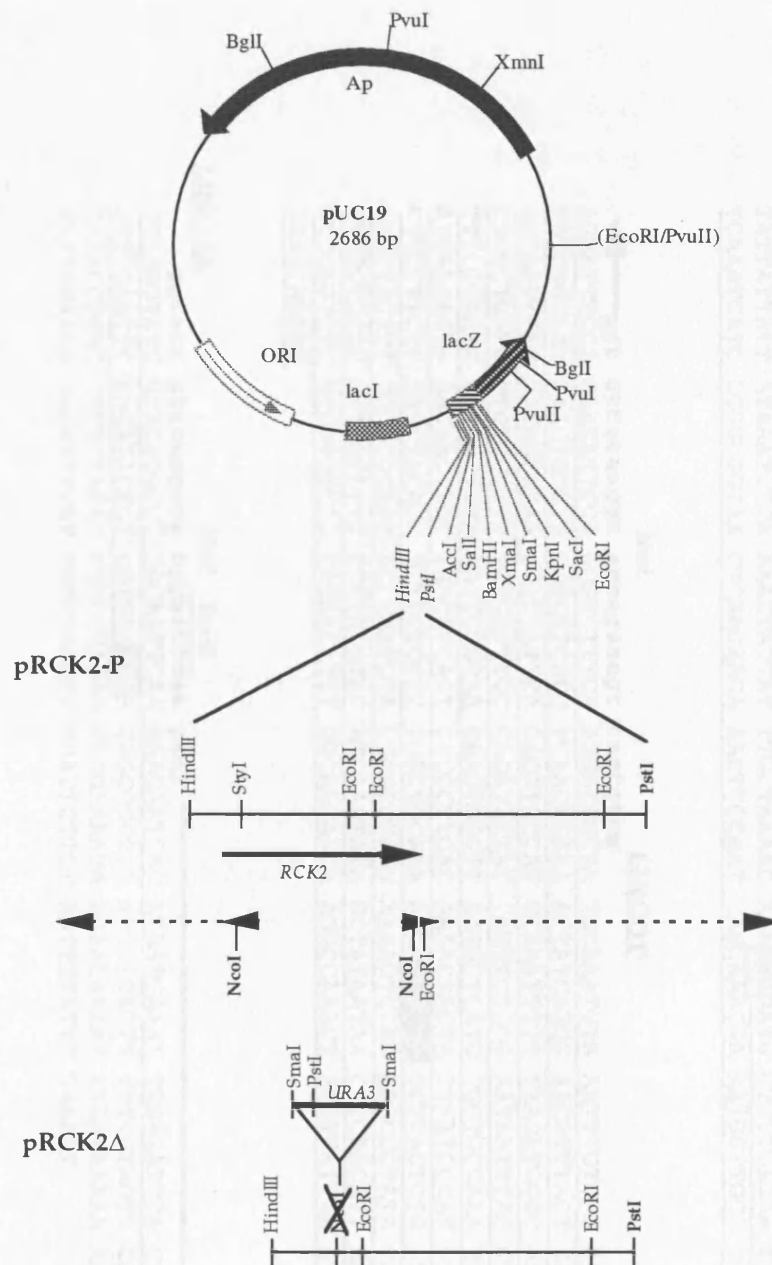


Figure 5.3

Construction of an *RCK2* deletion. pRCK2-P (see table 2.4) was used as a template for PCR amplification using primers designed against *RCK2* sequence. Oligonucleotides oLJNCO1 and oLJNCOA (see table 2.5) hybridised to sequences approximately 100bp within the *RCK2* ORF and directed amplification away from the gene and into the vector. Both primers incorporated an *NcoI* site, and LJNCOA also gained an *EcoRI* site (see text for details). The PCR product, of ~4.5kb, was digested with *NcoI* and self-ligated. A 1.1kb *SmaI* fragment containing *URA3* was ligated subsequently into the blunt-ended *NcoI* site to form pRCK2Δ. This final plasmid was used as a source of DNA for subsequent yeast transformations (see text for details).

HindIII

AGCT TTTTACTC CGGGCATTAT AAAACAGAA TCTCTTAGCA

GTTACTCTTT TCCCTTTTCT CTTGTGCCGT CATATTAGAG TAATTATAAT TTCTTCTTTT TCGGCTTCCC

CTTGTCTTCTA CAAAATCTCG ATTTTATTAT CTAACACTAT CATAACTACT ACTATTACTA TTACTATTAT

TATTATTATT ATAAATCTTA AAACCTATTAT TTGCTAAAAC AAATC GATG CTTTGTAGAA TATGTCTGTA

TCAAATCATC CGGGCGGTAA CGCAAGAAGA AACTCCCAAT CAGCAAACGA AATGCTTGCA TCGCAAATTC

NcoI LJNCO1'

← ggt cttataaggt accagaaagt tactgtcg

AGGATTTCCT GAATATTCCA AGGTCTTTCA ATGACAGCAA TGCAAATGTA AATTTGTCAA AAAATTGTAC

TGTCGGCAAT CAACTGCCTT TCTCATCACG TCAACAAAAA ATTATCATGG AACATTTACT GATAACGAAG

AACAATTCTC AACAGCAGAA GGACTATTCA CACGTGCCAT GCAAATTTTT TAAGATGGGC AATTGCCAAG

CTGGCTCGTC TTGTCCCTTT TCCCATTTCC CAGATATTAT CAGTTCTGCG AATAATTTAC CTTGCAAGTA

TTTCGCAAAG GGTAACGTGA AATTCGGTAA CAAGTGTGTT AATGCTCATG TGCTGCCAAA TGGGTTCAAG

ATGAACAGTA AAGAACCTAT TGACATCACT CCCCTTCAC AAAACAATA CTTATCCCAT GCTAGATCAG

CTTCTTTCTC TACCTACACG TCTCCTCCTC TGTCTGCACA AACG TCTCACTCTG CTTGCAATGC

AAACTACTTT TCCTCGCAAT ACCTAATGTA TTCGCCTCAG AAAAGTCCAG AGGCCCTATA TACT CAATTC

TTTTCTCCCC CTTCGTCTTC TTCTTCGTAC ATCAACTATA GCTATAATAA CTCCAACATT AATGCCTATT

CTCCAGTCTC TTCGTCTTCA TCTAACATTT GGCAAGAGCA AGGCCAAACT ACGTTATCGA ATCCGAGTGT

GAATCAGAAT

LJNCOA *NcoI* *EcoRI*

ggtatc gtacaggcca tgggaattcaa gaag →

TTAAGGTATC GTACAGGCCC TGCAATTCAA GAAGAATCAG ATAATGAAAT TGAAGATTTA CTTATTTCACA

ATTTCAATTC CAGATACTGT CATGAATGAA GAGCCGCCCC ATCCATAGTT TTTCTAGTT CAAGCATTA

TATACCGGAC CACTCTACAT CTTCCGAAAG CCGGAAAATA AGAACAAAA ATAATAAAAA AAAGCACGTT

TAATATAAAA AACATTTTAT TGTATGAGTT GGATTCTTTT AATTTTATTT TAAAAA

Figure 5.4

LJNCO primers used for the *RCK2* deletion. Two primers were designed to enable reverse PCR production of an *RCK2* deletion. LJNCOA (ggtatcgtacaggccatggaattcaagaag) and LJNCO1 (gctgtcattgaaagaccatggaatattctgg) both contained *NcoI* restriction sites and annealed to sequences within *RCK2*. Amplification out from *RCK2* and into pRCK2-P vector produced a ~4.5kb product which could be digested with *NcoI* and religated (see text). Underlined sequence represents the *RCK2* ORF; boxed CAATTC represents deleted *EcoRI* sites.

fragment (figure 5.5a). In wild-type strains, the *HindIII-PstI* probe would hybridise to four *EcoRI* fragments of 2.4kb, 1.2kb, 1.1kb and 0.1kb (figure 5.6b). The size of the 1.2kb *EcoRI* fragment of strains in which the *RCK2* allele is disrupted would increase by 1.1kb due to the *URA3* insertion, producing a fragment of 2.3kb as seen on the Southern blot (figure 5.6c). Deletion of *RCK2*, using the oLJNCO oligonucleotides (see table 2.5), removes the two *EcoRI* sites within *RCK2* (figure 5.6d). Consequently, the 1.2kb, 1.1kb and 0.1kb fragments found of the wild-type DNA are lost (figure 5.5a). However, a new *EcoRI* site was incorporated into the deletion construct by the oLJNCOA primer (see figure 5.4). The introduction of this site, and the *URA3* fragment, would produce three *EcoRI* fragments of 2.4kb, 0.8kb and 1.8kb which should hybridise to the radioactively labelled probe (figure 5.5a).

The results of the Southern blot, along with the deduced genetic organisation, indicated that HDs (a or α) [His⁻] and HDI (a or α) [His⁻] were genetically correct. HDI α [His⁺] transformants also appeared to be correct although HDs α [His⁺] appeared to be identical to the wild-type profile (figure 5.5a). Both HDs a [His⁺] and HDI a [His⁺] transformants contained DNA which were also identical to wild-type DNA suggesting that genomic manipulations in these strains were unsuccessful (figure 5.5a). In addition, the profiles of HDI a [His⁺], HDs a [His⁺] and HWT a [His⁺] DNAs were different from the other W303 haploid DNAs in that they had two larger fragments which hybridised to the probe (figure 5.5a). The a [His⁺] strains were therefore considered unsuitable for further analysis.

5.5 Disrupted/Deleted Diploids

As well as characterising the effect of mutant *rck2* alleles on growth, mutant homozygote diploid strains should assist in assessing the effect of the mutant alleles on sporulation, tetrad formation, and spore germination. The construction of diploid strains from mutant haploids would also help to assess the requirement of *RCK2* for mating. This was particularly important considering the PRE elements and the SCB sequence found upstream of the *RCK2* ORF (see section 4.15). These two consensus sequences could be involved in the pheromone-stimulated transcription of *RCK2* and/or its possible cell cycle regulation.

The Southern blot analysis of the disrupted/deleted haploid strains described above indicated that α [His⁺] cells should be mated with a [His⁻] cells. These matings would generate a $\Delta rck2/\Delta rck2$ homozygous deleted diploid strain and a *rck2/RCK2* heterozygous disrupted diploid strain. The problem with this strategy was the selection against the haploid strains. The positive selection of diploids on histidine-lacking media would select against the a [His⁻] strains, but a selection against the α [His⁺] haploid strains was also essential. Consequently, α [His⁺] haploid strains were made ρ^- (or ρ^0 ; see below) so they were not able to utilise glycerol as a carbon source. α [His⁺] haploids were grown on solid media in the

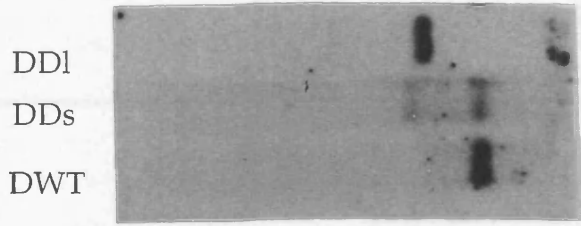
Figure 5.5

Southern blots of DNA from W303 mutant strains. A) Both mating types of haploid W303 (α and **a**) were transformed with disrupted (Ds) and deleted (Dl) forms of *RCK2* gene. Both histidine auxotrophic (His^-) and prototrophic (His^+) haploid cells were used for transformation. Genomic DNA was prepared from selected transformants, digested with *EcoRI*, and used for Southern blot analysis. The labelled probe used for hybridisation was the *HindIII-PstI* fragment containing the complete *RCK2* gene (see text). KEY: Haploid (H); wild-type (WT); histidine auxotroph (-); histidine prototroph (+); **a** mating-type (**a**); α mating type (α); *RCK2* disruptant (Ds); *RCK2* deletion (Dl).

B) HDs **a** (His^-) was mated with HWT α (His^+) to produce a heterozygous diploid disruption, DDs (*rck2/RCK2*); HDl **a** (His^-) was mated with HDl α (His^+) to produce a homozygous diploid deletion, DDl ($\Delta rck2/\Delta rck2$); WT **a** (His^-) was mated with WT α (His^+) to produce a diploid wild-type strain DWT (*RCK2/RCK2*). Genomic DNA was isolated from all three diploids and digested with *EcoRI*. A radioactively labelled *EcoRI* fragment containing *RCK2* was hybridised against this digested DNA.

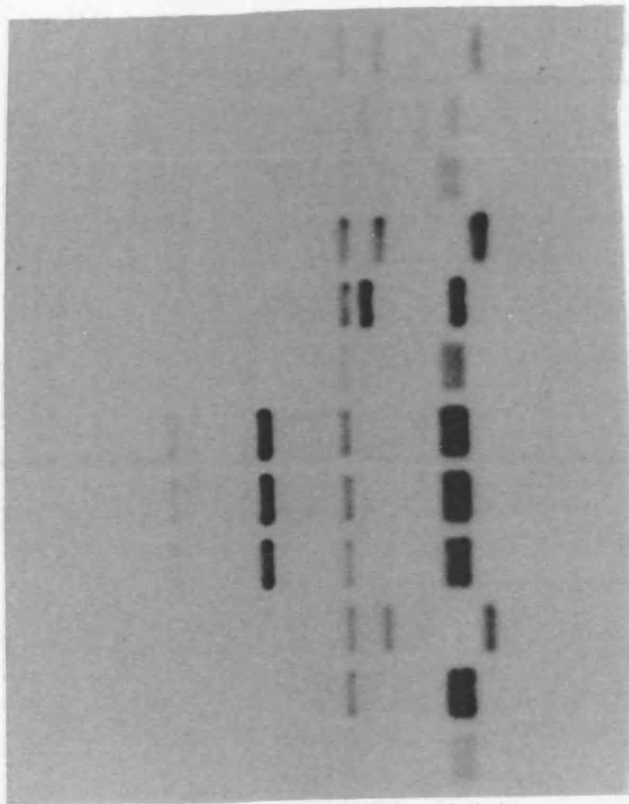
High stringency hybridisation and washing was carried out for both Southern blots (65°C , 0.1M NaHPO_4), and exposure was for 4-7 days.

B



2.3kb
1.8kb
1.2kb

HDI⁻ a
HDs⁻ a
HWT⁻ a
HDI⁻ α
HDs⁻ α
HWT⁻ α
HDI⁺ a
HDs⁺ a
HWT⁺ a
HDI⁺ α
HDs⁺ α
HWT⁺ α



2.4kb
2.3kb
1.8kb
1.2kb
1.1kb
0.8kb
0.1kb

A

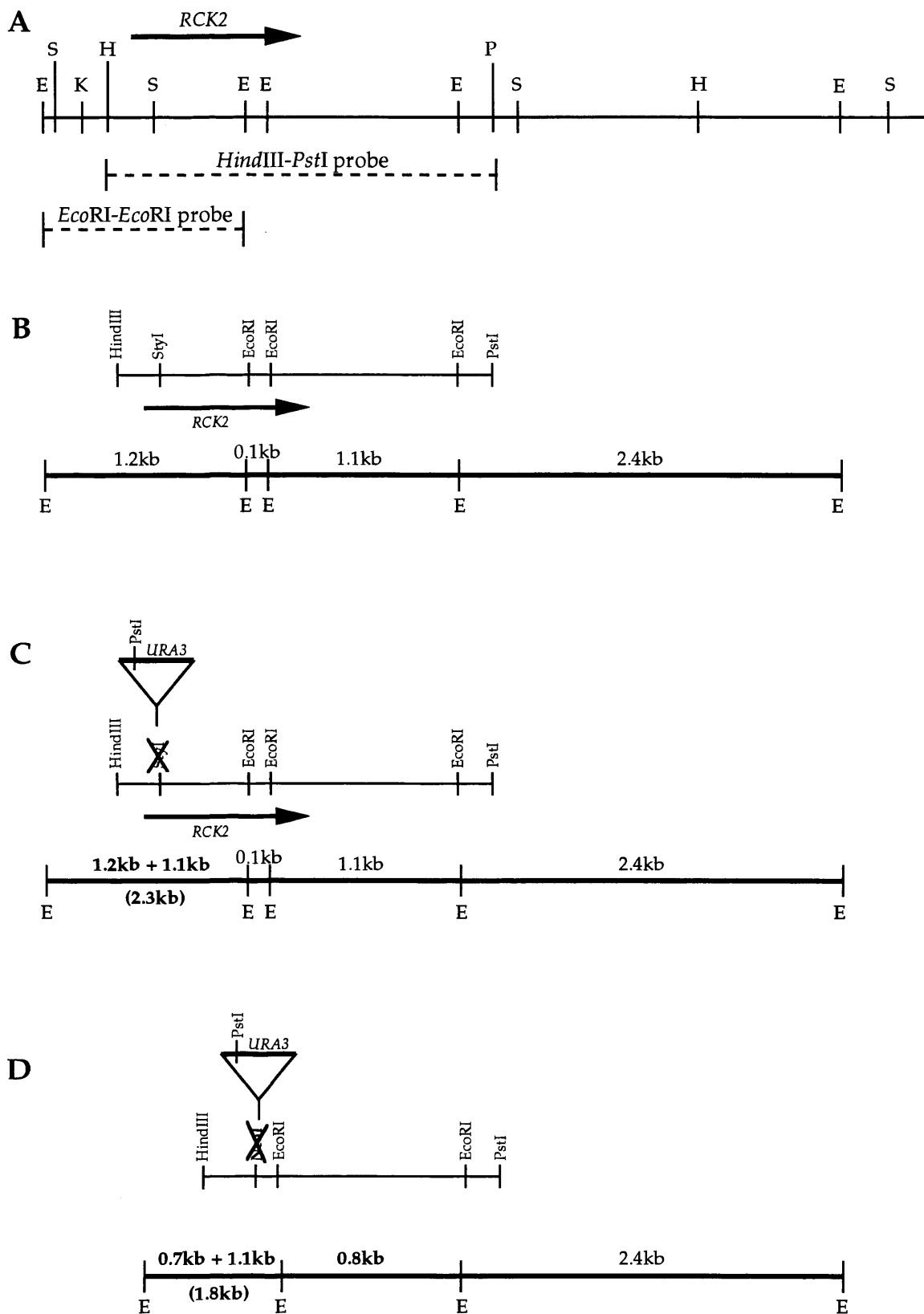


Figure 5.6

Explanation of Southern blot of *RCK2* mutants. **A)** Genomic restriction map showing the position of the *RCK2* ORF, and the fragments used as radioactive probes (E=*EcoRI*, P=*PstI*, H=*HindIII*, S=*StyI*, K=*KpnI*). **B)** Wild-type genomic DNA indicating the sizes of the *EcoRI* fragments generated after genomic digestion. **C)** Genomic DNA after *RCK2* disruption, indicating the change in *EcoRI* fragment sizes. **D)** Genomic DNA after *RCK2* deletion, indicating the change in *EcoRI* fragment sizes.

presence of a drop of ethidium bromide (10mg/ml) which was added onto the surface. The ethidium bromide diffuses away from its initial point of contact and generates a gradient. When the concentration of ethidium bromide is sufficiently high it intercalates with the mitochondrial DNA and promotes the loss of mitochondrial DNA from daughter cells. Such cells are termed *rho* zero (ρ^0) if all the mitochondrial DNA is lost, or *rho* minus (ρ^-) if some mitochondrial DNA remains. ρ^-/ρ^0 strains are unable to grow on a non-fermentable sugar such as glycerol as their respiration is impaired due to the absence of functional mitochondria. If the concentration of ethidium bromide is too high, the chromosomal DNA is affected leading to cell lethality, observed as a clear halo around the ethidium bromide spot. Cells growing around the clear halo were isolated and checked for their inability to grow on glycerol as a sole carbon source.

After the mating of a [His⁻] with α [His⁺] ρ^0/ρ^- diploid cells were selected on media containing glycerol as the sole carbon source, and lacking histidine. Several His⁺ diploid cells were isolated and analysed through Southern blot hybridisation (figure 5.5b). A radioactively labelled *Eco*RI fragment, containing the *RCK2* ORF, would hybridise to *Eco*RI fragments of 1.8kb if an $\Delta rck2/\Delta rck2$ homozygous deleted (DDI) strain was successfully constructed (figure 5.5b). In *rck2/RCK2* heterozygote disrupted diploid (DDs) this radioactively labelled probe would hybridise to *Eco*RI fragments of 2.3kb, derived from the disrupted haploid, and to a 1.2kb fragment, derived from the wild-type haploid. These hybridisation profiles were indeed detected as illustrated in figure 5.5b.

Disrupted, deleted and wild-type diploids were plated onto sporulation media (see section 2.4.18). All three diploid strains produced a high percentage (>80%) of sporulating cells. Subsequent dissection of asci produced four viable spores which developed into colonies of equivalent size and indicated that Rck2p has negligible function with respect to mating, sporulation, spore germination and growth under the conditions used.

5.6 Search For A Phenotype Of $\Delta rck2/\Delta rck2$ and *rck2/RCK2* Diploids

The natural habitat of yeast is decaying fruit and vegetable matter which provide a range of conditions to which the organism must respond. The available nitrogen source, for example, may range from simple ammonia and amino acids, to complex nucleic acids and their derivatives. The temperature of the environment not only varies but fluctuates and the availability of nutrients may often be limited. With such a broad spectrum of environmental conditions, yeast has evolved an equally broad array of pathways to utilise nutrients efficiently so as to accommodate conditions of nutrient deprivation most effectively.

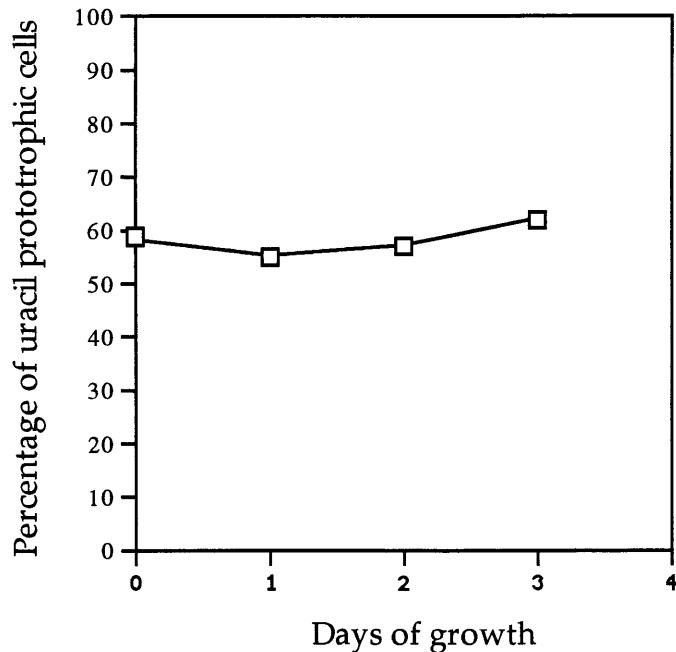


Figure 5.7

Competition experiment to compare growth rates of *rck2* mutant yeast cells with wild-type cells. Approximately equal numbers of DD1 (diploid cells homozygous for an *RCK2* deletion) and DWT (diploid wild-type cells) were used to inoculate 100mls of minimal media. The culture was grown to OD600=1.0 in a 30°C shaking waterbath (approximately one day). 100µl of culture was removed and used to inoculate a fresh 100mls of minimal media. This culture was again left to grow to OD600=1.0. This re-inoculation procedure was carried out for a total of three times. Samples of culture were taken immediately following re-inoculation. These three samples, in addition to a sample taken after the initial inoculation (time zero), were assessed for the numbers of mutant and wild-type cells. Mutant, DD1 cells are uracil prototrophs, while wild-type cells are uracil auxotrophs. Consequently, the numbers of cells which grow on uracil supplemented media can be compared with to the number of cells growing on media lacking uracil. The results showed that the percentage of uracil prototrophs (DD1 cells) remained relatively constant over the period of ~3 days.

