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Structure and function of the protein kinase C gene family

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Abstract. Protein kinase C is a serine/threonine protein kinase which is activated in the cell in response to production of diacylglycerol. Gene cloning has revealed the presence of a highly related family of enzymes, which can be sub-divided into groups on the basis of sequence conservation. Differences are seen in both isoform distribution and associated biochemical activity, for example in substrate specificity and activator requirements. Comparison of the protein sequences and *in vitro* activities of the protein kinase C isoforms has identified regions important for particular aspects of kinase function. Some of these regions are also found associated with other proteins, allowing confirmation of the assigned activity. Site-directed mutagenesis has confirmed the presence of an autoinhibitory sequence involved in protein kinase C regulation and generated constitutively activated proteins which can be used to study differential isoform function. These same sequences have been shown to play a role in substrate selection, perhaps by competition for binding to the active site. Protein kinase C is known to be a phosphoprotein and the identification of regulatory sites phosphorylated by a 'PKC-kinase' suggest a possible alternative route for regulation of protein kinase C activity.

Keywords. Protein kinase C; structure; function.

1. Introduction

The protein kinase C (PKC) family of serine/threonine protein kinases plays a major role in signal transduction in response to the production of diacylglycerol (reviewed in Nishizuka 1988; Parker *et al* 1989). The major sources of diacylglycerol are the agonist-induced hydrolysis of phosphatidylinositol bisphosphate (PIP_2) by a phospholipase C (Meldrum *et al* 1991) and the hydrolysis of phosphatidylcholine by a phospholipase D (Billah and Anthes 1990). PKC is also the major receptor for the class of tumour-promoting phorbol esters. These molecules appear to act in a manner analagous to DAG and activate the kinase function of PKC (Castagna *et al* 1982) and thus have been used experimentally to investigate PKC function. The activation of PKC has been implicated in the response to a wide range of effectors in many cell types (reviewed in Woodgett *et al* 1987) as the responses can either be elicited by short-term treatment with phorbol esters [e.g., induction of gene expression through the transcription factor AP1 (Angel *et al* 1987)] or abolished by long-term treatment which leads to the down regulation and loss of endogenous PKC from the cell (e.g., see Woodgett *et al* 1987). PKC is known to regulate cell motility and cytoskeletal function as treatment with the phorbol ester TPA causes profound alterations in cell shape and PKC is known to phosphorylate cytoskeletal components. The activation of PKC has also been implicated as playing

an important role during development; TPA induces severe disturbances in the behaviour and growth of the nematode *Caenorhabditis elegans* and resistant mutants isolated by transposon tagging contain insertions in a gene with high homology to PKC (Tabuse *et al* 1989). PKC has been implicated in many other signal transduction pathways including regulation of neuronal ion channels (Knox and Kaczmarck 1992), and in carcinogenesis (Borner and Fabbro 1992). The activation of PKC in the intact cell is often implied as phospholipid turnover is known to occur, and can be directly established by examination of PKC translocation to the membrane (Kawahara *et al* 1980; Kikkawa *et al* 1982). As diacylglycerol is generated in the membrane, PKC membrane association is stabilized by interaction with its activator and, under certain extraction conditions, will co-purify with membrane vesicles.

Although originally defined as a single activity, the PKC gene family consists of an expanding number of gene products. A family of at least eleven genes has been described in mammalian cells and many PKCs from non mammalian sources have also been identified as activities or as gene sequences (reviewed in Stabel and Parker 1991). Alternative splicing gives rise to a potentially larger number of gene products (Coussens *et al* 1987; Housey *et al* 1987; Ono *et al* 1988). The high degree of heterogeneity raises the question of whether family members have overlapping or distinct functions within the cell. The conservation of a particular isoform between species certainly provides circumstantial evidence that each is playing a specific role within the organism. There is increasing evidence for functional differences associated with activation of distinct isoforms. Selective down regulation or membrane translocation of PKC has been observed suggestive of differential activation (Kiley *et al* 1991; Olivier and Parker 1994). Variation in the ability of expression of PKC isoforms to activate transcription from serum responsive promoters in co-transfection studies has been reported (Ohno *et al* 1994) and a difference in the ability of PKCα and ß to induce neural competence in Xenopus embryos has also been reported (Otte and Moon 1992). In a different approach, PKCs can be added back to permeabilized rat basophilic RBL-2H3 cells to reconstitute phorbol ester-induced exocytosis (Ozawa *et al* 1993a, b). Addition of PKCß or δ restored the response whereas PKCα and PKCε did not, although the latter isoforms did appear to mediate feedback control on phosphatidylinositol turnover.

The isoforms can be divided into four groups on the basis of sequence homology (figure 1) and biochemical activity. The originally identified conventional cPKCs (α,ß and γ) (Coussens *et al* 1986; Parker *et al* 1986), the novel nPKCs (δ,ε,θ and η) (Ono *et al* 1987, 1988; Osada *et al* 1990, 1992; Bacher *et al* 1991; Baier *et al* 1993) and the atypical aPKCs (ζ and λ) (Ono *et al* 1987, 1988 1989; Akimoto *et al* 1994) have recently been joined by an unusual family member which is predicted to have a transmembrane domain (PKCμ) (Johannes *et al* 1994).

2. Differences in isoform distribution

Differences between isoforms have been reported in tissue distribution and subcellular localization as well as *in vitro* kinase -activity characteristics. Some isoforms (such asPKCα, δ and ζ) are expressed in a wide variety of tissues whereas others seem to show greatly restricted distribution (PKCγ is found exclusively in the brain and spinal cord) (Nishizuka 1988). This pattern of expression means that most cell

types express only a subset of isoforms suggestive of a functional diversity. Differences in subcellular localization have also been reported. Thrombin causes rapid nuclear translocation of PKCα in IIC9 fibroblasts but not of PKCε and ζ which are also expressed in these cells (Leach *et al* 1992). Translocation of PKC to the nucleus seems to be a cell-type specific effect (Thomas *et al* 1988) and depends on the activator used as bryostatin (another artificial activator of PKC) causes PKC nuclear association in HL60 cells whereas phorbol esters do not (Hocevar and Fields 1991). PKC has also been reported to be associated with the cytoskeleton and, again, such association can be isoform specific. For example, PKCα has been found associated with focal contact points in rat embryo fibroblasts whereas there is no evidence for such a localization for PKCε and ζ present in the same cells (Jaken *et al* 1989). Such differences might be expected to reflect specificity in activating molecules which could be generated at different membrane locations. For example different sources of diacylglycerol due to hydrolysis of PIP_2 or phosphatidylcholine (PC) could lead to selective activation of one particular isoform. The phospholipases which hydrolyse PIP_2 are associated with the plasma and nuclear membranes, whereas the phospholipase D involved in PC breakdown is associated with microsomal fractions. As the former hydrolysis is associated with transient changes in Ca^{2+} concentration and the latter is not, different PKC isoforms may be activated in the two circumstances (see below). Intracellular receptors for activated PKC (RACKs) have also been identified (Mochly-Rosen *et al* 1991) and the sequence of a cDNA clone encoding one such receptor, RACK1 (Ron *et al* 1994), reveals sequence homology to the β subunit of the heterotrimeric GTP-binding proteins. RACK1 has been shown to bind PKC in a saturatable manner but not other kinases tested. If these RACKs show a distinct subcellular localization, coupled with a specificity for certain PKC isoforms, then they could prove to play a major role in selective translocation on activation.

