PICK1, an Anchoring Protein That Specifically Targets Protein Kinase Ca to Mitochondria Selectively upon Serum Stimulation in NIH 3T3 Cells*

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PICK1 binds to protein kinase Ca (PKCa) through the carboxylate-binding loop in its PDZ (PSD95/Disc-large/ZO-1) domain and the C terminus of PKCa. We have previously shown that PICK1 modulates the catalytic activity of PKC selectively toward the antiproliferative gene TIS21. To investigate whether PICK1 plays a role in targeting activated PKCα to a particular intracellular compartment in addition to regulating PKC activity, we examine the localization of PICK1 and PKCα in response to various stimuli. Double staining with organelle markers and anti-rPICK1 antibodies reveals that PICK1 is associated with mitochondria but not with endoplasmic reticulum or Golgi in NIH 3T3 cells. Deletion of the PDZ domain impairs the mitochondria localization of PICK1, whereas mutations in the carboxylate-binding loop do not have an effect, suggesting that PICK1 can bind PKCa and mitochondria simultaneously. Upon serum stimulation, PICK1 translocates and displays a dense ring-like structure around the nucleus, where it still associates with mitochondria. A substantial portion of PKCa is concomitantly found in the condense perinuclear region. The C terminal-deleted PKCa fails to translocate and remains a diffuse cytoplasmic distribution, indicating that a direct interaction between PICK1 and PKCa is required for PKCa anchoring to mitochondria. 12-O-Tetradecanoylphorbol-13-acetate stimulation, in contrast, causes translocation of PKCa to the plasma membrane, whereas the majority of PICK1 remains in a cytoplasmic punctate pattern. Deletion at the C terminus of PKCa has no effect on 12-O-tetradecanoylphorbol-13-acetate-induced translocation. These findings indicate a previously unidentified role for PICK1 in anchoring PKCa to mitochondria in a ligand-specific manner.

Targeting of signaling molecules to specific intracellular sites through interactions with anchoring proteins allows activation of particular pools of protein kinases and phosphatases and thus plays a crucial role in determining the specificity of signal transduction cascades (1, 2). The anchoring proteins not only bring kinases/phosphatases to the proximity of their substrates but can also modulate the activity of these enzymes upon binding (3).

Protein kinase C (PKC)1 is a family of at least 11 isoforms that have been implicated in a variety of cellular responses (4, 5). Upon activation, each isozyme differentially translocates to distinct subcellular structures including plasma membrane, cytoskeleton, and other organelles (1, 6). Translocation is a cell type-specific event. In addition, different stimuli cause PKC isoforms to move to different intracellular sites in the same cell (7, 8). A number of proteins, including RACKs, myristoylated alanine-rich C kinase proteins (MARCKs), and PICKs, have been found to bind activated PKC (9–11), but yet only a few are shown to be responsible for selective targeting of particular PKC isoforms to particular subcellular compartments in cells. RACK1 (receptors for activated protein kinase C) selectively binds the active form of βII PKC (12, 13). Treatment of Chinese hamster ovary cells with phorbol 12-myristate 13-acetate causes movement of βII PKC and RACK1 to the same intracellular sites that resemble Golgi apparatus (13). Peptides that prevent binding of βPKC to RACK1 inhibit insulin-induced βPKC translocation and function in Xenopus oocytes and phorbol 12-myristate 13-acetate-induced hypertrophy in cardiac myocytes (12, 14), demonstrating a correlation between PKC redistribution and cellular responses and the importance of anchoring proteins in mediating PKC functions. Activated PKC co-localizes with the coatomer protein β′-COP in cardiac myocytes and binds to Golgi membranes in a β′-COP-dependent manner. β′-COP is thus identified as a PKC- and selective RACK (15).

