

PICK1: A Perinuclear Binding Protein and Substrate for Protein Kinase C Isolated by the Yeast Two-hybrid System

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Abstract. Protein kinase C (PKC) plays a central role in the control of proliferation and differentiation of a wide range of cell types by mediating the signal transduction response to hormones and growth factors. Upon activation by diacylglycerol, PKC translocates to different subcellular sites where it phosphorylates numerous proteins, most of which are unidentified. We used the yeast two-hybrid system to identify proteins that interact with activated PKC α . Using the catalytic region of PKC fused to the DNA binding domain of yeast GAL4 as "bait" to screen a mouse

T cell cDNA library in which cDNA was fused to the GAL4 activation domain, we cloned several novel proteins that interact with C-kinase (PICKs). One of these proteins, designated PICK1, interacts specifically with the catalytic domain of PKC and is an efficient substrate for phosphorylation by PKC in vitro and in vivo. PICK1 is localized to the perinuclear region and is phosphorylated in response to PKC activation. PICK1 and other PICKs may play important roles in mediating the actions of PKC.

PROTEIN kinase C (PKC)¹ is a calcium and phospholipid-dependent serine/threonine protein kinase that is rapidly activated in response to a variety of hormones, mitogens, and neurotransmitters (Nishizuka, 1992). At least nine isoforms of PKC have been identified, most of which share a common structural organization in which an amino-terminal regulatory domain is separated from the carboxyl-terminal catalytic domain by a central hinge region. In resting cells, a pseudosubstrate sequence near the amino terminus is thought to occupy the active site, thereby maintaining the enzyme in an inactive state (House and Kemp, 1987). Upon stimulation of cells with activators of phosphatidylinositol turnover, diacylglycerol and calcium are released intracellularly and bind to the regulatory domain leading to allosteric activation of the enzyme.

Activation of PKC is accompanied by its translocation from the cytoplasm to the plasma membrane and other subcellular sites, including the Golgi apparatus (Saito et al., 1989), the perinuclear region (Chen et al., 1987; Halsey et al., 1987; Thomas et al., 1988; Fields et al., 1989; Mochly-Rosen et al., 1990; James and Olson, 1992), and the nucleus

(Kraft and Anderson, 1983; Jaken et al., 1989; Mochly-Rosen, 1990; Saraste et al., 1990). Certain isoforms of PKC have also been shown to translocate to the nuclear envelope upon activation, where they may regulate nuclear envelope breakdown or changes in gene expression that accompany a mitogenic response (Cambier et al., 1987; Huang et al., 1988; Thomas et al., 1988; Leach et al., 1989; Masmoudi et al., 1989; Fields et al., 1989, 1990; Rogue et al., 1990; Divecha et al., 1991; Heccevar and Fields, 1991; Block et al., 1992; Eldar et al., 1992; James and Olson, 1992; Matter et al., 1993).

Mutagenesis of PKC α has revealed regions in the hinge and catalytic domain that mediate translocation to the nucleus (Eldar et al., 1992; James and Olson, 1992). These domains appear to be masked in the inactive enzyme, but they become exposed upon enzyme activation in response to phorbol esters. Deletion of the regulatory domain of PKC α releases the enzyme from its dependence on cellular cofactors for activity and renders it constitutively active (Kaibuchi et al., 1989; Muramatsu et al., 1989; James and Olson, 1992). In some cell types, such deletion mutants are translocated to the nucleus, presumably because of the unmasking of one or more nuclear targeting sequences (Eldar et al., 1992; James and Olson, 1992). How PKC becomes targeted to the nucleus or the plasma membrane remains unknown, but the specificity in targeting suggests that it may involve specific proteins at these subcellular sites that recognize and bind PKC. In this regard, Mochly-Rosen and coworkers (Mochly-Rosen et al., 1991a, b; Ron et al., 1994) have described "receptors" for PKC, termed RACKS, that bind acti-

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1. *Abbreviations used in this paper:* DB, DNA binding domain; PICK, proteins that interact with C kinase; PKC, protein kinase C; RACK, receptor for activated C-kinase; SC, synthetic complete; TA, transcription activation.

vated PKC and could play a role in targeting of PKC to different subcellular sites. Intracellular PKC-binding proteins have also been identified by Jaken and coworkers (Chapline et al., 1993).

Despite a great deal of information on the structure, function, and regulation of PKC, relatively little is known of the proteins with which PKC interacts in vivo to elicit its diverse cellular responses. At least three types of proteins can be envisioned that would interact with PKC in vivo; those that are substrates for the enzyme, those that are inhibitors, and those that direct the enzyme to different subcellular sites by serving as chaperons or intracellular receptors. In an attempt to identify cellular proteins that may interact with activated PKC and participate in the PKC signal transduction pathway, we used the yeast two-hybrid system to isolate cDNA clones encoding proteins that interact with C kinase (PICKs). Here we describe one such protein, PICK1, which is localized primarily to the perinuclear region where it serves as a substrate for PKC. PICK1 and other PICKs are likely to participate in transduction of PKC signals in vivo.

Materials and Methods

Construction of GAL4 (DB)-PKC Chimeras and Library Screening

The GAL4 (DB)-PKC7 fusion plasmid was constructed from a deletion mutant of bovine PKC α (Coussens et al., 1986), termed PKC7 (James and Olson, 1992), which encodes amino acids 302–672. PKC7 in pCDM8* (James and Olson, 1992) was digested with *Bam*I, the 1.1-kb fragment encoding PKC7 was isolated, and the ends were filled with Klenow polymerase and deoxynucleotide triphosphates followed by digestion with *Bam*HI. The fragment was then inserted into the *Sma*I and *Bam*HI sites in pAS-1 (Durfee et al., 1993), which encodes the DNA binding domain (DB) of yeast GAL4 encompassed by amino acids 1–147. The GAL4(DB)-PKC regulatory domain plasmid (GAL4(DB)-PKC-R) was constructed by digesting PKC α in pCDM8* with *Nco*I, gel purifying the 1.2-kb fragment, and ligating it to pAS-1 linearized with *Nco*I. The GAL4(DB)-E12 fusion plasmid was constructed as described (Staudinger et al., 1993). The GAL4(TA) cDNA fusion library contains cDNA fused to the GAL4 transcription activation (TA) domain and was constructed from mouse T cell poly(A)⁺ mRNA and was converted to plasmid form using λ -act as described (Durfee et al., 1993). GAL4(DB)-PKC α was created by cloning the complete coding region of bovine PKC α in frame to GAL1-147 using an *Nco*I site at the initiating methionine codon in PKC.

