Identification of an Amino Acid Residue in the Protein Kinase C C1b Domain Crucial for Its Localization to the Golgi Network*

Anna Schultz, Mia Ling, and Christer Larsson‡

From the Department of Laboratory Medicine, Molecular Medicine, Lund University, 205 02 Malmö, Sweden

Protein kinase C (PKC) isoforms have been reported to be targeted to the Golgi complex via their C1 domains. We have shown recently that the regulatory domain of PKCθ induces apoptosis in neuroblastoma cells and that this effect is correlated to Golgi localization via the C1b domain. This study was designed to identify specific residues in the C1 domains that mediate Golgi localization. We demonstrate that the isolated C1b domains from PKCα, -δ, -ε, -η, and -θ are targeted to the Golgi complex, whereas the corresponding C1a domains localize throughout the cell. Sequence alignment showed that amino acid residues corresponding to Glu-246 and Met-267 in PKCθ are conserved among C1b but absent from C1a domains. Mutation of Met-267, but not of Glu-246, to glycine abolished the Golgi localization of the isolated C1b domain and the regulatory domain of PKCθ. The mutated PKCθ regulatory domain constructs lacking Golgi localization were unable to induce apoptosis, suggesting a direct correlation between Golgi localization and apoptotic activity of PKCθ regulatory domain. Mutation of analogous residues in the C1b domain of PKCε abrogated its Golgi localization, demonstrating that this effect is not restricted to one PKC isoform. The abolished Golgi localization did not affect neurite induction by PKCε. However, the PKCε mutant did not relocalize to the Golgi network in response to ceramide and ceramide did not suppress the neurite-inducing capacity of the protein. Thus, the specific mutations in the C1b domain influence both the localization and function of full-length PKCε.

The members of the protein kinase C (PKC) family are central components in pathways regulating a wide variety of cellular processes. This family of serine/threonine kinases contains at least 10 different isoforms, which can be subgrouped into classical PKCs (α, βI, βII, and γ), novel PKCs (δ, ε, η, and θ), and atypical PKCs (ζ and ι) (1, 2). The functions of the different isoforms are believed to be largely controlled by the localization of the proteins. Activation of both classical and novel isoforms, for instance, is frequently correlated to a translocation to the plasma membrane (3, 4). The Golgi apparatus is another site at which different PKC isoforms can be located. In some cases, the Golgi localization has been suggested to be crucial for the effect of the PKC isoform (5–7). Several reports indicate that the targeting to the Golgi network is mediated by the C1 domains in the PKC molecule (5, 8, 9).

C1 domains are found in many different proteins and they are generally divided in two subgroups: typical C1 domains that bind phorbol esters and atypical C1 domains that do not bind phorbol esters (10). The classical PKCs and novel PKCs have in their regulatory domains (RD) a tandem repeat of typical C1 domains C1a and C1b. A C1 domain consists of a conserved amino acid sequence of about 50 residues, with six cysteine and two histidine residues that co-ordinate two Zn2+ ions per C1 domain (11, 12). At the tip of typical C1 domains, there is a hydrophilic ligand-binding cleft that is surrounded by hydrophobic residues. Binding of diacylglycerol or phorbol esters caps the hydrophilic cleft and generates a continuously hydrophobic surface, which enables the C1 domain to penetrate membranes (13). Several studies have investigated the relative importance of residues within C1 domains for structural maintenance and ligand interaction (14–17). The Zn2+-coordinating residues are essential for the integrity of the tertiary structure, and the loops of amino acids 6–12 and 20–27 in PKCθ C1b domain have been characterized as essential for phorbol ester binding (15, 16).

In addition to PKC, typical C1 domains have also been found in proteins such as chimaerins, RasGRP1, protein kinase D, and Munc-13 (4, 10, 17–19). Several C1 domain-containing proteins have been shown to be targeted to the Golgi complex, and this is dependent on their C1 domains. Thus, the C1a domain of protein kinase D (20), the C1b domains of PKCθ (9, 21) and PKCε (8), and the C1 domain of β2-chimaerin (22) all mediate Golgi localization.

For several of these proteins, targeting to the Golgi network has been suggested to be important for the function of the protein. For instance, RasGRP1 translocates to the Golgi apparatus and activates Ras at this site (23). Protein kinase D regulates the fission of transport carriers destined for the cell surface (24). Furthermore, PKCε modulates secretion from the Golgi complex (5, 6), whereas PKCβ has been implicated to be involved in the regulation of coat assembly on Golgi membranes (7). We have recently shown that the RD of PKCθ induces apoptosis in neuroblastoma cells and this effect is correlated to a Golgi localization via the C1b domain (9).

The tendency of many C1 domains to localize to the Golgi led us to hypothesize that these domains have residues in common that mediate a specific interaction with structures in the Golgi network. Our aim was to identify such residues. This study shows that mutation of Met-267 in the PKCθ C1b domain or
Ap3-257 and Met-278 in the PKCε C1b domain abolishes their Golgi localization. The mutations also inhibit the Golgi localization and apoptosis-inducing capacity of PKCζ/RD and suppress the responsiveness of full-length PKCζ to ceramide.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Plasmids encoding PKCζ/RD and PKCeFL (25), the C1b regions of PKCe and -ε (26), and the C1a, C1b, and C1ab domains of PKCζ (9) fused to EGFP have been described. cDNA encoding the C1a and C1b domains of PKCζ, -δ, -ε, and -η and the C1b regions of PKCζ and -η were generated by PCR using plasmids encoding the full-length PKC isoforms as template. The PCR reactions were performed with Pfu polymerase (Promega) to minimize introduction of mutations. Restriction enzyme sites were introduced in the primers (Table I) enabling the ligation of the PCR product in the EGFP-N1 vector (BD Biosciences Clontech). cDNA encoding the PKCζ mutants E246A, M267G, and the double mutant E246A/M267G and the PKCe mutants D257G, M278G, and the double mutant D257G/M278G were generated by QuikChange site-directed mutagenesis kit (Stratagene) according to the supplier’s protocol, using plasmids encoding PKCζ-EGFP and PKCe-EGFP as templates. Table I lists the primers used to generate the PKCζ and PKCe mutants fragments. To create myc-tag fusions of the PKCζC1b domain, PKCζ/C1b and PKCe/C1b-M267G cDNA were cut out from the pEGFP-N1 vector, digested with SalI and BglII and ligated into pcDNA4/myc-His vector (Invitrogen). All PKC cDNAs were sequenced to ensure that only the desired mutations were introduced in the mutagenesis reactions.

**Cell Culture and Transfections**—Human neuroblastoma SK-N-BE (2)C cells were cultured in minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For transfection experiments, cells were trypsinized and seeded at a density of 150,000 cells per 35-mm cell culture dish on glass cover slips in medium containing serum and antibiotics. Transfections were initiated 24 h after seeding. Before transfection, cells were washed with serum-free medium. Cells were transfected using 3 μl of LipofectAMINE 2000 (Invitrogen) and 1.6 μg of DNA essentially according to the supplier’s protocol. When indicated, transfection, cells were washed with serum-free medium. Cells were examined using a 60× objective (numerical aperture, 1.4) and a Bio-Rad Radiance 2000 confocal system with excitation wavelengths at 488 nm (EGFP and Alexa Fluor 488) and 543 nm (Alexa Fluor 546) and emission filters 515/30 for EGFP and Alexa Fluor 488 and 560/50LP (Alexa Fluor 546).

**Live Confocal Experiments**—Live SK-N-BE(2)C cells were examined by confocal microscopy on the day after transfection. The cover slips of PKCζ-GFP were followed for 2 min. The cells were examined as described for fixed cells.

**Western Blot**—Cell lysates, SDS-PAGE, blotting, and detection were done as described previously (9). Proteins were analyzed with monoclonal antibodies toward EGFP (Zymed Laboratories Inc.) diluted 1:250 or actin (ICN Biomedicals) diluted 1:1000.

**RESULTS**

**PKC C1 Domains Localize to the Golgi Complex**—It has previously been demonstrated that overexpressed C1 domains of PKCζ (5, 8) and PKCδ (9) are enriched in the Golgi complex. To identify a putative specific motif that targets C1 domains to the Golgi network, we first compared the localization of C1 domains from several PKC isoforms. SK-N-BE(2)C neuroblastoma cells were therefore transfected with plasmids encoding the two C1 domains of PKCζ, -δ, -ε, -η, and -θ fused to EGFP. The cells were fixed and the trans-Golgi network (TGN) was visualized by immunofluorescence toward the TGN marker syntaxin 6 (Fig. 1). Examination of the cells with confocal microscopy confirmed the previous observations that both

**TABLE I**

| Construct   | Primers used to generate PKC constructs |
|-------------|-----------------------------------------|
| PKCζ/C1a   | GCGAGATCTGGACCATGTCAGATGTGAGGTTTCTTT   |
| PKCζ/C1b   | GCGAGATCTGGACCATGTCAGATGTGAGGTTTCTTT   |
| PKCζ/C1a   | GCGAGATCTGGACCATGTCAGATGTGAGGTTTCTTT   |
| PKCζ/C1b   | GCGAGATCTGGACCATGTCAGATGTGAGGTTTCTTT   |
| PKCζ/C1a   | GCGAGATCTGGACCATGTCAGATGTGAGGTTTCTTT   |
| PKCζ/C1b   | GCGAGATCTGGACCATGTCAGATGTGAGGTTTCTTT   |
| PKCζ/C1a   | GCGAGATCTGGACCATGTCAGATGTGAGGTTTCTTT   |
| PKCζ/C1b   | GCGAGATCTGGACCATGTCAGATGTGAGGTTTCTTT   |
| PKCζ/M267G | GCGCTGAGAATGTCGGAGCTGAGCTGTGGGACC      |
| PKCζ/D257G | GCGCTGAGAATGTCGGAGCTGAGCTGTGGGACC      |
| PKCζ/M278G | GCGCTGAGAATGTCGGAGCTGAGCTGTGGGACC      |
| PKCζ/E246G | GCGCTGAGAATGTCGGAGCTGAGCTGTGGGACC      |
| PKCζ/M267G | GCGCTGAGAATGTCGGAGCTGAGCTGTGGGACC      |

**Morphology Studies**—Sixteen hours after end of transfections, cells were fixed in 4% paraformaldehyde in PBS for 4 min, washed in PBS, and mounted on microscopy slides using polyvinyl alcohol-diazabicarbocyclic octane. 200 transfected cells per experiment, visualized by the fluorescence of EGFP, were counted. The transfected cells were considered to have neurites if the length of the process exceeded that of two cell bodies.
PKCαC1 (5) and PKCθC1 (9, 21) almost exclusively localize to the TGN. Furthermore, the C1 domains of PKCα, -δ, -ε, -η, or -θ fused to EGFP. Transfected and fixed cells were stained with antibodies toward the TGN marker syntaxin 6 using Alexa Fluor 546-conjugated secondary antibodies, and the cells were thereafter analyzed with confocal microscopy. One set of cells was treated with 16 nM TPA for 16 h after the transfection and were analyzed with confocal microscopy after fixation.

The C1b Domains of PKCs Are Important for Golgi Localization—Because no differences in localization pattern could be detected between the tandem C1 domains of the different isoforms, our next approach was to compare the localization of the individual C1a and C1b domains. SK-N-BE(2)C cells were transfected with vectors encoding the isolated C1a and C1b domains of PKCα, -δ, -ε, -η, and -θ fused to EGFP. Transfected cells were fixed, and the TGN was visualized by staining with antibodies toward syntaxin 6 (Fig. 2). Analysis with confocal microscopy revealed that the PKC C1a fusion proteins generally localized throughout the cell, and there was no detectable localization to the Golgi complex. In contrast, PKCδC1b, PKCεC1b, and PKCηC1b all showed a distinct colocalization with the TGN marker, and these fusion proteins were not found in the nuclear matrix. PKCαC1b and PKCθC1b both localized throughout the cell, but there was an enrichment of the fusion proteins in the TGN. Cells expressing the PKC C1a and C1b domains were also treated with TPA, and the localization of the fusion proteins was examined with confocal microscopy. The C1a domains of PKCα, -ε, and -η were translocated from...
the nuclear matrix, whereas neither PKCδC1a nor PKCεC1a responded to TPA. PKCαC1b and PKCδC1b were translocated from the nuclear matrix after TPA treatment. PKCαC1b, PKCεC1b, and PKCηC1b lost their distinct Golgi localization and distributed to other cytoplasmic structures as well after treatment with TPA. There was also a tendency to increased localization to the nuclear and plasma membrane of some C1b domains. These results indicate that a Golgi localization motif...
in PKC mainly resides in the C1b domain. Such a motif is conceivably common for all the investigated C1b domains but not present in the C1a domains.

Sequence Alignment of PKC C1 Domains—The differential localization pattern of the C1a and C1b domains led us to search for divergences between the domains. We aligned the protein sequences of the C1a and C1b domains of PKCα, -δ, -ε, -η, and -θ and observed that subsequently were mutated in the C1b domains of PKCα and -θ are highlighted in the figure. B, three-dimensional structure of the C1b domain of PKCγ (13). The amino acid residues analysis by site-directed mutagenesis, corresponding to PKCγ Asp-245 and Met-266, are indicated in the figure. Hydrophobic residues are shown in white, basic in blue, acidic in red, uncharged polar in lilac, and the backbone in purple.