Analysis of growth rates

In order to assess the growth of the mutant and wild-type yeast cells, a competition experiment was carried out. Approximately equal numbers of DDl (*Δrck2/Δrck2*) and DWT (*RCK2/RCK2*) cells were used to inoculate 100mls of SD minimal media (see section 2.1) which contained 2% glucose and the appropriate amino acids for growth of both cell types. The culture was then incubated in a shaking water bath at 30°C. Once the culture reached OD₆₀₀≈1.0 (after approximately 24 hours), a sample of 100μl was removed and used to inoculate another 100mls of fresh media. This re-inoculation procedure was carried out over a time period of three days. Samples were taken at time zero, the point of initial inoculation and immediately after each subsequent dilution. Cells were plated onto uracil-containing SD minimal media (see section 2.1) and incubated at 30°C for 2-3 days. Colonies were replica plated onto uracil-lacking media and the number of colonies on each medium calculated. The number of colonies on the former medium should represent both DDl and DWT strains, whereas those on the latter medium would only represent the DDl diploid (Ura⁺). The results, indicated that the percentage of wild-type and mutant cells remained relatively constant over the period of 3-4 days of growth (figure 5.7). This result strongly suggests that the growth rate of yeast cells is independent of *RCK2* under the conditions used.

Effects of altered carbon source

Glycolysis is the anaerobic degradation of glucose which generally yields lactic acid. In yeast, however, glucose is degraded to yield ethanol through fermentation. More energy can be obtained from the oxidation of glucose to CO₂ and H₂O through respiration under aerobic conditions. Growth is fastest on glucose than on other carbon sources, but only approximately 13% of the carbohydrate is assimilated into the cell material, while the remaining carbohydrate mostly accumulates as fermentation products (ethanol). Growth on galactose is slower but more efficient, approximately 26% of the carbohydrate is assimilated into the cell material (Fraenkel, 1982). Aerobic growth on ethanol is slow, but more efficient with 39% assimilation of the carbon source into cell material.

Yeast can utilise different carbon sources although glucose is the one routinely used in the laboratory. Other monosaccharides, such as fructose and galactose, can be converted to glucose-6-P and subsequently enter the glycolysis pathway. Maltose, a disaccharide of two glucose moieties, sucrose made up of glucose and fructose, and raffinose a trisaccharide made up of glucose, fructose and galactose can all be metabolised by yeast. Glycerol, unlike the carbohydrates above, cannot be fermented, but can be utilised as a sole carbon source in aerobically grown strains.

[illegible]

MEDIA	Haploid						Diploid		
	HWT a [His ⁻]	HDs a [His ⁻]	HDI a [His ⁻]	HWT α [His ⁺]	HDs α [His ⁺]	HDI α [His ⁺]	DWT	DDs	DDI
1% SDS	X	X	X	X	X	X	X	X	X
0.1% SDS	X	X	X	X	X	X	X	X	X
0.05% SDS	X	✓✓	✓✓	X	✓✓	✓✓	X	✓	✓
0.01% SDS	✓X	✓✓	✓✓	✓X	✓✓	✓✓	✓X	✓✓	✓✓
0.005% SDS	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓
0.001% SDS	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓
0.01% SDS (Defined media)							✓X	✓✓	✓✓
0.01% SDS (SD Media)							X	X	X
0.01% (SDS YPD)							X	X	X

Table 5.1

Results of *rck2* phenotype search. Wild-type W303 cells (WT), cells containing a disrupted *RCK2* gene (Ds), and cells containing a deleted *RCK2* gene (DI) were analysed under controlled conditions (see text). Both haploid and diploid cells were tested. Growth is represented by (✓) while no significant growth is represented by (X).

Different carbohydrates were used as sole carbon sources to assess their effects on the metabolism of the *RCK2* disrupted/deleted strains. This was achieved by replacing the 2% glucose, normally added to M63 defined yeast media (see section 2.1), with either 2% of galactose, raffinose, glycerol, sucrose or maltose (table 5.1). In addition, the effect of carbon starvation was also monitored. It was found that all cells grew well irrespective of the carbohydrate source used. Furthermore, cells grew well in media containing as little as 0.01% glucose. Preliminary results, therefore, failed to link the loss of *Rck2p* with carbon metabolism.

Effects of altered nitrogen metabolism

Saccharomyces cerevisiae cells grow equally well when the sole nitrogen source is an amino acid, purine derivative, urea or ammonia (Cooper, 1982). When logarithmically growing yeast are transferred from a rich medium to distilled water, they are able to divide for one to two generations indicating their ability to adapt to adverse conditions. Such adaptation is achieved by the shut down of protein synthesis, increased protein degradation, and the induction of a 'salvaging' pathway. The 'salvaging' pathway involves the oxidative degradation of the carbon skeletons of amino acids to compounds that can enter the tricarboxylic acid cycle (Cooper, 1982; Hinnebusch and Liebman, 1991).

The yeast nitrogen starvation response can be studied by reducing the concentrations of the required amino acids/nucleotides in the media (Hinnebusch and Liebman, 1991). Reduction in the total concentration of available nitrogen source present in the defined M63 yeast media (see section 2.1) affected both mutant and wild-type strains equally (table 5.1). All strains could grow in the presence of 4% of the routinely used concentration (0.002% w/v normally used), but failed to grow at 1% the normal concentration of amino acids, purine and pyrimidine present in defined yeast media.

Effects on the cell wall

The yeast cell wall is a dynamic organisation of carbohydrates, lipids and proteins providing a protective layer and structural integrity to the organism. Glucan, mannoprotein and chitin make up over 90% of the cell wall, whereas only a small amount of lipid was found in this structure (Ballou, 1982). Although the composition of cellular membranes remains relatively constant, the proportion of individual lipids varies according to temperature, nutrient supply, and growth rate. As implied in the introduction, phospholipids associated with the cell wall may play an essential role in signal transduction.

Growth on M63 defined media containing increasing concentrations of the ionic detergent SDS, was carried out to analyse the integrity of the cell membrane. Interestingly, initial studies suggested that in the presence of ~0.05% SDS, wild-type

cells were sensitive to the detergent while mutated cells were resistant (see table 5.1). However, this phenotype was somewhat inconsistent, and could not be reproduced in richer media (YPD; see section 2.1). It was possible that the uracil present in the minimal media became limiting for the uracil auxotrophic wild-type cells, while mutant cells containing a functional *URA3* gene were unaffected. Consequently, a yCP50 (see table 2.4) yeast centromeric plasmid containing a functional *URA3* gene was transformed into wild-type diploid cells. This strain was subsequently found to be resistant to 0.05% SDS on M63 medium displaying similar sensitivity to that of mutant cells. It is unclear why uracil starvation would lead to SDS sensitivity. An additional experiment to confirm the importance of uracil concentration in the media would be to simply supplement the media with a greater concentration of the purine. Although this method of maintaining uracil in the media is less controlled than that of the plasmid-based, it negates any effect of the plasmid itself on the phenotype of the yeast.

So as to analyse the integrity of the yeast cell wall/membrane further, a series of spot tests were carried out (see section 2.4.19b). A drop of substance, applied to agar plates, diffuses away from the initial point of contact and generates a gradient on which the yeast grow. A halo of non-growth around the added substance can be measured and used to assess the sensitivity of the yeast to this compound. This has proven to be a relatively reliable assay which provides quantitative results. Spots of SDS (10%), and the glucanase enzymes zymolyase (10mg/ml, with 1% β -mercaptoethanol), and β -glucuronidase, were added to plates containing cultures of either wild-type or mutated diploid strains on M63 medium. The halos on plates containing DDs, DDI and wild-type strains showed no significant differences with any of the substances tested. These results indicate that the structural integrity of the yeast envelope is unaffected by the *rck2* mutation under these conditions. In addition, the lack of an observed phenotype with the 10% SDS spot test further suggests that the apparent sensitivity of wild-type strains in defined media containing 0.05% SDS could be independent of the *rck2* mutations.

Effects of altered environmental conditions

The pH of the cell protoplasm is kept relatively constant at around pH7. Cells grown in markedly acidic or alkaline media die if they cannot adjust their internal pH. Many of the most important cellular components (nucleic acids, proteins, phospholipids) are acidic, basic or amphiprotic and a dramatic change in their ionisation state, due to altering the pH of their environment, may change their conformation and biological activity.

Wild-type and mutant strains were grown at various pH ranging from pH4.0 to pH10. Although there were no significant differences between the growth of wild-type and mutant strains, it was noticed that diploid cells were sensitive to pH9.0 and

above while haploids strains could still grow under these conditions. It is possible that the greater surface area:volume ratio of diploid cells exposes more pH-sensitive components, such as lipids and proteins, present within the cell surface. Both mutant and wild-type strains grew at 26°C and 30°C, although neither grew at 37°C.

Thermotolerant is a term used to describe cells which have been exposed to a brief, mild temperature rise and can subsequently endure a lethal heat shock. It is believed that the pre-treatment of cells to heat induces the stress-response genes and a subsequent exposure to excessive heat shock is consequently anticipated (Lindquist, 1986, Lindquist and Craig, 1988). It was possible that cells containing a mutant *rck2* would be more sensitive to heat shock, and/or unable to become thermotolerant. Consequently, thermotolerance and heat shock were tested in mutant and wild-type diploid strains.

Cells of each strain were incubated at 25°C to a Log phase ($OD_{600} \approx 0.1$). They were then divided into two; one half remained at 25°C (non-treated), while the other half was incubated at 37°C (pre-treated). After 90 mins, both pre-treated and non-treated cultures were shifted to 51°C. Samples of both non- and pre-treated cultures were taken after a number of time points. Samples were suitably diluted and spread onto YPD agar plates. The plates were incubated at 30°C for 24-48 hours after which the number of colonies were counted (figure 5.8).

The results indicate that: 1) an approximately 10-fold loss of viability occurred in all pre-treated cells after incubation at 51°C for 12 mins, when compared with time zero; 2) an approximately 100-fold loss of viability occurred in non-treated cells under the same conditions; 3) there was no significant differences between wild-type and mutant strains (figure 5.8). The results corroborate what is known about thermotolerance and heat shock *i.e.* exposure to excessive heat promotes rapid cell death, while pre-treatment with mild heat limits the lethality (Lindquist, 1986, Lindquist and Craig, 1988). There is no indication, therefore, that Rck2p is involved in the heat shock response, or in thermotolerance. Nevertheless, additional experiments with more frequent sampling over a greater time period and at a range of stress temperatures, could be informative.

5.7 OVEREXPRESSION OF Rck2p IN YEAST

Overexpressing a protein of interest can complement the phenotypic analysis of mutants defective in this polypeptide. A subsequent alteration of the intracellular protein concentration can have dramatic and revealing consequences. A receptor-like protein, for instance, when present in excessive quantities, could sequester its ligand and effectively nullify the function of the ligand. The overproduction of several cytoskeletal proteins such as tubulin, actin, and myosin, leads to cell lethality (F. Sweeney, PhD thesis; *MYO1* overproduction).

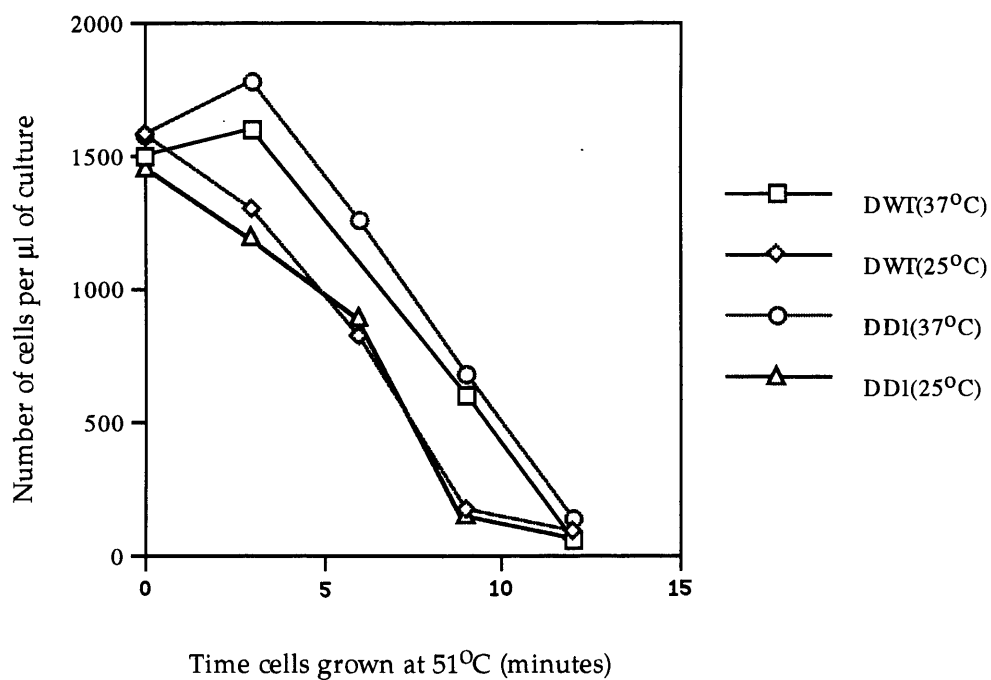


Figure 5.8

Thermotolerance of W303 diploid yeast cells. Two W303 diploid yeast strains were used for a thermotolerance assay; a wild-type strain (DWT), and a homozygous *rck2* deletion mutant (DD1). Cultures of diploid yeast were grown at 25°C until an absorbance of $OD_{600}=0.1$ was obtained. Each of the two cultures were then divided into two separate cultures, one of which was incubated for 90 minutes at 37°C, while the other remained at 25°C. The four cultures were subsequently transferred to 51°C and samples taken after 0, 3, 6, 9, and 12 minutes. Several dilutions of each sample were spread onto YPD agar and incubated at 30°C for 2-3 days. Cell numbers were then calculated. The graph represents data from a single experiment.

5.8 Cloning RCK2 Into pEMBLyex4

pEMBLyex4 is a commonly used shuttle vector for overexpression of proteins in yeast (figure 5.9). The plasmid contains both yeast and bacterial origins of replication and confers ampicillin resistance and uracil prototrophy for selection in both organisms. Transcription of the gene of interest occurs due to the hybrid promoter made up of the GAL upstream activator sequence (UAS) and non-translated leader region of the yeast *CYC1* gene. The UAS contains the intergenic region of the divergently transcribed *GAL1* and *GAL10* genes. Induction of transcription *via* this intergenic region occurs in the presence of galactose which overcomes the activity of the inhibitory protein, Gal80p, allowing the positively regulating Gal4p to function. The vector lacks a translational start ATG and consequently translation begins at the first ATG encountered in the fragment ligated into the polylinker.

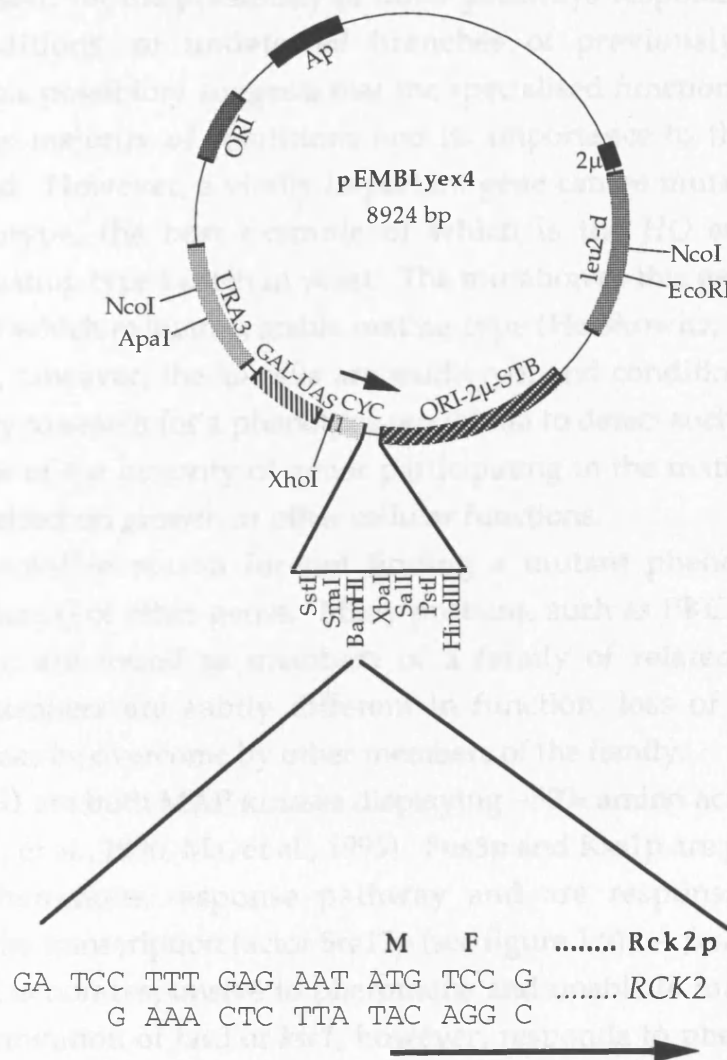
A *Bam*HI-*Pst*I fragment isolated from the plasmid pLJBAM (see table 2.4), containing the *RCK2* gene, was cloned into pEMBLyex4 (see section 6.2 for details of pLJBAM construction). The resulting construct was named pRCK2yex (see table 2.4). Prior to its cloning into pLJBAM this *Bam*HI-*Pst*I fragment had been modified by PCR to allow Rck2p to be expressed as a fusion protein in expression vectors (see section 6.1). This modification resulted in the loss of the first ATG of the *RCK2* ORF. However, a second ATG (encoding a methionine residue) occurs 6 residues downstream of the first ATG and it is this ATG that should be recognised for translational initiation by RNA polymerase in the pEMBLyex4 vector (figure 5.9).

Plasmids pEMBLyex4 and pRCK2yex were each transformed into an 842 diploid strain (see table 2.1) and the induction of transformants was assessed in the presence of galactose as a sole carbon source. Cultures containing either pEMBLyex4 or pRCK2yex appeared to grow similarly in both glucose- and galactose-containing media. Microscopical observation of pRCK2yex cells grown in either glucose- or galactose-containing media, and pEMBLyex4 cells grown under identical conditions, failed to detect any differences. In the absence of a detectable *RCK2* transcript by the Northern blot analysis (see section 4.18) or anti-Rck2p antibodies, it was impossible to confirm that *RCK2* was indeed transcribed in excess or that Rck2p was overproduced.

5.9 Discussion

The idea that *RCK2* is a redundant gene is hard to accommodate as non-functional genes would be expected to accumulate mutations. There are several other reasons why a phenotype may not be detected when *RCK2* is mutated, however. The first possibility is that Rck2p has a specialised function which is not induced under laboratory conditions. Yeast responds to a vast array of environmental stimuli in the wild, many of which can not be reproduced under experimental conditions, and

A



B

M D A F E N M F Rck2p
AAC AAA ATG GAT GCT TTT GAG AAT ATG TCC G RCK2
TTG TTT TAC CTA CGA AAA CTC TTA TAC AGG C

Figure 5.9

pRCK2yex construction. A) The *Bam*HI fragment from pLJBAM (see chapter 6) was cloned into the *Bam*HI site of pEMBLyex4. The construction of the *Bam*HI site of this fragment removed the first ATG of the *RCK2* ORF (B) but translation in pRCK2yex originated from a second ATG present 6 residues downstream (see text for details).

consequently, the role of some genes can be overlooked. Yeast signal transduction pathways which respond to osmotic stress, heat shock, and nutrient starvation have been well characterised, yet the possibility of other pathways responding to other environmental conditions, or undetected branches of previously described pathways, exists. This possibility suggests that the specialised function of Rck2p is redundant under the majority of conditions and its importance to the yeast cell should be questioned. However, a vitally important gene can be mutated without displaying a phenotype, the best example of which is the *HO* endonuclease responsible for the mating type switch in yeast. The mutation of this gene results in a heterothallic strain which exhibits a stable mating type (Herskowitz, et al., 1992). In all other respects, however, the *ho* cells are wild-type, and conditions routinely used in the laboratory to search for a phenotype would fail to detect such a mutation. Similarly, null alleles of the majority of genes participating in the mating pathway have no deleterious effect on growth or other cellular functions.

The second possible reason for not finding a mutant phenotype is the compensatory function(s) of other genes. Many proteins, such as PKC, G proteins, and phospholipases, are found as members of a family of related molecules. Although family members are subtly different in function, loss of one protein function can sometimes be overcome by other members of the family.

FUS3 and *KSS1* are both MAP kinases displaying ~50% amino acid identity to each other (Bardwell, et al., 1996, Ma, et al., 1995). Fus3p and Kss1p are positioned at the base of the pheromone response pathway and are responsible for the phosphorylation of the transcription factor Ste12p (see figure 1.9). A *fus3/kss1* double mutant is viable but is non-responsive to pheromone and unable to mate. A strain containing a single mutation of *fus3* or *kss1*, however, responds to pheromone and mates almost as a wild-type strain (Herskowitz, et al., 1992). Comparison of the completed yeast genome sequence with *RCK2* rules out the presence of homologous proteins/genes and thus the possibility of *RCK2* belonging to a family of homologous genes. However, it is possible that a closely related gene, homologous in function rather than sequence, could compensate for many of the functions, lost in an *RCK2* mutant cell, in which case a phenotype would be difficult to detect.

Additional phenotype analyses could include greater stress on the integrity of the yeast cell wall, as *PKC1* function has been linked to cell wall metabolism (see introduction). The use of different detergents in the defined yeast media could highlight a defect in the cell envelope components mediated by an *RCK2* mutation. Alternatively, changing the osmotic balance of the media could promote morphological defects if the cell wall is altered in some way. Experiments carried out to investigate inositol starvation of *Saccharomyces cerevisiae* have suggested that the lack of inositol prevents cell membrane expansion and eventually lead to cell death (Henry, et al., 1977). The importance of inositol phospholipids in signal

transduction is also well recognised. Analysis of the inositol requirements of *RCK2*-mutated cells could perhaps reveal an inositol-related function for this gene.

The thermotolerance experiment suggested that *RCK2* was not involved in heat shock response. Preliminary data also analysed the osmotolerance of *RCK2*-mutant cells. Log phase cells were subjected to osmotic shock of 1.4M NaCl and the number of surviving cells assessed. It was found that cells pre-treated with 0.7M NaCl were more tolerant to the stress than untreated cells, but that *RCK2*-mutated cells behaved identically to wild-type cells (data not shown).

A more focused analysis could include the production of synthetic mutations *i.e.*, introducing a mutated *RCK2* allele into strains already mutated for another locus. The most obvious candidates for such investigation would be strains mutated for components of known signal transduction pathway, particularly those involved in the *PKC1* pathway.

Rck2p association with Pkc1p may have been hypothesised to occur in *Saccharomyces cerevisiae* when considering the proposed and acknowledged functions of the proteins, respectively. The overexpression of Rck2p, *via* pRCK2yex, may have subsequently been expected to produce a phenotype similar to that observed with a deleterious *PKC1* mutation if Rck2p was to mop up Pkc1p within the cell. However, such a phenotype was not observed. The phenotypic consistency of mutants defective in components of the *PKC1* signal transduction pathway (including cell lysis and arrested cell division) indicates that *RCK2* may not be part of this pathway. However, *RCK2* may function on a branch of this pathway which is utilised less often under rarely encountered conditions.

The search for a phenotype did not seem to be pressing at this time as it was thought that the analysis of the Rck2p association with PKC would be more informative (see chapter 7).

CHAPTER 6.

