Importance of C1B Domain for Lipid Messenger-induced Targeting of Protein Kinase C*

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The molecular mechanisms by which arachidonic acid (AA) and ceramide elicit translocation of protein kinase C (PKC) were investigated. Ceramide translocated ePKC from the cytoplasm to the Golgi complex, but with a mechanism distinct from that utilized by AA. Using fluorescence recovery after photobleaching, we showed that, upon treatment with AA, ePKC was tightly associated with the Golgi complex; ceramide elicited an accumulation of ePKC which was exchangeable with the cytoplasm. Stimulation with ceramide after AA converted the AA-induced Golgi complex staining to one elicited by ceramide alone; AA had no effect on the ceramide-stimulated localization. Using point mutants and deletions of ePKC, we determined that the C1B domain was responsible for the ceramide- and AA-induced translocation. Switch chimeras, containing the C1B from ePKC in the context of δPKC (δ1C1B) and vice versa (εδC1B), were generated and tested for their translocation in response to ceramide and AA. δ(εC1B) translocated upon treatment with both ceramide and AA; ε(δC1B) responded only to ceramide. Thus, through the C1B domain, AA and ceramide induce different patterns of ePKC translocation and the C1B domain defines the subtype specific sensitivity of PKCs to lipid second messengers.

The PKCε family of serine/threonine protein kinases contains at least 10 subtypes. They are divided into three subgroups based on structural differences and requirement for activators (1–4). The conventional PKCs (εPKC; α, βI, βII, and γ) are Ca2+-dependent and activated by diacylglycerol or phorbol esters. The novel PKCs (nPKC; δ, ε, η, and θ) are activated by diacylglycerol (DG) or phorbol esters, but are Ca2+-independent (5–7). The atypical PKCs (aPKC; γ and λ/ι) are insensitive to DG/phorbol ester, and are Ca2+-independent (8–10).

All PKCs possess an amino-terminal regulatory domain and a catalytic domain in the carboxyl terminus. The regulatory domain of the PKCs contains a variable region 1 (V1), a pseudosubstrate motif (PS), and a conserved region 1 (C1). The V1 of ePKC has been reported to be a selective inhibitor of ePKC translocation (11, 12). In the resting state, the PS is bound in the active site of the catalytic domain, keeping the enzyme inactive by blocking the catalytic site. The binding of activators to the regulatory domain causes a conformational change which releases the PS from the active site and activates the enzyme (13). DG and phorbol ester binding have been localized to the C1 domain (2, 8, 14, 15). Additionally, the C1 domain mediates protein-protein interactions: that of ePKC binds actin (16–18).

The C1 domain of ePKCs and nPKCs has two cysteine-rich loops (C1A and C1B), each consisting of ~50-amino acids including six cysteine and two histidine residues arranged in a zinc finger motif. The C1B of ePKCs and nPKCs showed strong phorbol esters binding, but all C1A except for γPKC showed very weak affinity for phorbol esters (19). GFP-tagged C1A-C1B or C1A translocated to the plasma membrane in response to receptor or phorbol esters stimuli, whether significant plasma membrane translocation of C1B was only observed in phorbol esters stimulation (20). In addition, distinct roles for the C1A and C1B domains in the activation of the enzyme have been shown (21). These results suggest that the C1A and C1B domains of PKCs are functionally distinct.

The activity of PKC can be regulated not only by DG and phorbol ester but also by other lipids such as arachidonic acid (AA) (22, 23) and ceramide (24, 25). Like DG/phorbol esters, these lipid second messengers also induce translocation of PKCs. Immunoblot analysis and immunocytochemistry in fixed cells have shown that AA induces translocation of ePKC (26) and ceramide translocates ePKC and δPKC from the plasma membrane to the cytoplasm (27). Using green fluorescent protein (GFP)-tagged PKCs and live cell imaging, we have shown that AA translocates ePKC, but not δPKC, from the cytoplasm to the Golgi complex (28, 29) and that ceramide translocates δPKC from the cytoplasm to the Golgi complex (30). However, little is known about the mechanism underlying these translocations. Here we identified the intramolecular domains of ε- and δPKC that respond to ceramide and AA to clarify the molecular mechanisms responsible for the lipids-dependent translocation of nPKCs.