3. Differences in biochemical activities

Much of what we know of the biochemical activities of the individual isoforms of PKC comes from purification after overexpression in either insect cells or cell lines. Purification of individual isoforms from tissue sources has not been performed in all cases. There are several major differences in the biochemical characteristics of the families of isoforms. The most quoted difference in the properties between isoforms is that of calcium dependency. The cPKCs show Ca^{2+} dependency in the physiological range (Jaken and Kiley 1987; Huang *et al* 1988; Marais and Parker 1989) whereas the nPKC activities are independent of the Ca^{2+} concentration. It is worth noting that the Ca^{2+} dependency of the *in vitro* kinase activity of the cPKCs can vary depending on the substrate used. Support for this constituting a functional difference between families comes from investigation of the properties of their membrane association. Purified cPKCs interact with plasma membranes in Ca^{2+} dependent manner (Wolf *et al* 1985). Extraction from cells in the presence or absence of Ca^{2+} leads to a differential distribution of the isoforms consistent with the implied Ca^{2+} dependency of their activities. PKCα will associate with the membrane in the presence of Ca^{2+} alone whereas PKCδ and ε require prior treatment of the cell with phorbol ester (Kiley *et al* 1990; Olivier and Parker 1991).

Studies using a range of phorbol esters on purified isoforms of PKC suggest variation in their responsiveness which may help to explain differences in the physiological effects of these molecules (Ryves *et al* 1991). PKCζ and PKCλ are not activated by phorbol esters/diacylglycerol unlike all the other isoforms tested so far. There have been no reports so far of diacylglycerols with different acyl side chains specifically interacting with particular isotypes but the heterogeneity in the diacylglycerol population generated in the intact cell in response to stimulus suggests that there is a potential for such selective activation to occur. Fatty acids and lysophosphatidic acid show differential isotype stimulation in synergy with diacylglycerol, but the physiological relevance of this is unknown (reviewed in Asaoka *et al* 1992). Various isoforms of PKC have also been reported to be activated by the product of phosphatidylinositol 3-kinase (Nakanishi *et al* 1993; Singh *et al* 1993) but there is no data suggesting co-ordinate activation in the intact cell.

PKC shows a preference for substrates with basic residues surrounding the serine or threonine which is to be phosphorylated. A detailed analysis of the primary structural requirements for substrates of PKCs α, ß1 and γ has been made using a series of peptide substrates differing in the number and position of N- and C-terminal basic residues (Marais *et al* 1990). This failed to reveal any significant differences for PKCs α and ß but suggested that PKC γ had a strong preference for substrates with a C-terminal basic amino acid. The nPKCs show a greatly reduced *in vitro* substrate range. For example they show no activity towards histone IIIS, a commonly used assay substrate for PKCs, although they will phosphorylate peptide substrates based on the pseudosubstrate sequence of PKCs (Schaap *et al* 1989; Bacher *et al* 1991; Olivier and Parker 1991). Neither of these substrates is physiologically relevant but some differences in the ability of PKC isoforms to act on true PKC substrates has been reported; for example the EGF receptor makes a better substrate for PKCs α and ß than γ, PKC α, ß1 and γ show a much higher activity towards glycogen synthase kinase 3ß (GSK3ß) than PKC ß2 (Goode *et al* 1992). This is particularly striking as PKC ß2 is a splice variant of PKC ß1 differing only in the C-terminal 50 amino acids, suggesting that these residues must play an important role in substrate selection. PKCε shows no activity towards GSK3ß. Differential activity towards a few other substrates has been reported [e.g., GAP-43, a prominent PKC substrate in neurons (Sheu *et al* 1990)] but information is not available for a large number of substrates tested with all available isoforms. The biochemical characteristics of the aPKCs are not well defined. The lack of a readily available activator has meant that it has not been possible to identify good substrates for these kinases.

4. Information from sequence comparisons

Sequence comparison of the PKC family members has facilitated identification of regions important to common characteristics as well as highlighting differences which may well reflect divergence in biochemical activity and function. Sequence comparison (figure 1) reveals four highly conserved domains (Cl-4) interspersed by five variable regions (Vl-5). The C3 and C4 domains contain sequences which are conserved in all protein kinases (Hanks *et al* 1988) and thus the C-terminal

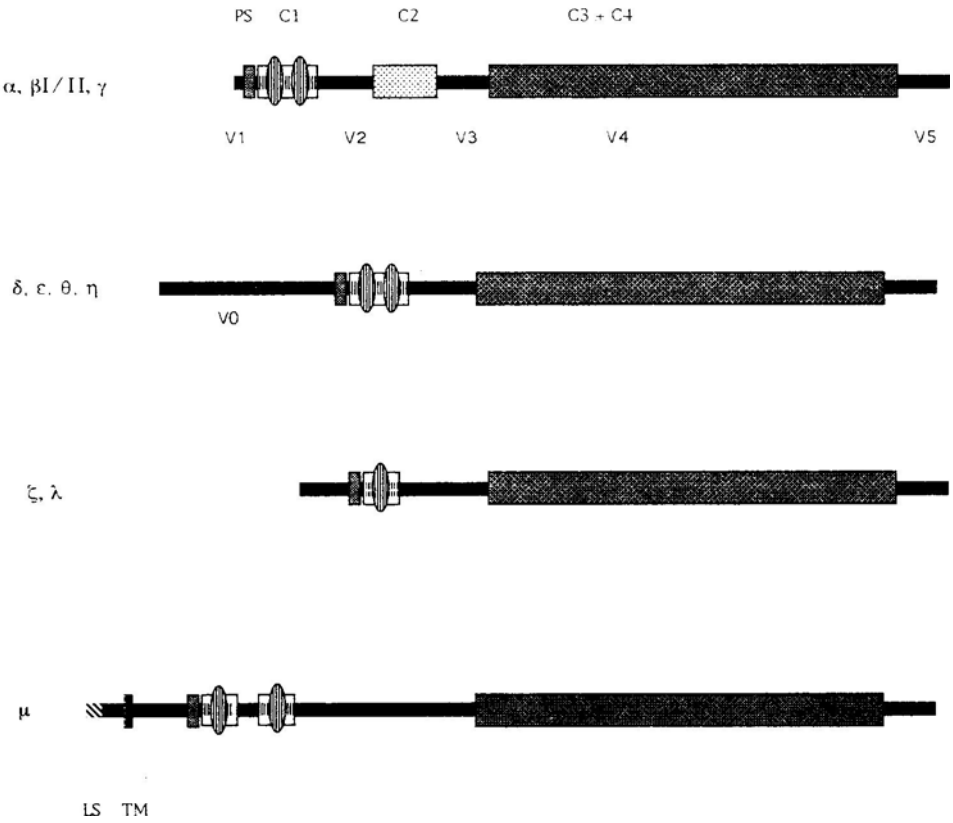


Figure 1. Domain structure of mammalian protein kinase C.