Fig. 3. Sequence alignment of PKC C1 domains. A, the protein sequences of the human C1a and C1b domains of PKCα, -δ, -ε, -η, and -θ were aligned. Positions that subsequently were mutated in the C1b domains of PKCα and -θ are highlighted in the figure. B, three-dimensional structure of the C1b domain of PKCγ (13). The amino acid residues analyzed by site-directed mutagenesis, corresponding to PKCγ Asp-245 and Met-266, are indicated in the figure. Hydrophobic residues are shown in white, basic in blue, acidic in red, uncharged polar in lilac, and the backbone in purple.

Mutations of Glu-246 and Met-267 in PKCδ and PKCθ—Our previous results have indicated a correlation between localization to the Golgi and induction of apoptosis by the isolated RD of PKCδ (9). We therefore focused on the C1b domain of PKCδ and altered the residues in the domain (Glu-246 and Met-267) corresponding to PKCγ Asp-245 and Met-266 with site-directed mutagenesis. Three mutated variants of δC1b-EGFP were created in which Glu-246, Met-267, or both residues were replaced by glycine (denoted δC1b-E246G, δC1b-M267G, and δC1b-DM, respectively). These vectors and a vector encoding the wild-type δC1b domain fused to EGFP (δC1b-wt) were transfected into SK-N-BE(2)C cells and expression of the fusion proteins was confirmed by Western blotting (Fig. 4B).
whether TPA could alter the subcellular localization of the EGFP-fused C1b mutants (Fig. 4A). As previously seen, C1b-wt localized throughout the cell but was clearly enriched in perinuclear structures. TPA treatment influenced the localization as seen by the clearance of nuclear fusion proteins. The same pattern was observed for the C1b-E246G mutant. In contrast, C1b-M267G and C1b-DM were evenly distributed throughout the cells with no perinuclear enrichment. Treatment with TPA translocated the fusion proteins from the nuclear matrix to the cytoplasm and gave rise to a minor perinuclear enrichment.

The phorbol ester responsiveness of the C1b domains was further confirmed in a live confocal experiment. SK-N-BE(2)C cells expressing C1b-wt, C1b-M267G, or C1b-DM were treated with 1 μM phorbol 12,13-dibutyrate (PDBu) and the localization of the fusion proteins was followed for 2 min. SK-N-BE(2)C cells were transfected with expression vectors encoding myc-tagged wild-type C1b or C1b-M267G. For visualization of the PKC constructs, transfected and fixed cells were stained with antibodies toward the myc tag using Alexa Fluor 488-conjugated secondary antibodies. The cells were thereafter analyzed with confocal microscopy.

To establish whether mutation of Met-267 also abolishes the Golgi localization of the entire C1b domain, we made three new constructs encoding C1b fused to EGFP, in which Glu-246, Met-267, or both residues were replaced by glycine (denoted C1b-wt, C1b-M267G, and C1b-DM, respectively). SK-N-BE(2)C cells were transfected with these vectors or a vector encoding the wild-type C1b (C1b-wt), and fixed cells were subjected to staining with antibodies toward syntaxin 6. Co-localization of the fusion proteins with the TGN marker was then analyzed with confocal microscopy. This experiment showed a clear enrichment of C1b-wt and C1b-E246G in syntaxin 6-positive structures, whereas only small amounts of C1b-M267G and C1b-DM were present in the TGN (Fig. 5). Thus, Met-267 seems to be crucial for proper Golgi localization of the C1b domain.

Mutation of Met-267 Abolishes the Apoptotic Effect of PKCεRD—We next sought to determine whether the abrogated Golgi localization caused by mutation of Met-267 is correlated to a diminished apoptotic activity of C1b. SK-N-BE(2)C cells were transfected with vectors encoding C1b-wt, C1b-E246G,
Golgi Localization of the PKC C1b Domain

Fig. 5. Met-267 is necessary for localization of PKCθC1b to the Golgi complex. SK-N-BE(2)C cells were transfected with expression vectors encoding wild-type θC1b (θC1b-wt), θC1b-E246G, θC1b-M267G, or the double mutant θC1b-E246G/M267G (θC1b-DM), wild-type θRD (θRD-wt), θRD-E246G, θRD-M267G, or the double mutant θRD E246G/M267G (θRD-DM), all fused to EGFP. For visualization of the TGN, transfected and fixed cells were stained with antibodies toward syntaxin 6 using Alexa Fluor 546-conjugated secondary antibodies and the cells were thereafter analyzed by confocal microscopy.

