Association of Protein Kinase C\(\mu\) with Type II Phosphatidylinositol 4-Kinase and Type I Phosphatidylinositol-4-phosphate 5-Kinase*  

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Protein kinase C\(\mu\) (PKC\(\mu\)), also named protein kinase D, is an unusual member of the PKC family that has a putative transmembrane domain and pleckstrin homology domain. This enzyme has a substrate specificity distinct from other PKC isoforms (Nishikawa, K., Toker, A., Johannes, F. J., Songyang, Z., and Cantley, L. C. (1997) *J. Biol. Chem.* 272, 952–960), and its mechanism of regulation is not yet clear. Here we show that PKC\(\mu\) forms a complex in vivo with a phosphatidylinositol 4-kinase and a phosphatidylinositol-4-phosphate 5-kinase. A region of PKC\(\mu\) between the amino-terminal transmembrane domain and the pleckstrin homology domain is shown to be involved in the association with the lipid kinases. Interestingly, a kinase-dead point mutant of PKC\(\mu\) failed to associate with either lipid kinase activity, indicating that autophosphorylation may be required to expose the lipid kinase interaction domain. Furthermore, the subcellular distribution of the PKC\(\mu\)-associated lipid kinases to the particulate fraction depends on the presence of the amino-terminal region of PKC\(\mu\) including the predicted transmembrane region. These results suggest a novel model in which the non-catalytic region of PKC\(\mu\) acts as a scaffold for assembling of enzymes involved in phosphoinositide synthesis at specific membrane locations.

Protein kinase C (PKC)\(^{1}\) family members play crucial roles in the signal transduction of a variety of extracellular stimuli, such as hormones, neurotransmitters, and growth factors (1). Molecular cloning has demonstrated the presence of at least three groups, conventional PKC (cPKC) comprising \(\alpha, \beta, \beta I,\) and \(\gamma\) isoforms (activated by calcium, acidic phospholipid, and diacylglycerol (DAG)), novel PKCs (nPKC) comprising \(\delta, \epsilon, \eta,\) and \(\theta\) (activated by DAG and acidic phospholipid but insensitive to calcium), atypical PKCs \(\lambda/\zeta\) (mechanism of regulation not clear) (1–6). Another subgroup of PKCs may be defined by PKC\(\mu\) (human)/PKD (mouse homologue of PKC\(\mu\)) (7–11). PKC\(\mu\) differs in some structural features from other PKC isoforms in that PKC\(\mu\) contains a putative transmembrane domain and a pleckstrin homology (PH) domain in its amino-terminal regulatory region providing a mechanism for constitutive association with the membrane (7, 8). Recent investigations using various approaches such as overexpression and down-regulation of specific isoforms indicate that each PKC isoform plays a unique role in signal transduction processes (1, 5). It is highly likely that the distinct functions of these isoforms are a consequence of isoyme-specific substrates and/or interacting proteins. However, information about the substrate specificities of each PKC family member has been quite limited.

Recently, we determined optimal substrate motifs for all classes of PKC isoforms using an oriented peptide library technique and found that each PKC isoform has a unique optimal substrate sequence (12). Interestingly, PKC\(\mu\) has very different substrate specificities from other isoforms in that PKC\(\mu\) showed extreme selectivity for peptides with Leu at position −5 amino-terminal of the phosphorylated Ser. This finding suggested that PKC\(\mu\) is involved in a unique signaling pathway distinct from those mediated by other PKC family members. PKC\(\mu\)/PKD was recently shown to be activated by mitogenic regulatory peptides and platelet-derived growth factor (PDGF) by a pathway that requires cPKC or nPKC activation upstream (13). In lymphocytes, PKC\(\mu\) is shown to regulate signaling via Syk and phospholipase C\(\gamma\)1 (14). However, substrate proteins or interacting proteins of PKC\(\mu\) in intact cells are poorly understood.

Of interest, recent data indicate that PKC\(\mu\) is located in the Golgi apparatus and is involved in basal transport processes (15). Lipid kinases, such as phosphatidylinositol 3-kinase (PtdIns 3-K), phosphatidylinositol 4-kinase (PtdIns 4-K), and phosphatidylinositol-4-phosphate 5-kinase (PtdIns-4-P 5-K), have also been implicated in membrane trafficking (16). One of the PtdIns 4-K isoforms, PtdIns 4-K\(\beta\), is concentrated in the Golgi apparatus (17). The yeast *VPS34* gene product, Vps34p, which is a PtdIns-specific 3-kinase, is required for the efficient sorting and delivery of proteins from the Golgi to the vacuole (18). Vps34p exists as a complex with Vps15p, a 160-kDa Ser/Thr protein kinase, which recruits Vps34p to the membrane of the Golgi complex and enhances Vps34p PtdIns 3-kinase activity (19). A human homologue of Vps34p has recently been characterized as part of a complex with a human homologue of Vps15p, an adapter protein called p150 (20). Interestingly, this
complex also has a protein kinase activity, and the association of Vps34p with p150 increases the PtdIns 3-kinase activity (21). These observations prompted us to investigate the possibility of the specific interaction of PKCγ and lipid kinases.

In this study we found that PKCγ associates with at least two lipid kinases, a type II PtdIns 4-K and type I PtdIns 4-P 5-K, in an isoform-specific manner. Analysis using various deletion mutants of PKCγ suggest that these two lipid kinases effectively bind to PKCγ through its amino-terminal region and that the protein kinase activity of PKCγ is essential to this association. These results suggest that PKCγ plays a role in assembly of specific phosphatidylinositol-phosphorylating enzymes at the membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—Isozyme-selective antipeptide antibodies for PKCα, -ε, and -μ were purchased from Santa Cruz Biotechnology. Two anti-PKCγ antibodies were used as follows: one raised against amino acids 6–25 mapping at the amino terminus of PKCγ (α-γ-N) and one against amino acids 893–912 mapping at the carboxyl terminus of PKCγ (α-γ-C). Anti-FLAG M2 monoclonal antibodies were purchased from Eastman Kodak Co. LipofectAMINE reagents were purchased from Life Technologies. Immunoprecipitation and polyacrylamide gel electrophoresis were performed as described previously (12). Purified PtdIns 3-K was prepared as described previously (12). Purified PtdIns 3-K was prepared and used as the primary antibody. Antirabbit IgG or antiamouse IgG conjugated to horseradish peroxidase was used as a secondary antibody. The membranes were finally visualized by chemiluminescence as described by the manufacturer (NEC Life Science Products).

PKC Assay—PKC activity was assayed in vivo essentially as described previously using the standard PKC vesicle assay (25). The reaction mixture (30 μl) contained 100 μM ATP with [γ-32P]ATP (5 μCi), 1 mM dithiothreitol, 5 mM MgCl2, 25 mM Tris-HCl, pH 7.5, 20 μg/ml PS, 10 μg/ml DAG, 200 μM CaCl2 (for PKCa), 0.5 mM EGTA (for PKCε, ζ, and μ), and the indicated amount of synthetic substrate peptides. Reactions were started by the addition of immunoprecipitated PKC and incubated at 30 °C for 10 min. Reactions were terminated by spotting 2-μl samples from the reaction mixture onto Whatman 3MM paper and washed 4 times in 500 ml of 1% phosphoric acid. Incorporation of 32P was determined by liquid scintillation counting. For each experimental condition, values for control reactions lacking substrate peptide were subtracted as blanks. Autophosphorylation of PKCγ was carried out essentially as described above, but no substrate was added. Proteins of the reaction mixture were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

