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Influence of Various Domains of Protein Kinase C ϵ on Its PMA-Induced Translocation from the Golgi to the Plasma Membrane

Csaba Lehel,*,[1] Zoltán Oláh,* György Petrovics,* Gábor Jakab,† and Wayne B. Anderson*,[2]

**Laboratory of Cellular Oncology, NCI, NIH, and †Clinical Neuroscience Branch and Laboratory of Experimental Neuropathology, NINDS, NIH, Bethesda, Maryland 20892*

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Subcellular redistribution (translocation) was initiated by treatment of NIH 3T3 cells overexpressing different epitope-tagged fragments of PKCϵ with PMA, and was analyzed by immunocytochemistry. The PMA-induced translocation of holo PKCϵ, as well as fragments ϵ2 (zinc finger domain + pseudosubstrate domain) and ϵ7 (zinc finger domain + hinge region) from the Golgi to the plasma membrane was rapid (<10 min), while translocation of fragment ϵ3 (zinc finger domain) was much slower (30–60 min). These results, combined with results of studies carried out at 20°C to inhibit exocytotic vesicle traffic, indicated that PMA-induced translocation from the Golgi to the plasma membrane may proceed by two distinct mechanisms: a rapid, vesicle independent process noted with holo PKCϵ (which requires the presence of the pseudosubstrate and/or hinge regions), and a slow, vesicle-dependent pathway observed with the zinc finger fragment.

Protein kinase C (PKC) is a multigene family of phospholipid-dependent, serine/threonine phosphotransferases shown to play a crucial role in many signal transduction pathways (1). The PKC family consists of at least 10 closely related isozymes (1,2). Thus, it is important to determine the specific regulatory properties of each, and to identify cellular targets which might be modulated by the different isoforms of PKC to regulate specific cellular functions.

The ϵ isoform of PKC has been shown to have unique properties among the PKC isotypes in terms of its membrane association and oncogenic potential (3–6). Thus, studies were initiated to examine the specific regulatory and biological properties of PKCϵ. Recently, we showed that holo PKCϵ is found at the Golgi, as well as at the plasma membrane, whereas the zinc finger domain of PKCϵ is localized predominantly to the Golgi (7). Other studies identified the pseudosubstrate and hinge regions as PKCϵ domains which also appear to contain putative localization signals (8).

The tight association (translocation) of PKC to membranes in response to activator binding has been well documented (1, 9). While agonist-induced translocation is a rapid, dramatic subcellular redistribution of PKC, little is known concerning the PKC domains involved in mediating this process. Here we describe studies carried out to characterize the influence of the different domains of PKCϵ on activator (PMA)-induced translocation. We have utilized a series of stably transfected NIH 3T3 cell lines, each overexpressing a different truncated epitope-tagged domain fragment of PKCϵ. These overexpressed mutant proteins were used to identify regions of the enzyme possibly involved in mediating PMA-induced translocation of PKCϵ from the Golgi to the plasma membrane.

MATERIALS AND METHODS

Materials. The sources for the following materials were: Life Technologies (Gaithersburg, MD) for Dulbecco's Modified Eagles Medium (D-MEM), and the antibody recognizing the C-terminal 12 amino acids of PKCϵ; ICN Flow (Costa Mesa, CA) for fetal calf serum; