Specific Interaction of the PDZ Domain Protein PICK1 with the COOH Terminus of Protein Kinase C-α*

(Received for publication, July 30, 1997, and in revised form, September 18, 1997)

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PICK1 is a protein kinase C (PKC) α-binding protein initially identified using the yeast two-hybrid system. Here we report that PICK1 contains a PDZ domain that interacts specifically with a previously unidentified PDZ-binding domain (QSAV) at the extreme COOH terminus of PKCα and that mutation of a putative carboxylate-binding loop within the PICK1 PDZ domain abolishes this interaction. The PDZ-binding domain in PKCα is absent from other PKC isoforms that do not interact with PICK1. We also demonstrate that PICK1 can homooligomerize through sequences that are distinct from the carboxylate-binding loop, suggesting that self-association and PKCα binding are not mutually exclusive. A Caenorhabditis elegans PICK1-like protein is also able to bind to PKCα, suggesting a conservation of function through evolution. Association of PKCα with PICK1 provides a potential mechanism for the selective targeting of PKCα to unique subcellular sites.

Protein kinase C (PKC)α belongs to a family of serine/threonine protein kinases that are activated in response to a wide variety of hormones, mitogens, and neurotransmitters (1, 2). The PKC family is comprised of eleven isoforms, encoded by 10 genes. Individual cells typically express multiple isoforms of PKC (3). Although purified PKC proteins can phosphorylate many different substrates in vitro, individual PKC isoforms appear to mediate distinct and separate functions in vivo, due to their selective localization to different subcellular sites.

In unstimulated cells, PKCα resides primarily in the cytoplasm, and upon stimulation of cells with signals that promote the hydrolysis of membrane phospholipids, it translocates to the plasma membrane (4), as well as the nuclear envelope (5–7). It has been proposed that the localization of PKCα to different intracellular sites is mediated by specific PKC-binding proteins that can discriminate between different PKC isoforms. A number of PKC-binding proteins have been cloned and characterized (8, 9), but the specific amino acid sequences involved in interaction of these proteins with different PKC isoforms have not been defined, nor has it been demonstrated how various PKC-binding proteins might distinguish between PKC isoforms.

Recent studies have identified the PDZ domain (PSD-95, dishwelled and ZO1) as a modular protein-protein interaction motif that serves to localize proteins to specific subcellular sites (10–14). This domain binds to proteins containing the tripeptide motif (S/T)XX (X = any amino acid) at their COOH termini (10, 11, 15–22). PDZ domain-containing proteins have been shown to mediate protein-protein interactions during receptor and ion channel clustering and to recruit kinases and phosphatases to their membrane-associated substrates. Certain PDZ domains have also been shown to homooligomerize, thereby contributing to the formation of multiprotein complexes at specific subcellular sites (23, 24).

PICK1 is a PKCα-binding protein cloned using the yeast two-hybrid system (25). Here we report that PICK1 contains a PDZ domain at its NH2 terminus, which is required for interaction with a PDZ-binding motif (QSAV) at the extreme COOH terminus of PKCα. This PDZ-binding motif is specific to PKCα and can account for the selective interaction of PICK1 with activated PKCα and not with other PKC isoforms. PICK1 can also homooligomerize through its PDZ domain. We also describe a PICK1-like protein from Caenorhabditis elegans that can function as a PKCα-binding protein, suggesting a conservation of function through evolution. We speculate that the function of PICK1 is to localize activated PKCα to the plasma membrane, thereby bringing the kinase in proximity with specific substrates and lending isoform specificity in vivo to the kinase.

EXPERIMENTAL PROCEDURES

Expression Vectors—Construction of GAL4(DB)-PKC7 was described previously (25, 26). The resulting plasmid fuses amino acids 302–672 of bovine PKCα to the DNA-binding domain (amino acids 1–147) of yeast GAL4 in the yeast expression vector pAS1. GAL4(DB)-PKC7(3671–672) was constructed using PCR primers to remove the indicated amino acids and introduce a BamHI site using GAL4(DB)-PKC7 as a template. The amplified fragment contained an EcoRI restriction site from the pAS1 vector at the 5′ end and a termination codon preceding a BamHI restriction site immediately following the glutamine at position 669 of bovine PKCα. The fragment was digested with EcoRI and BamHI and cloned back into pAS1 that had been digested with EcoRI and BamHI. The GAL4(DB)-PICK1 K27A and GAL4(DB)-PICK1 K27,28AA plasmids were constructed using the QuickChange site-directed mutagenesis kit (Stratagene) as directed. The GAL4(DB)-PKC7(QSAV669–672AAA) mutation was generated by digesting pECE-FLAG-PKC7(QSAV669–672AAA) with BamHI and EcoRI and gel purifying the 1.0-kilobase insert. This DNA fragment was cloned into pAS1 that had been digested with EcoRI and BamHI. The GAL4(DB)-PICK1(1–358) yeast expression construct was constructed using PCR primers to introduce an NcoI site at amino acid 1 and a Smal site following a termination codon after amino acid number 358 in mouse PICK1 using GAL4(AD)-PICK1 as a template. The resulting fragment was digested with NcoI and Smal and inserted into pAS1 that had been digested with