**Lipid Kinase Assay and HPLC Analysis**—Lipid kinase assays were performed as described previously with a slight modification (26). Briefly, the reaction mixture (100 μl) contained 50 μM ATP, 20 mM HEPES, pH 7.5, 10 mM MgCl2, 0.2 mg/ml sonicated lipids, 20 μg/ml of anti-PKCα, -ε, and -μ antibodies as described previously (12). The 110-kDa immunoprecipitated PKCγ (a-γ-N) was used to coat to nitrocellulose membrane. Isozyme-specific anti-PKC antibodies were used as the primary antibody. Antirabbit IgG or antiamouse IgG conjugated to horseradish peroxidase was used as a secondary antibody. The membranes were finally visualized by chemiluminescence as described by the manufacturer (NEC Life Science Products).

**RESULTS**

**Association of PtdIns 4-K and PtdIns 4-P 5-K Activities with PKCγ**—To examine whether PKCγ associates with lipid kinases, phosphoinositide kinase activities were measured in immunoprecipitates of PKCγ. A PKCγ expression vector or control vector were transiently transfected into COS-7 cells, and cell lysates were immunoprecipitated with an anti-PKCa-specific antibody raised against the amino terminus of PKCa. The resultant immunocomplexes were subjected to Western blot analysis and visualized by autoradiography and quantitated using a molecular imager (Bio-Rad). The lipid products on the TLC plate were collected, deacylated, and analyzed by HPLC as described in detail elsewhere (27). Standards were made from tritiated PtdIns-4-P and PtdIns-4,5-P2 (NEN Life Science Products), which were deacylated and included with the 32P-labeled product in the HPLC run. PtdIns-3-P, PtdIns-3,4-P2, and PtdIns-5-P standards were made as described previously (28).

**Expression of PKCγ in vivo and in vitro**—Western blot analysis was done as reported previously (24). The immunoprecipitated proteins were dissolved in 20 μl of sodium dodecyl sulfate (SDS) buffer (62.5 mM Tris, 2% SDS, 5% mercaptoethanol, and 5% glycerol, pH 6.8) and boiled for 5 min. This solution was subjected to 7.5% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were electroeluted from nitrocellulose membrane. Isozyme-specific anti-PKC antibodies were used as the primary antibody. Antirabbit IgG or antiamouse IgG conjugated to horseradish peroxidase was used as a secondary antibody. The membranes were finally visualized by chemiluminescence as described by the manufacturer (NEC Life Science Products).

**Association of Lipid Kinases from COS-7 Cell Lysate with GST-fused Proteins**—COS-7 cells were washed with PBS and lysed in lysis buffer. Crude cell extract was clarified by centrifugation (14,000 rpm for 10 min). GST or GST-fused proteins (5 μg each) were incubated with the cell lysate at 4 °C for 2 h with constant rocking. The beads were washed twice with 1-ml volume of ice-cold PBS, twice with 1-ml volume of ice-cold washing buffer (0.1 mM Tris-HCl, pH 7.4, 0.1 mM LiCl), and twice with 1-ml volume of TNE (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA). Lipid kinase assays were then performed on the beads as described below.

**Western Blot Analysis**—Western blot analysis was done as reported previously (24). The immunoprecipitated proteins were dissolved in 20 μl of sodium dodecyl sulfate (SDS) buffer (62.5 mM Tris, 2% SDS, 5% mercaptoethanol, and 5% glycerol, pH 6.8) and boiled for 5 min. This solution was subjected to 7.5% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were electroeluted from nitrocellulose membrane. Isozyme-specific anti-PKC antibodies were used as the primary antibody. Antirabbit IgG or antiamouse IgG conjugated to horseradish peroxidase was used as a secondary antibody. The membranes were finally visualized by chemiluminescence as described by the manufacturer (NEC Life Science Products).
results are consistent with our previous finding that PKC\(\mu\) preferentially phosphorylates the \(\beta\) and \(\mu\) peptides compared with the \(\alpha\) peptide (12). These results confirm the overexpression of functional PKC\(\mu\) in COS-7 cells. These immunocomplexes were subjected to a lipid kinase assay using crude brain phosphoinositides (CBP) as lipid substrates in the presence or absence of 0.3% of Triton X-100. As shown in Fig. 1.