EXPERIMENTAL PROCEDURES

Materials—Arachidonic acid and C6-ceramide were purchased from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ) and Molecular Probes, Inc. (Eugene, OR), respectively. 12-O-Tetradecanoylphorbol-13-acetate (TPA) was obtained from Sigma. All the other chemicals used were of analytical grade.

Cell Culture—COS-7 and CHO-K1 cells were purchased from the Riken cell bank (Tsukuba, Japan) and Health Science Research Re-
solutions Bank (Osaka, Japan), respectively. COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium, and CHO-K1 cells in Ham’s F-12 medium (Invitrogen, Grand Island, NY) at 37 °C in a humidified atmosphere containing 5% CO₂. Both media contained 25 mM glucose, were buffered with 44 mM NaHCO₃, and were supplemented with 10% fetal bovine serum (100 units/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml amphotericin B). The fetal bovine serum used was not heat-inactivated. For transfection experiments, CHO-K1 cells were trypsinized and seeded at a density of 1 × 10⁶ cells/3.5-mm glass-bottomed culture dishes (Mattek Corp., Ashland, MA) and incubated for 16–24 h before transfection.

Transfection of the GFP-tagged PKCs—CHO-K1 cells were transfected with 3 μg of plasmid DNA (Roche Biochemicals) and 1 μg of DNA according to the manufacturer’s protocol. Transfected cells were cultured at 37 °C for 16–48 h prior to imaging.

Construction of Plasmids Encoding the GFP-tagged PKCs—The constructs encoding GFP-conjugated εPKC (εPKC) and δPKC (δPKC) were previously described (28, 31). The cDNA for the GFP-tagged proteins used in these studies are diagrammed in Fig. 5; the primers used are shown in Table I. The cDNA encoding the amino-terminal deletion mutants of εPKC were generated by PCR using BS 495 (rat εPKC in pCPT72) (28) as the template. The primers were synthesized with BglII sites on the both 5’ and 3’ terminus to facilitate subcloning. cDNAs encoding domain-deleted and point-mutated εPKCs were produced using the ExSite™ PCR-based Site-directed Mutagenesis kit (Stratagene) with BS495 as a template. Chimeras of εPKC containing δC1B (εC1B) was produced by two-step PCR using two plasmids as templates at one reaction. For the first step, BS495 and BS751 (rat δPKC in pCPT72) (31) were used as the templates with ΔR845ΔF882 as the primers using the ExSite™ PCR-based Site-directed Mutagenesis kit. The product of the first reaction was a chimera having the δPKC regulatory domain and the εPKC kinase domain (BS758). For the second step, BS758 and BS495, and εR718ΔF882 were used as the templates and the primers, respectively. Similarly, chimeras of δPKC containing εC1B (εδC1B) was generated by two-step PCR. BS495 and BS751 as the templates and εR881ΔF846 as the primers were used for the first step to produce a chimera having the regulatory domain of εPKC and the kinase domain of δPKC (BS759). For the second step, BS759/BS751 and RS683/FT7120 were used as the templates and primers, respectively.

The PCR products for deletion mutants of εPKC, point-mutated εPKCs and εδC1B were digested with BglII, and subcloned into the BglII sites of the EGFP expression vector (BS340). δC1B was digested with EcoRI/BamHI and subcloned into the EcoRI/BamHI sites of BS340. All plasmids were sequenced prior to use.