Conserved (C) regions are indicated as Cl-4 and variable as Vl-5. The pseudosubstrate sites (PS) are indicated. Putative signal sequence (LS) and transmembrane domain (TM) of PKCμ are also shown. See text for references.

portion of the molecule is presumed to contain the kinase active site (Parker *et al* 1989).

The Cl domain contains two copies of a cysteine rich motif with similarity to the so-called zinc finger regions of DNA binding proteins. Compared to the zinc fingers found in a variety of transcription factors and associated with DNA binding, the PKC repeat has an extra two cysteine and two histidine residues which are also highly conserved. Thus the PKC finger can be thought of as an extended zinc finger motif of general structure His-X_{12}-Cys-X_2-Cys-$X_{13/14}$-Cys-X_2-Cys-X_4-His-X_2-Cys-X_7-Cys. This extended motif is also found to be conserved in diacylglycerol kinase (Schaap *et al* 1990a; Sakane *et al* 1990). These protein sequences are responsible for the diacylglycerol/phorbol ester binding properties of PKC as defined by deletion analysis (Ono *et al* 1989; Cazaubon *et al* 1990) as point mutation of two central cysteines abolishes phorbol ester binding. These mutations would destroy any potential zinc finger. PKC has been shown to be a zinc binding protein (Hubbard *et al* 1991) but there is as yet no evidence for the existence of a

finger-like structure. A bacterially expressed segment encompassing a single repeat motif from PKCγ has been shown to bind zinc (Quest *et al* 1994a). The authors proposed a model in which a single extended finger co-ordinates two zinc ions and also binds phorbol ester (Quest *et al* 1994b). Deletion analysis defined a minimum phorbol ester binding sequence extending from the first conserved histidine residue to a valine residue (V_{144}) immediately downstream of the last but one cysteine (figure 2). The presence of the most C terminal cysteine is, however,

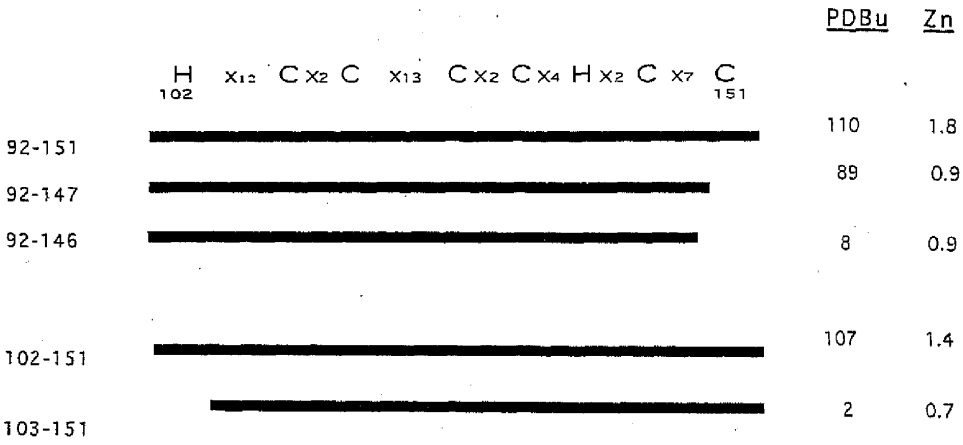


Figure 2. Phorbol ester and zinc binding to an isolated cysteine motif from PKCγ.

A single cysteine motif (C_6H_2) from PKO*y* was expressed in *E. coli* as a fusion protein (Quest *et al* 1994a, b). The purified protein was able to bind the phorbol ester phorbol dibutyrate (PDBu) and co-ordinate two zinc ions. The relative binding of fusion proteins containing a series of N- and C-terminal deletions was determined. Binding of PDBu is expressed in pmol/nmol of fusion protein and the zinc binding in mol zinc/mol protein. Loss of the most N-terminal conserved histidine residue abolished phorbol binding and reduced the stochiometry of zinc binding to one. Deletion of the C-terminal conserved cysteine residue reduced the stochiometry of zinc binding but did not abolish PDBu binding. It did enhance the latter interaction in some fusion proteins tested, perhaps by providing additional interaction sites with phosphatidylserine, as this region contains some positively charged residues. Deletion of a valine residue at position 144 rendered the purified fusion protein unable to bind PDBu. This residue is conserved in many isoforms of PKC which bind phorbols, but also in PKC ζ which does not. Conversely *unc*-13 and a PKC homologue *tpa*-l from *C. elegans* both bind phorbols but do not contain a valine in this position.

required for co-ordination of the second zinc ion. Deletion of the first histidine residue abolishes phorbol binding and reduces the co-ordination of zinc to one, suggesting that the two zinc ions are not functionally equivalent. The valine residue found to be essential for phorbol binding is conserved in many isoforms of PKC which bind phorbols, but also in PKCζ which does not. Conversely *unc-13* and a KC homologue *tpa*-l from *C. elegans* both bind phorbols but do not contain a valine in this position (Tabuse *et al* 1989; Maruyama and Brenner 1991).

cDNA clones have been isolated containing sequences which would give rise to proteins predicted to bind to diacylglycerol as they contain sequences highly homologous to the diacylglycerol-binding domain of PKC (figure 3). However they contain no sequences with homology to protein kinase domains and so are not