CLONING OF RCK2 INTO EXPRESSION VECTORS

6.1 Introduction

The expression of cloned sequences is an important procedure for a geneticist as it allows a more direct analysis of a gene by focusing on its product. The controlled overexpression of a gene in its natural environment, in this instance yeast, may provide informative insights into its function. The recent development of expression systems which allow a protein of interest to be expressed as a fusion has provided another tool for the geneticist. The fused polypeptide can be utilised for its affinity purification, which is the prerequisite for several experimental strategies, especially for the production of antibodies. Furthermore, there are examples of fusion polypeptides which allow the protein of interest to be targeted to particular intracellular structures, *e.g.* to mitochondria. Others could provide a fluorescent tag for identifying the protein of interest in microscopical studies.

Affinity chromatography can be used for purifying overexpressed proteins (Phizicky and Fields, 1995). In this case, the bait protein is covalently coupled to a matrix contained within an affinity chromatography column. Ligand proteins present in an extract which is applied to the column are retained, whilst the non-binding proteins are washed through in low-salt buffers. The ligand proteins can then be released from the column in high-salt buffers, or *via* the use of cofactors or denaturing agents such as SDS and urea.

Affinity chromatography is a sensitive purification method, detecting the weakest protein-protein interaction which could be physiologically significant. Unlike immunoblotting techniques, affinity chromatography does not disrupt multisubunit interactions and therefore such associations can be detected. Care must be taken, therefore, to distinguish whether an observed interaction is a direct one or indirect *via* an intermediary protein. An additional consideration has to be the fact that proteins which appear to interact with high specificity may never encounter one another in the cell, as exemplified by the strong affinity between actin and DNase I (Phizicky and Fields, 1995).

6.2 CLONING RCK2 ORF INTO pUC19 VECTOR

The pMAL (New England Biolabs) and pGEX (Pharmacia) vectors were used for Rck2p expression (see table 2.4) as they provide a means of generating workable quantities of fusion proteins and allow their partial purification. Both vectors contained *Bam*HI cloning sites which were utilised for the cloning strategy. To make use of these sites and to ensure that the *RCK2* fusion creates a contiguous ORF with the respective bacterial genes (see below), the *RCK2* gene was manipulated using PCR amplification. A primer was designed so that the amino terminal end of *RCK2*

would be fused, in frame, to the required ORF in the vector. Care was taken to avoid any significant loss of coding sequence (figure 6.1). Substitution of cytosine by guanine generated a *Bam*HI site immediately downstream of the first ATG codon of *RCK2* (figure 6.1). In addition, for the cloning of *RCK2* into pDMR32 (see table 2.4) it was necessary to delete a thymine base downstream of the *Bam*HI site to retain the correct open reading frame of *MBP-RCK2*. A 30-mer oligonucleotide was designed accordingly. This primer, oLJBAM (see table 2.5), incorporated several features; an engineered *Bam*HI site, additional nucleotides surrounding the *Bam*HI site to increase the efficiency of digestion, the loss of a thymine base to preserve *RCK2* translation and additional nucleotides complementary to the template to ensure the efficient hybridisation of the primer during PCR (figure 6.1).

Using pRCK2-P (see table 2.4) as the template, oLJBAM and the commercially available forward pUC primer (see table 2.6) produced a PCR product of approximately 2.1kb which, when digested with *Bam*HI, could be cloned into a suitable vector. For several reasons it was advantageous to construct a recombinant pUC19 plasmid, pLJBAM (see table 2.4), as an intermediary, subsequent to the cloning of *RCK2* into the expression vectors. Firstly, it is a stable and easily manipulated source of DNA. Secondly, its high-copy number makes its amplification easy and negates the further use of PCR. Finally, the construct can be sequenced using universal primers should such data be necessary.

As the PCR product was designed to be used for the expression of *RCK2* it was obviously imperative that the fidelity of the amplification was maintained. Consequently, *Pfu* DNA polymerase, rather than *Taq* polymerase, was utilised in these cloning experiments (see section 4.7).

6.3 CLONING *RCK2* INTO THE pDMR32 EXPRESSION VECTOR

The pMAL expression system (New England Biolabs) allows a gene of interest to be expressed in large quantities in an *E. coli* host as a fusion protein with the maltose-binding protein (MBP). The expression is driven by the strong P_{tac} promoter and the translation initiation signals of the MBP (*malE* gene). The C-terminus of *MalE* is fused in frame to *lacZ*α (figure 6.2). MBP is a periplasmic protein essential for the energy-dependent translocation of maltose and maltodextrins through the cytoplasmic membrane of *E. coli* (Bankaitis, et al., 1984, Duplay, et al., 1984). An MBP fusion can be purified by its ability to bind an amylose resin. The *malE* signal sequence, required for membrane localisation, is missing from the vector pMAL-c2. This results in the cytoplasmic expression of the fusion protein. A spacer sequence encoding 10 asparagine residues, which increases the binding efficiency of the fusion protein to the amylose resin, was introduced between the MBP and the cloning site for the gene of interest. In addition, a sequence recognised by the protease Factor Xa (Ile-Glu-Gly-Arg) is present within the junction of the fusion, allowing the cleavage

A

Rck2p expression >>>>>										
		M	D	A	F	E	N	M	F	Peptide sequence
AAC	AAA	ATG	GAT	GCT	TTT	GAG	AAT	ATG	TCC	G
				:						
AAC	AAA	ATG	<u>GAT cC</u>	.	TTT	GAG	AAT	ATG	TCC	G
				<i>Bam</i> HI						
										Primer sequence

B

MBP				Rck2p					
X	X	X	S	F	E	N	M	F	
GAA	TTC	GGA	TcC	TTT	GAG	AAT	ATG	TCC	G
CTT	AAG	<u>CCT</u>	<u>AGG</u>	AAA	CTC	TTA	TAC	AGG	C
				<i>Bam</i> HI					

C

GST				Rck2p					
X	X	X	S	F	E	N	M	F	
CCG	CGT	GGA	TcC	TTT	GAG	AAT	ATG	TCC	G
GGC	GCA	<u>CCT</u>	<u>AGG</u>	AAA	CTC	TTA	TAC	AGG	C
				<i>Bam</i> HI					

D

pET				Rck2p					
X	X	X	S	F	E	N	M	F	
GGT	CGC	GGA	TcC	TTT	GAG	AAT	ATG	TCC	G
CCA	GCG	<u>CCT</u>	<u>AGG</u>	AAA	CTC	TTA	TAC	AGG	C
				<i>Bam</i> HI					

Figure 6.1

Strategy for cloning RCK2 into expression vectors. A PCR primer was designed which facilitated the subcloning of a single RCK2-containing fragment into 3 different expression vectors. By substituting a cytosine for a guanine a *Bam*HI site was created, and by omitting a thymine base the correct ORF was maintained (A). This primer (LJBAM) was used with universal pUC forward primer to amplify an RCK2-containing fragment from pRCK2-P. The PCR product was subsequently digested with *Bam*HI and subcloned into pDMR32 (see table 2.4) (B), pGEX-2T (see table 2.4) (C), and pET-3a (see table 2.4) (D) respectively. This strategy is predicted to lose only 3 amino acids normally encoded from the N-terminus of Rck2p (MDA).

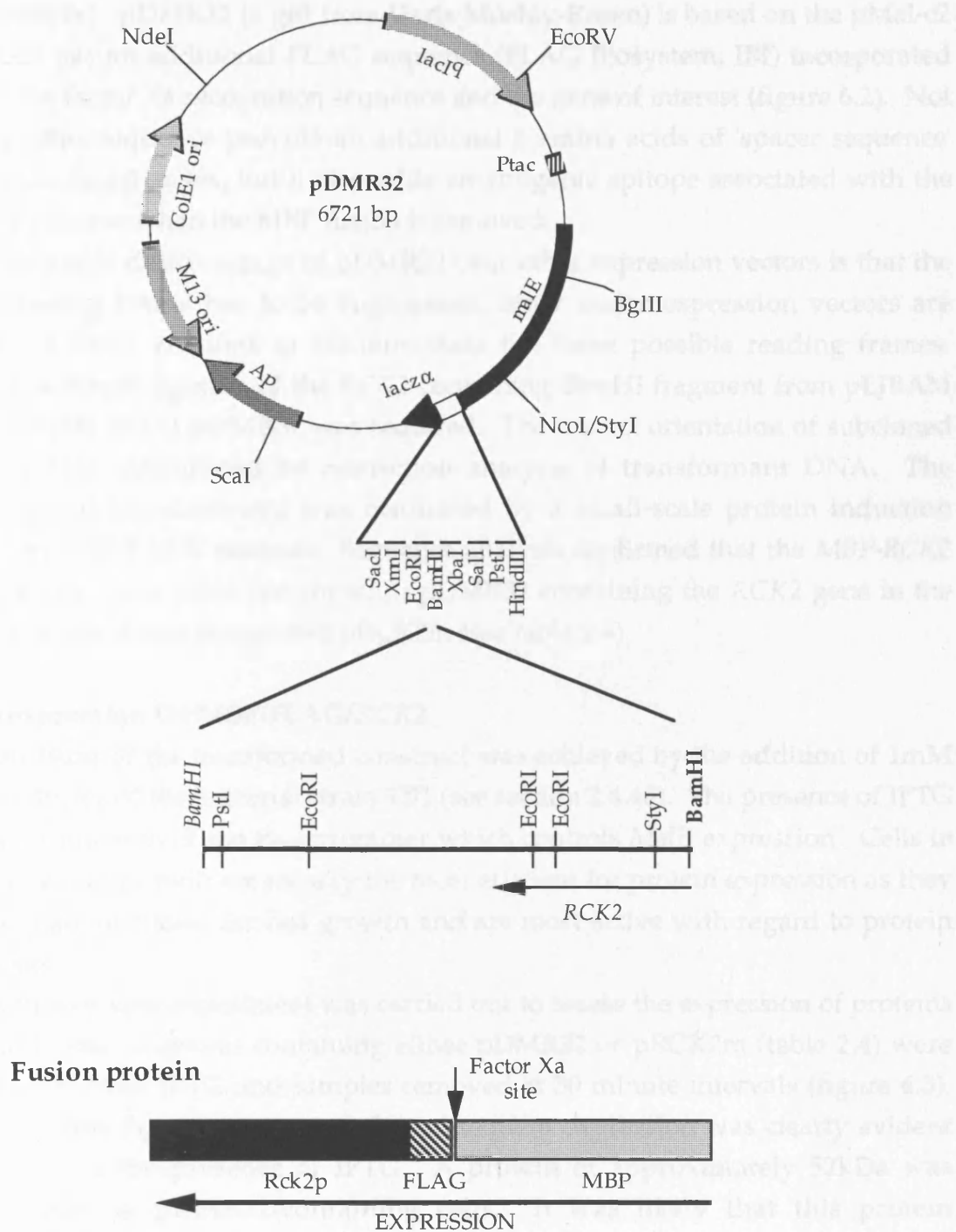


Figure 6.2

Construction of pRCK2m expression vector. A ~2.2kb *BamHI* fragment from plasmid pLJBAM (see table 2.4) was ligated into the *BamHI* fragment of pDMR32 (see table 2.4). This derived construct generated a continuous ORF containing: a) MBP polypeptide, b) FLAG epitope tag, c) Rck2p polypeptide, and d) a Factor Xa protease cleavage site. The construct was confirmed by restriction analysis, and sequencing data (see text).

of the protein of interest from the MBP (see New England Biolabs manual for details and references). pDMR32 (a gift from Daria Mochly-Rosen) is based on the pMal-c2 vector, but has an additional FLAG sequence (FLAG Biosystem, IBI) incorporated between the factor Xa recognition sequence and the gene of interest (figure 6.2). Not only does this sequence provide an additional 8 amino acids of 'spacer sequence' between the fused genes, but it also adds an antigenic epitope associated with the protein of interest when the MBP fusion is removed.

The major disadvantage of pDMR32 over other expression vectors is that the correct reading frame has to be engineered; other major expression vectors are available in three versions to accommodate the three possible reading frames. However, a simple ligation of the RCK2-containing *Bam*HI fragment from pLJBAM into the *Bam*HI site of pDMR32 was required. The correct orientation of subcloned fragments was determined by restriction analysis of transformant DNA. The authenticity of transformants was confirmed by a small-scale protein induction followed by SDS-PAGE analysis. Sequence analysis confirmed that the MBP-RCK2 junction was correct (data not shown). pDMR32 containing the RCK2 gene in the correct orientation was designated pRCK2m (see table 2.4).

6.4 Overexpression Of MBP/FLAG/RCK2

Overexpression of the transformed construct was achieved by the addition of 1mM IPTG to cultures of the bacterial strain TB1 (see section 2.4.46). The presence of IPTG allows the induction of the P_{tac} promoter which controls *MalE* expression. Cells in the Log phase of growth are usually the most efficient for protein expression as they have adequate nutrients for fast growth and are most active with regard to protein metabolism.

A time course experiment was carried out to assess the expression of proteins in induced cells. Cultures containing either pDMR32 or pRCK2m (table 2.4) were induced with 1mM IPTG and samples removed at 30 minute intervals (figure 6.3). Induction of the P_{tac} promoter and the subsequent expression was clearly evident after 1 hour in the presence of IPTG. A protein of approximately 52kDa was overexpressed in pDMR32-containing cells. It was likely that this protein represented the MBP-β-gal-α polypeptide (figure 6.2). A protein of approximately 75kDa was overexpressed in the pRCK2m-containing cells (figure 6.3), presumably representing the MBP-FLAG-Rck2p fusion; the approximate molecular mass values of MBP and Rck2p are 42.7kDa and 33kDa, respectively. The overexpression of both proteins continued throughout the 3 hours of the experiment; the overexpressed proteins displayed a greater proportion of the total protein content as time progressed (figure 6.3).

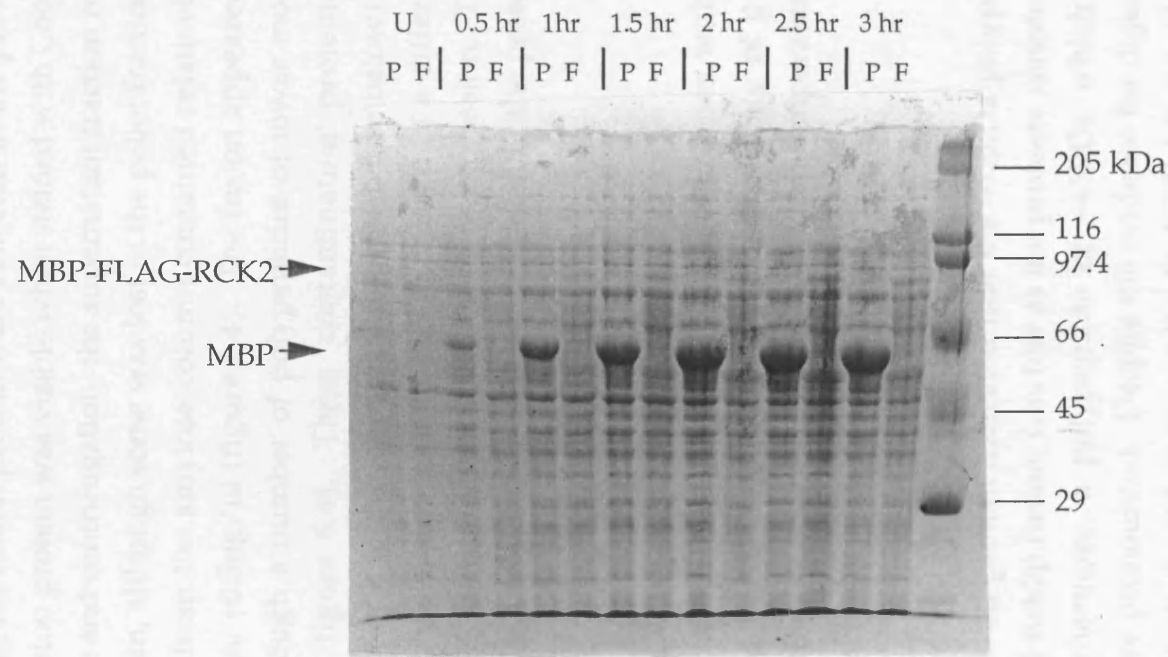


Figure 6.3

Time course of pRCK2m induction. Plasmid pRCK2m (see table 2.4) was transformed into a bacterial host (TB1). Cells were subsequently grown to $OD_{600}=0.6$ at $37^{\circ}C$ and then induced for 3 hrs with 1mM IPTG. Samples were taken from the induced culture at 30 minute intervals and analysed by SDS-PAGE. Cells containing pRCK2m (F) were compared with cells containing pDMR32 without an insert (P). Induction of each culture was clearly evident after 1 hour.

6.5 Purification Of MBP-FLAG-Rck2p Fusion Protein

A culture containing pRCK2m (table 2.4) was induced with 1mM IPTG for 3 hours at 37°C (see section 2.4.47). Cells were sonicated and then spun at 9000g for 30 minutes. Both the supernatant and pellet fractions were analysed by SDS-PAGE (figure 6.4). The supernatant fraction was then applied to an amylose agarose column and fractions containing the fusion protein were collected in the presence of maltose. MBP-FLAG-Rck2p fusion protein was visible when stained with Coomassie (figure 6.4). After cell breakage and centrifugation, the supernatant fraction retained the majority of the fusion protein, although some was lost in the pellet fraction (data not shown). Fractions eluted from the amylose column contained relatively high concentrations of fusion protein, ~0.5µg/µl (figure 6.4). The fusion appeared to be the major protein eluted, although a number of polypeptides of lower molecular mass could also be detected (figure 6.4). These 'contaminating' proteins were assumed to be degradation products of the larger fusion protein (see below). In an attempt to reduce degradation, the fusion protein was expressed in a different *lon* strain, BL21 (see table 2.2), which is routinely used for protein expression. The low molecular mass proteins could still be detected in this strain even in the presence of protease inhibitors.

6.6 Factor Xa Digest Of MBP-FLAG-Rck2p Fusion Protein

As described above, a Factor Xa protease recognition sequence lies within the junction between the MBP and FLAG domains. Approximately 5µg of purified protein was digested with dilutions of Factor Xa protease at room temperature for 3 hours:

(i) Coomassie stain analysis

SDS-PAGE analysis of the digested protein indicated that the ~75kDa polypeptide, presumed to be the MBP-FLAG-Rck2p fusion, was lost as the protease concentration increased (figure 6.5a). Furthermore, a polypeptide of ~43kDa which could represent the MBP, became more pronounced. Despite the evidence for digestion, a clear polypeptide of ~33kDa which should represent the FLAG-Rck2p could not be observed, although a diffuse smear was apparently generated by a number of similarly sized polypeptides (figure 6.5a). This data suggested that the Rck2p could be unstable when cleaved from the fusion. The low molecular mass 'contaminating' proteins in the purified fractions were of two types. The first type, represented by a prominent ~50kDa polypeptide, appeared to be digested by the protease and was consequently believed to be a degradation product of the MBP-FLAG-Rck2p fusion. The second type were polypeptides which remained unaffected in the presence of

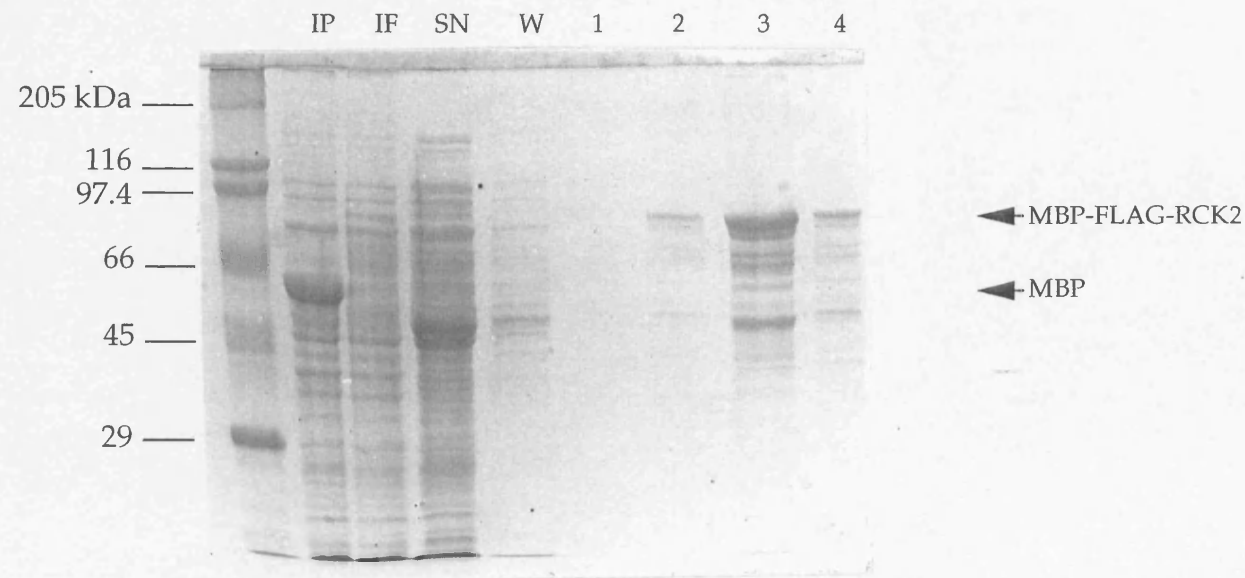
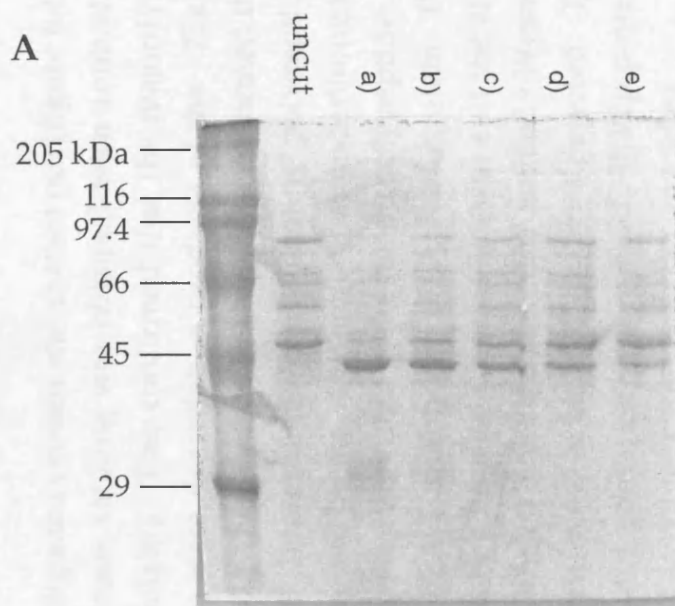


Figure 6.4

MBP-Rck2p purification. A 200ml culture of pRCK2m (see table 2.4) was induced with 1mM IPTG for 3hrs at 37°C. The cells were spun at 3000g for 15 mins and resuspended in 10mls of column buffer (see materials and methods). After freezing the cells at -20°C o/n and thawing at room temp., the cells were sonicated for 6X 30secs. The broken cells were spun down at 9000g for 30 mins and the supernatant kept. The SN was then added to an amylose column. 400ul fractions were eluted from the column with 10mM maltose. (IP), induced plasmid [pDMR32] without insert; (IF), induced fusion [pRCK2m]; (SN), supernatant before column; (W), washthrough of column before addition of 10mM maltose; (1-4), fractions eluted from column. The results suggest that good induction was achieved, and that the SN contained a good quantity of fusion protein. A negligible amount of fusion protein was lost during the washthrough procedure, whilst the eluted fractions contained a relatively high protein concentration. Several smaller protein bands were observed in these fractions, possibly degradation products of the fusion protein.