PICK1, a PDZ-containing protein, is first cloned as a PKCa-binding protein by the yeast two-hybrid screening (11). The association of PICK1 and PKCa was subsequently demonstrated by different approaches including co-immunoprecipitation (16–18). PDZ domains are protein-interacting motifs implicated in association with plasma membrane, cell-cell junctions, cytoskeletal proteins, and signaling molecules (19, 20). Studies have revealed a complexity in PDZ-target interactions (19, 20). PICK1 selectively binds to PKCa through interaction with the QSAV sequence at the extreme C terminus of PKCa (12, 13). This PDZ domain then mediates localization of PKCα to mitochondria (7, 8).

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1 The abbreviations used are: PKC, protein kinase C; PICK, proteins that interact with C-kinase; RACK, receptors for activated protein kinase C; COP, coat protein; PDZ, PSD95/Disc-large/ZO-1; PMA, phorbol 12-myristate 13-acetate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; GluR, glutamate receptor; ER, endoplasmic reticulum; TPA, 12-O-Tetradecanoylphorbol 13-acetate; EYFP, enhanced yellow fluorescent protein; FITC, fluorescein isothiocyanate.
PKCa (16). The carboxy-terminal binding loop within the PDZ domain of PICK1 is required for the interaction (16). In addition to PKCa, PICK1 interacts and co-localizes with several membrane proteins including Eph receptor tyrosine kinases and ephrin-B ligands (21), AMPA receptor GluR2 (22) metabotropic receptor mGluR7a (17), the dopamine transporter (23), and ion channels (24). Functionally, PICK1 can induce clustering of Eph receptor and its ligands (21), AMPA receptors (22), and mGluR7a (17) in heterologous expression systems. PICK1 is also shown to target PKCs to AMPA receptor clusters in hippocampal neurons (18) and to regulate phosphorylation of mGluR7 by PKC (17). It is therefore proposed that PICK1 plays a role in synaptic transmission mediated by PKCa in the central nervous system and that the function of PICK1 depends on the cell types and its interacting partners.

Functions of PICK1 in cells other than neurons are not yet documented; however, certain features of PICK1 suggest that functions of PICK1 should not be limited to synaptic transmission in neurons only. PICK1 interacts with, in addition to neuronal membrane-bound proteins, class I ADP-ribosylation factors, which are essential for vesicle formation in Golgi apparatus (25), and the immediate-early gene TIS21, whose expression is induced by a variety of extracellular stimuli including those known to activate PKC (26, 27). Moreover, phosphorylation of TIS21 by PKC is selectively modulated by PICK1 (27). What further supports this notion is that the message and protein of PICK1 are ubiquitously expressed in all tissues examined (11, 22). Thus, a full understanding of PICK1 functions will require further investigation in cells other than neurons.

In this study, we examine the intracellular localization of endogenous PICK1 in NIH 3T3 cells and its role as a PKCa targeting component in response to different stimuli. We show for the first time that PICK1 is specifically localized to mitochondria and that an intact PDZ domain is essential for the mitochondrial localization. We demonstrate that activated PKCa co-localizes with PICK1 selectively upon serum stimulation and that the co-localization requires a direct interaction between these two proteins. This indicates that PICK1 targets PKCa to mitochondria and is likely to be responsible for mediating PKCa functions associated with mitochondria. 12-O-Tetradecanoylphorbol 13-acetate (TPA) treatment causes a translocation of PKCa to the plasma membrane that appears to be independent of PICK1 binding. These findings suggest that the differential translocation of PKCa isoforms upon distinct stimulations may be achieved through differential responses of targeting molecules such as PICK1 to the extracellular stimuli.