θRD-M267G, θRD-DM, or empty EGFP vector (EGFP). After transfection, cells were fixed and scored for apoptosis using a TUNEL assay. As seen before, expression of θRD-wt caused a significantly higher rate of apoptosis compared with cells expressing EGFP alone (Fig. 6A). A similar effect was obtained by the θRD-E246G variant. However, expression of neither θRD-M267G nor θRD-DM led to an altered rate of apoptosis compared with control cells. The apoptotic effects of θRD-wt and θRD-M267G was further analyzed in the presence of TPA. This experiment revealed that although TPA causes an increased rate of apoptosis in cells expressing θRD-M267G, the percentage of apoptotic cells is significantly lower than for TPA-treated cells expressing θRD-wt (Fig. 6B). To exclude that the apoptotic effects of θRD-wt and θRD-E246G, compared with the θRD-M267G mutant, were caused by higher expression levels, SK-N-BE(2)C cells were also transfected with half or a quarter of the amount of these vectors. Analysis of the protein expression by Western blotting demonstrates that transfection with a smaller amount of vector reduces the amount of expressed protein (Fig. 6A). Although a lower expression level of the fusion protein correlates with a slightly lower rate of apoptosis, θRD-wt still induced a significantly higher rate of apoptosis than θRD-M267G or θRD-DM. θRD-E246G also maintained apoptotic activity at lower expression levels.

To confirm that the mutations do not impede the capacity of θRD to respond to phorbol esters and thus that the general characteristics of the C1 domains are maintained, we examined whether the θRD mutants translocate after TPA treatment. θRD-wt primarily localizes to the perinuclear region in untreated cells, and TPA treatment does not alter this localization (Fig. 6C). A similar pattern is observed for θRD-E246G. However, θRD-M267G and θRD-DM, which both primarily localize to the nucleus before TPA treatment, are translocated to the plasma membrane and to punctuate cytoplasmic structures after addition of TPA. In some cells, these structures are slightly enriched in a perinuclear region. Because the mutated θRD fusion proteins were able to respond to TPA treatment, the protein conformation is probably maintained. The fact that the two PKCeθ constructs lacking Golgi localization (i.e. θRD-M267G and θRD-DM) were unable to induce apoptosis implies that Golgi localization is important for the apoptotic activity of PKCeθRD.

Analogous Mutations of PKCeC1 Alter the Localization but Not the Neurite-inducing Capacity of the Protein—The finding that mutation of Met-267, and Glu-246/Met-267 in combination, abrogates the Golgi localization of both the RD and the isolated C1b domain of PKCeθ, raised the question of whether this effect is isoform-specific. To test this, mutant variants of PKCeC1ab-EGFP and PKCeC1b-EGFP were created in which the residues analogous to PKCθ Met-267 and PKCeθ Met-267, PKCeθ Asp-257, and PKCeθ Met-278, were replaced by glycine (denoted cC1ab-DM and cC1b-DM). We chose to study the double mutant because initial experiments indicated that PKCeC1ab with only Met-278 mutated still displayed some minor enrichment in the Golgi network (data not shown). SK-N-BE(2)C cells were transfected with these vectors or the corresponding wild-type vectors (cC1ab-wt and cC1b-wt) and protein expression was confirmed by Western blot analysis (Fig. 7B). We next investigated the subcellular localization of the PKCeC1 mutants and compared it with the wild-type variants. Transfected SK-N-BE(2)C cells were examined with confocal microscopy (Fig. 7A). As previously shown, both cC1b-wt and cC1ab-wt have distinct Golgi localization. In contrast, cC1b-DM is located throughout the cell, whereas cC1ab-DM localizes uniformly in the cytoplasm and is absent from the nucleus. Treatment with 16 nM TPA increases the enrichment of cC1b-wt in the perinuclear region, whereas cC1b-DM translocates from the nuclear matrix. Both the wt and DM of cC1ab translocate to the plasma membrane in response to TPA.
It has previously been shown that a structure encompassing the C1 domains of PKC\textsubscript{H9280} induces neurites independently of the kinase activity of the enzyme (25). To investigate whether the mutations that abolish Golgi localization of PKC\textsubscript{H9280}C1 fusion proteins also affect the neurite-inducing capacity, SK-N-BE(2)C cells overexpressing PKC\textsubscript{H9280}C1ab-wt, PKC\textsubscript{H9280}C1ab-DM, or empty EGFP vector were treated with 16 nM TPA or vehicle for 16 h. The morphological effects were visualized with fluorescence microscopy and quantified by counting the number of transfected cells with neurites longer than two cell bodies. The results demonstrate that both PKC\textsubscript{H9280}C1ab-wt and PKC\textsubscript{H9280}C1ab-DM induced neurites in more than 30% of the transfected cells, compared with 2% of cells expressing EGFP alone (Fig. 7C). TPA treatment further potentiated the neurite-inducing effect of both PKC\textsubscript{H9280}C1ab constructs.