To characterize further the lipid kinase(s) associated with PKC\(\mu\), the lipid products obtained from the experiment in Fig. 1C were separated by TLC, deacylated, and analyzed by HPLC. As shown in Fig. 2A, the PKC\(\mu\) immunoprecipitate contained activity that phosphorylated lipids to produce products that migrate on thin layer chromatography in the region of PtdIns-4-P and PtdIns-4,5-P\(_2\), standards. This activity was enhanced in the presence of Triton X-100. Since Triton X-100 is known to inhibit PtdIns 3-kinase activity (29), these results argue against PtdIns 3-kinase being responsible for the phosphorylation detected.

For this purpose, cPKC\(\alpha\), nPKC\(\epsilon\), and atypical PKC\(\xi\) were used, each of which is classified into a different subfamily of PKCs from PKC\(\mu\). The expression vectors of PKC\(\alpha\), PKC\(\epsilon\), and PKC\(\xi\) were transiently transfected into COS-7 cells, and the enzymes were immunoprecipitated with isozyme-specific antibody. Endogenous PKC\(\alpha\) was investigated since this enzyme is highly expressed in COS-7 cells. The expression of each isozyme was confirmed by Western blot analysis (Fig. 3A) and protein kinase assays of immunoprecipitates (data not shown). Each immunocomplex was also subjected to PtdIns 4-K (Fig. 3B) and PtdIns 4-P 5-K (Fig. 3C) assays. Both lipid kinase activities were normalized to the \(\beta\) peptide phosphorylating activity, because \(\beta\) peptide is a relatively good substrate for all the PKC isozymes as described previously (12). Significant PtdIns 4-K activity was detected in the PKC\(\mu\) immunoprecipitate, whereas less activity was detected in the PKC\(\epsilon\) immunoprecipitate, and no PtdIns 4-K activity was detected in PKC\(\xi\) immunoprecipitate.
Association of PKC\(\mu\) with Lipid Kinases

**FIG. 3.** Lipid kinases specifically associate with PKC\(\mu\) compared with other PKC isoforms. Expression vectors of PKC\(\epsilon\), \(\zeta\), and \(\mu\) or control vectors were transiently transfected into COS-7 cells and immunoprecipitated with an isotype-specific antibody. Immunoprecipitated PKC\(\mu\) was prepared from control COS-7 cells using a specific antibody. The resultant immunocomplexes were subjected to Western blot analysis (A). A PtdIns 4-K assay using PtdIns/PtdSer (1:1) (B) or a PtdIns-4-P 5-K assay using PtdIns-4-P/PA (1:1) (C) was performed in the presence of 0.3% Triton X-100, as described under “Experimental Procedures.” Phosphoinositides were visualized by autoradiography and quantitated as described under “Experimental Procedures.” The data are presented as the percentage of the activity present in the immunoprecipitated PKC\(\mu\) fraction. Both lipid kinase activities were normalized to the \(\beta\) peptide-phosphorylating activities present in the immunocomplexes. The data are representative of three experiments. The PtdIns 4-K to protein kinase ratio for PKC\(\mu\) was 16.9 and the PtdIns-4-P 5-K to protein kinase ratio was 18.5.