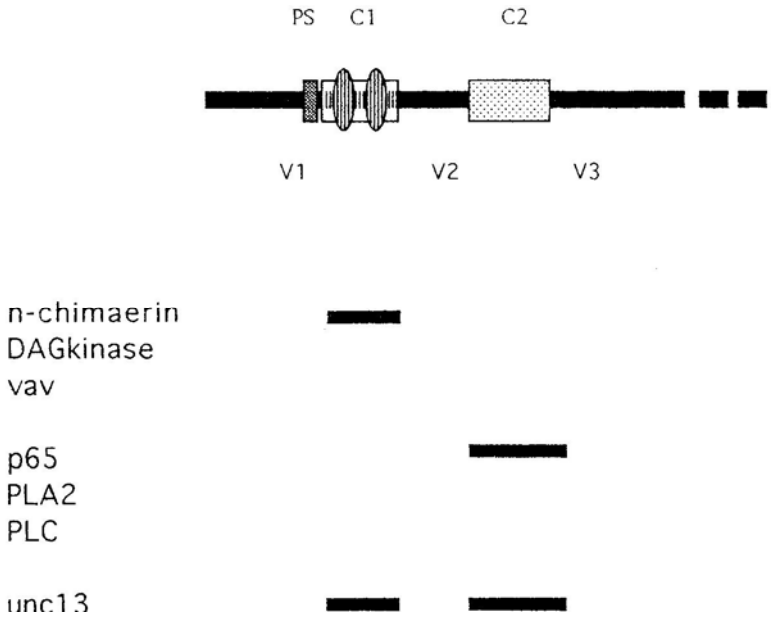


Figure 3. Homologous regions between PKCs other proteins.
Areas of shared homology between PKC and other proteins are highlighted relative to the conserved regions C1 and C2. See text for references.

members of the PKC family. Such proteins include n-chimaerin (Ahmed *et al* 1990), the proto-oncogene *vav* (Katzav *et al* 1989) and *unc-13* (Maruyama and Brenner 1991) a protein whose mutation is known to cause developmental abnormalities in *C. elegans.* These proteins may or may not bind phorbol esters in addition to diacylglycerols as the residues essential for binding may differ slightly between the two ligands. Some recently identified PKC homologues in *Saccharomyces cerevisiae* are activated by diacylglycerol but do not bind phorbol esters (Ogita et *al* 1990).

n-Chimaerin was isolated as a brain-specific human cDNA sequence (Ahmed *et al* 1990). The N-terminal portion of the predicted protein contains a single copy of a sequence which has 50% homology to the cysteine-rich motif found duplicated in the regulatory domains of the PKC family members. This cysteine-rich region has been shown to confer phorbol ester binding activity on the n-chimaerin protein (Ahmed *et al* 1990) but its ability to bind diacylglycerol has not been investigated. The C-terminal portion of n-chimaerin contains sequences with high homology to regions of the p85 subunit of the phosphatidylinositol 3-kinase (reviewed in Fry 1992) and the breakpoint cluster region (BCR) gene product (Hall *et al* 1990), which is involved in Philadelphia chromosome translocation (Heisterkamp *et al* 1985), This region has since been found to contain GTPase activating (GAP) activity for the $p21^{rac}$-family of $p21^{ras}$-like proteins (Diekmann *et al* 1991). Thus n-chimaerin may play an important role in signal transduction by providing a link between diacylglycerol generation and regulation of the activity of $p21^{rac}$. The human proto-oncogene *vav* contains two copies of a highly homologous cysteine repeat

(Katzav *et al* 1989) but little is known about its function. In *C. elegans, unc-13* mutant organisms have uncoordinated movement, apparently due to a defect in the nervous system rather than the musculature. Isolation of the *unc-13* gene revealed a protein with a central region containing a single copy of a diacylglycerol-binding motif (Maruyama and Brenner 1991). This region has been shown to bind both phorbol esters and diacylglycerol when expressed in bacteria. The phenotype of *unc-13* mutants suggests that this protein may play a role in neurotransmitter release from neurons (Hosono *et al* 1989). Phorbol esters are known to enhance neurotransmitter release (Matthies *et al* 1987) suggesting that physiological effect of phorbol esters may be due to the alteration in function of diacylglycerol-binding proteins other than PKC.

The function of these putative diacylglycerol binding proteins and the consequences of their interaction with diacylglycerol is unknown. The GAP activity associated with n-chimaerin suggests that it may be a molecule involved in cross-talk between pathways. Both *vav* and *unc-13* are significantly larger than diacylglycerol-binding requires and so have the potential to encode other functions. It is possible that such proteins could act as 'sinks' for diacylglycerol to limit the activation of PKC, as 'cross-talk' molecules to allow coordination between separate signalling pathways or they could be alternative effectors for diacylglycerol. The ability of these proteins to respond to diacylglycerol generation in the intact cell, for example by translocating to the membrane, has not been described. The existence of such proteins casts some doubt over the interpretation of experiments which they rely on phorbol ester stimulation as the sole criterion for PKC activation.

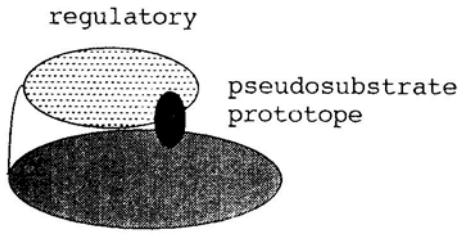
The aPKCs ζ and λ contain only a single copy of the cysteine motif. Despite the fact that a single copy of the motif from PKCγ is sufficient to bind phorbol esters (Quest *et al* 1994a), neither of the aPKCs have been shown to bind phorbol esters or to respond to diacylglycerols as assayed by activation of kinase function *in vitro* or translocation to the membrane in response to activation of phospholipase C in the intact cell. It may be that a different lipid effector is required for activation of these isoforms as has been suggested for PKC ζ (Nakanishi *et al* 1993).

The C2 domain is only found in the cPKCs and correlates with an *in vitro* dependence on Ca^{2+} for kinase activity. Furthermore the phorbol ester-induced translocation of PKCs lacking the C2 domain is independent of Ca^{2+} (Kiley *et al* 1990; Olivier and Parker 1991). Deletion of this region from a cPKC renders the kinase activity independent of Ca^{2+} (Kaibuchi *et al* 1989; Ono *et al* 1989). A sequence resembling a known Ca^{2+} binding motif known as an 'EF hand', has been noted just downstream of the C2 region in PKCα (Parker *et al* 1986). However, although this is conserved in PKCß it is absent from PKCγ which still displays Ca^{2+} dependency, so the C2 sequences are a more likely Ca^{2+} binding domain. Sequences homologous to the C2 region (figure 3) have been identified in p65 and phospholipases C and A_2. Bacterially expressed p65 has a strong association with sphingolipids and phosphatidylserine but not lipids in general (Perin *et al* 1990). A corresponding sequence in phospholipase A_2 has been shown to be responsible for the calcium dependent translocation of this enzyme to the membrane (Clark *et al* 1991).