Yeast strain Y153 (MATa, gal4, gal80, his3, trp1-901, ade2-101, ura3-52, leu2-3, -112, URA3::GAL \rightarrow lacZ, LYS2::GAL \rightarrow HIS3) was used. Transformation was by the method of Schiestl and Gietz, (1989). A typical transformation yielded 3×10^4 transformants/ μ g of plasmid DNA. Briefly, cells were grown in one liter of YPD or the appropriate selective minimal media to an OD₆₀₀ of 0.5–0.8. The cells were harvested by centrifugation and resuspended in 250 ml of water and centrifuged again. The washed pellet was resuspended in 100 ml of LISORB (100 mM LiOAc, 10 mM Tris, pH 8.0, 1 mM EDTA, 1 M Sorbitol) and shaken gently at 30°C for 30 min. Cells were then pelleted as before and resuspended in 2.0 ml LISORB in a 50-ml conical tube and incubated on ice. Denatured salmon sperm carrier DNA (800 μ l of a 10 mg/ml solution) was mixed with 1.6 ml of LISORB and the mixture was allowed to cool to room temperature. 80 μ g of library plasmid DNA was added to the cooled carrier DNA-LISORB mixture and mixed thoroughly. This mixture was added to the chilled cells, mixed briefly, and incubated without shaking for 30 to 60 min at 30°C. After incubating, 20 ml of LIPEG (40% PEG 3350, 1.0 M LiOAc, 1 \times TE, pH 8.0) was added and mixed by inversion. This suspension was incubated for 30 min at 30°C, after which it was placed in a 42°C water bath and heat shocked for 10 min. The cell suspension was then added to 0.5 l of synthetic complete (SC) media lacking the amino acids tryptophan, leucine, and histidine (6.7 g/L yeast extract minus amino acids, 20 g/l dextrose, 0.87 g/l amino acid powder, \pm 20 g agar/l) and shaken at 250 rpm at 30°C for 3 h. The cells were harvested as before and resuspended in 10 ml of SC media lacking tryptophan, leucine, and histidine. 30 300- μ l aliquots were plated

on 15-cm plates containing SC-try, leu, and his, + 25 mM 3-amino-1,2,4-triazole (A-8056; Sigma Chem. Co., St. Louis, MO) and grown for 10 d before assaying for β -galactosidase activity.

Expression Vectors and Yeast Screen

Yeast harboring both GAL4(DB) and GAL4(TA) fusion proteins were monitored for β -galactosidase activity using plate and liquid assay methods. Yeast transformants were transferred to nitrocellulose membrane, permeabilized in liquid nitrogen, and placed on Whatman no. 3 filter paper that had been soaked in Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM MgCl₂, 50 mM β -mercaptoethanol) containing 1.0 mg/ml X-gal. Positive colonies appeared in 10 min to 10 h. Liquid cultures of 2.5 ml were grown in the appropriate selective media to an OD₆₀₀ of 1.0 to 1.2 for quantitative ONPG assays as described (Guarente, 1993). To rescue GAL(TA) fusion plasmids, total yeast DNA was isolated by the method of Hoffman and Winston (1987), except that the DNA was precipitated with one half volume of 7.5 M ammonium acetate and one volume of isopropanol. The pellet was washed twice in 70% ethanol and resuspended in 20 μ l of sterile distilled water and used to electroporate *E. coli* DH5- α cells as recommended in the Bio-Rad users guide for the Gene-Pulser.

Construction of GST-PICK1, pRSET-PICK1, and PECEFLAG-PICK1 Fusion Protein

GAL4(TA)-PICK1 was digested with *Bgl*II and the resulting 1.7-kb cDNA insert was gel purified and used to create all of the following plasmids. To obtain the GST-PICK1 fusion, the cDNA was ligated to pGEX-3X (27-4803-01; Pharmacia LKB Nuclear, Gaithersburg, MD) which had been linearized with *Bam*HI. For in vitro transcription and translation, the cDNA was ligated to pRSET A (V350-20; Invitrogen, San Diego, CA) which had been digested with *Bgl*II. To obtain an expression vector for transfections, the cDNA was ligated to PECE-FLAG (Ellis et al., 1986) which had been digested with *Bgl*II. All plasmids were sequenced to ascertain reading frame and orientation using Sequenase 2.0 according to the manufacturer's specifications (Un. States Biochem. Corp., Cleveland, OH).

In Vitro Transcription and Translation and Expression of Bacterial Fusion Proteins

In vitro translation of PICK1 was performed using the TNT rabbit reticulocyte lysate kit (Promega Corp.) according to manufacturer's instructions. The myogenin and MEF2C cDNAs used as negative controls have been described (Edmondson and Olson, 1989; Martin et al., 1993).

The GST-PICK1 fusion protein was isolated from DH5 α cells transformed with the GST-PICK1 plasmid. The GST-myo fusion protein contained amino acids 33–224 of mouse myogenin, cloned in frame to GST (Chakraborty et al., 1991). Following induction of the fusion protein with 0.5 mM IPTG (0.24 g/l) for 4 to 6 h, the GST-PICK1 fusion protein was purified on glutathione agarose beads (Pharmacia), as described (Chakraborty et al., 1991).

Binding of GST-PICK1 and In Vitro Translated PKC7

To test for interactions between PICK1 and PKC7 or PKC α in vitro, \sim 15 μ g GST-PICK1 was incubated with 20,000 cpm of [³⁵S]-labeled in vitro translation products in 300 μ l of incubation buffer (50 mM KCl, 40 mM Hepes pH 7.5, 5 mM mercaptoethanol, 0.1% Tween-20, 0.5% nonfat dry milk). The effects of PKC cofactors on binding of PKC α to GST-PICK1 were determined in the presence of 1 mM calcium and 80 μ g/ml PS. The slurry was allowed to rock at 4°C for 30 min. The beads were then washed five times in 1 ml of NETN and five times in 1 ml of incubation buffer containing 100 mM KCl. After washing and removing the final supernatant, the samples were resuspended in 50 μ l of 2 \times SDS sample buffer. The samples were boiled for 10 min and resolved on 10% SDS-polyacrylamide gels. The gels were dried and autoradiography was performed overnight at -80° C.