Figure 6.5

Digest and Westerns of MBP-FLAG-Rck2p (batch). Eluate containing the MBP-FLAG-Rck2p fusion protein was purified from amylose resin. 5 μ g of purified protein was digested with dilutions of Factor Xa protease a) 1 μ g, b) 0.5 μ g, c) 0.25 μ g, d) 0.13 μ g, e) 0.6 μ g. Digestion was carried out at room temp. for 3 hrs. **(A) Digested protein was analysed by SDS-PAGE.** The number of polypeptides observed, and the lack of a distinct FLAG-Rck2p polypeptide suggests degradation of the fusion protein (see text). Digested protein was transferred to nitrocellulose membrane for Western blot analysis. **(B)** Western of digested proteins using MBP primary antibodies (1:100,000) and goat anti-rabbit HRP as secondary IgGs (1:50,000), **(C)** Western using FLAG primary antibodies (1:100,000) and goat anti-mouse as secondary IgGs (1:50,000) with normal exposure and **(D)** overexposure. **Note that the acrylamide gel shown after staining with Coomassie stain was a replica of that used for the Western analysis. The same Western blot filter was used for both the MBP and FLAG antibodies (see materials and methods for stripping protocol).**

A

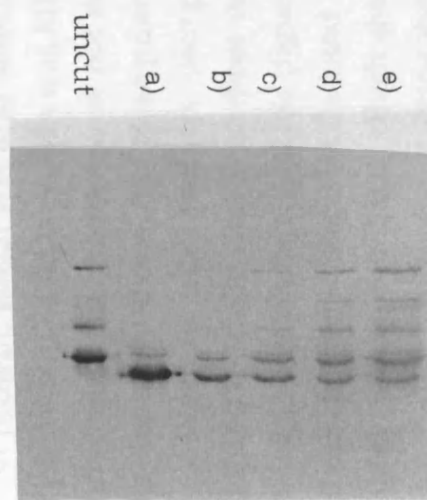
◀ MBP-FLAG-RCK2 ▶

◀ Breakdown ? ▶

◀ MBP ▶

◀ FLAG-RCK2 ? ▶

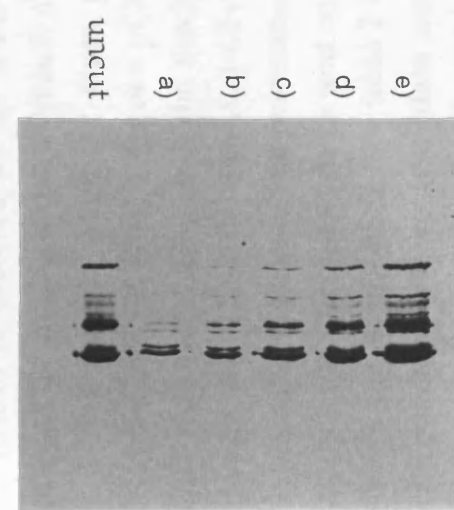
? ▶

B

◀ MBP-FLAG-RCK2 ▶

◀ Breakdown ? ▶

◀ MBP ▶

D**C**

the protease and could therefore represent unrelated proteins carried through the purification procedure (see chapter 7).

(ii) Western blot analysis

Western blot analysis of the Factor Xa digested protein was carried out (figure 6.4b). A profile mirroring that of the Coomassie staining was obtained with antibodies raised against MBP (New England Biolabs). This confirmed that the majority of polypeptides of lower molecular mass were degradation products of the ~75kDa fusion protein, which was also detected. The Western analysis also confirmed that the prominent ~43kDa protein was MBP. Interestingly, 'contaminating' polypeptides unaffected by the protease were not detected by the MBP antibodies underlining the suggestion (see above) that these proteins are unrelated to the fusion polypeptide.

Western blot analysis using FLAG antibodies (FLAG Biosystem, IBI) produced a profile similar to those described for the Coomassie stain and the anti-MBP antibody Western analysis (figure 6.5c). Unlike the anti-MBP antibody Western analysis, however, the ~43kDa band, presumed to be MBP, was not detected. The ~33kDa polypeptide band, which should have represented a FLAG-Rck2p protein, was also absent further suggesting that this polypeptide is rapidly degraded.

6.7 CLONING RCK2 INTO pGEX-2T EXPRESSION VECTOR

Several expression vectors which direct the synthesis of foreign polypeptides as C terminal fusions with Sj26 in *E. coli* have recently been constructed (Smith and Johnson, 1988). Sj26 is a 26kDa glutathione *S*-transferase (GST) encoded by the parasitic helminth *Schistosoma japonicum*. A variety of pGEX vectors (Pharmacia), approximately 4.9kb in size, provide three open reading frames necessary for the successful translation of a foreign gene as a GST fusion protein. Transcription of the GST fusion is directed by an IPTG-inducible *tac* promoter which is efficiently repressed by an over-expressed *lacIq* allele of the *lac* repressor.

The *Bam*HI fragment from pLJBAM (table 2.4) containing *RCK2* was subcloned into the *Bam*HI site of a pGEX-2T vector (figure 6.6). This vector produced a GST fusion in frame with the Rck2p polypeptide, retaining a thrombin protease cleavage site (Leu-Val-Pro-Arg-Gly-Ser) within the junction between the two polypeptide moieties. The orientation of the subcloned fragment was determined initially by restriction analysis and confirmed by overexpressing the fusion proteins in the *E. coli* strain, BL21 (see table 2.2). Sequence analysis confirmed that the GST-*RCK2* junction was correct (data not shown). Plasmids producing a GST-Rck2p fusion were designated pRCK2g (table 2.4; figure 6.6).

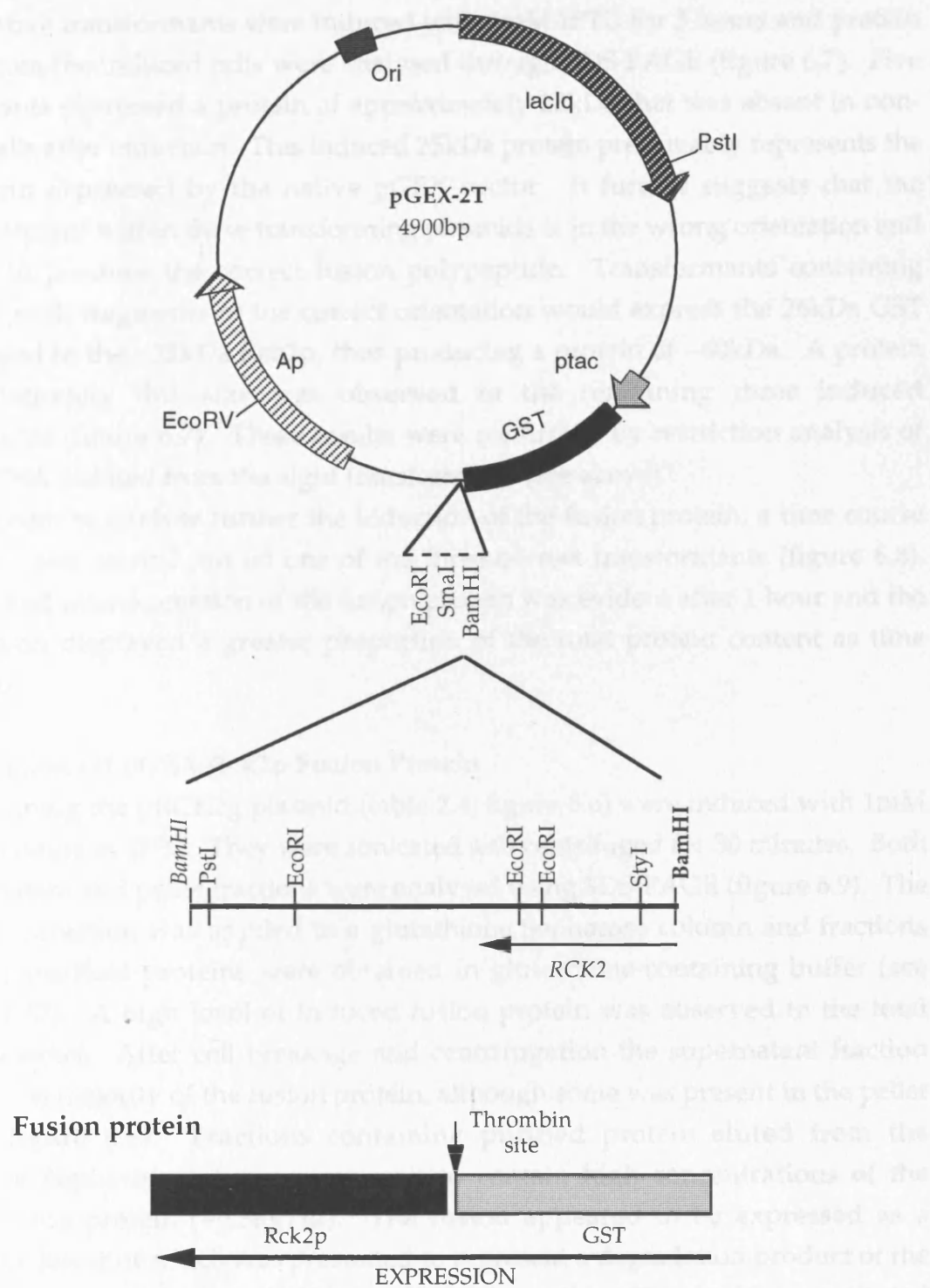


Figure 6.6

Construction of pRCK2g expression vector. A ~2.2kb *BamHI* fragment from plasmid pLJBAM (see table 2.4) was ligated into the *BamHI* fragment of pGEX-2T (see table 2.4). This derived construct generated a continuous ORF containing: a) GST polypeptide, b) Rck2p polypeptide, and c) a Thrombin protease cleavage site. The construct was confirmed by restriction analysis, and sequencing data (see text).

6.8 Overexpression Of GST-Rck2p

Eight putative transformants were induced with 1mM IPTG for 3 hours and protein samples from the induced cells were analysed through SDS-PAGE (figure 6.7). Five transformants expressed a protein of approximately 25kDa that was absent in non-induced cells after induction. This induced 25kDa protein presumably represents the GST protein expressed by the native pGEX vector. It further suggests that the fragment present within these transforming plasmids is in the wrong orientation and is unable to produce the correct fusion polypeptide. Transformants containing constructs with fragments in the correct orientation would express the 26kDa GST protein fused to the ~33kDa Rck2p, thus producing a protein of ~60kDa. A protein of approximately this size was observed in the remaining three induced transformants (figure 6.7). These results were confirmed by restriction analysis of plasmid DNA isolated from the eight transformants (see above).

In order to analyse further the induction of the fusion protein, a time course experiment was carried out on one of the three correct transformants (figure 6.8). Induction and overexpression of the fusion protein was evident after 1 hour and the 60kDa fusion displayed a greater proportion of the total protein content as time progressed.

6.9 Purification Of pGEX-Rck2p Fusion Protein

Cells containing the pRCK2g plasmid (table 2.4; figure 6.6) were induced with 1mM IPTG for 3 hours at 37°C. They were sonicated and centrifuged for 30 minutes. Both the supernatant and pellet fractions were analysed using SDS-PAGE (figure 6.9). The supernatant fraction was applied to a glutathione Sepharose column and fractions containing purified proteins were obtained in glutathione-containing buffer (see section 2.4.47). A high level of induced fusion protein was observed in the total bacterial extract. After cell breakage and centrifugation the supernatant fraction contained the majority of the fusion protein, although some was present in the pellet fraction (figure 6.9). Fractions containing purified protein eluted from the glutathione Sepharose column were seen to contain high concentrations of the ~60kDa fusion protein (~0.5µg/µl). The fusion appeared to be expressed as a doublet, the lower of which was presumed to represent a degradation product of the ~60kDa fusion protein into a slightly smaller polypeptide. The doublet constituted the major proteins eluted, although a number of lower molecular mass polypeptides could also be observed. These proteins were assumed to be degradation products of the larger fusion proteins (see below).

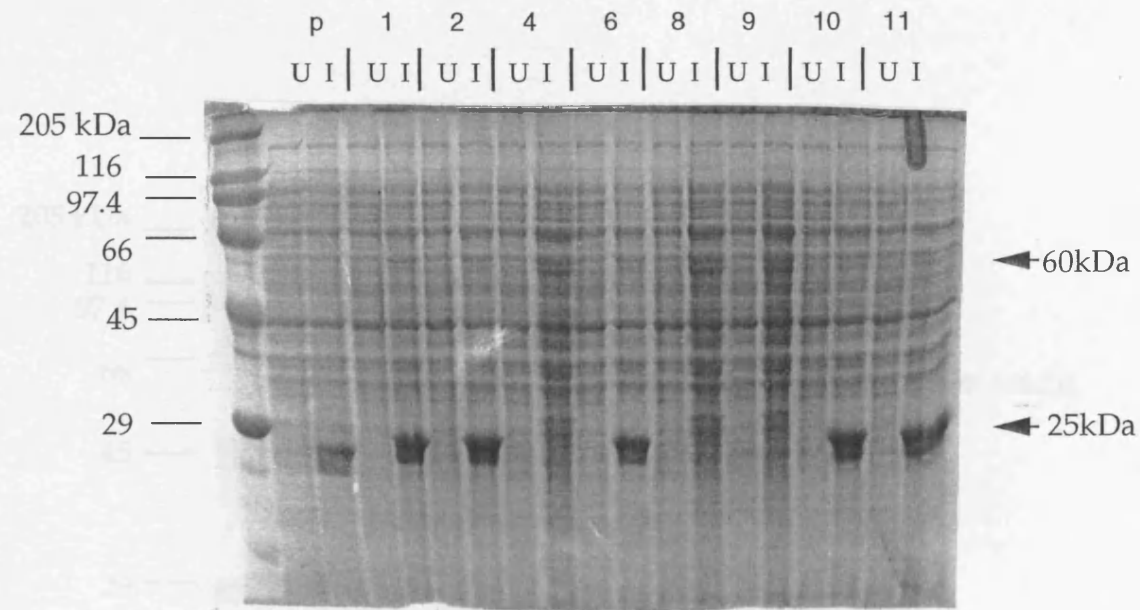


Figure 6.7

Induction of pRCK2g fusion constructs. The eight transformants containing inserts were induced with 1mM IPTG for 3 hrs at 37°C. Induced cells were resuspended in SDS sample buffer and ran on a 12% SDS polyacrylamide gel. Plasmid control (p) was pGEX-2T without an insert, and induced (I) and uninduced (U) samples were run side by side for each construct. Constructs 4, 8, and 9 all contain an inducible protein band of approximately 60kDa thought to be the GST-Rck2p fusion polypeptide. The remaining constructs have an inducible protein band of approximately 25kDa, thought to be the GST polypeptide alone.

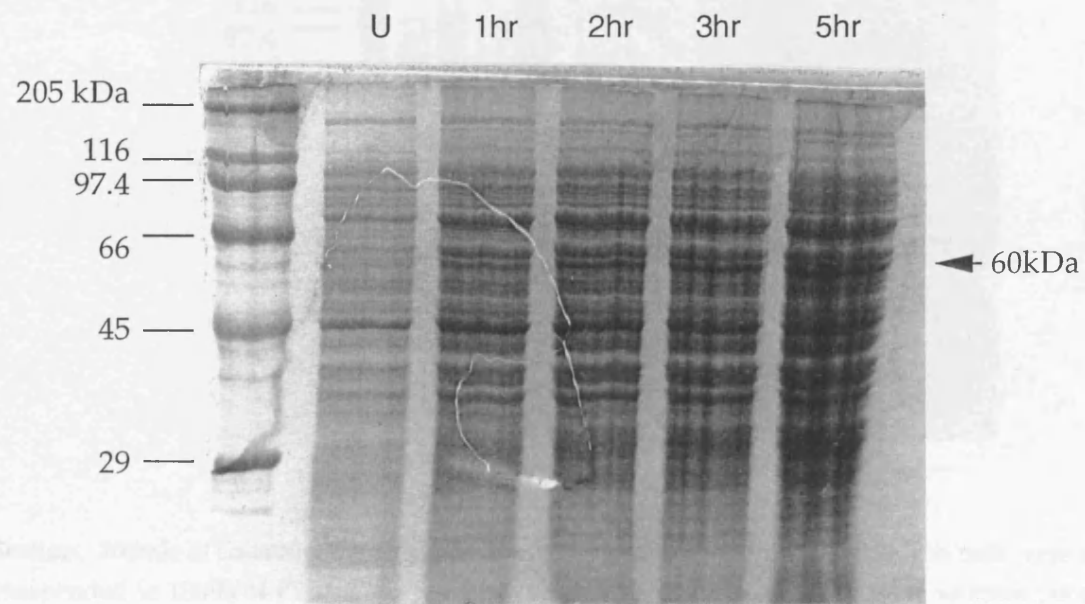


Figure 6.8

Time course of pRCK2g induction. Bacterial cells (TB1) containing the correct pRCK2g construct (see table 2.4) were induced for 5 hrs with 1mM IPTG at 37°C and samples taken at intervals. Induction was clearly evident after 1hr as compared with the uninduced (U) control, and the induced protein appears to become a greater proportion of the total protein content as time progressed.

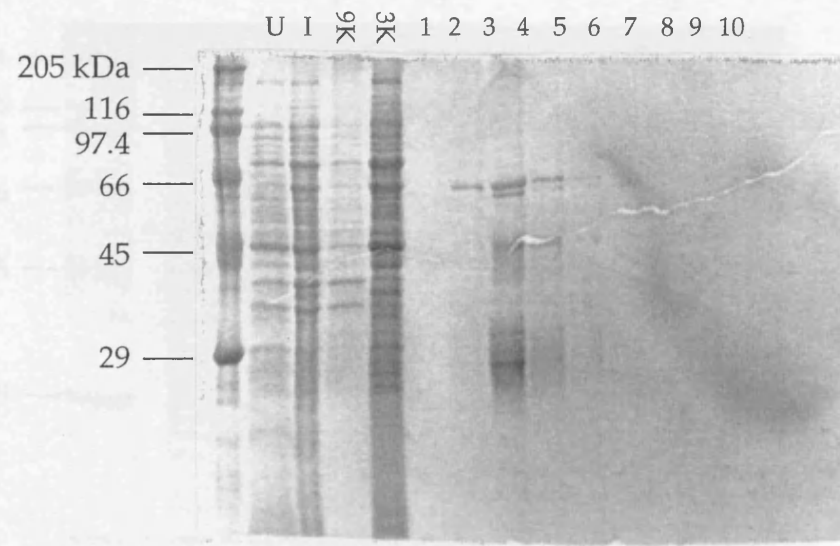


Figure 6.9a

pRCK2g purification. 200mls of construct 9 was induced with 1mM IPTG for 3hrs at 37°C. The cells were spun at 3000g for 15 mins and resuspended in 10mls of PBS. After freezing the cells at -20°C o/n and thawing at room temp., the cells were sonicated for 6X 30secs. The broken cells were spun down at 9000g for 30 mins and the supernatant kept (9K). The 9000g pellet was resuspended in 5mls PBS and subjected to a 3000g spin for 30 mins. The supernatant from this spin (3K) was also kept. Both SN (9K and 3K) were then applied to a glutathione sepharose column. 500ul fractions were eluted from the column with 10mM glutathione and a sample (5ul) run on SDS-PAGE, along with uninduced (U) and induced (I) total bacterial cell extract, and samples of the 9K and 3K SNs. The bacterial extracts show a good level of induction. Both the 9K and 3K spins release a proportion of protein from the pellet to the SN, including the induced 60kDa protein. Fractions 2-4 inclusive from the glutathione sepharose column contain a high quantity of 60kDa protein along with several smaller proteins, possibly degradation products.

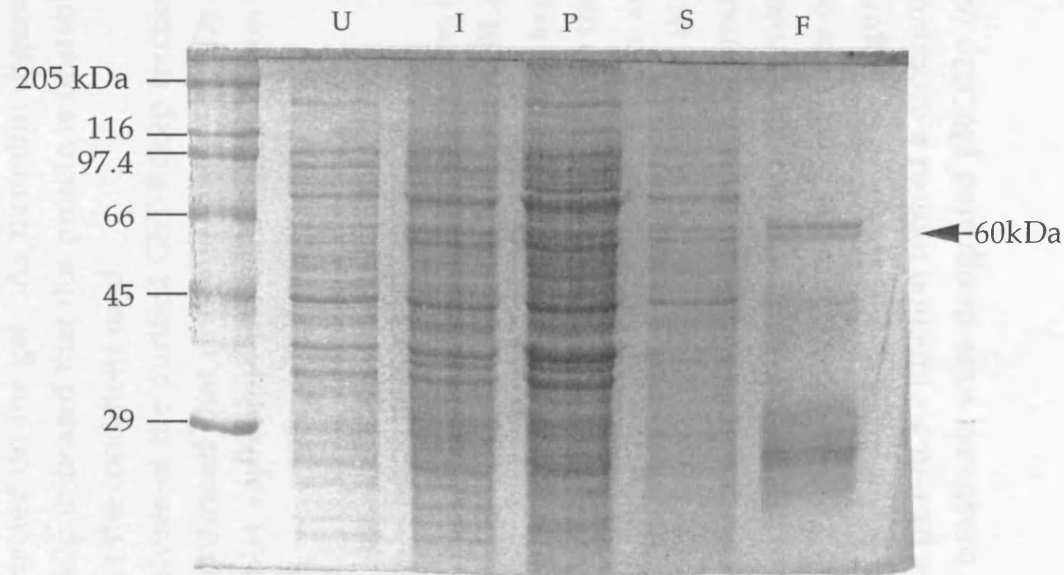


Figure 6.9b

pGEX-RCK2 purification. 200mls of a culture containing pRCK2g was induced with 1mM IPTG for 3hrs at 37°C. The cells were spun at 3000g for 15 mins and resuspended in 10mls of PBS. After freezing the cells at -20°C o/n and thawing at room temp., they were sonicated for 6X 30secs. The broken cells were spun at 9000g for 30 mins and the SN added to a glutathione sepharose column. 500ul fractions were eluted from the column with 10mM glutathione. Uninduced bacterial extract (U), induced bacterial extract (I), pellet obtained from 9000g spin (P), 9000g spin supernatant (S), and fraction number 3 from the column (F) were run on SDS-PAGE. The gel shows that induction of the pGEX-RCK2 fusion gives rise to a doublet of approximately 60kDa which is found in both the pellet and SN fractions after a 9000g spin. This doublet is also purified from a glutathione column along with other smaller proteins/degradation products.

6.10 Digestion Of GST-Rck2p Fusion Protein With Thrombin Protease

Digestion of the purified GST-Rck2p fusion protein was carried out as described by Smith and colleagues (Smith and Johnson, 1988). Approximately 5µg of fusion protein was digested with 100ng of thrombin for up to 4¹/₂ hours at room temperature. No significant digestion of the upper ~60kDa protein was noticed and only the lower ~60kDa protein of the doublet was affected (figure 6.10). Subsequent studies indicated that the lower ~60kDa molecular mass protein is, in fact, the intact GST-Rck2p fusion while the larger ~60kDa protein is of bacterial origin (see chapter 7). No ~33kDa Rck2p could be detected on the gel. As thrombin digestion was effective, the lack of the ~33kDa Rck2p indicated that this protein is unstable when cleaved from the fusion protein under the conditions used.

Subsequent Western blot analyses of the purified GST-Rck2p fractions with anti-GST antibodies (Pharmacia) confirmed that only the lower ~60kDa protein associated with the observed doublet represents the GST-Rck2p fusion protein (figure 6.11b).