*This work was supported by grants from the Robert A. Welch Foundation and the National Institutes of Health (to E. N. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1The abbreviations used are: PKC, protein kinase C; PDB, polymerase chain reaction; ACTD, activation domain; PAGE, polyacrylamide gel electrophoresis; DB, DNA-binding domain; GST, glutathione S-transferase.
The GAL4(AD)-PICK1 deletions were constructed using PCR primers that introduced BglII restriction sites to amplify the respective fragments using GAL4(AD)-PICK1 as a template. The resulting fragments were cloned into the BglII restriction site of pACT.

The C. elegans expressed sequence tag accession number Z14656 was obtained from R. Waterston (Washington University), and the full-length sequence was determined by automated sequencing. The C. elegans PICK1 activation domain fusion expression vector was constructed by using GT10 insert-screening amplimers (CLONTECH) to amplify the fragment from the phage clone. The resulting fragment was cloned into the pCRII plasmid (Invitrogen-TA cloning kit C2020–03) and sequenced in its entirety. Using PCR primers that introduce BglII restriction sites at the NH2 terminus and the COOH terminus, the cDNA was amplified and cloned into pACT for expression in yeast. The resulting plasmid fuses the full-length C. elegans PICK1 protein COOH-terminal of the GAL4 activation domain (AD) in pACT for expression in yeast.

Two-hybrid Assays—Yeast two-hybrid assays were performed as described previously (25, 26). Interactions between bait and prey were monitored by β-galactosidase activity in colonies on plates.

GST Binding Assays—Binding of in vitro translated PICK1 and PKCα to GST-PICK1 fusion protein was constructed using a GST pull-down procedure (25). The C. elegans PICK1 expression plasmid was used as bait in yeast two-hybrid interactions. The GST-PICK1 fusion protein was expressed in E. coli and purified by glutathione-agarose bead affinity chromatography. The purified GST-PICK1 was then incubated with 20,000 cpm of35S-labeled in vitro translation products in 300 μl of incubation buffer (50 mM KCl, 40 mM Hepes, pH 7.5, 5 mM mercaptoethanol, 0.1% Nonidet P-40). Beads were then washed five times in 1 ml of NETN (250 mM NaCl, 20 mM Tris-Cl, pH 8.0, 0.5 mM EDTA, 1% Nonidet P-40). After washing and removing the final supernatant, the pellet was resuspended in SDS sample buffer and resolved by SDS-PAGE.

Immunoprecipitations—To test for interactions between PICK1 and PKCα in vivo, COS cells were transiently transfected with 2 μg of expression vectors encoding PICK1 and wild-type PKCα or PKCα mutant QSAV669–672AAAA. Two days later, cells were harvested and lysed on ice in 0.5 ml of lysis buffer (50 mM Tris-Cl, pH 8.0, 50 μm NaCl, 1% Nonidet P-40 with complete protease inhibitors (Boehringer Mannheim 1697–498)). Following centrifugation for 10 min at 12,000 rpm in a microfuge, the supernatant was removed, and 2 μg of nonimmune mouse IgG was added, followed by incubation for 30 min at 4 °C. 30 μl of protein A/G was then added, and the extract was mixed for 30 min followed by centrifugation. The supernatant was washed twice with ice-cold lysis buffer and centrifuged, and the supernatant was removed. The pellet was then resuspended in 2× SDS sample buffer with dithiothreitol, boiled for 5 min, and separated by 10% SDS-PAGE. Proteins were then transferred to nitrocellulose by Western blotting, and PICK1 was detected using a rabbit polyclonal antibody raised against a GST-PICK1 fusion protein. Detection was accomplished using goat anti-rabbit horseradish peroxidase-conjugated antibody and a chemiluminescence detection kit (Amersham RPN.2108).