Immunoblotting for GFP-tagged PKCs—COS-7 cells were transiently transfected by electroporation and cultured for 2 days. The transfected cells were harvested with phosphate-buffered saline (PBS) and concentrated by centrifugation. The pellet was resuspended in 200 µl of homogenization buffer containing 1% Triton X-100 (250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 20 mM Tris-HCl, 20 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and homogenized by sonication. After centrifugation, 20 µg of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% polyacrylamide gel (Invitrogen, Grand Island, NY) at 37 °C, followed by transfer to nitrocellulose paper. The membranes were probed with anti-εPKC monoclonal antibody (Transduction Laboratories, Lexington, KY) (diluted 1:1,000), or anti-GFP polyclonal antibody (CLONTECH Laboratories, Inc., Palo Alto, CA) (diluted 1:1,000) for 1 h at 25 °C. After washing with PBS-T, the blots were incubated with peroxidase-conjugated AffiniPure goat anti-mouse IgG (for PKC antibody) or anti-rabbit IgG (for GFP antibody) (1 h, at 25 °C). The immunoreactive bands were visualized with an enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, England).

Confocal Microscopy—CHO-K1 cells transfected with the GFP-tagged PKCs were cultured for 16–48 h for maximal GFP expression. The media was then replaced with Ringer’s solution composed of 135 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, and 10 mM glucose, pH 7.3. Translocation of the GFP-tagged PKCs was triggered by the addition of the various stimuli to the Ringer’s solution to obtain final concentrations. All experiments were done at 37 °C. The GFP fluorescence was monitored by confocal laser scanning fluorescence microscopy (Carl Zeiss, Jena, Germany) at 488-nm argon excitation with a 515-nm long pass barrier filter. Time series images were recorded before and after stimulation.

Fluorescent Recovery after Photobleaching Study (FRAP)—After recording 1 or 2 images, a 10 × 10 pixel subregion of the cells was scanned with the maximal power of the 488-nm laser for 30 s to bleach the fluorescence. To monitor the recovery of fluorescence in the photobleached spot, a time series of 30–50 images was taken with 2.25-s time intervals. The fluorescence intensity of the region was quantified for each image in the time series using LSM510 softwear (Carl Zeiss). The media was then replaced with Ringer’s solution composed of 135 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, and 10 mM glucose, pH 7.3. Translocation of the GFP-tagged PKCs was triggered by the addition of the various stimuli to the Ringer’s solution to obtain final concentrations. All experiments were done at 37 °C. The GFP fluorescence was monitored by confocal laser scanning fluorescence microscopy (Carl Zeiss, Jena, Germany) at 488-nm argon excitation with a 515-nm long pass barrier filter. Time series images were recorded before and after stimulation.

RESULTS

Effects of Ceramide, AA, and TPA on εPKC Translocation—Ceramide has been shown to translocate δPKC from the cytoplasm to the Golgi complex in HeLa cells (30), but the effect of ceramide on εPKC translocation has not been examined. The ability of C₃-ceramide (ceramide), a membrane permeable ceramide analog, to alter subcellular localization of εPKC in CHO-K1 cells was investigated and compared with that of AA and TPA.

In resting cells, wild type εPKC-GFP (εPKC) was detected throughout the cytoplasm, but was excluded from the nuclei. Within the cytoplasm, εPKC was diffusely distributed with slight enrichment in the perinuclear region (Fig. 1A, before). After treatment with ceramide (10 μM), εPKC accumulated in the perinuclear region (Fig. 1A, left), peaking within 20 min and remaining for more than 60 min; the cytoplasmic fluorescence did not change significantly after ceramide stimulus. In contrast, the homogeneous fluorescence of εPKC in the cytoplasm became heterogeneous within 1 min after AA addition (100 μM) with a diffuse accumulation of fluorescence apparent in the perinuclear region (Fig. 1A, center). The timing of the AA-induced perinuclear accumulation was similar to that seen upon ceramide treatment, reaching a maximum within 20 min and remaining at least for 60 min. In contrast, TPA (1 μM) translocated εPKC from the cytoplasm to the plasma membrane within 10 min, where it remained for at least 60 min (Fig. 1A, right).