Taken together, the results demonstrate that the mutated residues are important for localization of C1b domains to the Golgi complex and that this is not restricted to one PKC isoform. Furthermore, the fact that PKC\textsubscript{H9280}C1ab-DM induced neurites as efficiently as PKC\textsubscript{H9280}C1ab-wt, despite its lower expression levels, indicates that Golgi localization is not important for the neurite-inducing capacity of PKC\textsubscript{C1} domains.

**Localization of Full-length PKC\textsubscript{H9258}**—We have previously shown that TPA treatment induces apoptosis in neuroblastoma cells expressing full-length PKC\textsubscript{H9258} (9). It was of interest to determine whether this effect is correlated to a Golgi localization and to study the effect of the C1b mutations on the apoptosis-inducing capacity of PKC\textsubscript{H9258}. We therefore investigated the localization of PKC\textsubscript{H9258}FL in TPA-treated cells, the conditions under which it induces apoptosis (Fig. 8). However, examination of the cells with confocal microscopy revealed that PKC\textsubscript{H9258}FL does not localize to the Golgi complex. This experiment suggests that Golgi localization is not important for the apoptotic effect of PKC\textsubscript{H9258}. Thus, PKC\textsubscript{H9258}FL and PKC\textsubscript{H9258}RD are probably exerting their apoptotic effects via different mechanisms in neuroblastoma cells.

**Mutation of the C1b Domain Makes Full-length PKC\textsubscript{H9280} Less Responsive to Ceramide**—Because we could not study the effect of the C1b domain mutations on the apoptotic effect of PKC\textsubscript{H9280}, we used a different approach to investigate the role of the C1b domain in PKC\textsubscript{H9280} activity. We previously showed that PKC\textsubscript{H9258}RD, a mutant that lacks the C1b domain, is more responsive to ceramide than wild-type PKC\textsubscript{H9258} (9). To examine whether this effect is specific to PKC\textsubscript{H9258}, we transfected SK-N-BE(2)C cells with expression vectors encoding wild-type PKC\textsubscript{H9258}RD (wt), PKC\textsubscript{H9258}RD-E246G, PKC\textsubscript{H9258}RD-M267G, and the double mutant PKC\textsubscript{H9258}RD-E246G/M267G (PKC\textsubscript{H9258}RD-DM) all fused to EGFP. To reduce the expression levels of the fusion proteins, cells were transfected with different amounts of PKC\textsubscript{H9258}RD-wt or PKC\textsubscript{H9258}RD-E246G constructs as indicated in the figure. At 16 h after transfection, cells were fixed and a TUNEL analysis was performed. TUNEL-positive EGFP-expressing cells were visualized by fluorescence microscopy. Data (mean ± S.E., n = 3) are presented as percentage of TUNEL-positive EGFP-expressing cells. * indicates statistically significant difference from the value obtained with PKC\textsubscript{H9258}RD-wt (analysis of variance followed by Duncan’s multiple range test, p < 0.05). The formation of protein products was analyzed by Western blotting with antibodies directed toward EGFP (top) or actin as a loading control (bottom). The positions of the molecular markers 66 and 45 kDa are shown to the left of the blot. C, cells expressing the different PKC\textsubscript{H9258}RD variants were treated with 16 nM TPA or vehicle for 16 h and the localization of the fusion proteins was thereafter analyzed by confocal microscopy.