In Vitro Phosphorylation of GST-PICK1

For in vitro phosphorylation, 5 μ g of GST-PICK1 was brought to a final volume of 40 μ l with water. Ten μ l of solution A (120 mM Tris, pH 7.5, 60 mM MgCl₂, 6 mM CaCl₂) was added, followed by 5 μ l of phosphatidylserine (1 mg/ml) and 2.0 μ l of PKC α (Promega). After mixing, 1 μ l of γ -[³²P]ATP (10 mCi/ml) was added and the reaction was incubated for 5 min at 30°C. One μ l of 1 mM unlabeled ATP was then added and the incuba-

tion was continued for an additional 30 min. Samples were diluted with 2× SDS-sample buffer, boiled for 10 min, and then resolved by SDS-PAGE. Gels were dried and autoradiography was performed for 1 to 5 h at room temperature.

Transfection and Immunofluorescence of PICK1 and COS1 Cells

COS1 cells were grown to 50–80% confluency in DMEM containing 10% FBS and transfected by calcium phosphate precipitation using 20 µg of PECE FLAG-PICK1 expression vector. Cells were refed with fresh medium 18 h after transfection and were analyzed for exogenous PICK1 expression 2 d later.

For immunostaining, cell monolayers were washed with PBS and fixed for 5 min at room temperature in ice cold absolute alcohol. The cells were then rinsed three times quickly with PBS followed by incubation for 30 min at room temperature in 3% BSA, 0.1% NP-40 in PBS. The primary antibody (anti-FLAG M2 Mab, Kodak) was added at a 1:1,000 dilution and incubation was continued for an additional 30 min at 37°C. Two quick washes using 0.1% NP-40 in PBS were performed followed by one wash for 15 min with gentle agitation at room temperature. The secondary antibody (FITC-labeled horse anti-mouse, BA2000; Vector Labs. Inc., Burlingame, CA) was diluted in 3% BSA, 0.1% NP-40 in PBS as per manufacturer's instructions and incubated at 37°C in the dark for 30 min. The cells were washed as before and visualized using immunofluorescence microscopy.

In Vivo Labeling and Immunoprecipitation

For ³²P-labeling, transfected cells were transferred into phosphate-free medium (Sigma) two days after transfection. The cells were then incubated for 8 h before adding [³²P]ortho-phosphate (ICN Biomedicals Inc., Costa Mesa, CA) at 1 mCi/ml for an additional 4 h. After labeling, cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 0.1% SDS, 1% NP-40, 0.5% Sodium Deoxycholate, 150 mM NaCl, 5 mM EDTA, 30 mM Na pyrophosphate, 10 mM NaF, 1 mM PMSF, 1% Apoprotinin) and the lysate was homogenized by aspiration through a 26-gauge needle attached to a 1-ml syringe 10 times to reduce viscosity. The homogenate was the microfuged for 5 min at 4°C. The anti-FLAG antibody M2 was added to the supernatant at 2 µg/ml and incubated on ice for 1 h. 20 µl of protein A-agarose (Oncogene Science, Inc.) beads were added and incubated for an additional 30 min on ice. The beads were collected and washed three times in RIPA buffer containing 500 mM NaCl, followed by a final wash in PBS. The pellet was then resuspended in SDS sample buffer and heated to 95°C for three min. The samples were spun in a microfuge for 1 min at 4°C and the supernatant was resolved on 10% SDS-polyacrylamide gels. The gels were dried and autoradiography was performed overnight at –80°C.

DNA Sequencing and Sequence Analysis

DNA sequencing of PICK1 cDNAs was performed on both strands using an Applied Biosystems automated sequencer using oligonucleotide primers that annealed every 250 bp along the cDNAs. DNA sequences were analyzed using the GCG Sequence Analysis Software Package.

Results

Screening for PKC-Binding Proteins Using the Two-hybrid System

To search for cDNA clones encoding proteins that interact with PKC, we used the two-hybrid system for detection of protein–protein interactions in yeast (Fields and Song, 1989; Chien et al., 1991; Durfee et al., 1993). The yeast strain used for the screen harbored integrated *lacZ* and *HIS3* genes under control of the GAL4 upstream activating sequence (UAS). These marker genes are transcriptionally silent in this strain and are dependent on binding of a transactivator to the UAS for expression. As “bait”, we created a plasmid called GAL4(DB)-PKC7, which contained amino acids 302–672 of bovine PKCα fused in-frame to the DNA binding domain of yeast GAL4, encoded by amino acids 1–147

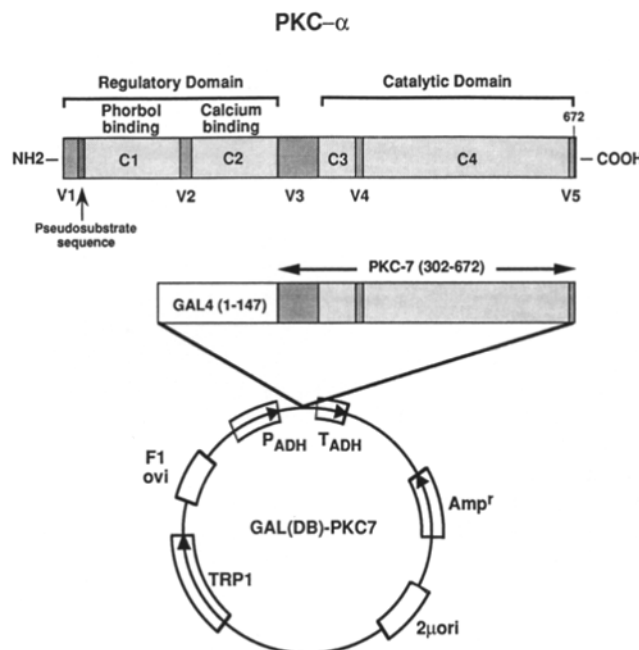


Figure 1. Structure of GAL4(DB)-PKC7. The structure of bovine PKCα is depicted at the top. Conserved (C) and variable (V) regions are indicated in light and dark grey, respectively. GAL4(DB)-PKC7 contains amino acids 302–672 of bovine PKCα fused to the DNA-binding domain of GAL4. This contains part of the V3 hinge region, which starts at amino acid 290. Details of the expression plasmid are in the text.

(Fig. 1). This region of PKCα, which corresponds to a PKC deletion mutant previously termed PKC7, contains part of the V3 hinge region and the complete catalytic domain. PKC7 shows constitutive kinase activity and becomes localized to the nucleus of transfected COS cells (James and Olson, 1992).

Because GAL4(DB)-PKC7 lacks transcriptional activity on its own (Table I) and is therefore undetectable by selection, we confirmed that it was expressed in yeast by Western blot analysis of extracts from yeast transformed with the GAL4-(DB)-PKC7 expression vector. Antibody against GAL1-147 detected a protein of 57 kD in cells transformed with the GAL(DB)-PKC7 plasmid, but not in untransformed yeast (data not shown). This agrees with the predicted size of the GAL4(DB)-PKC7 fusion protein.