6.11 CLONING RCK2 INTO pET3a EXPRESSION VECTOR

The pET vectors (table 2.4; New England Biolabs) allow protein expression in *E. coli* using a phage T7 promoter. Induction of a suitable bacterial host strain (BL21 [DE3] see table 2.2) containing an integrated phage T7 RNA polymerase allows expression of the gene of interest independently of the host RNA polymerase. Unlike the pMAL and pGEX vectors (see table 2.4), proteins expressed from the pET vectors are fused to a very short polypeptide moiety (only several amino acids). Although this fusion does not provide a means for the purification of an induced protein, it produces an almost unmodified polypeptide which may better represent the native protein. The *Bam*HI fragment from pLJBAM (table 2.4) was ligated into the *Bam*HI site of pET3a (table 2.4), a vector which is suitable for cloning the *RCK2* ORF (figure 6.12). Sequence analysis confirmed that the pET-RCK2 junction formed a continuous ORF (data not shown). Correct plasmid constructs were designated pRCK2p (see table 2.4; figure 6.12).

6.12 Induction Of pET-RCK2

IPTG induction of loga]rithmic phase BL21 (DE3) and subsequent expression of the T7 RNA polymerase, failed to produce a detectable Rck2p protein (data not shown). The addition of the antibiotic rifampicin to the induced culture inhibits the bacterial RNA polymerase and hence reduces the host polypeptide synthesis. However, overexpressed Rck2p was not detected even in the presence of rifampicin. An unrelated pET3a construct (yeast myosin) grown and induced under similar conditions (effectively a positive control) showed significant overexpression,

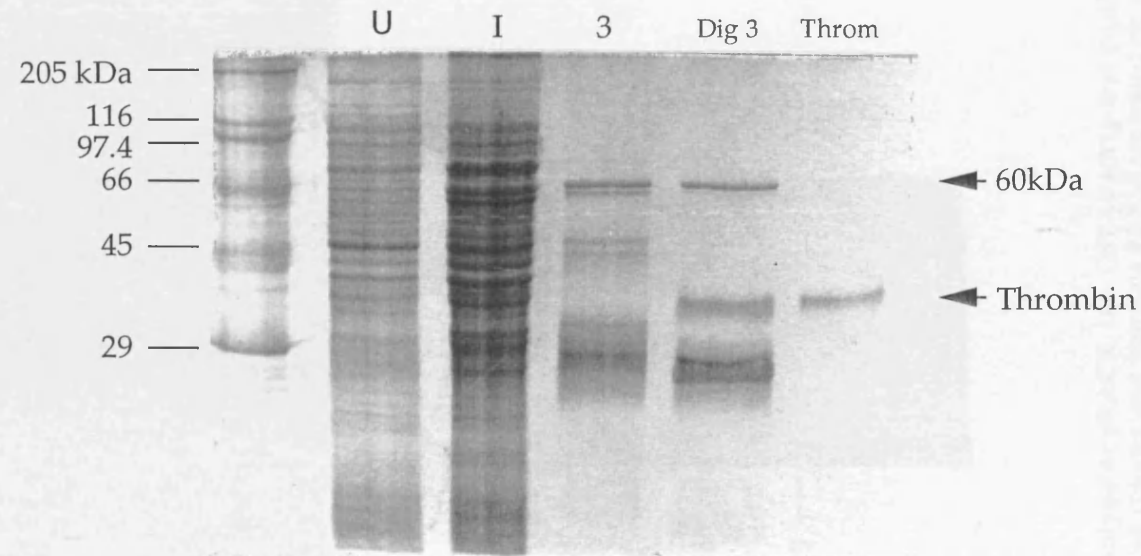


Figure 6.10

Digestion of pRCK2g protein with thrombin protease. Fraction 3 from the glutathione sepharose column was subjected to digestion o/n with thrombin (100ug) at room temp. Both undigested (3) and digested (Dig 3) samples were analysed using SDS-PAGE, along with a sample of thrombin protein (Throm) and uninduced (U) and induced (I) bacterial cell extracts. From the gel it is apparent that the 60kDa protein which is induced and purified from a column, remains undigested after the treatment with thrombin. Only the smaller contaminating proteins, which are possibly degradation products of the 60kDa protein, appear to be digested.

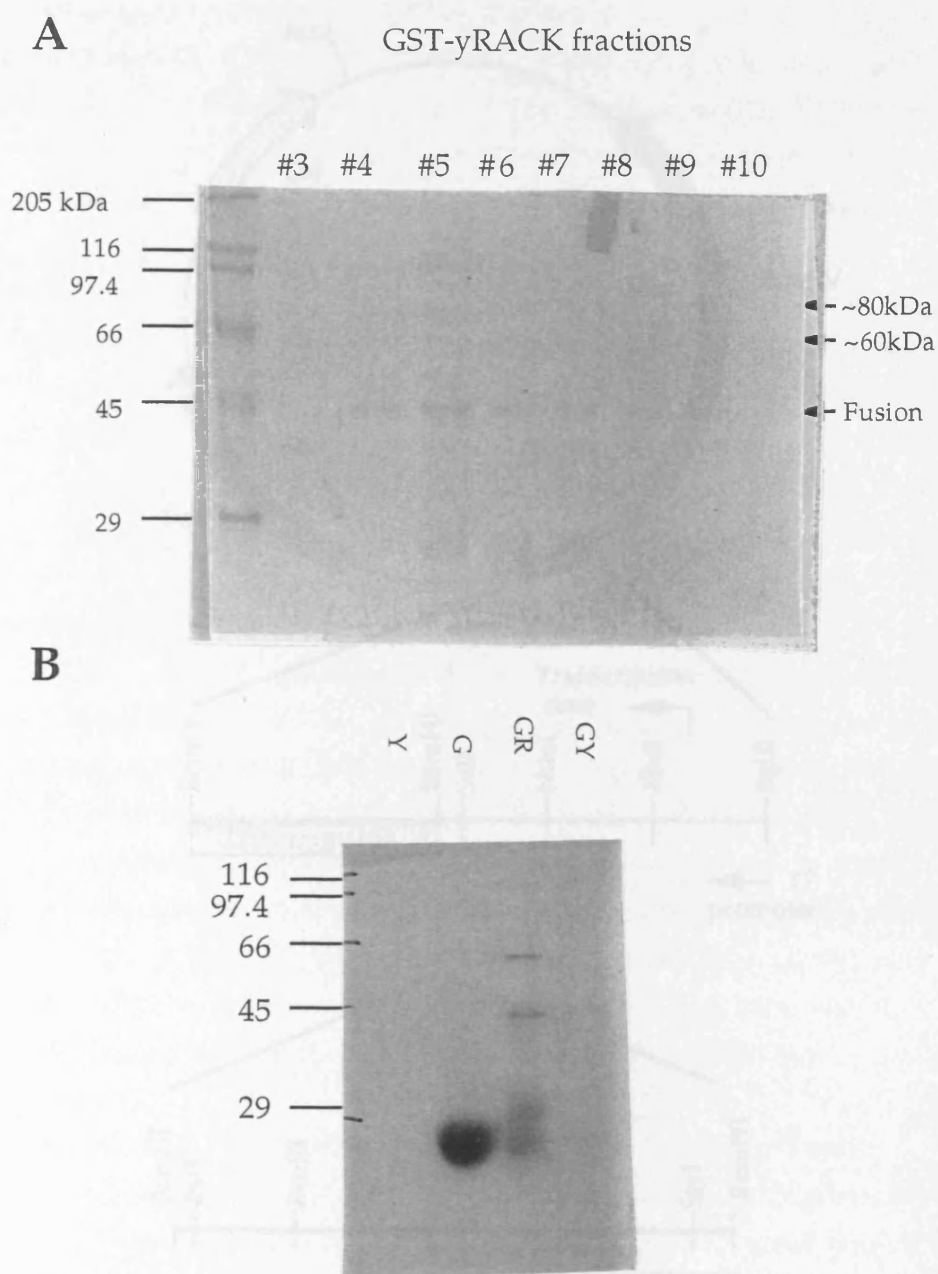


Figure 6.11

Purification and analysis of yRACK. (A) GST-yRack1p was purified from a glutathione sepharose column and fractions #3-#10 separated on a 12% SDS-PAGE gel. A prominent band of ~45kDa represented the fusion protein. Protein bands of ~60- and ~80kDa were also observed (see text). A fraction of purified GST-yRACK was subsequently used for Western blot analysis. (B) A Western blot was carried out on both GST-Rck2p and GST-yRack1p fusions with anti-GST antibodies. Y=Y1090 bacterial extract; G=GST without fusion; GR=GST-Rck2p fusion; GY=GST-yRack1p fusion. GST antibodies were used at 1:500, rabbit anti-goat secondary IgGs used at 1:20,000.

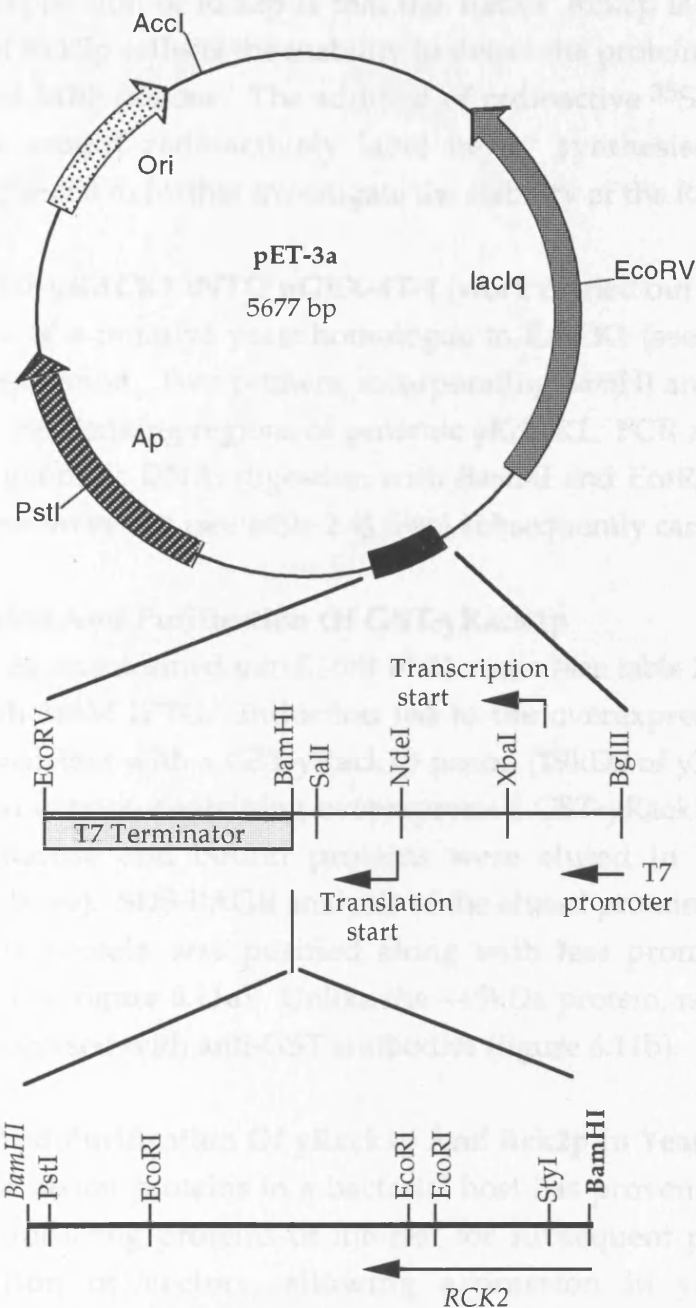


Figure 6.12

Construction of pRCK2p expression vector. A ~2.2kb *Bam*HI fragment from plasmid pLJBAM (see table 2.4) was ligated into the *Bam*HI fragment of pET3a (see table 2.4). The construct was confirmed by restriction analysis and sequencing data. pET constructs are transformed into a bacterial host (BL21) which has a λ DE3 prophage containing an IPTG inducible T7 polymerase gene. Induction of this polymerase promotes transcription of the pET plasmid *via* a T7 promoter present upstream of the cloning site. The Rck2p is generated as a fusion with fourteen pET-derived amino acids. The construct was confirmed by restriction analysis, and sequencing data.

however. The most likely explanation (although other possibilities exist) to the lack of a detectable expression of Rck2p is that the 'native' Rck2p is rapidly degraded. This instability of Rck2p reflects the inability to detect the protein when it is cleaved from the GST and MBP fusions. The addition of radioactive ³⁵S-methionine to the induced culture would radioactively label newly synthesised protein. This procedure should assist to further investigate the stability of the Rck2p protein.

6.13 CLONING OF *yRACK1* INTO pGEX-4T-1 (work carried out by Dr E. Orr)

The identification of a putative yeast homologue to RACK1 (see section 3.5) called for a further investigation. Two primers, incorporating *Bam*HI and *Eco*RI sites, were designed against the flanking regions of genomic *yRACK1*. PCR amplification of the gene from yeast genomic DNA, digestion with *Bam*HI and *Eco*RI, and cloning into pGEX-4T-1 expression vector (see table 2.4) were subsequently carried out.

6.14 Overexpression And Purification Of GST-yRack1p

pGEX-*yRACK1* was transformed into *E. coli* BL21 strain (see table 2.2) and expression was induced with 1mM IPTG. Induction led to the overexpression of a ~45kDa protein, a size consistent with a GST-yRack1p fusion (19kDa of yRack1p and 26kDa of GST). Bacterial extract, containing overexpressed GST-yRack1p, was applied to glutathione Sepharose and bound proteins were eluted in buffer containing glutathione (see above). SDS-PAGE analysis of the eluted proteins revealed that the abundant ~45kDa protein was purified along with less prominent proteins of ~60kDa and ~80kDa (figure 6.11a). Unlike the ~45kDa protein, none of these larger proteins were recognised with anti-GST antibodies (figure 6.11b).

6.15 Expression And Purification Of yRack1p And Rck2p In Yeast

The expression of fusion proteins in a bacterial host has proven to be a successful method of overproducing proteins of interest for subsequent purifications. The recent construction of vectors, allowing expression in yeast, provides a physiologically more realistic environment for eukaryotic protein expression, particularly when post-translational modifications occur. In addition, cloning of genes under the control of an inducible promoter may produce an observable phenotype.

6.16 Cloning Of *yRACK1* And *RCK2* Into pEG(KT)

Two vectors based on high-copy, galactose inducible shuttle vectors were constructed which allowed expression of a GST fusion in *Saccharomyces cerevisiae* (Mitchell, et al., 1993). Fortunately, the *Bam*HI fragment containing *RCK2* from pRCK2g (figure 6.6) and a *Bam*HI-*Sal*I fragment from pGEX-*yRACK1* could both be cloned directly into pEG(KT) (figure 6.13). These clonings resulted in Rck2p and

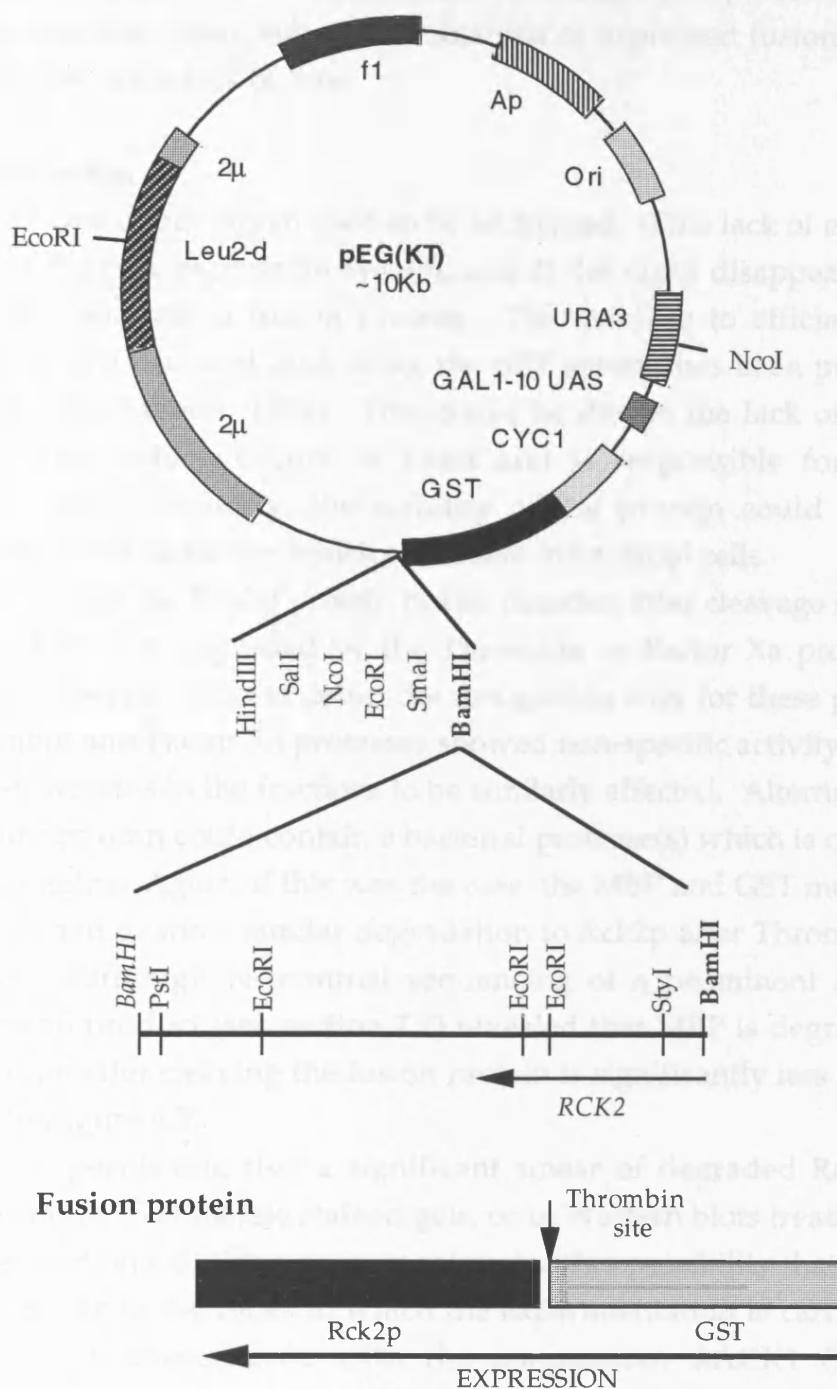


Figure 6.13

Construction of pEG/RCK2 yeast expression vector. A ~2.2kb *Bam*HI fragment from plasmid pLJBAM (see table 2.4) was ligated into the *Bam*HI fragment of pEG(KT) (table 2.4). The derived construct generated a continuous ORF containing: a) GST polypeptide, b) Rck2p polypeptide, and c) a Thrombin protease cleavage site. This construct, unlike pRCK2g (see figure 6.6), expresses a GST-Rck2p fusion in a yeast host. A *Bam*HI-*Sal*I fragment containing *yRACK1* was also ligated into this vector (not shown).

yRack1 being fused in frame to the GST moiety (figure 6.13). Restriction analysis confirmed correct vector constructs. Although the pEG(KT) constructs were transformed into yeast, subsequent analysis of expressed fusion proteins could not proceed due to the lack of time.

6.17 Discussion

There are two issues which need to be addressed: 1) the lack of expression *in vivo* of Rck2p in the pET expression system, and 2) the rapid disappearance of the Rck2p when cleaved from a fusion protein. The inability to efficiently express yeast sequences in a bacterial host using the pET system has been previously observed (Donnelly PhD thesis, 1993). This could be due to the lack of post-translational modification, which occurs in yeast and is responsible for a stable protein conformation. Similarly, the stability of the protein could be maintained by associated yeast co-factors which are absent in bacterial cells.

Why can the Rck2p protein not be detected after cleavage from a fusion? It is possible that it is degraded by the Thrombin or Factor Xa proteases. However, sequence analysis failed to detect the recognition sites for these proteases in Rck2p. If Thrombin and Factor Xa proteases showed non-specific activity, one would expect the other proteins in the fractions to be similarly affected. Alternatively, eluate from an affinity column could contain a bacterial protease(s) which is co-purified with the fusion proteins. Again, if this was the case, the MBP and GST moieties should have been expected to show similar degradation to Rck2p after Thrombin and Factor Xa cleavage. Although N-terminal sequencing of a prominent MBP-FLAG-Rck2p degradation product (see section 7.7) revealed that MBP is degraded, the extent of degradation after cleaving the fusion protein is significantly less than that observed for Rck2p (figure 6.5).

It is perplexing that a significant smear of degraded Rck2p could not be detected in the Commassie stained gels, or in Western blots treated with anti-FLAG IgGs (see section 6.6). This concern raises another possibility that the cleaved Rck2p proteins sticks to the tubes in which the experimentation is carried out. A similar problem was encountered with the mammalian RACK1 (D. Ron, personal communication). This problem may be solved by the addition of an unfolding reagent to the tubes, such as urea.

Western blot analysis of purified eluate from an amylose column indicated that the majority of polypeptides observed by Coomassie stain were breakdown products of MBP-Rck2p. However, despite thorough washing of the column before elution, several additional polypeptides were also observed. These polypeptides were not detected by anti-MBP antibodies and were resistant to the digestion by Factor Xa protease. The relatively tight association of Rck2p with similar polypeptides perhaps indicates that the interaction is physiologically significant.

Furthermore, the indication that these polypeptides are associated with both the GST-Rck2p and GST-yRack1p further points to some kind of protein-protein interaction (see chapter 7).

CHAPTER 7.

Rck2p ASSOCIATION WITH OTHER PROTEINS

7.1 Introduction

The Rck2p fusion proteins, described in chapter 6, made the study of protein interactions possible. One major aim of this project was to identify yeast proteins associating with Rck2p. It was also necessary to confirm the association of Rck2p with mammalian PKC. Previous overlay assay experiments showed that an MBP-RACK1 fusion protein failed to interact with exogenous mammalian PKC (Mochly-Rosen et al, personal communication). This lack of interaction was probably due to the MBP moiety interfering with the natural confirmation of RACK1, thus inhibiting its normal binding to PKC. It was noticed, however, that when bound to amylose resin, the MBP-RACK1 fusion protein was able to bind PKC in the presence of PKC activators. Under these conditions, the MBP moiety of the fusion is presumed to be tightly associated with the amylose matrix and therefore unable to inhibit the RACK1 interaction with PKC. This amylose-based association was consequently adopted to study the ability of the MBP-Rck2p fusion protein to bind other proteins from either yeast or mammalian extracts.

7.2 Western Blot Analysis Of MBP-Rck2p Column Eluate

Rck2p was originally identified as a polypeptide which bound mammalian PKC. A amylose-based assay was carried out to re-establish this interaction. Rat brain cytosol, in the presence and absence of PKC activators, was introduced to amylose resin bound to MBP-Rck2p. Western blot analysis was subsequently carried out to analyse the column eluates (figure 7.1). Several observations were made. Firstly, the cocktail of anti-PKC antibodies used for the Western blot identified two major polypeptides of approximately 60- and 80kDa in crude Y1090 (see table 2.2) bacterial extract, and the bacteria strain BL21 (see table 2.2) containing a pET vector (table 2.4) with no insert (figure 7.1). A third, less reactive band, of approximately 30kDa could sometimes be detected. Secondly, the 60- and 80kDa cross-reacting polypeptides could also be detected in MBP-Rck2p purified eluates, but not in the control MBP eluates (figure 7.1). This result suggested that these proteins were associated with the Rck2p polypeptide of the fusion rather than with the MBP polypeptide or the amylose resin. Thirdly, the profile of proteins cross-reacting with the anti-PKC antibodies remained unaltered with respect to the presence and absence of rat brain cytosol and/or PKC activators (figure 7.1). It appeared, therefore, that the 60- and 80kDa polypeptides were of a bacterial origin; that they associated with the Rck2p-moiety of the fusion; and that the exogenously added mammalian PKC neither interfered nor contributed to this association.