The pattern of εPKC concentration in response to ceramide resembles the Golgi complex staining induced by AA (Fig. 1B, bottom) (28). To test the hypothesis that ceramide induces translocation to the Golgi complex, CHO-K1 cells transfected with εPKC were stimulated with ceramide (10 μM), fixed, and the Golgi complex was visualized with Texas Red-conjugated wheat germ agglutinin (WGA) (Fig. 1B, upper). Intense fluorescence of Texas Red was seen in the perinuclear region (Fig. 1B, Cer, center). This staining resembled the perinuclear concentration of GFP (Fig. 1B, Cer, left). A merged image verified that the fluorescence of Texas Red and GFP co-localized in the perinuclear region (Fig. 1B, Cer, right). These results indicate that ceramide and AA both induced the translocation of εPKC to the Golgi complex, although the pattern of εPKC accumulation was slightly different (compare AA and Cer in Fig. 1B). The differences were not due to an effect of ceramide (10 μM) or AA (100 μM) on the structure of Golgi complex, since the shape of the organelle visualized by GFP-tagged galactosyltransferase (32) was not altered by 30 min treatment with each lipid (data not shown).

TPA Treatment and FRAP Identify Differences in the Association of εPKC with the Golgi Complex after Ceramide and AA Treatments—To compare the relative strength of εPKC–Golgi association in response to ceramide or AA, we tested the effect of subsequent TPA treatment on εPKC localization. Ceramide
was added to CHO-K1 cells for 10 min to induce ePKC translocation to the Golgi complex (Fig. 2, upper). Subsequent treatment with 1 μM TPA redistributed ePKC from the Golgi complex to the plasma membrane within 25 min (Fig. 2, upper). Cells treated with AA followed by TPA retained significant perinuclear staining for more than 40 min after TPA stimulus although some ePKC was translocated to the plasma membrane (Fig. 2, bottom). These results suggest that the AA-mediated ePKC-Golgi complex association is tighter than that induced by ceramide.

To further probe the interaction of ePKC with the Golgi complex, we used FRAP analysis. After photobleaching in the Golgi complex, the fluorescence in bleached or unbleached area was measured at 2–8-s intervals for 1–3 min (Fig. 3, A and B). In ceramide-treated cells, the fluorescence of ePKC in the photobleached area (Fig. 3A, blue) recovered to 80% within 30 s. The fluorescence in an unbleached Golgi complex area (Fig. 3A, yellow) did not change significantly over the course of the experiment. In contrast, the fluorescence in an unbleached region of the cytosol faded to 60% (Fig. 3A, red). These results suggest that after ceramide treatment, the fluorescence of the bleached area in the Golgi complex was recovered from the cytoplasm.

In cells treated with AA for 20 min, the fluorescence in the photobleached Golgi complex area (Fig. 3B, blue) recovered maximally by 60 s. However, this level was only 50% of the original signal. The recovery was accompanied by a corresponding decrease in the fluorescence level in an unbleached area of the Golgi complex (Fig. 3B, yellow). In contrast to the result in the ceramide-treated cell, the fluorescence in the unbleached cytosol was not significantly altered (Fig. 3B, red), suggesting that recovery in AA-treated cells was the result of the redistribution of the ePKC present in the Golgi complex.

AA and C6-ceramide Differently Regulate Translocation of ePKC—To determine whether one lipid mediator could alter...
the distribution of εPKC induced by the other, cells were stimulated sequentially with AA and Cε-ceramide. AA translocated εPKC to the perinuclear region and heterogeneous fluorescence was detected in the cytoplasm (Fig. 4A). A subsequent application of Cε-ceramide eliminated the accumulation of εPKC around nucleus and produced homogeneous cytoplasmic staining within 20 s. By 10 min, the perinuclear staining returned (Fig. 4A). Magnified images (bottom row, Fig. 4A) revealed that the Golgi complex staining elicited by ceramide and AA are different, suggesting that AA and ceramide selectively target εPKC to different compartments of the Golgi complex. Interestingly, AA failed to alter the localization of εPKC induced by ceramide (Fig. 4B). However, TPA was able to translocate εPKC to the plasma membrane after sequential treatment of ceramide and AA (Fig. 4B), indicating that the εPKC had not lost the ability to translocate.