To identify cDNA clones encoding proteins that interacted with PKC, we transformed the above yeast strain with GAL4(DB)-PKC7 and a mouse T cell cDNA library in which cDNA was fused to the GAL4 transcription activation domain. Screening of 3×10^6 independent transformants for activation of the *lacZ* and *HIS3* markers yielded 12 colonies that stained intensely for β-galactosidase activity within 30 min. Many colonies showed *lacZ* activity following overnight incubation. However, we focused only on the 12 strongly positive clones.

To determine whether activation of the UAS-dependent marker genes by the above clones reflected a specific interaction of the encoded proteins with the PKC portion of GAL4(DB)-PKC7, each cDNA clone was rescued from yeast and was retransformed into the same yeast strain in the ab-

Table I. Activation of *lacZ* Reporter Gene by *GAL4* Chimeras

Bait	Prey	β -Gal activity
None	None	<0.01
GAL4(DB)-PKC7	None	<0.01
GAL4(DB)-PKC-R	None	<0.01
GAL4(DB)-PKC α	None	<0.01
GAL4(DB)-E12	None	<0.01
GAL4(DB)-PKC7	PICK1	50.20
GAL4(DB)-PKC-R	PICK1	<0.01
GAL4(DB)-PKC α	PICK1	<0.01
GAL4(DB)-E12	PICK1	<0.01
GAL4(DB)-PKC7	PICK2	75.70
GAL4(DB)-PKC-R	PICK2	78.30
GAL4(DB)-PKC α	PICK2	<0.01
GAL4(DB)-E12	PICK2	<0.01
GAL4(DB)-PKC7	PICK3	100.00
GAL4(DB)-PKC-R	PICK3	<0.01
GAL4(DB)-PKC α	PICK3	<0.01
GAL4(DB)-E12	PICK3	<0.01
GAL4(DB)-PKC7	PICK4	83.60
GAL4(DB)-PKC-R	PICK4	<0.01
GAL4(DB)-PKC α	PICK4	<0.01
GAL4(DB)-E12	PICK4	<0.01
GAL4(DB)-PKC7	PICK5	97.20
GAL4(DB)-PKC-R	PICK5	<0.01
GAL4(DB)-PKC α	PICK5	<0.01
GAL4(DB)-E12	PICK5	<0.01

Yeast transformed with the indicated expression plasmids were grown to mid-log phase in SC lacking the appropriate amino acid(s). Extracts were prepared and assayed for β -galactosidase activity as described in the text. β -galactosidase activity is expressed as percent of activity in extracts with GAL4(DB)-PKC7 + GAL4(AD)-PICK3.

sence of GAL(DB)-PKC7. We also expressed each of the activating plasmids with an expression plasmid in which the GAL4 DNA binding domain was fused to a nonspecific protein; in this case we used the transcription factor E12, which would not be expected to interact with proteins that bind to PKC. 7 of the 12 clones were able to activate expression of the marker genes in the absence of GAL(DB)-PKC7 or in the presence of GAL4(DB)-E12, suggesting that the proteins they encoded interacted directly with the GAL4 DNA-binding domain or with the target genes themselves. The remaining five clones were all specific in that they activated *lacZ* expression only in the presence of GAL4(DB)-PKC7. We named the proteins encoded by these cDNAs PICKS. An indication of the relative strengths of the interactions between the above PICKs and PKC7 was obtained by quantitating β -gal activities in extracts of yeast harboring the DNA binding and activating plasmids. The five PICKs appeared to interact with GAL4(DB)-PKC7 similarly, yielding levels of β -galactosidase activity greater than 1,000-fold over background (Table I).

To further investigate the interaction between the above PICKs and PKC, we tested whether they could interact with full-length PKC α or with amino acids 1–382 of PKC. This portion of the protein contains the regulatory domain, the hinge region, and ATP-binding domain, which lies between residues 346 and 368 (Coussens et al., 1986). Expression of both of these GAL4 fusion proteins in yeast was confirmed by Western blot analysis using anti-GAL4 antibody (data not shown). None of the PICKs were able to interact efficiently with full length PKC α , suggesting that they interact with a

region that is cryptic in the native enzyme, but which becomes exposed when the enzyme is activated by deletion of the regulatory domain. Interestingly, PICK2 was able to interact with the regulatory domain of PKC to an extent similar to its interaction with the catalytic domain (Table I).

PICKs 1–5 Represent Novel Proteins

To determine whether PICKs 1–5 might represent previously identified proteins, we sequenced the 5' and 3' ends of the cDNAs and searched the data base for amino acid or nucleotide homologies. No exact matches were found, indicating that these 5 cDNAs represent novel gene products. However, the deduced open reading frame of PICK1 from residues 17–81 showed 53% identity to that of an expressed sequence tag cDNA from *Caenorhabditis elegans* called cml9cl (Watterson et al., 1992). This is the only region of this *C. elegans* cDNA that has been sequenced. It will be interesting to determine whether the homology between these two gene products extends beyond their amino termini.

We chose to focus further on the structure and function of PICK1 because it was the first PICK isolated and because, in contrast to the other cDNAs, Northern analysis suggested the PICK1 cDNA was near full-length. The 1771 bp PICK1 cDNA was sequenced in its entirety and was found to contain a long uninterrupted open reading frame in frame with GAL4 (1–147) (Fig. 2). Since the PICK1 mRNA is ~1,800 nucleotides in length (see below), it is likely that this cDNA lacks a short amount of sequence at one or both ends. A methionine codon, preceded by a sequence in reasonable agreement with Kozak's consensus for translation initiation (Kozak, 1987), was located 12 residues downstream of the cloning junction between GAL4 (1–147) and the PICK1 cDNA (Fig. 2). We believe this is the translation initiation site for PICK1 and that the additional residues between it and the cloning junction represent 5' untranslated sequences that are fortuitously in frame with both GAL4 and PICK1. Assuming this to be the case, the PICK1 protein would contain 416 amino acids ($M_r = 46,522$, $pI = 5.21$). Evidence to support the conclusion that the first ATG in the cDNA can initiate translation comes from the observation that in vitro transcription and translation of the PICK1 cDNA insert yields a polypeptide of $M_r = 55$ kD (see Fig. 4). In addition, the size of the PICK1 transcript suggests that all except 100–200 nt are contained in the original PICK1 cDNA (see below).