RESULTS

PICK1 Is a PDZ Domain-containing Protein—In searching the data base for proteins related to PICK1, we discovered that PICK1 contained a PDZ domain near its NH2 terminus (Fig. 1). The PDZ domain of PICK1 is most closely related to those of the receptor tyrosine kinase ROS1, the third PDZ domain in the post-synaptic density protein PSD95, and the Drosophila protein canoe, which has been implicated in wingless signaling (27). The PICK1 PDZ domain is also similar to the C. elegans PICK1 PDZ Domain-PKCα Interaction.
LIN-7 PDZ domain, which has been shown to mediate clustering of the receptor tyrosine kinase LET-23 (28). The PDZ domain in PICK1 appears to be relatively divergent when compared with other PDZ domains in general.

As we reported previously (25), the sequence of mouse PICK1 shares significant homology with an expressed sequence tag from *C. elegans*. We therefore obtained this cDNA and sequenced it in its entirety. Mouse PICK1 protein shares 44% identity and 63% similarity with the corresponding *C. elegans* protein (Fig. 1B). There are two primary regions of high identity between the two proteins. The first region includes the PDZ domain, which is 47% identical, as well as adjacent sequences. The carboxylate-binding loops of the two proteins, which in other PDZ domain proteins have been shown to mediate binding to the COOH-terminal carboxyl group of PDZ-binding proteins, are similar but not identical in the *C. elegans* and mouse proteins. The other region of high identity begins at amino acid 244 and ends at amino acid 331 in mouse PICK1. Mouse PICK1 protein contains a stretch of nine acidic amino acids (residues 382–390) at the COOH-terminal end of the protein that are not highly conserved in the *C. elegans* protein, although the homologous region is acidic overall.

**Detection of PKC-PICK1 Interactions Using the Yeast Two-hybrid System.**—Our previous studies showed that PICK1 interacted with the catalytic region of PKCα (25). To determine whether PICK1 could also interact with other PKC isoforms, we used the hinge and catalytic regions of PKCβI and PKCε fused to the GAL4 DNA-binding domain (DB) as bait in the yeast two-hybrid system. The COOH terminus of PKCa interacted strongly with PICK1, whereas no interaction was detected for the other PKC isoforms. The COOH-terminal residues of PKCa (QSAV) in PKC7 were deleted or mutated to alanines, and the resulting mutants were tested for their abilities to interact with PICK1 in the yeast two-hybrid system. Neither of the mutants were able to interact with PICK1. C, the complete *C. elegans* PICK1-like protein was fused to the GAL4 activation domain and tested for interaction with the wild-type and mutant PKCα proteins fused to the GAL4 DNA-binding domain. The locations of the variable (V) and constant (C) regions of the PKC isoforms are indicated.

**Fig. 2.** **Specificity of PICK1 for interaction with PKCα in the yeast two-hybrid system.** A, the COOH-terminal regions in PKCα (also referred to as PKC7 in B and C), PKCβI, and PKCε were fused to the GAL4 DNA-binding domain and tested for interaction with PICK1 fused to the GAL4 activation domain using the yeast two-hybrid system. The COOH terminus of PKCa interacted strongly with PICK1, whereas no interaction was detected for the other PKC isoforms. B, the COOH-terminal residues of PKCa (QSAV) in PKC7 were deleted or mutated to alanines, and the resulting mutants were tested for their abilities to interact with PICK1 in the yeast two-hybrid system. None of the mutants were able to interact with PICK1. C, the complete *C. elegans* PICK1-like protein was fused to the GAL4 activation domain and tested for interaction with the wild-type and mutant PKCα proteins fused to the GAL4 DNA-binding domain. The locations of the variable (V) and constant (C) regions of the PKC isoforms are indicated.
following the glutamine at position 669 in PKCα. This mutant, GAL4(DB)-PKC7(A670–672), failed to interact with PICK1 in the yeast two-hybrid assay (Fig. 2B). We then mutated the four COOH-terminal amino acids in PKCα to alanines. This mutant, GAL4(DB)-PKC7(QSAV669–672AAAA), also failed to interact with PICK1. These results indicate that the sequence QSAV in PKCα, which corresponds to the consensus for PDZ recognition, is required for interaction with PICK1.

To determine whether the C. elegans PICK1-like protein was also a PKCα-binding protein, we tested whether it could interact with PKCα in the yeast two-hybrid system (Fig. 2C). Indeed, C. elegans GAL4(AD)-PKC1 was able to activate the reporter gene only in the presence of GAL4(DB)-PKC7 and was unable to interact with either of the mutants that lacked the COOH-terminal PDZ-binding domain. These results show that the C. elegans PICK1-like protein exhibits the same PKC-binding properties as mouse PICK1.