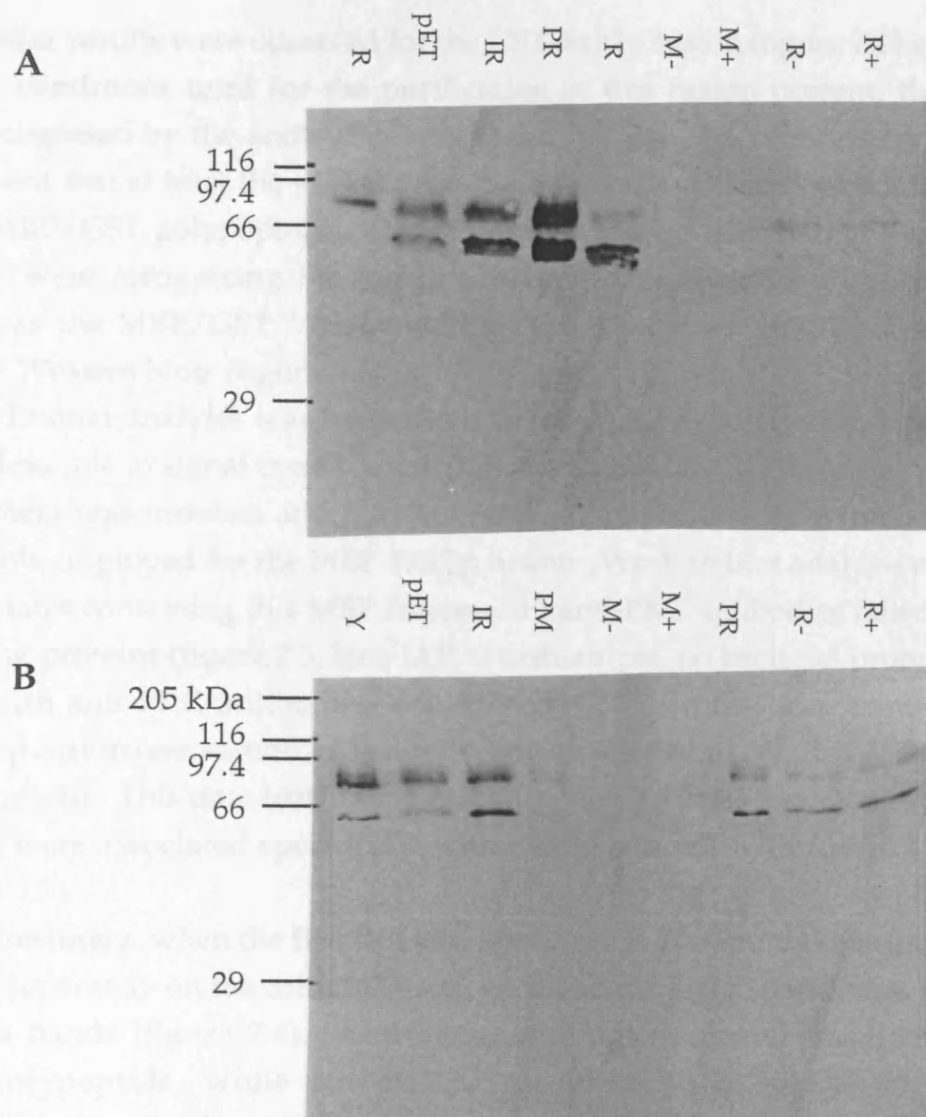


Figure 7.1

Western Blots of amylose resin eluates using anti-PKC antibodies. (A) 500µl of induced protein was added to 200µl of amylose resin in an eppendorf and aggitated at RT for 60 mins. Unbound protein was removed and the resin washed with column buffer. 50µl of rat brain cytosol (RBC) was added to the protein-bound resin in the presence of 2µg/ml DG, 60µg/ml PS, and 1mM CaCl₂ and incubated for 30 mins. Unbound material was removed and the resin washed with column buffer. 50µl fractions were eluted with 10mM maltose and 10µl used for SDS-PAGE. R=RBC extract; pET=pET3b-containing BL21 bacterial extract; IR=induced MBP-Rck2p/BL21 bacterial extract; PR=MBP-Rck2p pre-column; R=MBP-Rck2p purified from a previous amylose column; M-=MBP purified without incubation with RBC/DG/PS; M+=MBP purified with incubation of RBC/DG/PS; R-=MBP-Rck2p purified without incubation of RBC/DG/PS; R+=MBP-Rck2p purified with incubation of RBC/PS/DG. (B) As (A) except DG/PS did not include RBC; Y=Y1090 bacterial extract; PM=MBP pre-column. SIGMA antibodies (rabbit polyclonals against isozyme-specific peptides) used as a cocktail were: α (1:25,000); βI (1:35,000); γ (1:7,500); δ (1:5,000); ε (1:7,500) incubated o/n in TBS containing 3% BSA and 1% casein. Secondary antibodies were Jackson goat anti-rabbit HRP (1:100,000) and iLUMin8 used for development.

Similar results were observed for the GST-Rck2p fusion (figure 7.2) except that under the conditions used for the purification of this fusion protein, the ~80kDa protein recognised by the antibodies was absent. These observations strengthened the argument that at least the ~60kDa polypeptide was associated with Rck2p rather than the MBP/GST polypeptides. It also negated any possibility that the anti-PKC antibodies were recognising the fusion proteins themselves (or their breakdown products) as the MBP/GST fusions were of different sizes yet displayed similar profiles on Western blots (figure 7.2).

Additional analysis was carried out on MBP fused to another yeast protein which had no role in signal transduction (kindly donated by S. Elliston-Elhinn). This fusion protein was induced and purified from an amylose-resin column following the protocols employed for the MBP-Rck2p fusion. Western blot analysis of purified column eluates containing this MBP fusion with anti-PKC antibodies failed to detect any reacting proteins (figure 7.3; lane M?). Furthermore, no bacterial proteins, cross-reacting with anti-PKC antibodies, could be detected in fractions containing the MBP-Gcd6p fusion (see section 4.6) eluted from amylose resin (W. Richards, personal communication). This data further indicated that the proteins detected by anti-PKC antibodies were associated specifically with Rck2p and not with fusion proteins in general.

Interestingly, when the five isozyme specific anti-PKC antibodies (α , β I, γ , δ , ϵ) were used separately on the column eluate, each had different specificities for the 60- and 80kDa bands (figure 7.4). Anti-PKC α antibodies showed specificity for the ~60kDa polypeptide, while anti-PKC β I recognised the 80kDa polypeptide. Antibodies against PKC δ and PKC ϵ recognised both polypeptides, though antibodies against PKC δ showed a preference for the 80kDa polypeptide. Although the isozyme-specific antibodies recognise 60- and 80kDa polypeptides, it is unclear whether they are the same polypeptides or different proteins of similar size.

7.3 Lysozyme Preparation Of MBP-Rck2p

Over-sonication of host cells was reported to be the cause of purifying host proteins along with the fusion protein of interest (Pharmacia handbook for the GST gene fusion system). In order to address this possible problem, bacterial cells were lysed using lysozyme rather than sonication, prior to the protein purification. Western blot analysis confirmed that host polypeptides, cross-reacting with anti-PKC antibodies, were associated with Rck2p, irrespective of the cell lysis procedure (figure 7.3; lane MR).

7.4 Proteins Associated With yRack1p

A yeast homologue of mammalian RACK1 was identified (see chapter 3) and subsequently cloned into an expression vector (see section 6.13). The GST-yRack1p

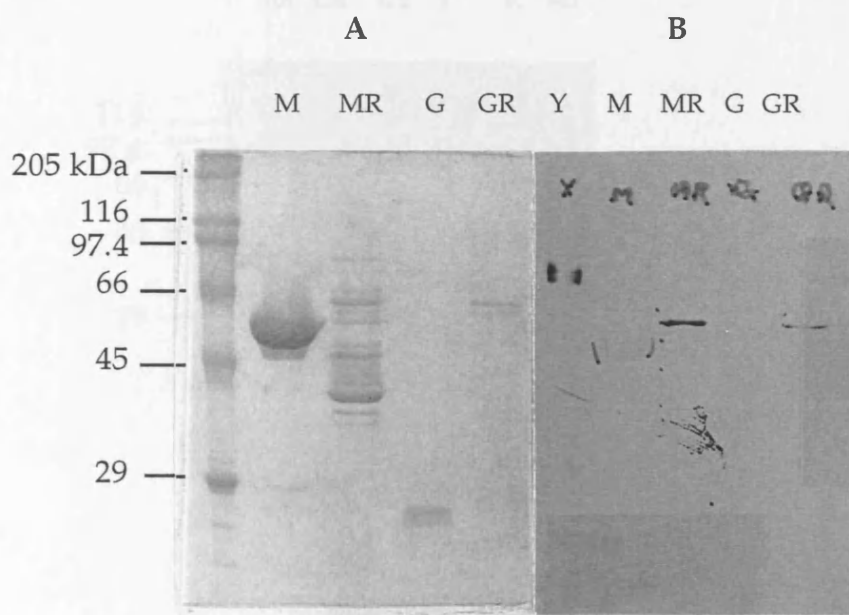


Figure 7.2

Proteins associated with Rck2p are recognised by anti-PKC antibodies. Purified GST (G), GST-RCK2 (GR), MBP (M) and MBP-RCK2 (MR) were analysed by 12% SDS-PAGE. Half of the gel was stained with Coomassie (A) while the other half was used for a Western blot (B) with a cocktail of SIGMA anti-mammalian PKC antibodies; α (1:25,000); β I (1:35,000); γ (1:7,500); δ (1:5,000); ϵ (1:7,500) incubated o/n in TBS containing 3% BSA and 1% casein. Secondary antibodies were Jackson goat anti-rabbit HRP (1:100,000) and iLumin8 used for development. Y1090 bacterial extract was used as a positive control for this Western (Y). It appears that a protein(s) present in the bacterial extract and recognised by the anti-PKC antibodies is associated with Rck2p.

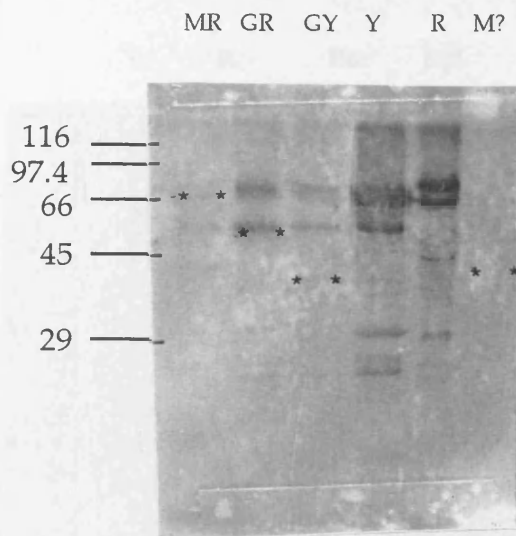


Figure 7.3

Western blot using anti-PKC antibodies. MR) MBP-Rck2p fusion eluted from an amylose affinity column after lysozyme lysis of bacterial cells and a wash with column buffer (pH10) containing 3M NaCl; GR) GST-Rck2p eluted from a glutathione affinity column; GY) GST-yRack1p eluted from a glutathione affinity column; Y) Y1090 bacterial extract; R) rat brain cytosol extract; M?) MBP fusion, unrelated to the Rck2p fusions, eluted from an amylose affinity column. Western blot with PKC SIGMA anti-mammalian PKC antibodies used as a cocktail [α (1:25,000); β I (1:35,000); γ (1:7,500); δ (1:5,000); ϵ (1:7,500) incubated o/n in TBS containing 3% BSA and 1% casein]. Secondary antibodies were goat anti-rabbit HRP (Jackson) 1:100,000 and the Western developed with iLumin8. Asterisk represent positions of the fusion proteins (*).

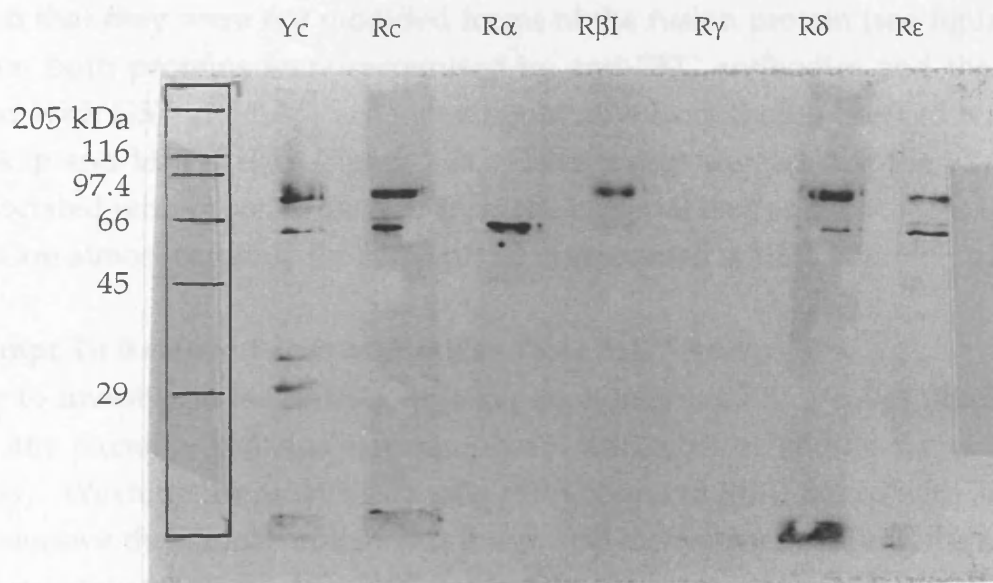


Figure 7.4

Western blot of MBP-Rck2p eluate using individual anti-PKC antibodies.

Eluate obtained after addition of 50 μ l of 10mM maltose to an MBP-Rck2p column (batch) was blotted onto nitrocellulose and incubated with individual anti-PKC antibodies. Yc= Y1090 bacterial extract incubated with a cocktail of all five PKC antibodies; Rc=MBP-Rck2p eluate incubated with the cocktail; R α =MBP-Rck2p incubated with anti-PKC α (1:25,000); R β I=incubated with anti-PKC β I (1:35,000); R γ =incubated with anti-PKC γ (1:7,500); R δ =incubated with anti-PKC δ (1:5,000); R ϵ =incubated with anti-PKC ϵ (1:7,500) antibodies. Secondary antibodies were goat anti-rabbit HRP (1:100,000) and iLUMin8 was used for development.

fusion protein was induced and purified using affinity chromatography on glutathione agarose. As is the case of the GST-Rck2p fusion, proteins of ~60kDa and ~80kDa were found to be associated with the fusion protein (see section 6.14). These ~60- and ~80kDa proteins were not recognised by anti-GST antibodies which indicated that they were not modified forms of the fusion protein (see figure 6.11). However, both proteins were recognised by anti-PKC antibodies and the profile observed with GST-yRack1p was indistinguishable from those observed with both GST-Rck2p and MBP-Rck2p (figure 7.5). These results suggest that the yRack1p is also associated with proteins derived from the bacterial host and that these bacterial proteins are almost certainly the same proteins associated with Rck2p.

7.5 Attempt To Remove Bacterial Proteins From MBP-Rck2p

In order to investigate the affinity of Rck2p to eukaryotic PKC, it was necessary to remove any bacterial proteins associated with Rck2p before adding the exogenous enzyme(s). Washing the amylose column resin bound to MBP-Rck2p with 3M NaCl did not remove the fusion protein, but it was also ineffective in eluting the bacterial proteins recognised by the anti-PKC antibodies (figure 7.6). Buffers containing 3M NaCl at pH2 (figure 7.7) and pH10 (figure 7.8) also failed to dissociate the bacterial proteins away from the Rck2p fusion suggesting they are very tightly associated. Interestingly, however, the 3M NaCl in the pH10 buffer eluted a prominent protein of ~70kDa which was not recognised by either anti-PKC or -MBP antibodies (figure 7.8). It is possible that this protein is DnaK (hsp70), previously reported to be co-purified with recombinant proteins (Pharmacia handbook for the GST gene fusion system).

7.6 Identification Of Rck2p-Associated Bacterial Proteins

Factor Xa protease digestion was efficiently used to digest MBP-Rck2p fusion polypeptides to their constituent parts which were smaller than the 42.7kDa MBP polypeptide (see figure 6.5). Coomassie staining of the proteins purified through the amylose resin, digested by factor Xa, and separated by SDS-PAGE, revealed several polypeptides of ~60kDa which remained unaltered after the protease treatment (figure 7.9). Western blot analysis with anti-PKC antibodies indicated that these protease-resistant polypeptides were responsible for the cross-reactivity with anti-PKC antibodies (figure 7.9). A larger (~80kDa) protein cross-reacted with the anti-PKC antibodies before the protease treatment but was missing after the treatment and was replaced by an additional ~60kDa polypeptide (figure 7.9).

7.7 N-Terminal Sequencing Of The ~60kDa Bacterial Protein

The surprising cross-reactivity of the ~60- and ~80kDa polypeptides with anti-PKC antibodies, as well as the evidence which suggested that these polypeptides

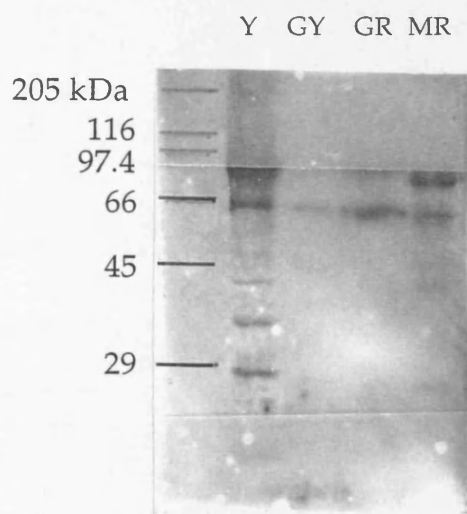
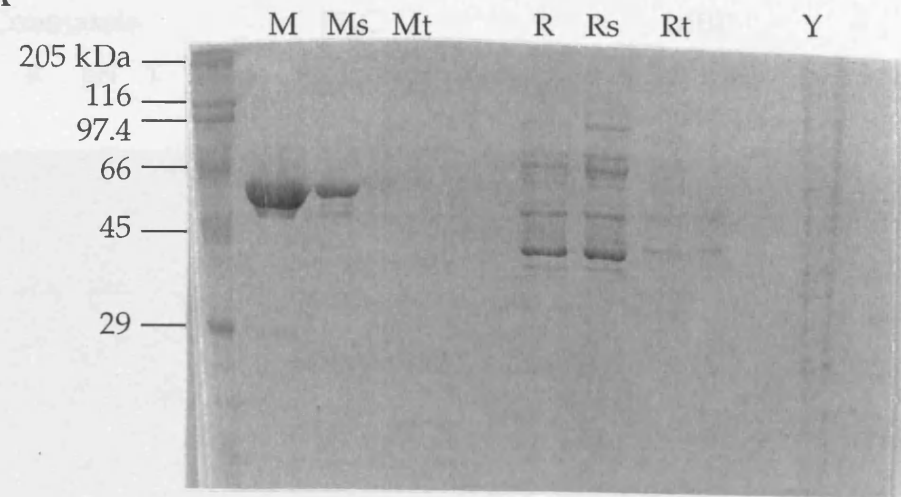
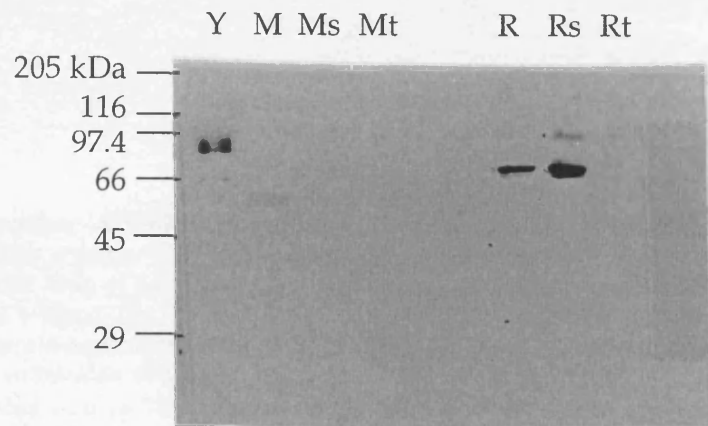
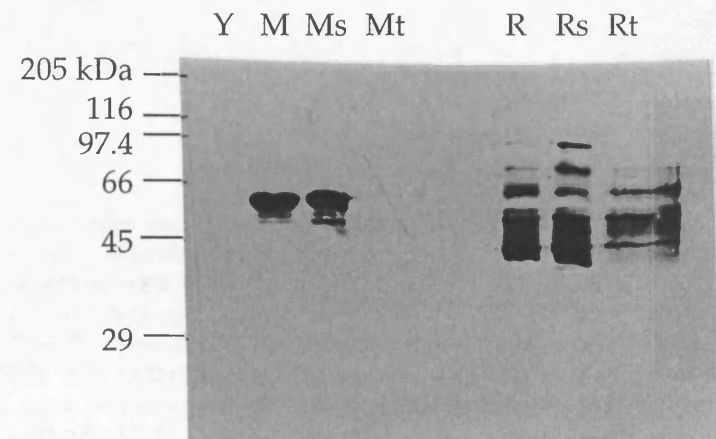


Figure 7.5

Western blot analysis with anti-PKC antibodies. Anti-PKC antibodies were used in a Western blot containing Y1090 bacterial extract (**Y**), GST-yRACK fusion (**GY**), GST-Rck2p fusion (**GR**) and MBP-Rck2p fusion (**MR**). PKC SIGMA anti-mammalian PKC antibodies used as a cocktail [α (1:25,000); β I (1:35,000); γ (1:7,500); δ (1:5,000); ϵ (1:7,500)] incubated o/n in TBS containing 3% BSA and 1% casein]. Secondary antibodies were goat anti-rabbit HRP (Jackson) 1:100,000 and the Western developed with iLUmin8. The results suggest that the bacterial proteins associated with Rck2p are also associated with yRack1p.

Figure 7.6

3M NaCl washes of MBP-Rck2p fusion. In an attempt to dissociate the MBP-Rck2p fusion from its bound bacterial proteins the effect of washing the amylose column with 3M NaCl before the elution with maltose was monitored. 400mls of sonicated extract was used for each column, packed with 2mls of amylose resin. MBP and MBP-Rck2p were purified without 3M NaCl washes as described previously (**M** and **R** respectively). MBP and MBP-Rck2p were also purified after the column was washed with 10mls of 3M NaCl (**Ms** and **Rs** respectively). Any proteins released from the columns by the 3M NaCl were precipitated with 20% TCA (**Mt** and **Rt**). Samples were analysed by 12% SDS-PAGE followed by Coomassie stain (**A**), or Western blot analysis. **B** is a Western blot using SIGMA anti-mammalian PKC antibodies used as a cocktail; α (1:25,000); β I (1:35,000); γ (1:7,500); δ (1:5,000); ϵ (1:7,500) incubated o/n in TBS containing 3% BSA and 1% casein. **C** is a the same Western blot which was stripped and re-incubated with anti-MBP antibodies (1:100,000). Secondary antibodies were Jackson goat anti-rabbit HRP (1:100,000) and iLumin8 used for development. The results suggest that the 3M NaCl wash fails to interfere with the MBP/amylose association, but also fails to dissociate the PKC cross-reactive bacterial proteins from the MBP-Rck2p fusion.