The PICK1 amino acid sequence contains several interesting motifs, including three potential PKC phosphorylation sites, R/KXXS/T, R/KXS/T (Pearson and Kemp, 1991). One of these is contained within a region that corresponds to a P-loop ATP/GTP binding motif (GXXXXGKS/T). This type of motif is contained in members of the RAS family of proteins, as well as guanylate kinase, thymidine kinase, and thymidylate kinase (Saraste et al., 1990). PICK1 does not contain any homology to the catalytic domains of known protein kinases. Near its carboxyl-terminus, PICK1 contains a stretch of acidic residues.

PICK1 mRNA is Expressed in a Wide Range of Tissues

Northern analysis of RNA from adult mouse tissues using the PICK1 cDNA as probe revealed a major transcript of ~1,800 nucleotides and a minor transcript of ~3,000 nt. PICK1 mRNA was present in all tissues examined, with

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CTCGAGATTTACCGTCGGCCCCAACTCTCGGAACCATGTTTGCAGACTTAGACTATGACA 60
      M F A D L D Y D I 9
TCGAAGAGGATAAATTAGGAATCCCCACTGTGCCTGGGAAGGTCACCTACAGAAGGATG 120
      E E D K L G I P T V P G K V T L Q K D A 29
CCCAGAACCTGATTGGGATCAGCATCGGAGGAGGGGCCCAATACTGTCCTTGTCTCTATA 180
      Q N L I G I S I G G G A Q Y C P C L Y I 49
TCGTCCAGGTATTTGATAACACGCCTGCAGCCTTGGATGGCACTGTGGCAGCTGGTGACG 240
      V Q V F D N T P A A L D G T V A A G D E 69
AGATCACTGGGGTCAATGGCAAGTCAATCAAAGGAAAAACCAAGGTAGAGGTGGCGAAGA 300
      I T G V N [G K S I K G K T K] V E V A K M 89
TGATTCAAGGAAGTGAAGGGGAGGTGACCATCCATTACAACAAGCTGCAGGCGGACCCTA 360
      I Q E V K G E V T I H Y N K L Q A D P K 109
AGCAGGGCATGTCCCTGGACATTGTGTTGAAGAAAGTCAAGCACCGGCTGGTAGAAAACA 420
      Q G M S L D I V L K K V K H R L V E N M 129
TGAGTTCAGGCACAGCTGATGCCCTGGGCCTAAGCCGCGCCATCCTGTGTAACGATGGCT 480
      S S G T A D A L G L S R A I L C N D G L 149
TGGTAAAGCGGCTGGAGGAGCTGGAACGCACTGCTGAGCTGTACAAAGGGATGACGGAAC 540
      V K R L E E L E R T A E L Y K G M T E H 169
ACACCAAGAATCTCTACGGGCCTTTTATGACGTGTCGACAGCCACCAGGGCCTTTGGGG 600
      T K N L L R A F Y D V S Q T H R A F G D 189
ACGTGTTCTCTGTGATTGGGGTGGCGGACGCGCAGCCAGCAGCAAGTGAAGCATTGTGA 660
      V F S V I G V R D A Q P A A S E A F V K 209
AGTTTGCAGACGCACACCGCAGCATTGAGAAGTTCGGCATTTCGGCTACTGAAAACCATCA 720
      F A D A H R S I E K F G I R L L K T I K 229
AACCGATGCTGACGGACCTGAACACGTACCTCAATAAAGCCATCCCTGATACACGCCTCA 780
      P M L T D L N T Y L N K A I P D T R L T 249
CCATCAAGAAGTACCTGGATGTGAAGTTTGAGTACCTGTCGTACTGTCTAAAGGTGAAGG 840
      I K K Y L D V K F E Y L S Y C L K V K E 269
AGATGGATGACGAGGAATACAGCTGCATTGCCGCTAGGCGAGCCCTATACCGAGTGAGCA 900
      M D D E E Y S C I A A R R A L Y R V S T 289
CAGGCAACTATGAGTACCGCCTCATTCTGCGCTGCCGCCAGGAGGCTGAGCCCGCTTCT 960
      G N Y E Y R L I L R C R Q E A R A R F S 309
CCCAGATGCGCAAAGATGTTCTGGAGAAGATGGAGCTGCTGGACAGCAAGCACGTCCAGG 1020
      Q M R K D V L E K M E L L D Q K H V Q D 329
ACATCGTGTTCAGCTGCAGCGCTTCGTATCCACCATGTCCAAGTACTATAATGACTGCT 1080
      I V F Q L Q R F V S T M S K Y Y N D C Y 349
ATGCAGTGTGTCAGGACGCCGACGTCTTCCCCATTGAGGTGGACCTGGCACACACCACTC 1140
      A V L Q D A D V F P I E V D L A H T T L 369
TAGCCTATGGCCCTAACCAAGGCAGCTTCACTGATGGAGAGGAGGAGGATGAAGAGGAGG 1200
      A Y G P N Q G S F T D G E E E D E E E E 389
AGGATGGGGCAGCTAGGGAAGTGTCCAAGGACGCATGTGGGGCCACTGGGCCTACGGACA 1260
      D G A A R R E V S K D A C G A T G P T D K 409
AGGGCGAAGCTGGTGTGACTCCTGAGTGCCCCCTCAAGTGGACCTGGGGGGCAGGGTAG 1320
      G G S W C D S 416
TGGGAGGACCGCAGTGGGAGTGGGGCGGGACCCTGTAGGACAGCGAAGGCATATAGTGG 1380
      CGCATAAAAGCCTGCTGGCTCGGGGCGCCTGCCTCCCTGCTCCTCTGCTCAAACAGTG 1140
AACCCAGGCTGCAGCCTGAGACCTGGACTCTAGCCCCCATCCATCCTTCCCATCTCCCAG 1500
      CTAGAGGGAGAGCTGGGCTTTGGACAGAAAAGAGAAGGGGCTGGAGCAGACAGAGCACAG 1560
GACCTGTCCCTGTGAGCGCCTCGGTGGTGCCAAGCCTGCAGGGAGTGGGAGCCGGGTGGA 1620
      CATCTGAGGCTGGGGCCAGTGATACAGGGCAGCCCTGAGTCCACATGCACACCTCAGCA 1680
GCCTATATTTATTTGTTTCTGGTTTAGCCTCAAGGGCTGGAAGCTGGGCCTGTATTGAAT 1740
      AAACAGAAACCCAGGGTAAATCTCGAGCAGC 1771

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Figure 2. Nucleotide sequence and predicted open reading frame of PICK1. The nucleotide sequence and predicted open reading frame of the PICK1 cDNA is shown. We believe that the first 33 nucleotides of the sequence represent 5'-untranslated region that is fortuitously in frame with GAL4(1-147) and PICK1. The methionine indicated as residue number 1 can serve as a translation initiation site in vitro. A sequence that matches the P-loop motif is indicated in brackets and potential PKC phosphorylation sites are underlined. These sequence data are available under EMBL/GenBank/DBJ accession number Z46720.

highest levels in brain and testes and lowest levels in lung (Fig. 3).