MATERIALS AND METHODS

Construction of Plasmids—The cDNA fragments encoding full-length rPICK1, the rat counterpart of PICK1, rPICK1Ala142–Ser416 or rPICK1Met1–Leu352 were excised from pCS86-rPICK1, pCS86-rPICK1Ala142–Ser416 or pCS86-rPICK1Met1–Leu352 plasmids (27) by ScaII/NcoI digestion and inserted into pFLAG-CMV2 plasmids by sequential subcloning. The resulting pFLAG-CMV2-rPICK1, pFLAG-CMV2-rPICK1Ala142–Ser416 and pFLAG-CMV2-rPICK1Met1–Leu352 plasmids were used for transient transfection. The Lys-27 and Asp-28 of rPICK1, which are essential for binding to the C terminus of PKCa, were mutated to alanines by a two-step PCR using pCS86rPICK1 as a template, and the rPICK1Ala142–Ser416 and rPICK1Met1–Leu352 mutant plasmids were used for transient transfection. The Lys-27 and Asp-28 of rPICK1, which are essential for binding to the C terminus of PKCa, were mutated to alanines by a two-step PCR using pCS86rPICK1 as a template, and the rPICK1Ala142–Ser416 and rPICK1Met1–Leu352 mutant plasmids were used for transient transfection. The Lys-27 and Asp-28 of rPICK1, which are essential for binding to the C terminus of PKCa, were mutated to alanines by a two-step PCR using pCS86rPICK1 as a template, and the rPICK1Ala142–Ser416 and rPICK1Met1–Leu352 mutant plasmids were used for transient transfection. The Lys-27 and Asp-28 of rPICK1, which are essential for binding to the C terminus of PKCa, were mutated to alanines by a two-step PCR using pCS86rPICK1 as a template, and the rPICK1Ala142–Ser416 and rPICK1Met1–Leu352 mutant plasmids were used for transient transfection. The Lys-27 and Asp-28 of rPICK1, which are essential for binding to the C terminus of PKCa, were mutated to alanines by a two-step PCR using pCS86rPICK1 as a template, and the rPICK1Ala142–Ser416 and rPICK1Met1–Leu352 mutant plasmids were used for transient transfection.

RESULTS

The PICK1 Protein Resides in Cytoplasm in a Punctate Pattern—To examine the intracellular distribution of PICK1 in NIH3T3 cells, we have performed immunofluorescence staining using anti-rPICK1 antibodies. The PICK1 protein resided mainly in the cytoplasm with a punctate pattern (Fig. 1A). The sera preabsorbed with recombinant rPICK1 did not detect PICK1 any more (Fig. 1B), suggesting a specific recognition to

The pcDNA3PKCa plasmid was also used as a template for construction of the pcDNA3PKCa-mQSAV plasmid by PCR.

Intracellular Distribution of Endogenous PICK1 Proteins—NIH 3T3 cells were routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For immunostaining, the cells were seeded on poly-L-lysine-coated coverslips 1 day before experiments, fixed in 4% paraformaldehyde for 5–10 min, permeabilized with Triton-X 100 (0.1%), washed with PBS (pH 7.4), and 1% bovine serum albumin containing 0.1% Triton X-100 for 30 min and then postfixed with acid ethanol for 10 min, and the non-specific binding was further blocked in Tris-buffered saline containing 0.1% Triton X-100 for 60 min. Cells were then incubated overnight at room temperature with the polyclonal anti-FLAG antibody (27) diluted in Tris-buffered saline containing 0.1% Triton X-100 (1:50), washed with PBS (pH 7.4) for three times, and incubated with either rhodamine- or Cy3-conjugated goat anti-rabbit IgG (1:600 and 1:400, respectively) (Jackson ImmunoResearch Laboratories) for 30 min to visualize PICK1. The samples were counterstained for DNA with Hoechst 33258 (1 µg/ml) for 30 min along with the secondary antibodies. Coverslips were mounted on slides with Fluoromount G (Southern Biotechnology Associates, Inc.). The cells were imaged using a fluorescent microscope (Olympus BX50) or a Leica TCS SP2 spectral confocal and multiphoton system. The image processing was performed using the Meta View software (Universal Imaging Corp.).