A**B****C**

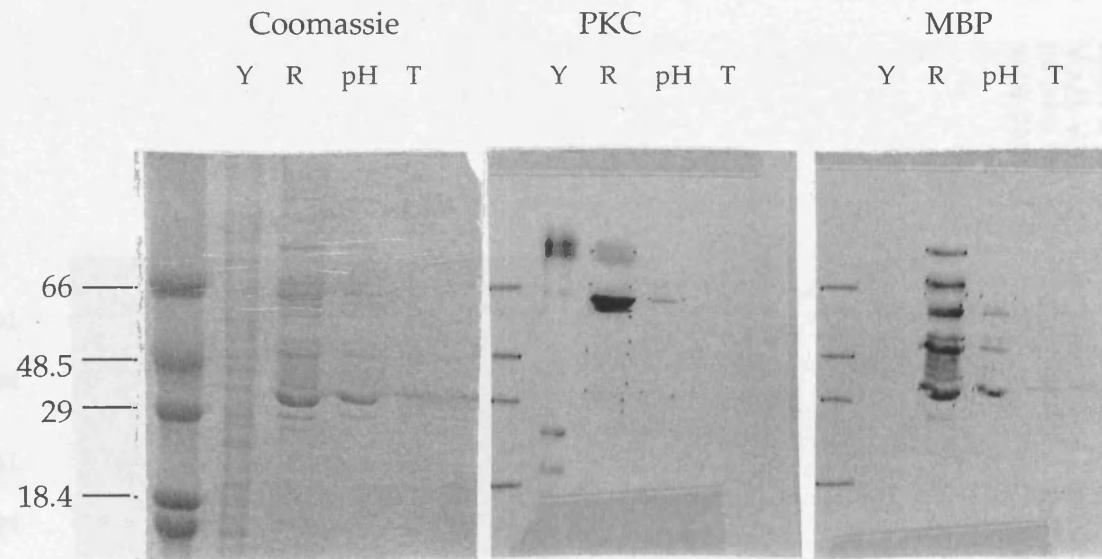


Figure 7.7

3M NaCl (pH2) washes of MBP-Rck2p fusion. In an attempt to dissociate the MBP-Rck2p fusion from its bound bacterial proteins, the effect of washing the amylose column with 3M NaCl of pH2 before the elution with maltose was monitored. 400mls of sonicated extract was used with a column packed with 2mls of amylose resin. MBP-Rck2p was eluted with 10mM maltose after washing with 20mls of 3M NaCl (pH2) washes (**pH**). MBP-Rck2p eluted without the 3M NaCl wash (**R**) and Y1090 bacterial extract (**Y**) were also analysed. Any proteins released from the column with the 3M NaCl wash were precipitated with 20% TCA (**T**). Samples were analysed by 12% SDS-PAGE followed by Coomassie stain, or Western blot using anti-PKC antibodies (SIGMA) used as a cocktail; α (1:25,000), β I (1:35,000), γ (1:7,500), δ (1:5,000), ϵ (1:7,500); or anti-MBP antibodies (1:100,000) incubated o/n in TBS containing 3% BSA and 1% casein. Secondary antibodies were Jackson goat anti-rabbit HRP (1:100,000) and iLUMin8 used for development. The results suggest that 3M NaCl at pH2 fails to interfere with the MBP/amylose association, but also fails to dissociate the PKC cross-reactive bacterial protein(s) from the MBP-Rck2p fusion.

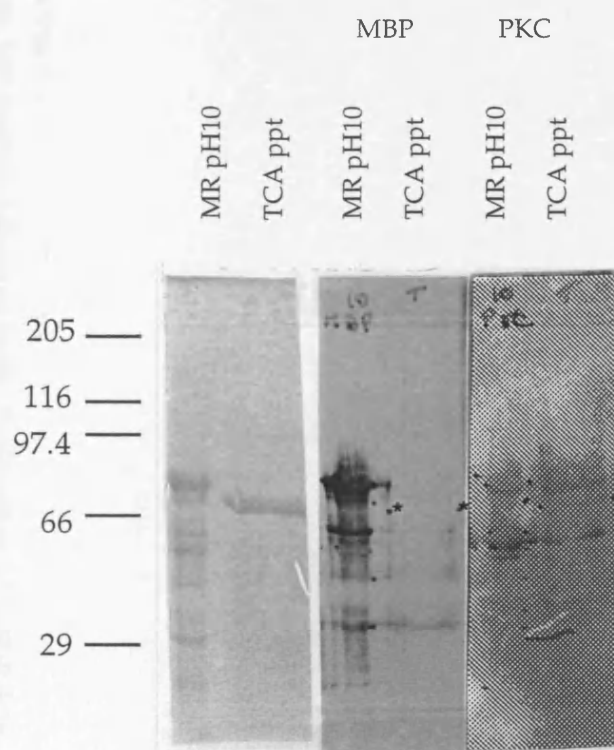


Figure 7.8

Release of ~70kDa protein from MBP-Rck2p. MBP-Rck2p was purified from BL21 bacterial extract using an amylose affinity column. The column was washed with 3M NaCl (pH10) and proteins removed precipitated with 20% TCA (**TCA ppt**). Proteins remaining bound to the column were eluted with 10mM maltose (**MR pH10**). 3M NaCl (pH10) washing did not interfere significantly with the MBP-Rck2p binding to the amylose column but did remove a ~70kDa protein. This protein was not recognised by either anti-MBP or anti-PKC antibodies.

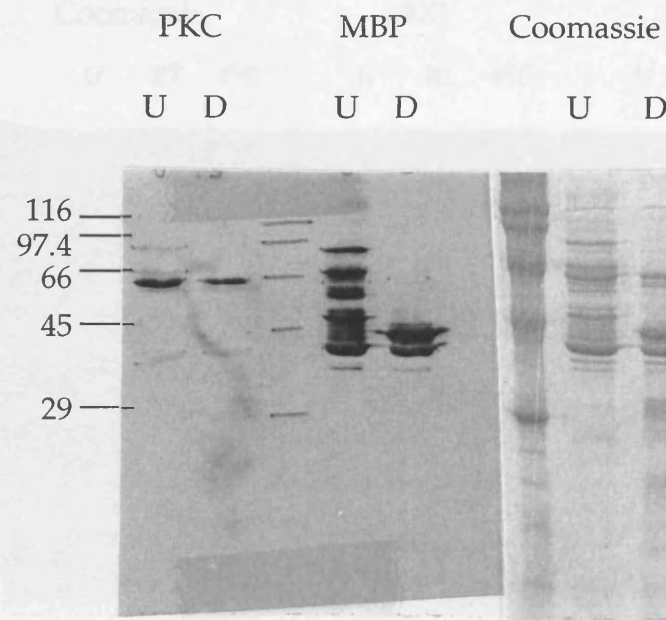


Figure 7.9a

Western blot analysis following Factor Xa digestion of MBP-Rck2p. MBP-Rck2p fusion protein was treated with 1 μ g of Factor Xa protease for 2hrs at room temp. Digested (D) and undigested (U) samples were separated for 0.7hrs by 12% SDS-PAGE in triplicate and then stained with Coomassie, or analysed by Western blot with anti-PKC antibodies (SIGMA) used as a cocktail [α (1:25,000); β I (1:35,000); γ (1:7,500); δ (1:5,000); ϵ (1:7,500) incubated o/n in TBS containing 3% BSA and 1% casein] or with MBP antibodies (1:100,000). It appears that Factor Xa digestion fails to alter the PKC-reactive profile, confirming that the fusion protein is not responsible for the cross-reactivity. It is possible that the heavy protein band of ~66kDa could be partly responsible for this PKC reactivity. Secondary IgGs were goat anti-rabbit HRP (Jackson) 1:100,000, and the Western developed with iLumin8.

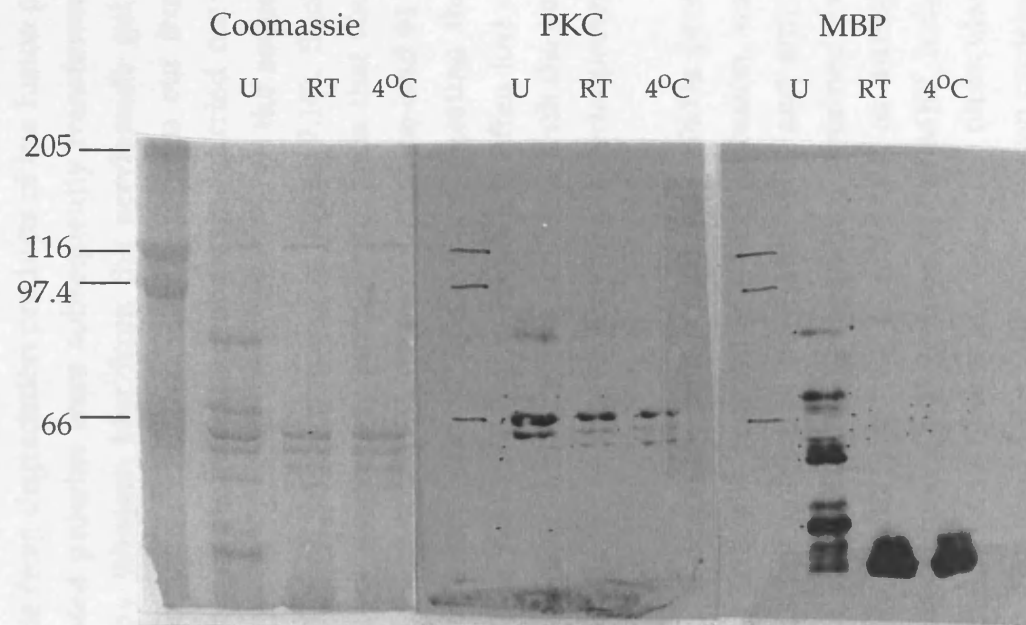


Figure 7.9b

Western blot analysis following Factor Xa digestion of MBP-Rck2p. MBP-Rck2p fusion protein was treated with 1 μ g of Factor Xa protease overnight at room temp (RT) or at 4 $^{\circ}$ C (4 $^{\circ}$ C). Digested and undigested (U) samples were separated for 1.5hrs by 12% SDS-PAGE in triplicate and then stained with Coomassie, or analysed by Western blot with anti-PKC antibodies (SIGMA) used as a cocktail [α (1:25,000); β I (1:35,000); γ (1:7,500); δ (1:5,000); ϵ (1:7,500)] or with MBP antibodies (1:100, 000) incubated overnight in TBS containing 3% BSA and 1% casein . Secondary IgGs were goat anti-rabbit HRP (Jackson) 1:100, 000, and the Western developed with iLUMin8. (Asterisks indicate the loss of an ~80kDa protein, recognised by anti-PKC antibodies, which is replaced by a ~60kDa protein after protease digestion).

specifically interact with Rck2p, called for the further characterisation of these polypeptides. Although both the ~60- and ~80kDa proteins were detected using anti-PKC antibodies, only the ~60kDa polypeptide could be detected with certainty by Coomassie stain (the fusion MBP-Rck2p is also ~80kDa). Efforts were therefore made to isolate the ~60kDa protein for N-terminal sequence analysis. Proteins, containing MBP-Rck2p purified from amylose resin, were digested with Factor Xa protease (figure 7.9). This digestion reduced MBP-Rck2p fusion polypeptides to their constituent parts which are smaller than 42.7kDa (see above). Unlike the ~80kDa protein, the ~60kDa protein was unaffected by the protease digestion (see above). SDS-PAGE separation of the digested proteins consequently resulted in the isolation of the ~60kDa polypeptide from degradation products of the fusion protein of similar size (figure 7.9). Digested proteins were subsequently transferred onto Immobilon™ PVDF membrane by Western blot from the acrylamide gel. The membrane was stained with Coomassie and the ~60kDa protein cut from the membrane. N-terminal sequencing of the ~60kDa protein was carried out at the GBF, Braunschweig, Germany. The 43 residues obtained through the sequencing showed 95% identity to the *E. coli* GroEL, hsp60 chaperonin (figure 7.10). Such high sequence similarity with a protein of similar size strongly suggests that GroEL is indeed the *E. coli* protein associated with Rck2p. Furthermore, amino-acid #1 of the obtained sequence corresponds to amino-acid #2 of GroEL suggesting that the protein was intact when isolated (the first methionine residue is often lost during sequencing). Comparison of the complete GroEL protein sequence with the peptides used to raise the anti-PKC antibodies failed to suggest why the bacterial protein was recognised (figure 7.11).

In addition to the N-terminal sequence analysis of the ~60kDa protein, a prominent polypeptide, associated with the purified MBP-Rck2p fusion, was also sequenced. This polypeptide was recognised by both anti-MBP and anti-FLAG antibodies, but not by anti-PKC antibodies. The sequence obtained for this polypeptide showed high homology (98%) to the *E. coli* MBP as anticipated. Interestingly, the first residue in the sequence was residue #27 of MBP, indicating that the fusion protein was degraded from its N-terminal end. The molecular mass of this protein was smaller (50kDa) than that of the MBP-Rck2p fusion protein. This strongly suggests that the fusion protein is degraded from both termini.

7.8 Discussion

Approximately 17 bacterial heat shock proteins have so far been characterised (for review see LaRossa and Van Dyk, 1991). These proteins are synthesised by an alternative sigma transcription factor, the product of the *rpoH* gene. A non-functional allele of *rpoH* leads to hypersensitivity of cells to stress responses due to the lack of synthesis of heat-shock proteins. DnaK, GroEL and GroES are three

A

Identity=42/44 (95%)

```
#2- .AAKDVKFGNDARVKMLRGVNVLADAVKVTLGPKGRNVVLDKXF -#44 60kDa?  
  |||||  
  MAAKDVKFGNDARVKMLRGVNVLADAVKVTLGPKGRNVVLDKSF      GroEL
```

B

Identity=43/44 (98%)

```
#27- KTEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKL -#69 MBP-Rck2p?  
  |.|||||  
  KIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKL      MBP
```

Figure 7.10

N-terminal sequencing data. A) A ~60kDa protein was found to be associated with Rck2p and was recognised by anti-mammalian PKC antibodies (SIGMA). This protein was isolated and its N-terminal sequence was obtained. The sequence data indicated that this protein was bacterial GroEL. In addition, sequence data began at residue #2 indicating that this protein was intact when isolated. B) A prominent MBP-Rck2p breakdown product, which was recognised by both ant-FLAG and anti-MBP antibodies, was also analysed by N-terminal sequencing. The sequence data obtained showed high homology to bacterial MBP, confirming that the protein isolated was a breakdown product of the fusion protein. Furthermore, sequence data began at residue #27 suggesting that degradation of the fusion protein probably occurs from both ends.

proteins which depend on *rpoH* transcription. GroE gene disruptions were shown to be lethal, whilst a DnaK disruption is viable under non-stressing conditions. These observations suggest that, unlike the GroE proteins which are also essential for normal growth, DnaK is functional only under stress conditions (LaRossa and Van Dyk, 1991). Interestingly, an *rpoH* disruption is viable under non-stress conditions as under these conditions the GroE genes are transcribed by the 'housekeeping' sigma factor *rpoD*.

It is thought that DnaK (and the co-chaperone DnaJ) and GroEL (and the co-chaperone GroES) cooperate in preventing the misfolding of proteins in *E. coli* (Gragerov, et al., 1992). Overproduction of either GroEL/GroES or DnaK/DnaJ partially prevents aggregation of a newly synthesised protein in *rpoH* mutants, while overproduction of all four heat shock proteins restores the phenotype of *rpoH* mutants to that of wild-type cells. It is thought that DnaK/DnaJ interacts with short polypeptides of newly synthesised proteins. Once the polypeptide chain is of a certain length, it becomes associated with GroEL/GroES which is more suited to large polypeptides. The cooperative interaction of these proteins may explain the association of both ~60kDa (GroEL) and ~70kDa proteins (suggested to be DnaK) with Rck2p.

GroEL is a cylindrical protein complex made up of 14 subunits of 57kDa which are arranged in two heptameric rings stacked back-to-back. Substrate protein binds in the central cavity of the cylinder, the so-called 'Anfinsen cage' (Ellis, 1994). GroES, which forms a single ring of seven 10kDa subunits, is essential for the activity of GroEL. ATP hydrolysis by GroEL leads to the dissociation of GroES and promotes the release of the native polypeptide from GroEL. An incorrectly folded polypeptide would re-associate once more with the hydrophobic domains of the GroEL cylinder ready for another cycle of folding and release.

Mutation analysis identified a putative tertiary structure, necessary for protein binding, on the inside surface of the GroEL cavity, consisting of hydrophobic residues (Fenton, et al., 1994). The same residues are also believed to be essential for the binding of GroEL to GroES. As yet, little is known about what determines the binding of GroEL to a particular protein and there seems to be little significant similarities between the known proteins that do bind to GroEL.

It is suggested (Neil Ransom, personal communication) that GroEL could bind relatively non-specifically to proteins overexpressed in bacterial cells. However, several lines of evidence suggest that the interaction of GroEL with Rck2p could be physiologically significant. Firstly, GroEL binds specifically to overexpressed MBP-Rck2p but not to overexpressed MBP- β -gal- α , and similarly to GST-Rck2p but not to overexpressed GST alone. As MBP- β -gal- α and GST are expressed in vastly greater concentrations than to the Rck2p fusions, it may have been expected that non-fusion proteins would stress the bacterial host cells more thus mediating the interaction of

chaperones. Secondly, there was no evidence for the presence of GroEL in purified fractions of non-Rck2p fusions. Finally, the GroEL-Rck2p interaction is extremely tight, withstanding washing with a high salt buffer at both acidic and alkaline pH, suggesting that the binding is unlikely to be non-specific.

Western blot analysis of MBP-FLAG-Rck2p fusion protein digested with Factor Xa revealed several polypeptides of ~60kDa which cross-reacted with anti-PKC antibodies (figure 7.9). It was reported that a heat-induced modification of GroEL occurs which is reversed when it is returned to low temperature (Sherman and Goldberg, 1992). This modification was found to be phosphorylation and was responsible for altering the mobility of GroEL on SDS-PAGE. GroEL polypeptides of 55- and 60kDa were often observed on polyacrylamide gels and a smear found between these polypeptides suggests modifications are either multiple and/or rapid (Sherman and Goldberg, 1992). Similarly, phosphorylation of DnaK was also reported suggesting that such a modification is a general mechanism for the control of chaperone activity. It is thought that phosphorylation increases the activity of GroEL and thus its ability to interact with and dissociate from unfolded proteins. It was also suggested that the heat-induced phosphorylation of chaperones contributes to thermotolerance, 'priming' the chaperones for a subsequent increase in temperature (Sherman and Goldberg, 1992). The multiple ~60kDa polypeptides detected by anti-PKC antibodies could represent modified forms of GroEL. These putative forms of GroEL may therefore be due to phosphorylation and perhaps indicates that the *E. coli* cells were subjected to stress conditions during the generation of the Rck2p fusion protein.

The interaction of anti-PKC antibodies with GroEL is perplexing. Preliminary analysis suggests that the peptides used to produce the antibodies (from the V5 region of PKC) show little homology to the sequence of GroEL and suggest the epitopes are missing from GroEL (figure 7.11). Because the peptides used for production of the antibodies are small it is likely that the antibodies would recognise the primary sequence of the protein rather than a conformational structure. An interesting possibility, therefore, is that it is the GroEL protein which 'recognises' the antibodies. This proposal appears feasible when considering the protein-binding properties associated with GroEL. Although blotted onto nitrocellulose, GroEL could still exhibit some affinity to PKC antibodies which is different from its normal binding activity *in vivo*. It is possible, for instance, that individual GroEL subunits possess binding activity, although no information regarding this possibility is available. This limited activity of the GroEL subunits could also reconcile the fact that the antibodies such as anti-MBP and anti-FLAG are not bound to the protein.

There is also uncertainty concerning the inconsistent presence of the ~80kDa protein which is recognised by anti-PKC antibodies. It appears that this protein is sensitive to Factor Xa protease digestion (figure 7.9). It would be interesting to

establish what this protein actually is. It is tempting to speculate that this protein is also a chaperone, perhaps hsp90 (HtpG), and binds Rck2p and PKC antibodies in a manner proposed for GroEL. Although it is possible that the ~80kDa polypeptide is a modified form of GroEL, the large size difference suggests that this is unlikely. Even more convincingly, monoclonal antibodies raised against GroEL failed to detect such a large polypeptide (see chapter 8).

Finally, the recent identification of yRack1p and the indication that it behaves similarly to Rck2p, in that it binds bacterial proteins recognised by PKC antibodies, is interesting. Does this suggest that both Rck2p and yRack1p are indeed RACK1 homologues and their binding to bacterial proteins is a result of some kind of RACK1-related function?

CHAPTER 8.

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST Rck2p (carried out at GBF, Braunschweig, Germany)

8.1 Introduction

Antibodies are synthesised primarily by plasma cells, a type of terminally differentiated B lymphocyte. A technique was developed which allowed the growth of clonal populations of cells secreting antibodies with a defined specificity (Kohler and Milstein, 1975). In this technique antibody-secreting cells are fused with myeloma cells to produce hybrids. These hybridomas can be maintained *in vitro* and will continue to secrete antibodies of a defined specificity. Antibodies produced from hybridomas are known as monoclonal antibodies.

Antibodies provide a useful tool for investigating the properties of an isolated protein. Antibodies against Rck2p can be used to assess the cellular concentration of this protein. In addition, they can be used to localise Rck2p to a particular intracellular compartment using indirect immunofluorescence microscopy and provide a further clue as to the function of the protein. Immunoprecipitation of Rck2p from yeast extract could assist in purifying the protein along with other molecules to which it associates, *i.e.* a yeast PKC homologue, a yeast chaperone(s) or others.

When the production of monoclonal antibodies was initiated, only the MBP-FLAG-Rck2p fusion protein was available (see section 6.5). In addition, the proteolytic digest of the fusion protein with Factor Xa failed to generate an intact Rck2p polypeptide as was hoped (see section 6.6). Consequently, the entire fusion protein was used for the immunisation (see below). Although the presence of minor concentrations of other polypeptides was observed in the fractions eluted from the amylose resin column, it was assumed that the majority of hybridoma cells would produce antibodies to the fusion protein.

8.2 Immunisation Of Mice With Rck2p Fusion Protein

MBP-FLAG-Rck2p fusion protein, was purified through affinity chromatography on amylose resin (see section 6.5). Approximately 20µg of fusion protein was used to immunise female BALB/c mice (see section 2.4.49a). Lymph node cells were subsequently removed from behind the knee of the mice and their lymphocytes fused with myeloma cells (carried out in the GBF by N. Boulton and W. Richards). The hybridomas were cultured in fifteen 24-well plates and examined for growth. The supernatants from these wells were assessed by ELISA against the injected protein (MBP-FLAG-Rck2p) in order to choose hybridomas which produce antibodies which strongly cross-react with the antigen. Fifteen hybridomas were initially selected, while the remaining cells were frozen and stored in liquid nitrogen.

The antibodies which were expected to be produced from the chosen hybridomas were against Rck2p, FLAG, MBP and the associated polypeptides observed in the purified eluate.

8.3 Preliminary Analysis Of Hybridoma Cells

Supernatants from all fifteen cell lines were tested by Western blot and ELISA in order to choose the most suitable for more detailed analysis. Samples containing the GST-Rck2p fusion, purified from a glutathione agarose resin column (see section 6.9), were used for this analysis. It was thought that antibodies cross-reacting with the GST-Rck2p fusion would be recognising the Rck2p polypeptide, as those recognising either MBP or FLAG polypeptides would fail to react with the GST-Rck2p fusion. The association of the bacterial proteins with both GST-Rck2p and MBP-FLAG-Rck2p was not known at that time. The supernatants from the four cell lines which showed the strongest positive response with the GST-Rck2p on both Western blot and ELISAs were subsequently selected for more detailed analysis. The remaining eleven cell lines were frozen and stored.