PICK1 Interacts Specifically with PKC In Vitro

To further investigate the nature of the interaction between PICK1 and activated PKC, we expressed PICK1 as a GST-fusion protein and tested whether it could interact with a [³⁵S]methionine-labeled in vitro translation product of PKC7. Incubation of GST-PICK1 with [³⁵S]methionine-labeled PKC7, followed by recovery of the fusion protein on

glutathione agarose beads, revealed that GST-PICK1 and PKC7 could interact in vitro (Fig. 4 A). In contrast, PKC7 showed no interaction with GST alone.

To assess the specificity of the interaction between PICK1 and PKC7 and to determine whether it might represent an enzyme-substrate interaction, we tested whether [³⁵S]methionine-labeled PKC7 could interact with a GST-myogenin fusion protein, which is efficiently phosphorylated by PKC (Li et al., 1992); no interactions were detected (Fig. 4 A). As an additional negative control, we incubated GST-PICK1 with [³⁵S]methionine-labeled MEF2C, a transcription fac-

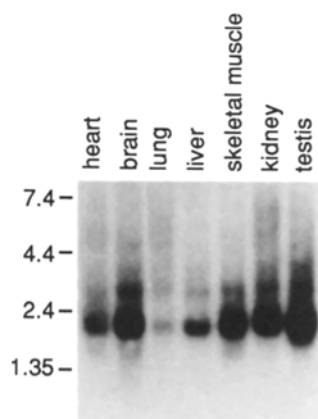


Figure 3. Northern blot of PICK1 mRNA in adult mouse tissues. A ^{32}P -labeled PICK1 cDNA probe was hybridized to poly(A)⁺mRNA from the indicated mouse tissues. A major species of ~1,800 nt and a minor species of ~3,000 nt are observed in all tissues.

tor containing several potential PKC phosphorylation sites, and detected no interaction (Fig. 4 *A*). These results confirm that the catalytic domain of PKC interacts directly with PICK1 and rule out the possibility that the activation of marker gene expression in yeast by the two proteins together is due to an indirect mechanism or to the involvement of an intermediary protein.

We also investigated whether GST-PICK1 could interact with full length PKC α in vitro. In the absence of cofactors, PKC α interacted weakly with GST-PICK1 and there was about a twofold increase in binding of PKC α to GST-PICK1 in the presence of cofactors for PKC activation (Fig. 4, *B* and

C). In the presence of PKC activators, PKC α bound less efficiently to GST-PICK1 than did PKC γ (Fig. 4 *B* and *C*), suggesting that the PICK1-binding site in PKC α was not fully exposed upon catalytic activation or that a portion of in vitro translated PKC α was not activatable.

PICK1 Is a Substrate for PKC In Vitro

We next examined whether PICK1 might be a substrate for PKC by incubating GST-PICK1 in the presence of purified PKC α , essential cofactors, and γ - ^{32}P ATP, followed by analysis of the reactions by SDS-PAGE. As shown in Fig. 5, the 72-kD GST-PICK1 fusion protein was efficiently phosphorylated by PKC in vitro. Because GST alone is not a substrate for PKC (Li et al., 1992), we conclude that ^{32}P is incorporated into the PICK1 portion of the fusion protein.

PICK1 Is Phosphorylated Following PKC Activation In Vivo

To test whether PICK1 can be phosphorylated in response to PKC activation in vivo, we transfected COS cells with an epitope-tagged PICK1 expression vector. 24 h following transfection, cells were labeled with [^{32}P]orthophosphate for 8 h, after which they were exposed to PDBu for 30 min. Extracts were then prepared and PICK1 was immunoprecipitated using an antibody directed against the epitope tag. In the absence of PDBu, the 55-kD PICK1 protein showed a low, but detectable level of labeling with ^{32}P (Fig. 6), indicating that it exists in vivo as a phosphoprotein. In the pres-