**C1B Is the Only Responsible Domain for the Translocation of εPKC Induced by Ceramide and AA**—We constructed cDNAs encoding a series of GFP-tagged deletion mutants of εPKC to identify the domains of εPKC required for the translocation by ceramide, AA, and TPA. Fig. 5A and Table I summarize the structures of the mutants and primers used to generate them. Immunoblotting of fusion proteins with anti-GFP antibody verified that molecular weight of each GFP-tagged mutant was appropriate and no significant degradation products were detected (Fig. 5B).

First, we compared the intracellular distribution of the deletion mutants with that of wild εPKC in resting CHO-K1 cells. Although all deletion mutants were localized in the cytoplasm as was full-length εPKC, differences in the intracellular distributions of some mutants were apparent (Fig. 6, before). For example, ΔV1-PS and ΔV1-PS-C1A were localized heterogeneously in the cytoplasm. When the regulatory domain was deleted (ΔV1-PS-C1A-C1B), the GFP fluorescence was homogeneous in the cytoplasm with no accumulation in the perinuclear region. ΔPS was localized heterogeneously in the cytoplasm with prominent accumulation in the perinuclear region. In contrast, deletion of ΔC1A, ΔC1B, or the entire C1 did not significantly change the distribution compared with εPKC.

The response of εPKC deletion mutants to ceramide, AA, and TPA was examined. Similar to full-length εPKC, ceramide translocated ΔV1, ΔV1-PS, ΔV1-PS-C1A, ΔPS, and ΔC1A to the perinuclear region; but mutants lacking the C1B domain (ΔV1-PS-C1A-C1B, ΔC1B and ΔC1A-C1B) failed to move. Similarly, AA induced the translocation of ΔV1, ΔV1-PS, ΔV1-PS-C1A, ΔPS and ΔC1A to the perinuclear region, but did not alter the distribution of mutants lacking the C1B domain (ΔV1-PS-C1A-C1B, ΔC1B, and ΔC1A-C1B). TPA induced translocation of ΔV1, ΔV1-PS, ΔV1-PS-C1A, ΔPS, and ΔC1A to the plasma membrane similar to that of wild type. ΔC1B also showed weak, but significant, translocation to the plasma membrane. In contrast, ΔV1-PS-C1A-C1B and ΔC1A-C1B, both of which lack the whole C1 domain, did not translocate in response to TPA. These results suggest that TPA can induce εPKC translocation via either the C1A or C1B domain, but both are not required. In contrast, the C1B domain is indispensable for ceramide- and AA-induced translocation.

We further studied the ceramide- and AA-induced translocation of εPKC mutated in the C1A and C1B domain (33). Mutation of 11th proline residue to glycine in the C1A or C1B domain of εPKC decreases the affinity of PDBu binding; mutation of 17th cysteine to glycine abrogates PDBu binding. Thus, we created two C1A mutants of εPKC (P180G, C186G) and two C1B mutants (P253G, C259G) which are predicted to weaken or lack PDBu binding to C1A and C1B domain (Fig. 5A). These mutants had the predicted molecular weights (Fig. 5B). Before the stimulation, the two C1A mutants (P180G, C186G) were expressed heterogeneously in the cytoplasm with...
some accumulation of fluorescence present in the perinuclear region (Fig. 7, before). The C1B mutants (P253G, C259G) were homogeneously distributed throughout the cytoplasm (Fig. 7, before). The C1A mutants (P180G, C186G) translocated similarly to wild type in response to ceramide or AA (Fig. 7). In contrast, distribution of the C1B mutants (P253G, C259G) were not altered by ceramide (10 μM) or AA (100 μM) treatment. TPA (1 μM) translocated all mutants to the plasma membrane with a pattern similar to that of wild type (Fig. 7). These results confirm that the translocation induced by TPA can be mediated through either C1A or C1B, but that the C1B domain is essential for AA- and ceramide-induced translocation.