**FIG. 4.** The amino-terminal region of the regulatory domain of PKC\(\mu\) is involved in the association with lipid kinases. Schematic drawing of the structure of wild type PKC\(\mu\) and the amino-terminal deletion mutants of PKC\(\mu\), delta 1–79 and delta 1–340 (A). TM, transmembrane domain; CYS, cysteine-rich domain; AD, activation domain; PH, pleckstrin homology domain. These constructs were transfected into COS-7 cells and immunoprecipitated with anti-PKC\(\mu\) antibody raised against the carboxyl terminus of PKC\(\mu\). The resultant immunocomplexes were subjected to Western blot analysis (B), in vitro protein kinase assay using \(\beta\) peptides as substrates (C), and autophosphorylation assay (D). PtdIns 4-K (E) and PtdIns-4-P 5-K (F) assays were performed as described in the legend for Fig. 3. Both lipid kinase activities were normalized to the \(\beta\) peptide phosphorylating activities present in the immunocomplexes. Autophosphorylation of various PKC\(\mu\) constructs and phosphorylation of phosphoinositides were visualized by autoradiography and quantitated as described under “Experimental Procedures.” The data are presented as the percentage of the activity present in immunoprecipitates of full-length PKC\(\mu\). Error bars represent the standard error from three independent experiments. The PtdIns 4-K to protein kinase ratio for full-length PKC\(\mu\) was 10.5, and the PtdIns-4-P 5-K to protein kinase ratio was 11.2.

against the amino terminus of PKC\(\mu\) (not shown). The autophosphorylation level of the delta 1–340 mutant was reduced by 75% compared with that of wild type PKC\(\mu\) and the delta 1–79 mutant. Since the delta 1–340 mutant has similar peptide kinase activity to wild type PKC\(\mu\) (Fig. 4C), these results suggest that the major autophosphorylation site might be present in the region between residues 79 and 340. The immunocomplexes were subjected to PtdIns 4-K (Fig. 4D) and PtdIns-4-P 5-K (Fig. 4E) assays. Both lipid kinase activities associated with the delta 1–340 mutant were reduced by 80% compared with activities associated with wild type PKC\(\mu\) and the delta 1–79 mutant. These results indicate that the region
between residues 79 and 340 of PKC\(\mu\), which contains cysteine-rich domain I and II, is involved in the association with both lipid kinases.

**Characterization of the PKC\(\mu\)-associated PtdIns 4-K and PtdIns-4-P 5-K**—In order to confirm the above observations and further characterize these lipid kinases, the GST-fused amino-terminal region of PKC\(\mu\) coding for amino acids 34–290 (GST-CYS domain), GST-fused pleckstrin homology domain coding for amino acids 380–545 (GST-PH domain), or GST only were incubated with COS-7 lysate and assayed for PtdIns 4-K and PtdIns-4-P 5-K activities. As shown in Fig. 5B, the GST-CYS domain associated with a PtdIns kinase activity while very little activity associated with GST alone or the GST-PH domain. This PtdIns kinase activity was inhibited by adenosine in a dose-dependent manner (85% by 500 \(\mu\)M adenosine) (Fig. 5C) but was not inhibited by 100 \(\mu\)M wortmannin which caused 100% inhibition of PtdIns 3-K activity (Fig. 5D). This result indicates that this PtdIns kinase has properties similar to type II PtdIns 4-K which is markedly stimulated by detergent and inhibited by adenosine (30). Similar characteristics were obtained using wild type PKC\(\mu\)-associated PtdIns kinase (data not shown).

The GST-CYS domain also bound PtdIns-4-P 5-K activity, whereas the GST-PH domain and GST alone had very little associated activity (Fig. 5E). In agreement with results in Fig. 1C, the GST-CYS domain-associated PtdIns-4-P 5-K activity was activated by phosphatidic acid, consistent with this being a type I PtdIns-4-P 5-K (31). All of these results suggest that PKC\(\mu\) associates with a type II PtdIns 4-K and a type I PtdIns-4-P 5-K through its amino-terminal region (amino acids 80–290).

**Protein Kinase Activity of PKC\(\mu\) Is Required for the Association of PKC\(\mu\) with the Lipid Kinases**—To determine whether the protein kinase activity of PKC\(\mu\) is required for the association with the lipid kinases, a kinase-deficient mutant of PKC\(\mu\), K612W, was transfected into COS-7 cells and immunoprecipitated with anti-PKC\(\mu\) antibody raised against the amino terminus of PKC\(\mu\). The resultant immunocomplexes were subjected to Western blot analysis (A), a protein kinase assay using \(\beta\)I peptide as a substrate (B), and an autophosphorylation assay (C). Lipid kinase assays (D) were performed as described in the legend for Fig. 3 using crude brain phosphoinositides as lipid substrates. Lipid products were visualized by autoradiography as described under “Experimental Procedures.”