The mitochondria were identified by staining with a monoclonal anti-cytochrome oxidase subunit I (a protein located in the mitochondria inner membrane) antibody, with the antibody diluted 1:50 (Molecular Probes), and the ER was identified by staining with a monoclonal anti-Bip/Gyrp78 (an ER-resident protein) antibody, with the antibody diluted 1:25 (BD Transduction Laboratories). The secondary antibody consisted of FITC-conjugated anti-mouse IgG antibodies, diluted 1:150 to 1:175 (Jackson ImmunoResearch Laboratories). For visualization of Golgi, cells were transfected with pEYFP-Golgi (Clontech) in which the EYFP (enhanced yellow fluorescent protein) was fused to an anchoring signal peptide that targets the fusion protein to the trans-medial region of the Golgi apparatus. Fluorescence from EYFP was observed directly on a fluorescent microscope. In some cases, cells were incubated with 10 µl of the rPICK1 (10 µg/ml), which disrupted Golgi structures, for 30 min to confirm the Golgi localization of EYFP. The endogenous RACK1 protein was detected using monoclonal mouse anti-RACK1 antibodies, diluted 1:50 (BD Transduction Laboratories).

Stimulation of Cells—NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum to 70–80% confluency, serum-starved overnight, and then stimulated with either 20% fetal bovine serum or TPA (Sigma) (200 ng/ml) for various times, as indicated.

Transfection and Detection of Heterologously Expressed Proteins—NIH 3T3 cells were seeded on coverslips overnight and transfected with various plasmids using Lipofectamine Plus reagent (Invitrogen) in accordance with the manufacturer’s instructions. The wild-type and mutant FLAG-tagged rPICK1 proteins were detected with anti-FLAG polyclonal antibodies (1:50) (Abcam Ltd.) and visualized with rhodamine-conjugated anti-rabbit IgG antibodies (1:600) (Jackson ImmunoResearch Laboratories). In some instances, the anti-cytochrome oxidase subunit I monoclonal antibody (1:50) and the FITC-conjugated anti-mouse IgG antibody (1:200) were used simultaneously to locate mitochondria. The PKCa was detected with monoclonal anti-PKCa antibodies (BD Transduction Laboratories), which recognize PKCa specifically.

Antibodies—The rabbit polyclonal anti-rPICK1 antibody was generated against the recombinant rPICK1 protein as described previously (23). The mouse monoclonal anti-FLAG M5 antibody was from Sigma, and the rabbit polyclonal anti-FLAG epitope antibody was from Abcam Ltd. Monoclonal mouse anti-RACK1, monoclonal anti-Bip/Gyrp78, and monoclonal anti-PKCa antibodies were from BD Transduction Laboratories. The mouse monoclonal anti-cytochrome oxidase subunit I antibody was from Molecular Probes. Rhodamine-, FITC-, and Cy3-conjugated anti-rabbit or anti-mouse IgG antibodies were from Jackson ImmunoResearch Laboratories.

RESULTS

The PICK1 Protein Resides in Cytoplasm in a Punctate Pattern—To examine the intracellular distribution of PICK1 in NIH3T3 cells, we have performed immunofluorescence staining using anti-rPICK1 antibodies. The PICK1 protein resided mainly in the cytoplasm with a punctate pattern (Fig. 1A). The sera preabsorbed with recombinant rPICK1 did not detect PICK1 any more (Fig. 1B), suggesting a specific recognition to
PICK1 Targets PKCα to Mitochondria