**C1B Domain of εPKC or δPKC Determines the Sensitivity to AA-induced Translocation of the PKC Subtypes**—Ceramide translocates both εPKC and δPKC (30); AA translocates εPKC but not δPKC (29). Our data demonstrate that the C1B domain of εPKC is important for the ceramide- and AA-induced translocation (Figs. 6 and 7). Taken together, these results suggest that the C1B domains of εPKC and δPKC determine their sensitivity to ceramide and AA. To test this hypothesis, we determined the effect of ceramide and AA on the translocation of GFP-conjugated chimeras of εPKC and δPKC. Chimeras of εPKC containing the C1B domain of δPKC (δεC1B) and δPKC having the C1B domain of εPKC (εδC1B) were made as described under “Experimental Procedures.” Western blots of the expressed chimeras with anti-GFP antibody showed the appropriate molecular weights and no degradation products were detected (Fig. 5B).

Both δεC1B and εδC1B were expressed in the cytoplasm and enriched in perinuclear structures (Fig. 8). Ceramide-induced translocation of both chimeras, as well as εPKC and δPKC, to the perinuclear region (Fig. 8). In contrast, δεC1B and εPKC, but not δεC1B and δPKC, accumulated in the perinuclear region upon addition of AA (100 μM). Additionally, like εPKC, δεC1B could be concentrated in the Golgi complex by sequential treatment with ceramide followed by AA (data not shown). Taken together, these results demonstrate that the C1B domain of εPKC is responsive to both ceramide and AA while the C1B domain of δPKC mediates ceramide, but not AA-stimulated translocation. Thus, although the C1B domains of εPKC and δPKC are very homologous, subtle differences in their sequences and/or structures determine their differential sensitivity to AA.

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**Table 1.**

| Assay                        | εPKC          | δPKC          |
|------------------------------|---------------|---------------|
| Western blot                 | εδC1B         | εδC1B         |
| Targeted protein             | Predicted molecular weight |
| 1 PKC                        | 113           | 113           |
| 2 εδC1B                      | 99            | 99            |
| 3 δεC1B                      | 94            | 94            |
| 4 εδC1B                      | 86            | 86            |
| 5 δεC1B                      | 79            | 79            |
| 6 εδC1B                      | 109           | 109           |
| 7 εδC1B                      | 105           | 105           |
| 8 εδC1B                      | 107           | 107           |
| 9 δεC1B                      | 98            | 98            |
| 10 δεC1B                     | 113           | 113           |
| 11 δεC1B                     | 113           | 113           |
| 12 δεC1B                     | 113           | 113           |
| 13 δεC1B                     | 113           | 113           |
| 14 δεC1B                     | 110           | 110           |
| 15 δεC1B                     | 110           | 110           |
| 16 δεC1B                     | 110           | 110           |

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![Image](http://www.jbc.org/doi/10.1074/jbc.M110.165470)
The importance of ceramide and AA as lipid messengers has only recently begun to be appreciated. Ceramide is involved in such processes as cell differentiation (34), outgrowth of neurons (35), apoptosis (27, 36, 37), and long-term depression of synaptic transmission (38). AA has been shown to act as a retrograde transmitter (39) in the generation of long-term potentiation, as a regulator of ion channels (40), a mediator of cell death (25, 41) and ceramide, is involved in such processes as cell differentiation (34), outgrowth of neurons (35), apoptosis (27, 36, 37), and long-term depression of synaptic transmission (38). AA has been shown to act as a retrograde transmitter (39) in the generation of long-term potentiation, as a regulator of ion channels (40), a mediator of cell death (25, 41) and is known to be necessary for superoxide generation (42). Similarly, different effects of ceramide and AA on PKC translocation have been reported for PKC-C1A (20). In those studies, pretreatment with AA reduced the DG-induced translocation of PKC-C1A to the plasma membrane but pretreatment with ceramide had no effect on the translocation.