![FIG. 5. Characterization of the PtdIns 4-K and PtdIns-4-P 5-K associated with the amino-terminal region of PKC\(\mu\). Schematic drawing of the structure of wild type PKC\(\mu\), GST-CYS domain, and GST-PH domain (A). Purified GST-CYS domain, GST-PH domain, or GST only were incubated with COS-7 lysate. After washing the beads, a PtdIns 4-K assay was performed (B). A PtdIns 4-K assay was also performed on the GST-CYS domain in the presence or absence of the indicated amount of adenosine (C) or 100 \(\mu\)M of wortmannin (D). The effect of 100 \(\mu\)M of wortmannin on purified PtdIns 3-K is shown for comparison (D). The assay conditions were the same as described in the legend for Fig. 3. The data are presented as the percentage of the activity in the absence of inhibitors. The PtdIns-4-P 5-K assay was performed in the presence of PS or PA as described in the legend to Fig. 3 (E). Phosphoinositides were visualized by autoradiography and quantitated as described under “Experimental Procedures.”](https://example.com/fig5)

![FIG. 6. A kinase-deficient mutant of PKC\(\mu\), K612W, does not associate with lipid kinase activities. Wild type PKC\(\mu\) or a kinase-deficient mutant of PKC\(\mu\), K612W, was transfected into COS-7 cells and immunoprecipitated with anti-PKC\(\mu\) antibody raised against the amino terminus of PKC\(\mu\). The resultant immunocomplexes were subjected to Western blot analysis (A), a protein kinase assay using \(\beta\)I peptide as a substrate (B), and an autophosphorylation assay (C). Lipid kinase assays (D) were performed as described in the legend for Fig. 3 using crude brain phosphoinositides as lipid substrates. Lipid products were visualized by autoradiography as described under “Experimental Procedures.”](https://example.com/fig6)
**Association of PKC\(\mu\) with Lipid Kinases**

The association of PKC\(\mu\) with lipid kinases is not affected by PDGF-induced activation of PKC\(\mu\) in Swiss 3T3 cells. Subconfluent Swiss 3T3 cells were incubated with 0.1% fetal calf serum for 24 h and stimulated with the indicated concentration of PDGF for 5 min. Endogenous PKC\(\mu\) was immunoprecipitated with anti-PKC\(\mu\) antibody (anti-amino terminus) or with control antibody. The immunocomplexes were used for Western blot analysis (A), protein kinase assay (B), and lipid kinase assays (C). PtdIns 4-K and PtdIns-4-P 5-K activities were measured using PtdIns/PtdSer (1:1) and PtdIns-4-PP/ (1:1) as substrates, respectively, as described in the legend for Fig. 3. Lipid products were visualized by autoradiography as described under “Experimental Procedures.”

**DISCUSSION**

In the present study, we found that PKC\(\mu\) associates with at least two lipid kinases, a type II PtdIns 4-K and a type I PtdIns 4-P 5-K. These lipid kinase activities could be immunoprecipitated with PKC\(\mu\)-specific antibodies from COS-7 cells overexpressing PKC\(\mu\) or from Swiss 3T3 cells that normally express PKC\(\mu\). In contrast, very little lipid kinase activity could be detected in immunoprecipitates of PKCa, PKCe, or PKCG. The protein kinase activity of PKC\(\mu\) was found to be essential for the association with these lipid kinase activities, because a kinase-deficient mutant of PKC\(\mu\), K612W, did not associate with either lipid kinase activity. Furthermore, the subcellular distribution of the PKC\(\mu\)-associated lipid kinases to the particulate fraction depends on the presence of the amino-terminal region of PKC\(\mu\), including the predicted transmembrane region. These results suggest that PKC\(\mu\) can act as a scaffold to locate phosphoinositide kinases at the membrane and that the association or activation can be regulated by autophosphorylation of PKC\(\mu\).