5. Autoinhibitory sequences in PKC

The arrangement of the conserved sequences in PKC has led to the proposal of a

two domain structure for PKC with the N-terminal regulatory domain normally acting to inhibit the C-terminal kinase activity (figure 4). This model is supported by the observation that partial proteolysis with trypsin or calpain leads to a preferential cleavage in the V3 hinge region and the generation of a 30 kDa fragment which can bind phorbol esters and a 50 kDa fragment that has constitutive kinase



RFARKGALRQKNVHEVKDH	α
RFARKGALRQKNVHEVKNH	β
LFCRKGALRQKVVHEVKSH	γ
TMNRRGAIKQAHIHYIKNH	δ
PRKRQGAVRRR-VHQVNGH	ε
LHQRRGAIKQAKVHHVKCH	θ
TRKRQRAMRRR-VHQVNGH	η
SIYRRGARRWRKLYRANGH	ζ
SIYRRGARRWRKLYCANGH	λ

Figure 4. Domain structure of protein kinase C.

The arrangement of conserved regions in PKC and the ability to generate constitutive kinase activity by partial proteolysis in the V3 region has led to a two domain model for protein kinase C structure. The pseudosubstrate sequence has been proposed to lie in the active site when the enzyme is catalytically inactive, preventing substrate access. A comparison of the pseudosubstrate sequences for the isoforms of rat PKC are shown for comparison (PKCθ is the human sequence). The alanine residue at the potential phosphorylation site is underlined in bold and conserved basic residues are underlined. For references please refer to the text.

activity (Lee and Bell 1986; Huang *et al* 1989; Young *et al* 1988; Kishimoto *et al* 1989). Low resolution analysis of crystals of PKCß confirms this two domain structure (Newman *et al* 1994). The inhibition in the unstimulated state has been proposed to be brought about by a short peptide stretch found just upstream of the Cl domain (House and Kemp 1987). Sequence analysis of several PKC substrates *in vitro* and studies using synthetic peptides as substrate has indicated a preference for basic residues surrounding the phosphorylated residue (Marais *et al* 1990). This pseudosubstrate site or autoinhibitory domain has all the sequence characteristics of a substrate for PKC but it lacks the serine or threonine residue to act as phosphate acceptor and instead contains a conserved alanine residue. Peptides containing this sequence are potent competitive inhibitors of PKC activity *in vitro* (House *et al* 1987) and inclusion of a serine residue in place of the alanine generates a good substrate. Comparison of this region in all the PKCs (figure 4) reveals that they all contain many basic, positively charged residues, presumably reflecting a common sequence requirement for substrates. There are variations which may reflect subtle differences in substrate preference. For example PKCη contains a basic arginine residue immediately preceding the conserved alanine and PKCμ, a serine, whereas other isoforms contain a conserved glycine (Stabel and Parker 1991). Although detailed kinetic data is often lacking, in general it has been found that the best substrates are not generated from an isoform's own pseudosubstrate prototope, but each displays a higher activity towards that of another. This may reflect a need to have a sequence of moderate affinity for the active site.

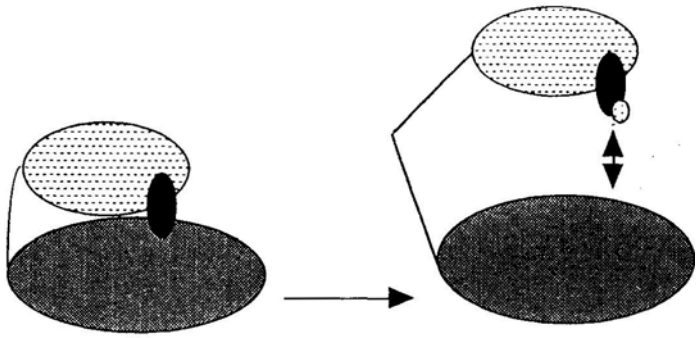
In the unstimulated state the autoinhibitory sequence is thought to lie in the active site and prevent substrate access. On binding of activators, a conformational change leads to the removal of this region from the active site and allows substrates to enter the site where phosphorylation can occur. This is supported by the observation that antibodies directed against this region cause activation of PKC *in vitro* (Makowske and Rosen 1989).

Sequence analysis of a receptor for activated C-kinase, RACK1, has revealed short regions of sequence homology between RACK1 and PKC. It has been postulated that PKC contains a RACK binding site which normally interacts with its internal RACK-homology domain (pseudo-RACK site) when the protein is inactive. The conformational change associated with activation would allow the exposure of the RACK-binding domain to allow it to interact with other proteins, such as RACK1. A peptide encompassing this internal pseudo-RACKl site has been shown to cause activation of PKC-β (Ron and Mochly-Rosen 1995). This suggests that the internal interaction between the RACK-binding domain and the pseudo-RACK site also contributes significantly to the stabilization of the inactive form of PKC, and disruption of this interaction may be sufficient to allow substrate access to the active site.

6. Mutagenesis of the pseudosubstrate site

To test this theory, a point mutation was made in PKCá to replace the alanine residue with a charged glutamic acid residue (Pears *et al* 1990). Inclusion of this substitution in the inhibitory peptide reduced the K_i for inhibition by approximately 100-fold, so in the mutant protein, E25PKCα, this region would have a comparably reduced affinity for the active site. The wild type and mutant proteins were expressed using a baculovirus expression system and the effector dependence of their kinase activities determined after partial purification. Consistent with the importance of the autoinhibitory region, the effector dependence of E25PKCα was reduced compared to that of the wild type protein (figure 5). This effect was more apparent when using a peptide substrate than when histone IIIS was used. This suggests that there are other regions which make important contributions to the interaction between the two domains and the mutation is not sufficient to completely abolish this interaction. A small peptide substrate can more easily enter the active site than a larger polypeptide which cannot diffuse into the site to easily. E25PKCα also showed an increased sensitivity to proteolysis by trypsin, again consistent with the protein being in a more activated conformation.

The increased effector independence of E25PKCα in the intact cell was confirmed by a co-transformation assay. Expression of chloramphenicol acetyl transferase (CAT) driven by a phorbol ester inducible promoter was determined after co-transformation with wild type or mutant PKCα. E25PKCα was able to increase expression in the absence of phorbol ester relative to PKCα. The co-expression of a construct containing a seven amino acid deletion spanning the pseudosubstrate site led to a greater increase in CAT activity, consistent with an increased effector-independence. No biochemical data is available for this mutant protein as it has a very short half life and cannot be expressed at significant levels. This suggest that it may be a substrate for the down regulation machinery which is responsible for removing active PKC from the cell.



	PKC α	E25PKC α
%activity - Activators		
a. peptide	32	64
b. histone	18	33
% activation by proteolysis at 30 secs	48	100
Induction of CAT activity - activators	100	210

Figure 5. Mutation of the pseudosubstrate site of PKCα.

Replacing the conserved alanine residue at position 25 of PKCα with a charged glutamic acid residue led to an increase in effector dependent kinase activity as judged by *in vitro* kinase activity towards a variety of substrates, increased sensitivity to proteolysis and ability to induce expression of a phorbol ester inducible chloramphenicol acetyl transferase (CAT) gene in co-transfection studies (Pears *et al* 1990).