To identify the domains of ePKC necessary for AA- and ceramide-induced ePKC translocation, we constructed a series of deletion mutants and studied their translocation characteristics. Loss of the V1, PS, and/or C1A domains did not alter the ceramide-induced activation of ePKC (25), O'Tailherty (43) demonstrated that ePKC, bPKC, and ePKC can be translocated by low concentrations of AA. In contrast, Oancea et al. (20) reported that AA inhibits translocation of the C1A domain of ePKC. Ceramide translocates ePKC, bPKC, and ePKC from the plasma membrane to the cytoplasm (27), and ePKC from the cytoplasm to the membrane (24). Taken together, these reports suggest that both ceramide and AA play important roles in signal transduction and implicate their involvement in the regulation of subtype-specific activation or translocation of PKCs.

In this study, we showed that ceramide translocates ePKC from the cytoplasm to the perinuclear region and identified this region as the Golgi complex by WGA staining (Fig. 1). We have previously shown that AA also induces the translocation of ePKC to the Golgi complex (Fig. 1B) (28). In those studies, 10 μM ceramide and 100 μM AA were used to detect translocation of PKCs clearly and constantly, although the translocation to the Golgi complex could be detected even at 25 μM AA and 1 μM ceramide. The concentrations of these lipids might be relatively higher than that of physiological condition. It, however, is noteworthy that ePKC is translocated to the Golgi complex when ceramide is generated by receptor stimuli with tumor necrosis factor-α as seen in the case of exogenous ceramide stimulation (data not shown), and that ePKC accumulates in the Golgi complex in the brain (data not shown). These findings suggest that translocation of ePKC to the Golgi complex occurs under physiological conditions.

Although both ceramide and AA translocated ePKC from the cytoplasm to the Golgi complex, the pattern of localization was subtly, but distinctly, different. First, in ceramide-treated cells ePKC was concentrated in the well defined Golgi complex with uniform distribution in the cytosol. In contrast, upon AA treatment ePKC accumulated in a diffuse pattern around the nucleus with heterogeneous fluorescence in the cytosol. Second, TPA application after ceramide or AA revealed differences in the dissociation of ePKC from the Golgi complex. The ceramide-stimulated interaction of ePKC with the Golgi complex was transient, as shown by the TPA-induced relocalization from the Golgi complex to the plasma membrane. On the other hand, interaction of AA-stimulated ePKC was strong enough to resist being translocated by TPA stimuli. Third, FRAP analysis also revealed distinct interaction of ePKC with the Golgi complex. The fact that, in ceramide-treated cells, fluorescence recovery in the Golgi complex was coincident with decreased cytosolic fluorescence suggests that ePKC exchanges with the cytosolic pool. As staining in the unbleached regions of the Golgi complex did not change, it is unlikely that there is significant movement of ePKC in the Golgi complex in response to ceramide. On the other hand, after AA treatment, the recovery came from unbleached regions of the Golgi complex rather than the cytosol. This suggests that AA mediates a tight association of ePKC with the Golgi complex resulting in low exchange with cytosolic ePKC pools. Finally, AA-translocated ePKC was sensitive to redistribution by ceramide but AA did not alter the ceramide-translocated ePKC. This indicates that AA-mobilized ePKC is responsive to ceramide, but the ceramide-treated ePKC cannot be further translocated by AA. Taken together, these differences imply that distinct mechanisms are involved in the translocation of ePKC mediated by ceramide and AA. Similarly, different effects of ceramide and AA on PKC translocation have been reported for γPKC-C1A (20). In those studies, pretreatment with AA reduced the DG-induced translocation of γPKC-C1A to the plasma membrane but pretreatment with ceramide had no effect on the translocation.