**Requirement of the Putative Carboxylate-Binding Loop of PICK1 for Binding to PKCα**—The carboxylate-binding loop is a 7–8-amino acid segment near the NH₂ terminus of the PDZ domain that interacts directly with the COOH-terminal peptides of PDZ-binding proteins (17). The first residue of a carboxylate-binding loop (amino acid 27 in mouse PICK1) is almost always either an arginine or lysine (Fig. 1A) that interacts with the carboxylate via a highly ordered water molecule (29). Additional hydrogen bonds are established between the carboxylate oxygens and other residues in the binding loop.

To test the importance of the carboxylate-binding loop of PICK1 for PKCα binding, we mutated lysine 27 alone or together with aspartic acid 28 to alanines (Fig. 3). The failure to detect PICK1 in vitro binding to GST-PICK1, which corresponds to the consensus for PDZ recognition, was mediated by the region of the protein containing the PDZ domain. We have been unable to obtain stable expression in yeast of shorter regions of the protein containing only the PDZ domain.

To determine whether the carboxylate-binding loop, which mediates interaction of PICK1 with PKCα, was also required for PICK1 homooligomerization, we tested the PICK1 mutants in which residues 27 and 28 were changed to alanine. Both mutants, GAL4(AD)-PICK1(K27A) and GAL4(AD)-PICK1(KD27,28AA), were able to homooligomerize with GAL4(DB)-PICK1(1–358). The finding that mutations in the putative carboxylate-binding loop of PICK1 (Lys²⁷→Asp²⁸), which disrupted interaction with PKCα, had no effect on homooligomerization suggests that PICK1 may be able to homooligomerize at the same time it is bound to PKCα.

**In Vitro Binding PICK1 Assays**—To determine whether the PICK1 interactions observed with the yeast two-hybrid assays could also occur in vitro, we tested the ability of a GST-PICK1 fusion protein to interact with [³⁵S]methionine-labeled PKC7 and PICK1 obtained from in vitro translation. As shown in Fig. 5A, when GST-PICK1 was incubated with labeled PKC7 and precipitated with glutathione agarose beads, the interaction between the two proteins was readily detected. In contrast, GST-PICK1 failed to interact with the PKC7 mutant QSAV669–672AAAA, in which the COOH-terminal PDZ-binding motif was eliminated. GST alone did not interact with PKC7 further demonstrating the specificity of the interaction.

GST-PICK1 was also mutated with the [³⁵S]labeled PICK1 in vitro translation product, as well as with the PICK1 mutants K27A and KD27,28AA, in the yeast two-hybrid assay (Fig. 5B). GST alone did not interact with PICK1. Thus, the specificity of the PICK1-PKCα and PICK1-PICK1 interactions observed with the yeast two-hybrid system is maintained in vitro.

**Detection of PICK1-PKCα Interactions in Transfected Cells**—We also tested whether binding of PICK1 to PKCα in mammalian cells required the PDZ-binding motif of PKCα, as was the case in the two-hybrid and GST-PICK1 binding assays. For these experiments, COS cells were transfected with expression vectors encoding PICK1 and either wild-type PKCα or PKCα mutant QSAV669–672AAAA using a PKCα-specific monoclonal antibody under low salt conditions. Following separation of immunoprecipitation reactions by SDS-PAGE, proteins were transferred to nitrocellulose, and PICK1 protein was detected by Western blot with a rabbit polyclonal antibody against PICK1. PICK1 was detected in extracts of cells transfected with wild-type PKCα but not with the mutant nor in mock transfected cells (Fig. 6A). The failure to detect PICK1 in

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**Fig. 3. Effects of carboxylate-binding loop mutations in PICK1 on binding to PKCα in the yeast two-hybrid system.** Wild-type PICK1 or PICK1 point mutants in the carboxylate-binding loop were fused to the GAL4 activation domain and tested for interaction with the catalytic domain of PKCα (called PKC7) fused to the GAL4 DNA-binding domain using the yeast two-hybrid system. The acidic region near the COOH terminus of PICK1 is indicated D/E.
extracts from cells transfected with the mutant form of PKCα was not due to a failure of this mutant to accumulate because Western blots of cells transfected with wild-type or mutant PKC showed equivalent amounts of protein (Fig. 6B). We conclude that the PDZ-binding motif of PKCα is required for interaction with PICK1 in mammalian cells, just as it is in yeast.