Multiple types of PtdIns 4-K exist in mammalian cells. The type II PtdIns 4-Ks were initially characterized as membrane-associated 55-kDa proteins whose lipid kinase activities are inhibited by adenosine and the monoclonal antibody 4C5G (30, 32). The gene for the 55-kDa PtdIns 4-K has not been cloned. However, two other mammalian PtdIns 4-K genes have been cloned (PtdIns 4-Kz and PtdIns 4-Kp) and shown to encode proteins with high homology to PtdIns 3-K (32, 33). These enzymes are not intrinsic membrane proteins, and the mechanism by which they are brought to the membrane where their substrates reside is not known (40). Interestingly, the recently cloned PtdIns 4-K\(\phi\) was shown to be concentrated in the Golgi (17). Since PKC\(\mu\) was reported to be concentrated in the Golgi (15), we investigated the possibility that the PtdIns 4-K activity associated with PKC\(\mu\) is due to PtdIns 4-K\(\phi\). However, we could find no evidence for association of either PtdIns 4-K or PtdIns 4-K\(\phi\) with PKC\(\mu\) using overexpression or isoform-specific antibodies (not shown). In addition, the adenosine...
sensitivity, detergent activation, and wortmannin resistance of the PKC\(\mu\)-associated PtdIns 4-K are consistent with the properties of the 55-kDa type II enzyme that has not yet been cloned (34). Only approximately 3% of the total cellular PtdIns 4-K and PtdIns-4-P 5-K activities co-precipitate with PKC\(\mu\) (not shown), indicating either that PKC\(\mu\) associates with isoforms that are in low abundance or that protein modification is required to assemble the complex.

Two classes of phosphatidylinositol phosphate kinases have been characterized, type I and type II. The type I enzymes phosphorylate PtdIns-4-P at the 5 position to form PtdIns-4,5-P\(_2\). Two different genes for type I enzymes have been cloned and expressed (type I\(a\) and type I\(b\)) (31, 35, 36). The PtdIns-4-P 5-K activity associated with PKC\(\mu\) is activated by phosphatidic acid, consistent with it being due to a type I enzyme. However, attempts to show association between PKC\(\mu\) and either type I\(a\) or type I\(b\) have thus far failed.\(^2\) The type II PtdIns-4-P 5-K was also thought to be a PtdIns-4-P 5-K; however, recent research in this laboratory showed that this enzyme phosphorylates PtdIns-5-P at the 4 position to make PtdIns-4,5-P\(_2\) (28). The activity associated with PKC\(\mu\) does not phosphorylate PtdIns-5-P.