To identify the domains of ePKC necessary for AA- and ceramide-induced ePKC translocation, we constructed a series of deletion mutants and studied their translocation characteristics. Loss of the V1, PS, and/or C1A domains did not alter translocation in response to ceramide or AA as compared with ePKC. However, deletion of the C1B domain rendered the mutants insensitive to both ceramide and AA. These results indicate that the C1B domain is necessary for the translocation induced by both ceramide and AA. Although the mechanisms causing the distinct translocation are unknown, they may include differences in phosphorylation, interaction partners, and/or specific conformation changes.

The fact that the C1B, but not the C1A domain, is involved in the AA- and ceramide-induced translocation suggests that C1A
and C1B have different roles in translocation. Even in the case of TPA, difference between C1A and C1B was observed. Unlike ceramide and AA, TPA induced translocation of both /H9004 C1A and /H9004 C1B but the mutants lacking both C1A and C1B ( /H9004 V1-PS-C1A-C1B and /H9004 C1A-C1B) were insensitive to TPA, indicating that either C1A or C1B can mediate TPA-induced translocation. However, the translocation of /H9004 C1B was weaker than that of /H9004 C1A (Fig. 6). This is consistent with the report that the C1B domain of ePKC has higher affinity for phorbol esters than the C1A domain (19). In addition, several reports suggest distinct contributions of C1A and C1B domain in the regulation of PKC. For example, Shieh et al. (44, 45) used mutants of αPKC lacking either C1A or C1B and showed no differences in TPA stimulated activity, suggesting that TPA regulates αPKC activity via either C1A or C1B. In contrast, mezerein regulation occurs predominantly via the C1A. Second, Bogi et al. (46) reported that translocation of δPKC by PMA requires the C1B domain but not C1A, although C1A and C1B domains of αPKC have equivalent roles for the PMA-induced translocation. Finally, the γPKC-C1A fragment was preferentially translocated to the plasma membrane compared with the γPKC-C1B or γPKC-C1AC1B fragment upon treatment of rat basophilic leukemia cells with IgE or ligands of PAF receptor. (20). Thus, there is a considerable body of literature consistent with our findings that C1A and C1B domains differentially regulate PKC translocation.

We used point mutations in the C1A (P180G and C186G) and C1B (P253G and C259G) domains to confirm that the C1B domain is responsible for the AA- or ceramide-induced translocation, and that either the C1A or the C1B domain is sufficient for the TPA-induced translocation. The proline mutants have a decreased affinity for PDBu, and the cysteine to glycine mutation eliminates PDBu binding (33). Ceramide and AA translocate the C1A mutants, but not the C1B mutants; TPA translocates all. Collectively, these results provide strong evidence that the εC1B domain is required for ceramide- and AA-stimulated translocation, while TPA has a less stringent
Ceramide and AA, but the absence of DG, although phenomena via ceramide are mediated by \( \alpha \), not \( \beta, \gamma \), or \( \delta \)PKC, to the cytoplasm, resulting in apoptosis in human leukemia cells (27). These results indicate that not only DG but also ceramide and AA regulate the activity and distribution of each PKC subtype, contributing to the subtype-specific physiological roles in long-term potentiation or apoptosis. In other words, even though several PKC isoforms are expressed in the same cell, each subtype of PKC can be regulated by specific activators and play a subtype-specific role in various signal transduction.

In conclusion, ceramide and AA translocate PKC to the Golgi complex by distinct mechanisms involving the C1B domain. In contrast, TPA requires only C1A or C1B domain for translocation. The subtle differences in the C1B domains of PKC and \( \delta \)PKC apparently account for their differential sensitivity to AA. These results indicate that different domains of PKC mediate translocation in response to different second messengers and the distinct characteristics of the domain determine the subtype-specific translocation, thereby contributing to the subtype-specific function.

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