**Mitochondria**—In addition to the PDZ domain, primary sequence analysis of PICK1 also predicts the existence of a coiled-coil domain (amino acids 139–166) and an acidic region (amino acids 380–390). Since no known mitochondria targeting sequence has been identified in PICK1, we speculated that mitochondria association might be mediated by an interaction with mitochondria-resident protein(s) residing in the outer membrane. To examine which sequence region is required for the mitochondria localization of PICK1, we transfected NIH 3T3 cells with pCMV2FLAG-rPICK1, pCMV2FLAG-rPICK1Ala142–Ser416, or pCMV2FLAG-rPICK1Met1–Leu352 for expression of the wild type and truncated rPICK1s with a FLAG epitope tag at the N terminus. Their intracellular distribution was examined by indirect immunofluorescence using anti-FLAG antibodies as primary antibodies and fluorescein-conjugated anti-mouse IgG antibodies as secondary antibodies. The wild type FLAG-rPICK1 displayed a punctate pattern in the cytoplasm resembling the one that was observed for endogenous PICK1 and was found to be coincident with mitochondria as detected with antibodies against cytochrome oxidase subunit I (Fig. 3), whereas the PDZ-deleted rPICK1 which still contains the coiled-coil domain, displayed a dense, juxtanuclear pattern, mostly concentrated in one or two regions that were not co-localized with the mitochondrial marker cytochrome oxidase (Fig. 3) indicating a loss of mitochondria association. The rPICK1Met1–Leu352, which contains an intact PDZ domain but lacks the C-terminal 64 residues, including the acidic region, displayed a mitochondria distribution pattern similar to the wild type (Fig. 3). These results indicated that the PDZ domain was essential for mitochondria association of PICK1, whereas the C-terminal region was dispensable and the coiled-coil region was not able to sustain mitochondria association.

Many PDZ domains bind directly to the C-terminal ends of their target molecules through the carboxylate-binding loop.

**Fig. 1. Intracellular distribution of PICK1 and RACK1 in NIH 3T3 cells.** NIH 3T3 cells were seeded on coverslips 16 h before fixation for immunostaining. PICK1 (A–C) and RACK1 (D) were detected with the polyclonal anti-rPICK1 antibody (1:500) and the monoclonal anti-RACK1 antibody (1:50) and visualized with Cy3-conjugated anti-rabbit antibodies and FITC-conjugated anti-mouse antibodies, respectively. In B, the anti-rPICK1 antibody was preabsorbed with recombinant rPICK1. Hoechst 33342 (1 μg/ml) was used to localize nuclei. Cells were observed under a fluorescent microscope using a ×40 objective lens (A and B) or a ×100 objective lens immersed in oil (D). Confocal scanning images are shown for a more detailed display of the PICK1 pattern (C).

PICK1. At higher magnifications, PICK1 protein typically exhibited as a rod-like network structure in the cytoplasm, with more concentration in a broad area surrounding the nucleus without significant indication of plasma membrane association (Fig. 1C). These results suggested that the PICK1 protein was associated with subcellular compartments in unstimulated cells. Similar distribution patterns were also observed in Rat-1 cells, H460 and H1299 (data not shown), indicating a common distribution pattern of PICK1 in these cells. The well characterized, PKCβII-specific targeting protein RACK1 displayed a distinct diffuse distribution pattern throughout the cytoplasm (Fig. 1D). Our results suggested that these two PKC-binding proteins have distinct roles in mediating PKC functions.

To identify which organelles the PICK1 protein is associated with, we localized ER, Golgi, and mitochondria in NIH 3T3 cells using organelle-specific markers. Mitochondria, detected with the anti-cytochrome oxidase subunit I antibody, displayed a rod-like or network structure in the cytoplasm, which was identical to that of the PICK1 protein (Fig. 2A). The structure was also recognized by the antibody to cytochrome c, an intermembrane resident of mitochondria, or Mito Tracker dye, whose uptake depends on mitochondria membrane potential in live cells (data not shown). The mitochondria and PICK1 signals overlapped almost completely, confirming the mitochondria localization of the PICK1 protein (Fig. 2A). Staining of NIH 3T3 cells with the anti-GRP78 (an ER-resident protein) antibody displayed a fine punctate/reticular pattern surrounding the nucleus, a typical ER distribution pattern (Fig. 2B). The pattern was distinct from that of PICK1, indicating that PICK1 did not associate with ER. The EYFP-Golgi-expressing cells, which expressed enhanced yellow fluorescent protein fused to a signal sequence targeting to the trans-medial Golgi, revealed intense fluorescence of a compact juxtanuclear structure, a characteristic feature of Golgi apparatus (Fig. 2C). Cells treated with brefeldin A, which disrupted Golgi structure, displayed a dramatic change of EYFP fluorescence into a diffuse distribution in the cytoplasm (Fig. 2C). The distribution of PICK1 remained unchanged in cells treated with brefeldin A (Fig. 2C). Taken together, these results indicated that PICK1 resided in mitochondria but not in ER or the Golgi apparatus.