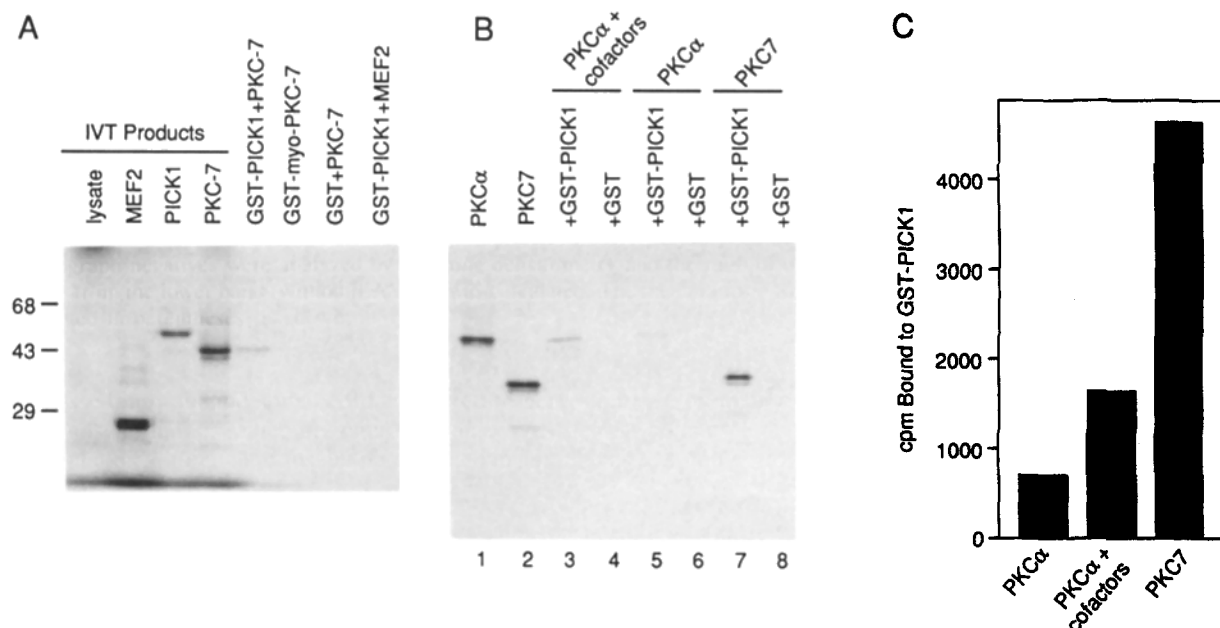


Figure 4. Interaction between PICK1 and PKC7 in vitro. cDNAs for PICK1, PKC α , PKC γ , and MEF2C were transcribed and translated in vitro and [^{35}S]methionine-labeled in vitro translation products were analyzed by SDS-PAGE followed by autoradiography (*A*, left four lanes; *B*, lanes 1 and 2). Unprogrammed lysate is shown in *A*, far left lane. The in vitro translation products were incubated with GST-PICK1, GST-myogenin (GST-my), or GST alone, as indicated. In *B*, incubations with PKC α were performed in the presence (lanes 3 and 4) or absence (lanes 5 and 6) of cofactors for PKC α and in the absence of cofactors for PKC γ (lanes 7 and 8). After recovery of the fusion proteins by binding to glutathione agarose beads, the reactions were analyzed by SDS-PAGE. Four times as much of the in vitro products were contained in the binding reactions as were applied to the lanes for total products. In *C*, the amount of in vitro product bound to GST-PICK1 was quantitated by excising the corresponding region of the gel and counting in a scintillation counter. The [^{35}S]methionine-labeled PKC γ and PKC α translation products bound specifically to GST-PICK1. Molecular weight markers are indicated at the left in *A*. Similar results were obtained in three separate sets of experiments.

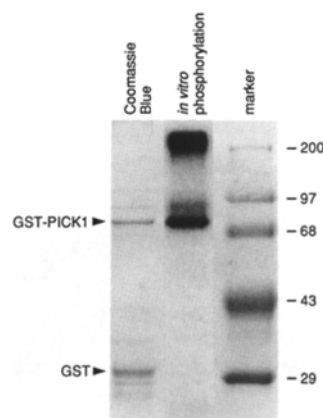


Figure 5. Phosphorylation of GST-PICK1 by PKC in vitro. 1 μ g of GST-PICK1 fusion protein was incubated in vitro with purified PKC α , cofactors and γ -[32 P]ATP as described in Materials and Methods. At the end of the reaction, proteins were separated by SDS-PAGE followed by autoradiography. The species migrating at 30 kD is GST, which arises from proteolysis. The lack of phosphorylation of GST alone can be seen by comparing the autoradiograph with the stained gel. Molecular mass

markers are indicated at the right. There is substantial proteolysis of the bacterial fusion protein near the junction of GST and PICK1, which yields GST migrating at 30 kD, but there is no detectable phosphorylation of this species. We believe that the labeled species migrating near the top of the gel is insoluble material trapped in the well of the gel.

ence of PDBu, multiple phosphorylated forms of PICK1 were observed. These results demonstrate that PICK1 is phosphorylated in vivo as a consequence of PKC activation. Given that PKC phosphorylates PICK1 in vitro, it is likely that the enhanced phosphorylation in response to PKC activation in vivo also reflects direct phosphorylation by PKC.

In some experiments, such as the one shown in Fig. 6, PICK1 migrated as a heterogeneous series of bands of \sim 45–55 kD. Because the same bands are observed with [35 S]methionine labeling in the presence and absence of PDBu, we do not believe they arise as a consequence of phosphorylation. It is more likely that PICK1 is sensitive to proteolysis in vivo.

PICK1 Is Localized to the Perinuclear Region

Using the epitope-tagged PICK1 expression vector, we also examined the subcellular distribution of PICK1 by immunostaining transiently transfected COS cells. Cells transfected

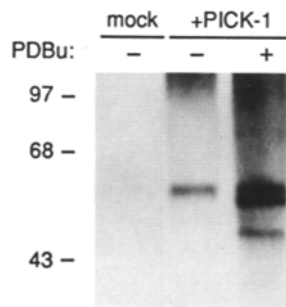


Figure 6. Phosphorylation of PICK1 in response to PKC activation in vivo. COS1 cells were transiently transfected with the PECE FLAG expression vector encoding epitope-tagged PICK1 or lacking a cDNA insert (*mock*). Cultures were labeled with 32 P and exposed to PDBu (500 μ g/ml) for 30 min before harvesting. PICK1 was immunoprecipitated from cell extracts

using anti-FLAG antibody, as described in Materials and Methods. Multiple PICK1 species, designated by arrowheads, were phosphorylated in response to PDBu. Molecular weight markers are indicated at the left.

with the PICK1 expression vector showed intense perinuclear staining, as well as a low level of diffuse staining throughout the cytoplasm (Fig. 7). In many cells, PICK1 staining appeared to be concentrated to one side of the nucleus, perhaps to the rough endoplasmic reticulum or Golgi. Using a nonspecific antibody or nonimmune serum, no staining was observed (data not shown).

Discussion

We have cloned five novel proteins that interact with PKC, using the yeast two-hybrid system. These proteins, called PICKs, interact with the catalytic domain of PKC, but not with the full-length enzyme when expressed in yeast. Activation of PKC following the binding of cofactors to the regulatory domain is accompanied by a conformational change in which the amino-terminal pseudosubstrate sequence is released from the active site (House and Kemp, 1987). The failure of our cloned PICKs to interact efficiently with full-length PKC suggests that they recognize determinants that are masked in the inactive enzyme, but which may become exposed when the enzyme becomes activated.