8.4 Subcloning Hybridoma Cell Lines To Single Cells

The four cell lines selected were subcloned (see section 2.4.49e). This procedure is carried out with the purpose of isolating a single hybridoma cell which produces the required monoclonal antibody. By counting the number of cells in a cell suspension, taken from each cell line, the correct dilution necessary to obtain single cells could be estimated. Subsequent dilutions of the cell suspensions into fresh media was carried out accordingly. After 1-2 days, the growing cells were examined under a microscope, and the presence of a single colony in a well noted. After more than one week the colonies were of suitable size to be tested. Supernatant containing antibodies was taken and tested both by Western blot and ELISA against the GST-Rck2p fusion. Anti-GST antibodies were used as a positive control. It was found that several clones, each originating from the four hybridomas, produced antibodies which appeared to positively cross-react with the GST-Rck2p fusion using either the Western blot or ELISA methodologies.

8.5 Analysis Of Monoclonal Antibodies

More detailed analysis of the antibodies obtained from the single clones was carried out. Antibodies were used individually with blots containing both GST-Rck2p and MBP-FLAG-Rck2p eluates. It was predicted that antibodies raised against Rck2p would produce the appropriate profiles on the Western blots of both the GST and MBP fusions, whilst antibodies against MBP, or FLAG would only recognise polypeptides found in the eluate containing MBP-FLAG-Rck2p.

Under normal circumstances, the antibodies present in supernatants taken from growing hybridomas are very dilute. Undiluted supernatant was used for the preliminary analysis described above but during the detailed analysis it was found that antibodies showed optimum cross-reactivity in Western blots after a dilution of 1:20. At this dilution, the majority of antibodies obtained from single hybridoma clones recognised the MBP-FLAG-Rck2p fusion, but not the GST-Rck2p fusion (figure 8.1a). The remaining antibodies produced by the single hybridoma clones recognised polypeptides in both the GST-Rck2p and MBP-FLAG-Rck2p samples (figure 8.1b). This pattern of antibody reactivities indicate that the primary screening was too crude and that each of the four hybridomas initially selected, contained two types of monoclonal antibody-producing cells. Interestingly, the polypeptides recognised in the fractions of both Rck2p and MBP fusion proteins appeared to be of the same size, ~60kDa, and did not correlate to the profiles expected from the fusions. This result, in addition to the parallel findings concerning the bacterial GroEL, indicated that these monoclonal antibodies recognised GroEL and not Rck2p, as was hoped. Further analysis confirmed that the antibodies recognised a similarly sized polypeptide in bacterial extract (figure 8.1b).

It appeared from the results that the anti-GroEL antibodies recognised two major polypeptides of ~60kDa (figure 8.1b). It is possible that these polypeptides represent modified forms of the GroEL protein as mentioned above (see section 7.8). Interestingly, the ~80kDa protein observed with the anti-PKC antibodies (see chapter 7) was not detected with the 'GroEL antibodies' under these conditions. The possibility that the ~80kDa protein is a modified form of GroEL is therefore unlikely. The other single hybridoma clones produced antibodies that cross-react with the MBP-Rck2p fusion protein. These antibodies would be expected to recognise either the bacterial *malE* product (MBP) or the FLAG peptide. As these antibodies fail to cross-react with proteins in a bacterial extract, it is likely that they recognise the FLAG peptide (figure 8.1b).

8.6 Discussion

The purified proteins eluted from the amylose column and used for the immunisation, contained GroEL due to its high binding affinity to Rck2p (see chapter 7). Consequently, monoclonal antibodies were raised against this protein. The screening procedure, used to identify hybridomas producing anti-Rck2p antibodies, revolved around a positive response with the proteins eluted from the glutathione column containing the GST-Rck2p fusion. However, the fractions eluted from this glutathione resin are now known to also contain GroEL (see chapter 7). Therefore, the screening procedure used would have selected for the bacterial GroEL as well as for Rck2p. In retrospect, the production of anti-Rck2p antibodies through the use of eluted fractions was doomed.

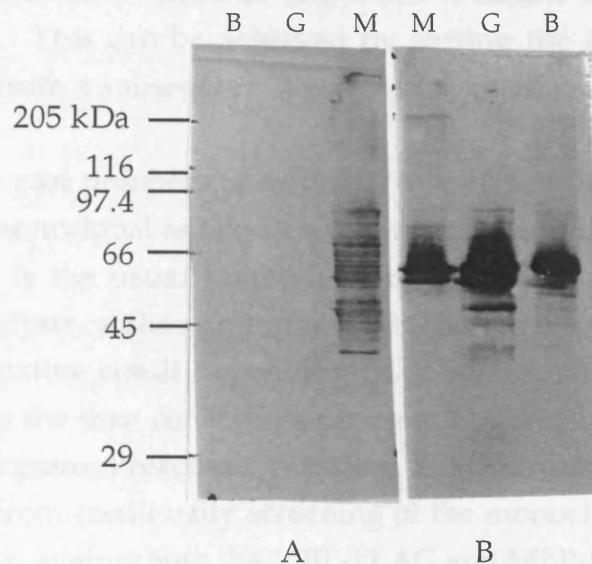


Figure 8.1

Western blot using monoclonal antibodies raised against MBP-Rck2p. Monoclonal antibodies were raised against MBP-Rck2p extract purified from an amylose column (see text for details). A number of antibodies isolated were tested by Western blot against Rck2p-containing fusions. M=MBP-FLAG-Rck2p fusion isolated from an amylose column; G=GST-Rck2p fusion isolated from a glutathione column; B=Y1090 bacterial extract. Monoclonal antibodies were used at 1:20; goat anti-mouse polyclonal secondary IgGs were used at 1:50,000. The antibodies tested cross-reacted with either MBP-FLAG (A), or with a bacterial protein(s) of ~60kDa (B) predicted to be GroEL (see text for details). None of the antibodies tested produced a Western profile which suggested cross-reactivity with Rck2p.

The high binding affinity of GroEL to Rck2p could prevent the generation of antibodies against Rck2p. As already discussed (see section 7.8) the GroEL/GroES complex forms a 'cage' within which a bound protein is folded (Ellis, 1994). Such an interaction would mask any antigenic domains present on Rck2p, necessary for the immune response. This could partially explain why clones producing antibodies against GroEL were identified, but clones producing antibodies against Rck2p were not. To overcome such GroEL-mediated difficulties when generating Rck2p monoclonal antibodies, it would be important to isolate the Rck2p fusion from the bacterial protein. This can be achieved by cutting the fusion protein from SDS-PAGE, eluting it from a subsequent Western blot, or attempting to elute GroEL from Rck2p with ATP.

Due to the vast numbers of colonies/cells which have to be screened during the selection for monoclonal antibodies, the emphasis should be placed on simplicity. An ELISA assay is the usual method for the quick screening of many samples. Western blot analysis, although more labour intensive, can provide a means of obtaining a qualitative result depending on the immunoreactive profile obtained. However, without the time consuming process of optimising antibody dilutions, the possibility of background reactivity remains. For this reason, some advantage could have been taken from continually screening of the monoclonal antibodies produced by the hybridomas, against both the MBP-FLAG and MBP-FLAG-Rck2p fusion.

The original cell lines obtained from the Rck2p immunisation were kept in liquid nitrogen and can be screened again using a modified screening protocol in an attempt to identify clones producing antibodies specifically against Rck2p. Later, if required, and if a suitable screening procedure can be devised, antibodies recognising specific domains of Rck2p can be obtained. This may be useful for investigating the domains of Rck2p which interact with GroEL and/or PKC, for instance.

The antibodies obtained against GroEL will allow a more direct investigation into the nature of the interaction between the bacterial protein and Rck2p. Immunoprecipitation of a bacterial extract with anti-GroEL antibodies would be predicted to purify Rck2p fusion proteins, along with other polypeptides to which GroEL is associated. It is possible that the monoclonal antibodies cross-react with the yeast homologue of GroEL, Mif4p. In this case, the antibodies could immunoprecipitate Rck2p directly from yeast extract, assuming that Rck2p associates with chaperones in its natural environment.

CHAPTER 9.

DISCUSSION

What are RACKs?

Do proteins which have acquired the sole purpose of localising PKC to particular cellular compartments exist in eukaryotic cells? Proteins having a common primary function could be expected to be descendants of a single protein which has diversified through evolution. This evolutionary process would result in a family of proteins displaying functional homology and overall sequence similarity. PKC isozymes and G proteins are good examples of such families of proteins. An alternative scenario is possible, where only the functional domain within the ancestral protein has been acquired by other proteins through gene reorganisation. In this case, domains which are functionally and/or structurally homologous would be found in proteins which are otherwise unrelated. SH2, SH3 and zinc-finger motifs are examples of domains which confer a particular function to unrelated proteins.

Little sequence homology has been detected between the limited number of proteins which are assumed to be receptors of PKC. There is certainly no evidence to suggest that PKC receptors are a family of proteins, and little indication to the existence a 'PKC-binding motif'. The possible presence of a 'PKC-binding structure', determined by the tertiary conformation of the protein, is nevertheless a valid possibility, but without more accurate tools to predict such structures it is difficult to comment on this possibility.

An alternative approach to understanding the RACK-PKC association would be to view it from the side of the PKC. In this case, instead of analysing proteins which bind PKC, proteins to which PKC could bind should be examined. PKC has diversified so that a number of distinct PKC isozymes have been generated. A means to regulate the activities of the different isozymes has been achieved by confining them to specific environments. It would be easy to speculate how a particular isozyme could attain an affinity for a particular cellular locale *via* an association with proteins unique to that site. For instance, an isozyme associating with the plasma membrane, initially *via* a phospholipid interaction, may have acquired the ability to bind G proteins, while an isozyme associating with the nuclear envelope may have gained the ability to bind lamins. As these isozymes would have obtained their new affinities to particular proteins independently of each other, the binding-domains involved (between the PKCs and/or between the associated proteins) would have little sequence homology.

This second hypothesis implies that proteins to which PKC bind would have a particular cellular location, be specific for a particular isozyme (or isozyme class), and show limited sequence homology to other proteins found to bind PKC. All these

characteristics are thought to be true of mammalian RACK1, for instance. This hypothesis does not mean that PKC isozymes could not bind a number of different proteins, however. A particular isozyme may have acquired the ability to translocate to different cellular compartments in different cell types. Such a situation could be explained by the presence of several different binding domains within the single PKC protein. These domains may display homology with other PKC isozymes and confer a general translocation of PKC or reflect isozyme diversity and confer an isozyme-specific translocation to a particular RACK within a particular cellular location. Recent data suggest that PKC isozymes may have several domains within a single protein which are necessary and sufficient for isozyme translocation (Ron, 1995; James, 1992).

The limited experimental data indicate that PKC is translocated, after cell stimulation, to different intracellular locations. The data also suggest that the sites of isozyme translocation depends upon the cell type (Disatnik, 1994; Goodnight, 1995). It is also possible that the destination of a particular translocated PKC isozyme depends on the specific cellular stimulation of the cell. Accordingly, conditions resulting from one stimulus may translocate a PKC isozyme to the nucleus, while another stimulus may lead to the translocation of the same isozyme to the cytoskeleton. For example, the altered conformation of a G protein due to a receptor stimulation could promote its binding to a specific PKC isozyme and mediate the translocation of the kinase to the plasma membrane. A different stimulus which triggers the onset of mitosis in the same cell could 'activate' the lamins and result in the translocation of the same isozyme to the nucleus. All these observations could be accommodated by the hypothesis in which PKC isozymes possess different domains for binding. Although this 'one-domain, one RACK' hypothesis may be seen as an inefficient method of regulating protein binding, it would provide a powerful mechanism of controlling and regulating isozyme-specific activity.

How does PKC 'find' its binding proteins?

The experimental data suggest that both active and inactive forms of PKC are localised. The localisation of inactive PKC often appears to be membrane associated (Disatnik, 1994; Goodnight, 1995). It is probable that such positioning facilitates the rapid activation of the PKC *via* phospholipid metabolism which occurs at the membranes. The presence of DG and PS in the membranes promotes their interaction with PKC and opens up the conformation of the kinase to expose its catalytic and RACK binding domains. It is proposed that this active PKC now translocates to a specific RACK, where it phosphorylates substrates within the vicinity of this target. What mediates the release of the active PKC molecule from the relatively tight association with membranes? What prevents the active PKC from phosphorylating any potential substrate on its journey to a specific RACK? *In vitro*

studies clearly indicate that PKC can phosphorylate a large number of substrates. Consequently, the tethering of PKC to its final destination within the cell could be an important means of restricting its activity. For this reason, the idea of PKC receptors is attractive, but it similarly highlights the importance for activating PKC near its site of action. If this argument was true it implies that the localisation of inactive PKC is crucial. It is unclear at the moment whether the localisation of inactive PKC affects the final destination of the active PKC.

It is possible that activated PKC is 'removed' from its tight association with the membranes through an interaction with a 'shuttle' protein. This shuttle protein would maintain the PKC in an open, active conformation but mask the catalytic site, preventing PKC-mediated phosphorylation. Perhaps it is this complex which translocates throughout the cell until it encounters a RACK which relieves the shuttle protein of the PKC. Such a shuttle protein should exhibit a ubiquitous role in transporting active PKCs throughout the cell, displaying no specificity for PKC isozymes. It should always be present in the cell, and its absence should produce a severe phenotype. The presence of such a shuttle protein would link PKC with its activators DG and PS at the membrane, to its source of substrate as determined by RACKs.

Presumably, once PKC is bound to RACKs, the kinase can only phosphorylate substrates to which it has direct contact. This is a very strict control over the PKC's activity, and though it facilitates a precise regulation over substrate phosphorylation, it means that the positioning of the RACK is crucial. For this reason it could be predicted that a RACK would be a structural protein, allowing PKC to bind throughout the structure and phosphorylate several substrates. The RACK could also be a member of a complex, promoting specific phosphorylations of a limited number of proteins.

Do RACKs exist *in vivo*?

Experimental data from *in vitro* studies suggested that purified mammalian RACK proteins behave as receptors for activated PKC (see introduction). To date, however, there is little evidence to show that PKC binds to RACKs in the cellular environment. Although it was observed that microinjected RACKs inhibit the normal translocation of PKC in *Xenopus* oocytes, it only confirms that RACKs have the potential to bind PKC, but does not indicate that such a binding has physiological meaning (see introduction). Without any *in vivo* evidence suggesting that PKC and RACKs co-localise, or any genetical data assessing the effects of RACK mutations on PKC translocation, the cellular function of RACKs remains questionable.

Are there PKC-binding proteins in yeast?

The apparent absence of PKC isozymes in *Saccharomyces cerevisiae* (only Pkc1p has been identified) presents an interesting situation as the diversification of PKC into distinct isozymes has not occurred in yeast as in higher eukaryotes. Pkc1p is presumed to have a number of *in vivo* substrates which are located at particular intracellular compartments. As in higher eukaryotes, the substrates which are phosphorylated by Pkc1p determine the intracellular response. Consequently, the targeting of PKC to intracellular locations could still be important, irrespective of the number of isozymes representing the PKC activity.

To date two proteins, Rck2p and Gcd6p (W. Richards, personal communication), have been isolated as putative yeast RACKs. Gcd6p is a member of a multisubunit complex involved in protein translation. The potential binding of Pkc1p to Gcd6p would position the kinase at a site where PKC activity could have a dramatic effect on the control of protein synthesis. However, as yet, no direct interaction between Gcd6p and Pkc1p could be detected, using immunoprecipitation or the yeast two-hybrid system (W. Richards, personal communication). The link between polypeptide synthesis, Gcd6p and Pkc1p, is interesting considering the possible interaction of Rck2p with GroEL (see below). This chaperone is thought to have a role at the ribosome in stabilising the elongating polypeptide chain until the complete protein is folded into its native conformation (Ellis, 1990). Perhaps the targeting of Pkc1p, by Gcd6p and/or Rck2p to the ribosomal machinery regulates protein translation in concert with protein stability *via* chaperones.

To date, there is little information regarding the translocation and localisation of Pkc1p in yeast cells. Such information could provide clues regarding the regulation of Pkc1p and its putative associated proteins.

Is Rck2p a yeast PKC-binding protein?

Although unlikely (see chapter 4) a concern was raised as to the possible PKC-binding activity of the 17 amino acids fused in frame to the *LacZ* of the recombinant λ gt11 phage, λ 7.2. This matter could be resolved by expressing or synthesising this polypeptide and assessing its ability to bind PKC. It would be preferable to express this polypeptide from a pET-like vector, where it would be fused to only a few amino acids. Expression of the 17 amino acid polypeptide when fused to a larger polypeptide moiety may mask any PKC-binding domain. In the event that this short polypeptide did fortuitously confer PKC-binding activity, its primary structure could provide useful information regarding the binding requirement of PKC isozymes.

Another uncertainty regarding the identification of the original λ gt11 recombinant phage was raised during the progression of this research. Were the phage identified because of their association with exogenously added PKC, or were

they identified due to the ability of the anti-PKC antibodies to recognise the GroEL from the bacterial host? It is anticipated that the cross-reactivity of GroEL with the anti-PKC antibodies would be unaffected by the presence or absence of the exogenous PKC. The fact that the antibodies failed to recognise the recombinant phage in the absence of exogenous PKC strongly indicates that the presence of the added PKC was essential. This observation consequently suggests that the expressed Rck2p polypeptide interacted with the exogenous PKC, and that the bacterial GroEL played no part in the screening results.

The only way to demonstrate that purified Rck2p associates with PKC *in vitro* is to remove the GroEL completely. The interaction of GroEL with unfolded proteins can be disrupted with ATP. It has been suggested that washing resin with 50mM TAE (pH7.5), 50mM KCl, 20mM MgCl₂ and 5mM ATP should be sufficient to remove any associated GroEL from an immobilised Rck2p fusion protein (Neil Ransom, personal communication). An Rck2p fusion protein, free of interacting polypeptides, can then be allowed to interact with cell extract, and protein associations analysed.

The direct expression of Rck2p in yeast is also possible using a pEG(KT)-based vector (see chapter 6). The purification of the fusion proteins using glutathione resin may indicate which yeast polypeptides associate with this protein. It would be hoped, if Rck2p is a true RACK, that Pkc1p or other yet unidentified kinases would be associated although it is plausible that the GroEL homologue, Mif4p, may also bind to it.

Is the association of Rck2p with GroEL biologically important?

Rck2p is not detected *in vivo* when expressed from a pET vector and rapidly disappears *in vitro* when cleaved from a fusion protein. The apparent Rck2p instability *in vivo* may account for its association with chaperones. If such instability would also be found in yeast, then chaperone association may protect Rck2p from degradation and thereby extend its functional life-span. It is also possible that the chaperone interaction with Rck2p is physiologically necessary for the correct conformation of the yeast protein and its subsequent function.

The previous observation of GroEL association with overexpressed recombinant proteins (Neil Ransom, personal communication) cast doubt on the biological importance of the Rck2p-GroEL interaction. However, the particularly strong affinity of the two proteins could indicate that some specificity is responsible for this stable association. Furthermore, the failure to detect GroEL association with other recombinant proteins (see chapter 7) suggests that it does exhibit specific affinity for Rck2p. A question that needs to be addressed though is whether the interaction is due to the affinity of GroEL for Rck2p, or vice versa, or the consequence of both.

The direct expression of Rck2p in yeast using the pEG(KT)-based vectors (see chapter 6) would indicate whether the yeast homologue of GroEL, Mif4p, interacts with the Rck2p fusion protein. Such an interaction would reinforce the suggestion that the association of chaperones with Rck2p is physiologically significant. Mif4p has been localised to the mitochondria and a heat-sensitive allele of *MIF4* results in defective mitochondrial protein assembly (LaRossa and Van Dyk, 1991). The β -subunit of F1-ATPase and cytochrome b2, for example, are both imported into the mitochondria of *mif4* mutants but are incorrectly assembled. Although Rck2p has no mitochondrial localisation sequence its whereabouts in the cell is unknown.

Chaperones, kinases, and RACKs

'Molecular chaperones are a ubiquitous family of cellular proteins which mediate the correct folding of other polypeptides' (Ellis, 1990). Chaperones function to prevent incorrect interactions of a growing polypeptide chain during translation, unfold and then refold proteins which enter organelles, and prevent the accumulation of protein aggregates during environmental stress conditions (Ellis, 1990). The crystal structure of GroEL has been analysed (Braig, et al., 1994) and regions of GroEL facilitating the binding of unfolded proteins were identified (Fenton, 1994). Nevertheless, little is known about the structural basis of GroEL 'substrates'. Until the mechanism by which chaperones bind a wide range of unrelated proteins is determined the true nature of the GroEL-Rck2p association will be difficult to establish.

The covalent modification of bacterial GroEL after a heat-shock response provides a possible connection between chaperones and kinases. The stress conditions mediate the reversible phosphorylation of GroEL, producing a more active GroEL (Sherman, 1992). This modified GroEL is therefore more able to refold damaged proteins and prevent protein aggregation. Presumably, this modification occurs in eukaryotic cells and it would be interesting to identify the kinase responsible for the activity. Could Rck2p, when associated with Mif4p, mediate the binding of a kinase necessary for the chaperone phosphorylation in yeast?

A recently published paper describes the regulation of a protein kinase, PKR, by a chaperone. PKR is a eukaryotic serine/threonine protein kinase which is activated by double-stranded RNA (dsRNA) (Melville, et al., 1997 and references therein). Active PKR mediates the phosphorylation of eIF-2 α and the subsequent block in protein synthesis. A cellular inhibitor of PKR, p58^{IPK}, displays homology at its carboxyl terminus to the DnaJ heat shock protein. Interestingly, the activity of p58^{IPK} is also regulated by its own inhibitor protein, I-p58^{IPK}, which was found to be the chaperone hsp40 (Melville, et al., 1997). It is believed, therefore, that regulation of PKR occurs *via* the heat shock stress pathway. It is interesting to speculate what comparisons may be found in yeast; perhaps a PKC which is regulated by an

association of Rck2p and Mif4p or through the association of Gcd6p with the eIF2 complex.

Future Work

It would be interesting to find *RCK2* homologues in other organisms. Although the comparison of *RCK2* with the computer databases identify no homologous sequences, it is probable that they do exist. The fact that Rck2p is predicted to have a specialised function could suggest that previous research has ignored Rck2p homologues. However, the possibility that Rck2p is unique to yeast should not be overlooked. Using a radioactively labelled *RCK2* fragment as a probe against DNA prepared from a range of organisms could be informative. However, a more useful experiment, when the acquisition of anti-Rck2p antibodies is achieved, would be Western blot analysis of protein extracts from various organisms. This immunological investigation has the advantage over DNA hybridisations as it compares protein structure, rather than primary DNA sequence, and is therefore less susceptible to evolutionary variations and differences in codon usage. In addition, similarities at a protein level suggests a more biological significance than that of DNA sequence.

As Rck2p and yRack1p are thought to be RACK-like proteins, it is possible that antibodies cross-reacting with mammalian RACK1 may recognise these yeast polypeptides. Preliminary analysis has implied that these antibodies may recognise these proteins, although further investigation is required. Such immunological evidence would indicate that these proteins show some structural homology to RACK1, even in the absence of convincing sequence homology.

The failure of Northern blot analysis to detect transcripts of *RCK2* may suggest that the RNA is rapidly degraded and/or present in small quantities. A method of amplifying transcripts, using PCR and reverse transcriptase, could be carried out for further assessment. Once a transcript can be identified its expression at particular points of the cell cycle in synchronised yeast cells could be determined.

Although bacteria containing null mutations of GroEL are lethal, conditional lethal mutants are available. Such mutants could help in purifying Rck2p without bound bacterial protein. The purification of GroEL from bacterial cells could also be carried out. Purified preparations of both GroEL and Rck2p could then be used for biochemical analysis of their interaction *in vitro*.

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False views, if supported by some evidence, do little harm, for everyone takes a salutary pleasure in proving their falseness; and when this is done, one path towards error is closed and the road to truth is often at the same time opened.

Charles Darwin (1809-1882)