Activating versions of various isoforms of PKC have now been synthesized (Ways K, Kiley S C, Pears C J and Parker P J, unpublished results; Dekker *et al* 1993a; Goode and Parker 1994) and these can be used to investigate the specific and overlapping functions of various isoforms in the same cell. Expression of a single activated isoform will reveal whether activation of that particular PKC isoform is sufficient to generate a signal in the absence of phorbol ester treatment.

7. Substrate selectivity

After isolation from tissue sources or by overexpression in mammalian cells, biochemical analysis has revealed significant differences in the substrate specificity of the various isoforms (Marais 1989; Marais *et al* 1990; Schaap *et al* 1989; Ono

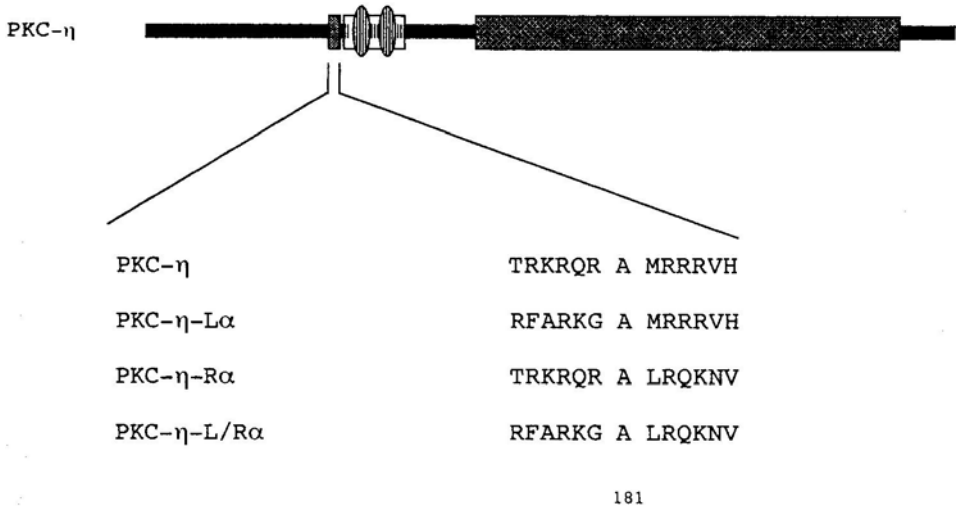
et al 1989; Olivier and Parker 1991). In particular the nPKCs have been demonstrated to show a reduced substrate range *in vitro,* being unable to phosphorylate a commonly used PKC substrate, histone IIIS. It might be expected that the substrate selectivity was determined by the catalytic domain and would depend on the structural features of the active site. However, partial proteolysis of PKCε was found to generate a constitutively active kinase domain fragment which was able to phosphorylate histone (Schaap *et al* 1990b). The structural basis behind the substrate specificity was further investigated by the creation of various chimaeric constructs containing sequences of one isoform fused to those of another. In one such construct the regulatory sequences of nPKCε were fused to the catalytic domain of the cPKC γ and the resulting chimaera expressed in COS cells (Pears *et al* 1991). After partial purification the substrate specificity of the chimaeric PKC was compared to that of its parental enzymes. The substrate exclusion was found to be determined by the regulatory domain of PKC as the chimaeric PKCε/γ was unable to phosphorylate histone, whereas it demonstrated a high level activity towards a peptide substrate.

A likely candidate for the substrate gating appeared to be the extended V1 (V0) domain found upstream of the C1 sequences of the nPKCs. However, detailed analysis has revealed a role for the pseudosubstrate site in substrate selection, perhaps acting via competition for the active site. Replacing the pseudosubstrate site of an nPKC (PKCη) with that of a cPKC (PKCα) was found to increase the substrate range of the chimaera to include histone (Dekker *et al* 1993a). Finer mapping (figure 6) revealed that substitution of only one half of the inhibitory sequences was sufficient to alter the specificity, while substitution of the other half had no effect on the substrate range (Dekker *et al* 1993b). The authors speculate that in effector-activated PKCη the pseudosubstrate site can effectively compete with histone and prevent its access to the active site (figure 7). When these sequences are removed or replaced with those of PKCα, they can no longer effectively compete with histone and phosphorylation occurs.

8. Atypical PKCζ

The characteristics of PKCζ activation are not well understood. It has been reported to be activated by PIP_3, the product of the reaction catalyzed by phosphatidylinositol 3-kinase (PI3kinase) (Nakanishi *et al* 1993). However, there is no supporting evidence that PKC ζ is activated in the cell in response to agonists which generate PIP_3. As PIP_3 is a lipid molecule associated with the membrane, it would be expected that PKCζ would translocate to the membrane on generation of its effector, in a manner comparable to that seen with other isoforms of PKC on generation of DAG. A dominant negative form of PKCζ has been described which will competitively inhibit PKC-dependent activation of the transcription factor NFκB (Diaz-Meco *et al* 1993). Using this approach PKCζ has been implicated in *Xenopus* oocyte maturation and mitogenic signalling in fibroblasts (Berra 1993).

The *in vitro* search for activators of PKCζ is hampered by the lack of detailed knowledge of its substrates and vice versa. The lack of detectable kinase activity could result from inappropriate, use of either of these two components, so the combinations and possibilities to be tried become prohibitive. The ability to fuse



Km values for Histone IIIS phosphorylation in presence of activators

	Km (μg/ml)	Vmax (nmol/min/ml)
PKC-η	50	1.2
PKC-η-Lα	178	1.2
PKC-η-Rα	11	1.3
PKC-η-L/Rα	11	1.2

Figure 6. Mutation of the pseudosubstrate site of nPKCη.

The pseudosubstrate site of nPKCη was replaced with that of cPKCα either completely (L/Rα) or only the N-terminal (Lα) or C-terminal (Rα) half (Dekker *et al* 1993b). Complete substitutionor replacement of the C-terminal half of the pseudosubstrate site was sufficient to allow the nPKC to phosphorylate histone efficiently (when activated by phorbol esters and phospholipids) unlike the parental PKCη. The V_{max} values did not change significantly but the K_m value was decreased 100-fold suggesting that the pseudosubstrate site of PKCη normally efficiently competes with histone for access to the active site, even in the presence of activators. The corresponding region from PKCα is unable to compete effectively, allowing efficient phosphate transfer to occur.

the regulatory domain of one PKC isoform with that of another to generate a functional, activatable kinase can be employed to reduce the number of variables and increase our knowledge of PKCζ biochemical activity. A chimaeric protein was created with the catalytic domain of PKCζ fused to the regulatory sequences of PKCδ and the resulting protein investigated after expression in *S. pombe* (Goode and Parker 1994). This expression system has the advantage that the cells contain no detectable endogenous phorbol ester stimulatable kinase activity, making the exogenous activity easier to characterize. As expected the chimaeric protein could