The PDZ Domain-deleted rPICK1 Fails to Associate with

**Fig. 2. Co-localization of endogenous PICK1 with mitochondria in NIH 3T3 cells.** The endogenous PICK1 in NIH 3T3 cells was stained with the polyclonal anti-rPICK1 antibody and visualized with either rhodamine-conjugated (1:600) (A) or Cy3-conjugated (1:400) (B and C) anti-rabbit antibodies. Cells were stained simultaneously with monoclonal antibodies against either cytochrome oxidase subunit I, a mitochondria marker (1:50) (A), or Grp78, an ER marker (1:250) (B), and then visualized with FITC-conjugated anti-mouse IgG antibodies. In C, fluorescence from cells transfected with the pEYFP-Golgi plasmid was observed directly under a fluorescence microscope. The Golgi pattern of EYFP was disrupted in cells treated with brefeldin A (BFA) (10 μg/ml) for 30 min (C). This treatment did not affect the distribution pattern of PICK1. Fluorescent images were observed using a ×100 objective lens immersed in oil (A and C) or by confocal scanning (B). The scale bar corresponds to 25 μm.
PK1 have distinct responses to the same stimulation in NIH 3T3 cells with respect to their intracellular localization. Our results support the notion that isozyme-specific targeting proteins mediate specific functions of each isozyme and that the mitochondria association and the redistribution upon serum stimulation were events specific to PICK1.

To investigate whether PICK1 directs PKCα to mitochondria in the perinuclear region in NIH 3T3 cells after activation, we expressed PKCα and examined its intracellular localization by indirect immunofluorescence. NIH 3T3 cells were co-stained with antibodies specific to PKCα and the anti-rPICK1 antibody. PKCα stained diffusely throughout the entire cytoplasm in unstimulated cells, whereas PICK1 displayed a punctate mitochondria pattern (Fig. 4D, serum). However, after cells were stimulated with serum, a substantial portion of PKCα translocated to the perinuclear region where it co-localized with PICK1 (Fig. 4D, serum). As shown with RACK1 in Fig. 4C, the distribution of endogenous PKCβ remained unchanged after serum stimulation (data not shown), indicating that the co-movement caused by serum stimulation was specific to PICK1 and PKCα.