**Fig. 6. Mutation of Met-267 abolishes the apoptotic effect of PKC\textsubscript{H9258}RD.** SK-N-BE(2)C cells were transfected with expression vectors encoding wild-type PKC\textsubscript{H9258}RD (wt), PKC\textsubscript{H9258}RD-E246G, PKC\textsubscript{H9258}RD-M267G, and the double mutant PKC\textsubscript{H9258}RD-E246G/M267G (PKC\textsubscript{H9258}RD-DM) all fused to EGFP. To reduce the expression levels of the fusion proteins, cells were transfected with different amounts of PKC\textsubscript{H9258}RD-wt or PKC\textsubscript{H9258}RD-E246G constructs as indicated in the figure. B, SK-N-BE(2)C cells expressing wild-type PKC\textsubscript{H9258}RD (wt) or PKC\textsubscript{H9258}RD-M267G fused to EGFP were treated with 16 nM TPA or vehicle. At 16 h after transfection, cells were fixed and a TUNEL analysis was performed. TUNEL-positive EGFP-expressing cells were visualized by fluorescence microscopy. Data (mean ± S.E., n = 3) are presented as percentage of TUNEL-positive EGFP-expressing cells. * indicates statistically significant difference from the value obtained with PKC\textsubscript{H9258}RD-wt (analysis of variance followed by Duncan’s multiple range test, p < 0.05). The formation of protein products was analyzed by Western blotting with antibodies directed toward EGFP (top) or actin as a loading control (bottom). The positions of the molecular markers 66 and 45 kDa are shown to the left of the blot. C, cells expressing the different PKC\textsubscript{H9258}RD variants were treated with 16 nM TPA or vehicle for 16 h and the localization of the fusion proteins was thereafter analyzed by confocal microscopy.
of the C1b mutations on full-length PKCe6, we turned to PKCe. We have previously shown that treatment with C2-ceramide induces a relocation of PKCe to perinuclear structures that is accompanied by a suppression of the neurite-inducing capacity of PKCe (29). The results in Fig. 7 suggest that the Asp-257/Met-278 residues of C1b are important for Golgi localization; whereas the C1a domains of the same isoforms are not enriched in this structure. This is in line with previous studies indicating an important role for the C1b domain of PKCe in the Golgi complex, whereas the C1a domains of the same isoforms are not enriched in this structure. This suggests that nuclear targeting motifs in many C1 domains may contribute to the nuclear localization that is sometimes observed for different PKC isoforms (35, 36). In many cases, however, the putative nuclear localization signal is overridden by other targeting determinants in the holoenzyme.

In this study, we investigated two amino acid residues that are conserved among Golgi-localizing PKC C1 domains, Glu-246 and Glu-248, with a minor enrichment in the perinuclear region (Fig. 9A). Treatment with TPA relocates the fusion protein to the plasma membrane, and, as reported previously (8, 29), treatment with C2-ceramide induces a clear enrichment of eFL in the perinuclear region. Combined treatment of C2-ceramide and TPA leads to a primarily cytoplasmic localization of eFL-wt, which is analogous with studies on other cell types (8). The C1b mutant, eFL-DM, also distributes evenly in the cytoplasm and translocates to the plasma membrane by TPA treatment. However, in contrast with the wild-type protein, eFL-DM still localizes in the cytoplasm after treatment with C2-ceramide. Moreover, C2-ceramide does not suppress the plasma membrane localization induced by TPA.

We next analyzed whether the PKCs mutants also become more resistant to the neurite-inhibiting effect of ceramide. The experiment confirmed that overexpression of eFL-wt induces neurites, an effect that is further potentiated by TPA (Fig. 9B). Treatment with C2-ceramide significantly inhibits the induction of neurites, and a combined treatment with TPA cannot restore the neurite-inducing capacity of eFL. Overexpression of eFL-DM induces neurites as potently as eFL-wt, and treatment with TPA enhances this effect. However, compared with the eFL-wt-expressing cells, a significantly higher percentage of eFL-DM-expressing cells have neurites after C2-ceramide treatment. Moreover, TPA treatment partially restores the neurite-inducing capacity of eFL-DM after C2-ceramide treatment. Taken together, these results indicate that Asp-257/Met-278 is important for the ability of eFL to relocate to the perinuclear region in response to C2-ceramide. Furthermore, the Asp-257/Met-278 residues are also important for the functional response of PKCe to ceramide.

**DISCUSSION**

Typical C1 domains, once identified as the phorbol ester binding site in PKC isoforms, have during the last years also been identified in several proteins outside the PKC family (19). This domain conceivably contributes to both the regulation and the localization of the protein. C1 domains are primarily thought to bind lipids, but recent studies have also identified proteins that directly interact with different C1 domains (30, 31). In addition to phorbol esters/diacylglycerol, C1 domains have been shown to directly bind and/or mediate intracellular targeting induced by mediators, including retinoic acid (32), arachidonic acid, and ceramide (8). Binding of these agents results in a translocation and/or an activation of the protein highlighting the importance of the C1 domain as a regulatory element. It has also become clear that C1 domains are differentially sensitive to lipids. For instance, the C1b but not the C1a domain of PKCe is a target for ceramide and arachidonic acid (8), whereas the C1a but not the C1b domain of PKCe is sensitive to diacylglycerol (33, 34).