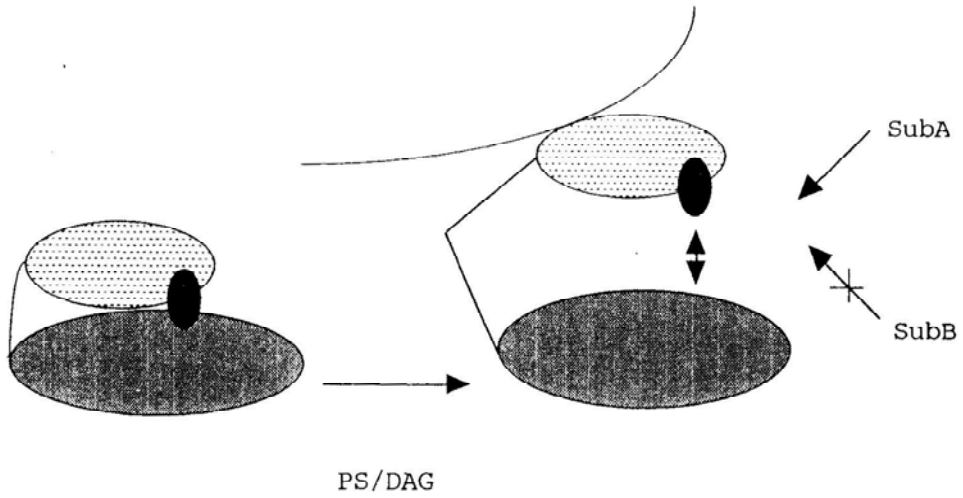


Figure 7. Model for substrate selectivity.

A model in which competition between pseudosubstrate site and substrate for access to the active site determines substrate selectivity has been proposed to explain the effect of swapping the pseudosubstrate sites between PKCs on their substrate ranges (Dekker *et al* 1993b).

now be activated by phorbol esters and phospholipids under conditions in which PKCδ shows regulated activity. This has allowed the substrate preference of PKCζ to be examined to identify a good *in vitro* peptide substrate for PKCζ.

Substitution of the conserved alanine in the pseudosubstrate site of PKCζ in a manner analagous to the point mutation in E25PKCα, leads to effector independent activation of the protein when assayed using the peptide substrate based on the pseudosubstrate site of PKCζ (Goode and Parker 1994). This mutant protein should prove an invaluable tool in determining PKCζ function. Its expression in the intact cell should lead to activation of NFκB -dependent gene expression and other, as yet undefined, responses which are stimulated by PKCζ activation. Identification of these responses and correlation with the known activators of these should confirm the identity of the lipid activator of PKCζ. The PKCδ/ζ chimaera should also prove a useful tool in probing PKOζ function as responses which are activated by PKCζ will become phorbol ester dependent in cells which are expressing this chimaeric protein.

9. Post translational modification of PKC

Translation of mRNA encoding PKCα in reticulocyte lysates gives rise to a 76 kDa polypeptide that has no associated kinase activity (Parker *et al* 1986; Cazaubon *et al* 1990). Active PKCα purified from tissue sources is a phosphoprotein of apparent molecular weight of around 80 kDa suggesting the existence of post-translational modification(s) which are essential for kinase activity. Treatment of this protein with potato acid phosphatase causes an increase in its migration rate on electrophoresis (Woodgett and Hunter 1987) but no loss in kinase activity and subsequent inhibition

of the phosphatase leads to a reversal in the mobility shift. This suggests that active PKCcan autophosphorylate and the addition of these phosphate groups causes an apparent increase in molecular weight on electrophoresis. This is consistent with the increase in phosphorylation of PKC seen on phorbol ester treatment of cells (Mitchell *et al* 1989). PKC will autophosphorylate *in vitro* (Huang *et al* 1986), with both the regulatory and catalytic domains becoming phosphorylated. Attempts to map the autophosphorylation site on PKCß2 revealed the existence of three regions, each containing a pair of potential phosphorylatable residues (Flint *et al* 1990). These sites are distributed throughout the molecule, one pair in the hinge region, one at the N-terminus and one at the C-terminus. Site directed mutagenesis of the equivalent sites in PKCß1, followed by expression in COS cells, did not reveal a function for the sites in the N-terminus and hinge regions (Zhang *et al* 1993). Mutation of the C-terminal sites led to production of an inactive form of PKC which showed reduced solubility and was comparatively resistant to down regulation. These sites are conserved in other PKC isoforms suggesting that they may play a functional role in physiological aspects of PKC activation.

A point mutation was introduced into the ATP binding site of PKCα which led to the replacement of a conserved lysine residue (Pears and Parker 1991). As has been found with a wide range of kinases, this mutation caused a complete loss of kinase activity. When the resulting protein was expressed in insect cells using a baculovirus expression vector, pulse chase experiments confirmed that the primary translation product did not increase in apparent molecular weight. This is consistent with the reduced mobility being the result of autophosphorylation. However, immunoprecipitation of this inactive protein after transient expression in COS cells labelled with [^{32}P]orthophosphate revealed that it is a phosphoprotein. This suggests the presence of a phosphorylation site on PKCα which is a target for a different kinase and which is a candidate for the post-translational modification required to reveal kinase function (figure 8). This trans phosphorylation is unlikely to be due to the endogenous PKC in the COS cells as the rate of activation of the primary translation product is reduced when cells are treated with phorbol ester. As this treatment activates the endogenous PKC, the initiating event must be a trans phosphorylation by a kinase whose activity is decreased on phorbol ester treatment or which is exclusively cytoplasmic in localization such that translocation of the PKC to the membrane prevents it being a substrate. The accumulation of an inactive precursor of PKCß on phorbol ester treatment (Borner *et al* 1988) suggests that this trans phosphorylation could be a physiologically relevant mechanism for regulation of PKC activity by limiting the availability of enzyme.

The requirement of a specific trans phosphorylation to reveal kinase activity has been confirmed by a series of mutagenesis experiments (Cazaubon and Parker 1993). Three threonine residues (Thr-494, Thr-495 and Thr-497) are found in PKCα at a site which is known to be phosphorylated in a wide range of protein kinases such as the cAMP dependent protein kinase. The crystal structure of cAMP-dependent protein kinase confirms the importance of this region which has been termed an activating loop (Knighton *et al* 1991). Replacement of these threonines with alanine residues in PKCα leads to the production of an immunoreactive polypeptide of 76 kDa in COS cells which has no detectable kinase activity associated with it (Cazaubon and Parker 1993). Further mapping (Cazaubon *et al* 1994) has identified threonine-497 of PKCα as being the most likely candidate for phosphorylation *in*

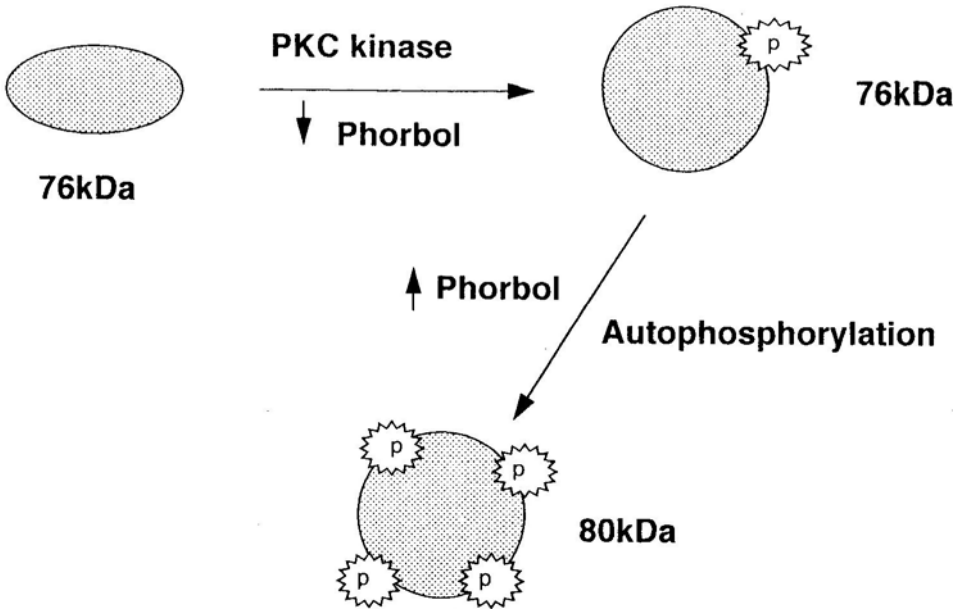


Figure 8. Phosphorylation state of PKCα.

The primary translation product of PKCα is the substrate for a trans phosphorylation reaction which is required for kinase activity. This necessary step is inhibited when cells are treated with phorbol ester (Stabel *et al* 1987). The conformation change induced by this phosphorylation allows the PKC to autophosphorylate, concomitant with an apparent increase in molecular weight on gel electrophoresis (Pears *et al* 1992). This reaction is stimulated by treatment of the cells with phorbol esters to activate the PKC.

vivo. Introduction of a charged glutamic acid residue in this region to mimic the effect of phosphorylation is sufficient to generate kinase activity when the polypeptide is expressed in *E. coli*. Expression of wild type sequences does not give activity as the kinase involved in trans phosphorylation is not present in bacteria. Similar experiments involving mutation of the equivalent threonine residue in PKCßl have confirmed the importance of the phosphorylation state of this residue (Orr and Newton 1994). In this case, replacing the threonine with an aspartic acid residue was not sufficient to generate activity, whereas a glutamic acid residue was, suggesting that the presence of a negative charge in this region is not sufficient to ellicit the exact conformational changes required.

10. Mechanism of down regulation of PKC

Prolonged activation of PKC leads to its down regulation and loss from the cell by proteolytic degradation (Young *et al* 1987) initiated by cleavage in the V3 hinge region (Young *et al* 1988). The protease responsible for this has not been defined; the rates of down regulation do not correspond to the rates of cleavage by known proteases and experiments to mutate the known cleavage sites for a variety of proteases have not been successful in preventing down regulation. Inactive versions of PKC can be down regulated with comparable half-lives to wild type

PKC (Pears and Parker 1991; Freisewinkel *et al* 1991) so autophosphorylation is not a prerequisite for recognition by the down regulation machinery. This does not rule out the possibility that this machinery must be activated by phosphorylation by active PKC as there is endogenous activity present in the cells during these experiments. Indeed, reports of inhibition of down regulation of inactive PKC suggest that extreme overexpression of the mutant protein may interfere with the down regulation process, perhaps by competition for activator or by binding to the relevant substrate (Ohno *et al* 1990). Some isoforms of PKC show phorbol ester dependent down regulation when expressed in *S. pombe* which correlates with growth inhibition (Goode *et al* 1994). Examination of these growth inhibited cells reveals the presence of a large number of clathrin coated vesicles in which the PKCs can be detected by immunolocalization. This suggests that PKC down regulation may occur via an endocytic mechanism. PKC activation leads to its translocation to the plasma membrane where it can accumulate in coated pits and be included in endocytic vesicles. The large accumulation of these vesicles in *S. pombe* cells overexpressing mammalian PKCs correlates with growth inhibition. Cells expressing PKCε do not show this phenotype, although phorbol ester dependent PKC activity can be isolated from the cytosol. The expressed PKCε does not down regulate, no accumulation of vesicles can be detected and no growth inhibition is observed. When PKCε is co-expressed with a different isoform, it will down regulate showing that it is capable of interacting with the relevant machinery in these cells. Instead it is hypothesized that PKCε is not able to phosphorylate the relevant substrate to activate the down regulation process in *S. pombe* whereas other isoforms can do this.

11. Conclusions

Comparison of the primary amino acid sequences of the known isoforms of PKC, in conjunction with a knowledge of their biochemical properties, has revealed much about the structure and function of PKC. Conserved areas have been assigned to common functions, such as diacylglycerol interaction, and calcium dependency. Manipulation of the available cDNA sequences by site directed mutagenesis, or the creation of chimaeras between isoforms, has confirmed the crude structural analysis and added finer detail to our knowledge of how the enzyme activity is regulated. These mutated versions which have defined biochemical characteristics will now provide sensitive tools with which to probe PKC function in intact cells. For example, the involvement of PKC in a response can now be confirmed by expression of an activated version of the kinase, by-passing the need to treat cells with phorbol esters which may alter the activity of other enzymes in the cell. These activated mutants can also be used to determine the extent of overlapping and distinct functions for the different isoforms by expressing each in turn in the same cell type. In the absence of transgenic mice lacking single isoforms of PKC, the range of inactive versions of PKC can be used in parallel in a ‘dominant negative’ manner to selectively interfere with PKC activation in cell lines, presumably by competition for activator or substrate. A PKC knockout has been described in *Drosophila* as the *inaC* locus is the gene for an eye specific PKC isoform. In this case the isoform shows a highly restricted pattern of expression, only being present in the tissues involved with visual response, and is required for inactivation and

rapid desensitization of the transduction cascade (Smith *et al* 1991). Expression of a mutant isoform of PKC which is not a substrate for the trans phosphorylation reaction caused accumulation of unmodified, inactive endogenous PKC (Cazaubon and Parker 1993) suggesting that this may be an alternative route to selectively abrogate PKC function. The activating kinase has yet to be identified and understanding of its regulatory properties will reveal a new dimension of PKC regulation.

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