We found that deletion of 79 amino acids from the amino terminus of PKC\(\mu\) (the delta 1–79 mutant) did not affect the binding of either PtdIns 4-K or PtdIns-4-P 5-K, whereas deletion of 340 amino acids (the delta 1–340 mutant) diminished binding of both enzymes by 80%. Furthermore, the CYS domain of PKC\(\mu\) coding for amino acids 34–290 could effectively bind both lipid kinase activities from COS-7 cell lysates. These results suggest that PKC\(\mu\) associates with these two lipid kinases through its amino-terminal region (amino acids 80–290). On the other hand, a kinase-deficient PKC\(\mu\) mutant, K612W, failed to associate with either lipid kinase, even though the K612W mutant has an intact amino-terminal region. These observations suggest that the protein kinase activity of PKC\(\mu\) is required for inducing a conformational change of PKC\(\mu\) that exposes the amino-terminal region which is involved in the association with lipid kinases. If so, what is the target substrate of PKC\(\mu\) in this model? The only major phosphorylated protein present in the immunoprecipitated PKC\(\mu\) following in vitro addition of \([\gamma-32P]ATP\) was PKC\(\mu\) itself (Figs. 4D and 6C). Furthermore, the autophosphorylation level of the delta 1–340 mutant was reduced by 75% compared with wild type PKC\(\mu\) under conditions in which the protein kinase activities (using \(\beta\)I peptide) of wild type PKC\(\mu\) and the delta 1–340 mutant were the same. All of these results suggest that the major target substrate of PKC\(\mu\), which is involved in the recruitment of lipid kinase activity, is PKC\(\mu\) itself and that the major in vitro phosphorylation sites are located in the amino terminus of PKC\(\mu\). The possibility that the kinase-inactive PKC\(\mu\) (K612W) associates with the lipid kinases but fails to activate them cannot be excluded by the present data. It will be necessary to clone the genes for the PKC\(\mu\)-associated PtdIns 4-K and PtdIns-4-P 5-K to resolve further the mechanism of recruitment and activation since they do not appear to be cloned genes.

It has been shown that Vps34p, which is a phosphatidylinositol-specific 3-kinase (PtdIns 3-K) and is involved in vesicle-
mediated transport of proteins to the vacuole, associates with Vps15p, a 160-kDa membrane-bound Ser/Thr protein kinase (19). Vps15p recruits Vps34p to the membrane of the Golgi complex and enhances Vps34p PtdIns 3-K activity, since mutational inactivation of Vps15p protein kinase activity stops its association with Vps34p and blocks activation of Vps34p lipid kinase activity (19, 37). A human homologue of Vps34p has also been characterized as part of a complex with a human homologue of Vps15p, called p150 (20). This complex also has a protein kinase activity, and the association of the Vps34p homologue with p150 increases the PtdIns 3-K activity (21). However, the structure of p150 is quite different from that of PKC, in that p150 contains an amino-terminal myristoylation site, a Ser/Thr protein kinase domain, a region with homology to the 65-kDa regulatory subunit of protein phosphatase 2A, and a region containing a WD40 repeat motif (21).

It has been demonstrated that both p150 and Vps15p are modified by myristoylation at the amino-terminal region (21, 38), which may allow their targeting to the cytoplasmic face of the Golgi membrane, thereby allowing PtdIns 3-K to gain access to the membrane. PKC\(\mu\) is the only PKC isoform that contains a putative transmembrane sequence (7). Consistent with this fact, membrane targeting of the PKC\(\mu\)-associated lipid kinases depends on the presence of amino-terminal region of PKC\(\mu\), including this transmembrane sequence (Fig. 8). Furthermore, PKC\(\mu\) was shown to be located in the Golgi membrane and involved in basal vesicle transport processes (15). The results presented here suggest that PKC\(\mu\) may play a role in vesicle transport processes by recruiting two different types of lipid kinases to the Golgi membrane to facilitate local production of PtdIns-4-P and PtdIns-4,5-P2. In addition to their roles as precursors of Ins-1,4,5-P3 and diacylglycerol, these lipids have been implicated in various vesicle transport events (16, 39).

Stimulation of Swiss 3T3 cells with PDGF caused marked activation of PKC\(\mu\) but did not affect the lipid kinase activities associated with PKC\(\mu\), suggesting that the basal protein kinase activity of PKC\(\mu\) is essential and sufficient for the association of both lipid kinases. Recently, PKD, a mouse homologue of human PKC\(\mu\), was shown to be activated not only by PDGF but also by other cell stimuli, such as vasopressin, endothelin, and Bradynkinin through a pathway that requires another PKC isoform upstream (13). Interestingly, bombesin was demonstrated to induce PKD phosphorylation upstream (13). Zugaza, J. L., Waldron, R. T., Sinnett-Smith, J., and Rozengurt, E. (1997) J. Biol. Chem., 272, 6146–6154.

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