**TTP Stimulation Causes a Translocation of PKCα to the Plasma Membrane but Does Not Affect the Cytoplasmic Distribution of PICK1**—To investigate whether the co-localization of PKCα and PICK1 is a common event upon activation of PKCα, we further examined the intracellular distribution of PICK1 and PKCα in response to stimulation of TPA, a potent PKC activator. After TPA treatment of NIH 3T3 cells, the distribution pattern of PICK1 was, in contrast to serum stimulation, largely unchanged (Fig. 5A). These results indicated that the redistribution of PICK1 to the perinuclear region was a serum-specific response and that translocation of PICK1 was dependent on the nature of extracellular stimuli. However, TPA treatment caused a translocation of PKCα to the plasma membrane (Fig. 5B, upper panel), in the same cell, as documented. The distribution pattern of PICK1 in cells either expressing PKCα (Fig. 5B, upper panel) or not expressing PKCα (Fig. 5B, lower panel) was similar, indicating that overexpression of PKCα alone did not cause redistribution of PICK1. These results indicated that the co-movement of PICK1 and PKCα was an event specific to serum stimulation.
PKCα That Lacks the C-terminal PDZ-binding Motif Fails to Translocate to the Perinuclear Region upon Serum Stimulation—To investigate the molecular mechanism of the targeting of PKCα to the perinuclear region in response to serum stimulation, we constructed PKCα mutant that lacked the 4 amino acid residues, QSAV, at the extreme C terminus. These 4 amino acid residues are not only the binding site for PICK1 but also provide the sequence specificity that discriminates PKCα from other PKC isozymes that do not interact with PICK1. The wild type PKCα co-localized with PICK1 in the perinuclear region after serum stimulation (Fig. 6, A and B), as shown in the previous figures. However, the QSAV-deleted PKCα failed to translocate to the perinuclear region after serum stimulation (Fig. 6D), whereas PICK1 still translocated in the same cell (Fig. 6C). After TPA treatment, the QSAV-deleted PKCα mutant was found predominantly in the plasma membrane (Fig. 6H) as seen for the wild type PKCα (Fig. 6F), indicating that the QSAV-deleted PKCα mutant was responsive to TPA but...
lost its capacity to translocate upon serum stimulation. TPA treatment did not change the distribution of PICK1 under these conditions (Fig. 6, E and G). Since PICK1 still moved in cells where mutant PKCα failed to when stimulated with serum, it was strongly suggested that PICK1 recruited PKCα to the perinuclear region when the kinase was activated by serum stimulation through interactions of the PDZ domain of PICK1 and the C-terminal QSAV of PKCα. Taken together, our results suggested a unique role of PICK1 in targeting active PKCα to mitochondria in a ligand-specific manner. The PICK1 protein may serve as a switch point in PKC-mediated pathways that lead to specific cellular processes in response to different stimuli.

**DISCUSSION**

PICK1 may mediate functions unique to mitochondria, as indicated by our results that PICK1 is in mitochondria but not in ER, Golgi apparatus, or plasma membrane. An identical distribution pattern of PICK1 observed in NIH 3T3, Rat-1, H460, and H1299 cells suggests a conservation of intracellular localization and, potentially, functions in these cells. Mitochondria association is through the PDZ domain near the N terminus, but mutations in the putative carboxyate-binding loop that abolish binding to PKCα (16) have no effect on its mitochondria localization. These findings indicate that PICK1 can associate with mitochondria and simultaneously interact with PKCα and thus suggest a role for PICK1 in mediating PKCα targeting to mitochondria. This notion is confirmed because of the fact that upon activation, a substantial amount of PKCα is targeted to mitochondria in the perinuclear region, where it co-localizes with PICK1.

As a targeting protein for C kinases, PICK1 is unique in its association with a particular intracellular organelle in unstimulated cells. RACK1 is not localized to any specific organelle in Chinese hamster ovary cells, NG108-15 (neuroblastoma and glioma hybrid cells) (13), or NIH3T3 cells (Fig. 1D). Activation of PKC by NPA (a dopamine D2 agonist) induces movement of both βIIIPKC and RACK1 to Golgi-like structures (13). β′-COP, a coatamer protein essential for Golgi budding and vesicle trafficking, is a selective anchoring protein for activated PKCε (15). Binding of β′-COP to Golgi membrane is dependent on activated ADP-ribosylation factor and accompanied by a corresponding in-
PKC be of particular importance in dissecting the distinct function of PKC.

Rum may be achieved via recruitment of PKC to mitochondria substrate(s) essential for cellular responses to se-

or the proapoptotic Bax, on mitochondria and therefore affects REH cells. Translocation is probably essential to bring PKC to PKC upon different stimulations and the signaling pathway leading to it. Mutant PKC with a deleted C-terminal QSAV sequence that abolishes interaction with PICK1 (16) fails to translocate on serum stimulation (Fig. 6). Serum stimulation may cause changes that lead to a “competent” conformation favoring interactions via the carboxylic-binding loop of PICK1 and the C-terminal QSAV peptide of PKC. The PICK1 protein may serve as a switch point in PKC-mediated pathways that lead to specific cellular processes in response to different stim-

ul. We propose that a direct interaction between PKC and its specific anchoring proteins, such as PKC and PICK1, is one of the mechanisms sustaining the mitochondria-specific localization and the mitochondria-associated function of PKC.