**DISCUSSION**

Our results show that PICK1 contains a PDZ domain that is required for interaction with the COOH terminus of PKCα. PICK1 is also able to homooligomerize through its PDZ domain, but mutations in the putative carboxylate-binding loop that abolish binding to PKCα have no effect on homooligomerization. The finding that determinants for interaction of PICK1 with itself and with PKCα are separable suggests that PICK1 can simultaneously self-associate and interact with PKCα. The fact that the extreme COOH terminus of PKCα mediates binding to PICK1 also suggests that the kinase can be catalytically active when bound to PICK1.

Among the vertebrate PKC isoforms, the consensus sequence for PDZ binding is contained only in PKCα. Thus, the specific interaction of this sequence with PICK1 provides a molecular

**FIG. 4. Detection of PICK1 homooligomerization in the yeast two-hybrid system.** A series of PICK1 deletion and point mutants fused to the GAL4 activation domain were tested for their abilities to interact with amino acids 1–358 of PICK1 fused to the GAL4 DNA-binding domain using the yeast two-hybrid system.

**FIG. 5. Detection of PICK1 interactions in vitro.** A, PKC7 or PKC7 mutant QSAV669–672AAAA (PKC7 MUT) was translated in a rabbit reticulocyte lysate in the presence of [35S]methionine. Labeled products were then incubated with GST or GST-PICK1 and precipitated with glutathione agarose, as described under “Experimental Procedures.” Labeled proteins were resolved by SDS-PAGE. Labeled PKC7 and PKC7 mutant proteins were also loaded directly onto the gel in adjacent lanes for comparison. The amount of labeled proteins used for the GST binding experiments in lanes 1–3 was four times more than the amount loaded in lanes 4 and 5. B, PICK1 or the indicated PICK1 mutants were translated in a rabbit reticulocyte lysate in the presence of [35S]methionine and then incubated with GST or GST-PICK1 as described in A. The amount of labeled proteins used in the GST binding experiments in lanes 1–4 was four times more than the amount loaded onto lanes 5–7. Molecular mass markers are indicated at the left.

**FIG. 6. Detection of PICK1 interactions in cell extracts.** A, COS cells were transfected with expression vectors encoding PICK1 and wild-type PKCα (lane 3) or PKCα mutant QSAV669–672AAAA (lane 2). Extracts were prepared from transfected cells and were immunoprecipitated with anti-FLAG antibody, which recognized the transfected PKC proteins. Following separation of immunoprecipitates by SDS-PAGE, Western blotting was performed using an anti-PICK1 antibody. The PICK1 protein, migrating at about 50 kDa, can be observed only in the lane containing wild-type PKCα and PICK1. Other bands are nonspecific background due to the relatively low affinity of the anti-PICK1 antibody. Lane 1 was performed with untransfected cells. B, to ensure that wild-type and mutant PKC accumulated to equivalent levels in transfected cells, Western blots were performed using anti-FLAG antibody. Molecular mass markers are indicated at the left.
mechanism for the selective targeting of PKCs to distinct subcellular sites, thereby allowing specific phosphorylation events to occur. In *Drosophila*, the subcellular location and substrate specificity of eye-specific PKC(InaC) has been shown to be regulated by the PDZ domain-containing protein InaD (30). The COOH terminus of InaC contains the sequence ITII, which is compatible with PDZ binding (17). Our results show that the *C. elegans* PICK1-like protein recognizes the COOH terminus of mammalian PKCs. There have been several PKC proteins identified thus far in *C. elegans*, but none contain a PDZ-binding domain at their COOH terminus. This suggests either that additional, yet unidentified PKC proteins exist in this organism or that this PICK1-like protein interacts with other PDZ-binding proteins.

It will be of interest to define the spectrum of proteins that interact with PICK1. It is possible that PDZ-PDZ interactions may enable PICK1 to interact with other PDZ domain-containing proteins. The NMDA receptor, for example, which clusters specifically at the postsynaptic density in the neuronal synapse, binds to PSD-95 and has been reported to be regulated by PKC activity (31, 32). The COOH terminus of InaC contains the sequence ITII, which is compatible with PDZ binding (17). It will be of interest to define the spectrum of proteins that interact with PICK1. Our results show that the PICK1-like protein interacts with PICK1. It is possible that PDZ-PDZ interactions may enable PICK1 to interact with other PDZ domain-containing proteins. The NMDA receptor, for example, which clusters specifically at the postsynaptic density in the neuronal synapse, binds to PSD-95 and has been reported to be regulated by PKC activity (31, 32). The COOH terminus of InaC contains the sequence ITII, which is compatible with PDZ binding (17).

**Acknowledgment**—We thank A. Tizenor for assistance with graphics.