We have focused in this study on PICK1, which is a

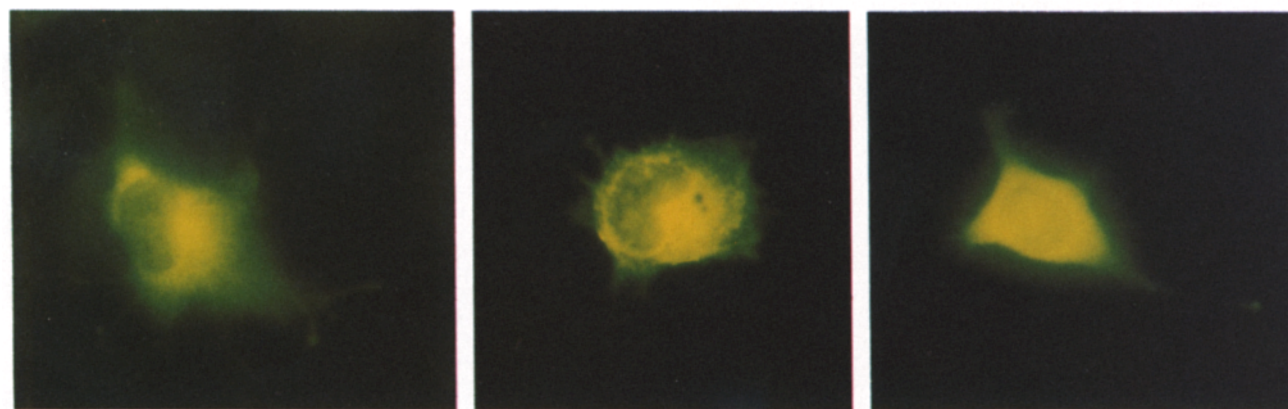


Figure 7. Immunolocalization of PICK1 in transfected COS cells. COS cells were transiently transfected with the PECE FLAG-PICK1 expression vector. Two days after transfection, cells were stained with a monoclonal antibody specific for the FLAG epitope. Three representative transfected cells are shown. FLAG-PICK1-expressing cells showed intense staining in the perinuclear region and diffuse staining in the cytoplasm. No staining was observed in cells transfected with the PECE FLAG expression vector lacking a cDNA insert.

perinuclear phosphoprotein that is a substrate for PKC *in vivo* and *in vitro*. PICK1 binds tightly to the PKC deletion mutant PKC7, which encompasses amino acids 302–672 of PKC α , but it does not interact with a PKC deletion mutant encompassing amino acids 1–382. Thus, it is likely that the binding site for PICK1 lies between residues 383 and 672 of PKC. This portion of PKC contains the active site, in conserved region 4, and variable regions 4 and 5. PICK1 contains a potential ATP/GTP binding motif, but it remains to be determined whether this is important for its function.

The properties of PICK1 suggest it may play a dual role in mediating the actions of PKC, by binding the enzyme and by serving as a substrate for phosphorylation. The binding site for PICK1 lies within the region of PKC that contains the active site. We believe, however, that PKC binding and phosphorylation of PICK1 may be independent activities because PKC7 failed to interact *in vitro* with a myogenin fusion protein, a known substrate for PKC (Li et al., 1992). GAL4(DB)-PKC7 also fails to interact with MyoD in the yeast two-hybrid system (Staudinger et al., 1993), even though MyoD is an excellent substrate for the enzyme (Li et al., 1992). Similarly, we have been unable to detect interactions in yeast between GAL4(DB)-PKC7 and MARCKS (Blackshear, 1993), a well defined PKC substrate (J. Staudinger, unpublished observations). These results suggest that interactions between PKC and its substrates may alone be insufficiently stable to detect using the two-hybrid system.

Numerous PKC isoforms have been shown to translocate to the perinuclear region (Chen et al., 1987; Halsey et al., 1987; Thomas et al., 1988; Fields et al., 1989; Mochly-Rosen et al., 1990; James and Olson, 1992). In fact, the subcellular distribution of PICK1 is remarkably similar to that of activated PKC α in COS cells (James and Olson, 1992). Whether PICK1 may play a role in localizing PKC to the perinuclear region or whether it simply interacts with PKC once it has been translocated there remains to be determined. Because different isoforms of PKC are translocated to different subcellular sites, it will be interesting to determine whether PICK1 shows a preference for interaction with certain PKC isoforms.

Previously we showed that PKC7 was localized specifically to the nuclei of transfected COS cells (James and Olson, 1992), a result confirmed by others (Eldar et al., 1992). This specific localization suggests the existence of a cryptic nuclear localization signal in PKC α that is exposed upon deletion of the regulatory domain (James and Olson, 1992). The subcellular distribution of PICK1 suggests that it is not an intranuclear receptor for PKC7. Whether other PICKs may play a role in localizing PKC to the nucleus remains to be determined.

The existence of intracellular PKC binding proteins, or RACKs, has been demonstrated in previous studies by binding assays of PKC to proteins in cell extracts (Mochly-Rosen et al., 1990, 1991a, b). Binding of RACKs to PKC is dependent on the presence of PS, diacylglycerol, and calcium, which are likely to result in exposure of the otherwise cryptic RACK-binding site in PKC. The binding site for RACKs on PKC appears to lie NH₂-terminal to the catalytic domain and is unaffected by enzyme–substrate interactions.

Ron et al. (1994) recently cloned a PKC-binding protein, called RACK1, using a PKC overlay assay. Sequence analysis showed RACK1 to be a homologue of the β subunit of G-pro-

teins. Binding of PKC to RACK1 is dependent on the presence of PKC activators and could be competed by synthetic peptides homologous to PKC-binding sequences in annexin I and the brain PKC inhibitor KCIP. Peptides derived from the pseudosubstrate sequence had no effect on binding, indicating that binding did not reflect enzyme–substrate interactions.

Chapline et al. (1993) have also isolated two PKC-binding proteins; one was the MARCKS-related protein F52, the other appeared to be novel, but showed homology to the carboxyl-terminus of β -adducin. Both proteins were substrates for PKC and bound PS, which was suggested to be an important component of PKC binding. Following phosphorylation by PKC, these types of PKC-binding proteins were no longer able to bind the enzyme (Chapline et al., 1993; Hyatt et al., 1994). Regions of high positive charge density in MARCKS and the β -adducin homologue were suggested to mediate their binding to PKC (Chapline et al., 1993). PICK1 contains no such region, nor does it contain homology to the PKC-binding motifs in annexin I and KCIP. It also appears that PKC binds equally well to phosphorylated and unphosphorylated PICK1 (J. Staudinger and E. Olson, unpublished results). This suggests the existence of multiple classes of PKC binding proteins with different functions in the PKC signal transduction pathway.

Several protein kinases in addition to PKC have been shown to bind intracellular receptors. For example, localization of protein kinase A to specific subcellular sites has been shown to be dependent on its association with A kinase-anchoring proteins (Carr et al., 1992). p60^{src} also binds a specific receptor on the cytoplasmic surface of the plasma membrane (Resh, 1994) and Raf-1 kinase associates specifically with the cytoplasmic domain of the PDGF receptor (Morrison et al., 1989). Further analysis of PICK1 and of PICKs 2–5 should yield additional insight into the functions of intracellular protein kinase-binding proteins in general and of the specific mechanisms whereby PKC regulates cell proliferation, differentiation, and oncogenesis.

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