The physiological significance of the association of PKC and mitochondria upon serum stimulation remains to be investi-
gated. Data indicate that PKC isoforms are localized on mito-

chondria and functionally associated with mitochondria under various conditions including apoptosis. Enhanced resistance to apoptosis induced by therapeutic drugs is reported in human pre-B REH cells when pretreated with the PKC agonist bryo-

statin-1 (29). The enhanced chemoresponse is coincident with the increased mitochondrial localization of PKC and aug-

mented phosphorylation of mitochondrial Bcl2, a key antiapop-
totic protein (29). These results support a role for mitochondrial PKC in Bcl2 phosphorylation and suppression of apoptosis in REH cells. Translocation is probably essential to bring PKC to the proximity of its substrates, such as the antiapoptotic Bcl-2 or the proapoptotic Bax, on mitochondria and therefore affects the apoptotic activity of mitochondria. Phosphorylation of mito-

chondria substrate(s) essential for cellular responses to se-

rum may be achieved via recruitment of PKC to mitochondria by PICK1.

Changes in intracellular mitochondria distribution are ob-

served in various cellular processes such as during oocyte mat-

uration, fertilization and embryo development (30, 31), viral infection (32), and apoptosis (33, 34). Clustering of mitochondria to a particular region may reflect a different local demand on energy and calcium flux or the need to facilitate transloca-

tion of mitochondrial proteins to a specific compartment. Tar-
geting of PKC to the perinuclear region by PICK1 upon serum stimulation may represent a need for translocation of phospho-

rylated mitochondria substrates or PKC itself to the nucleus upon serum stimulation.

The PDZ domain of PICK1 is required for association with both PKCs (16) and mitochondria (Fig. 3), but they appear to be through distinct modes of interaction because only PKCα requires the carboxylic-binding loop of PICK1 for interaction. PDZ domains are widespread protein interaction domains that bind primarily the C-terminal carboxylate group in a sequence-
specific way (19, 20, 28). In addition to the C-terminal peptide, PDZ domains can also bind internal non-C-terminal sequences or other PDZ. Functionally, PDZ domains have emerged as scaffolds for the organization and assembly of protein complexes at specific subcellular locations, particularly at the plasma membrane (20, 28). PDZ-containing proteins frequently interact with several different partners or transmembrane proteins simultaneously. Assembly of protein complex can be at-

distributed to the diverse modes of PDZ-target interaction. The PDZ in neuronal nitric oxide synthase can bind directly to the PDZ of α-syntrophin (35). Structural analysis reveals that there are two interaction surfaces in the PDZ of neuronal nitric oxide synthase (36). A β-finger motif docks in the syntrophin peptide-binding groove leaving the canonical peptide-binding groove of neuronal nitric oxide synthase free for interaction with the conventional C-terminal peptide ligand. PICK1, which contains only one PDZ domain, may interact with PKCs and mitochondria through different modes. There is no known mito-

chondria targeting sequence found in PICK1, so PICK1 prob-

ably associates with mitochondria via interaction with a mito-

chondria outer membrane protein or protein complex through PDZ-mediated interactions. Since PICK1 can self-associate through the coiled-coil domain (37), it is also possible that the PDZ from one PICK1 molecule interacts with PKCα and that from the other PICK1 molecule in the dimer interacts with the mitochondria outer membrane protein.

The co-localization of activated PKCα with PICK1 is specific to serum stimulation since TPA has no significant effect on the localization of PICK1, whereas it causes a translocation of PKCα to the plasma membrane. Taken together, we have dem-

onstrated that PICK1 is a specific targeting protein for PKCα that selectively recruits activated PKCα to mitochondria, thereby allowing specific phosphorylation events such as phos-

phorylation of PICK1 (27) to occur in a ligand-dependent way.

Our findings presented here indicate a novel, previously unrec-

ognized regulatory role for PICK1 as a specific mitochondria anchoring protein of PKCα.

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