Our study further underscores the differences between C1 domains, because the C1b domains of PKCa, -δ, -ε, -η, and -θ localize to the Golgi complex, whereas the C1a domains of the same isoforms are not enriched in this structure. This is in line with previous studies indicating an important role for the C1b domain of PKCe (5, 8) and PKCθ (9, 21) for Golgi localization. Furthermore, all C1a domains and the C1b domains of PKCa and PKCθ also localize to the nucleus, whereas the tandem domains (C1ab) of all isoforms are extranuclear. This suggests that nuclear targeting motifs in many C1 domains may contribute to the nuclear localization that is sometimes observed for different PKC isoforms (35, 36). In many cases, however, the putative nuclear localization signal is overridden by other targeting determinants in the holoenzyme.
Mutation of the C1b domain makes full-length PKCε less responsive to ceramide. SK-N-BE(2)C cells were transfected with expression vectors encoding wild-type full-length PKCε (wt) or the double mutant D257G/M278G (DM) thereof, both fused to EGFP. After transfection, cells were treated with 16 nM TPA and/or 50 μM Ceramide or vehicle for 16 h, fixed, and mounted on object slides. A, the localization of the fusion proteins was analyzed by confocal microscopy. B, transfected cells with neurites longer than two cell bodies were counted. Data (mean ± S.E., n = 3) are presented as percentage of transfected cells with neurites. *p < 0.05. The formation of protein products was analyzed by Western blotting using an antibody toward EGFP (top) or actin as a loading control (bottom). The positions of the molecular mass markers 220 and 97 kDa are shown to the left of the blot.
been shown to be a site of ceramide production (40), and another PKC isoform, PKCδ, was recently shown to depend on its Golgi localization for induction of apoptosis in HeLa cells (41).

However, the induction of apoptosis by PKCδFL does not seem to depend on a localization to the Golgi, because no enrichment in the Golgi network was observed in the presence of TPA. Thus, PKCδFL and the isolated PKCδRD probably induce apoptosis via separate pathways in neuroblastoma cells. It is difficult to assess the physiological importance of these two effects. We could detect an increase in immunoreactivity toward the regulatory domain of PKCδ in perinuclear structures during Fas-induced apoptosis in Jurkat cells. We do not know whether this represents the isolated regulatory domain, which is formed upon Fas stimulation (42), or if it represents a translocation of endogenous PKCδFL. Nevertheless, it illustrates a correlation between localization of the PKCδRD to the Golgi network and induction of apoptosis in a setting resembling a physiological situation.

Mutation of analogous residues in PKCεC1b (i.e. Asp-257 and Met-278) abolished the Golgi localization of the protein. However, PKCεC1ab Asp-257/Met-278 retained the ability to induce neurites. Thus, Golgi localization is not important for PKCε-mediated neurite outgrowth. This is in line with previous results from our group, indicating that localization of PKCε to the plasma membrane and/or the cortical cytoskeleton is important for its ability to induce neurites (43). Furthermore, mutation of Asp-257 and Met-278 in PKCεCFL abolished its translocation to the Golgi network induced by ceramide exposure. These C1b residues are thus crucial for a proper localization to the Golgi apparatus, not only for isolated PKC domains, but also for PKCεFL. We have also seen that ceramide suppresses the neurite-inducing capacity of PKCε (29), but PKCε with the D257G and M278G mutations is more resistant to this effect of ceramide. Thus, the residues are important both for the localization and function of PKCε.

In conclusion, this study identifies one amino acid residue, conserved among Golgi-localizing C1 domains, that is critical for the localization of the PKCεC1b domain to the Golgi complex. This residue is a prominent candidate as a common mediator of Golgi-targeting of C1 domains. The data also further support the hypothesis that localization to the Golgi network is critical for apoptosis induction by PKCεRD. The corresponding residue in PKCδ is, together with Asp-257, important for ceramide-induced localization and effects of PKCδFL, illustrating that the identified residues are important also for the holoenzyme.

REFERENCES

1. Newton, A. C. (1995) J. Biol. Chem. 270, 28495–28498
2. Nishizuka, Y. (1992) Science 258, 607–614
3. Nishizuka, Y. (1995) FASEB J. 9, 484–496
4. Meller, H., and Parker, P. J. (1998) Biochem. J. 332, 281–292
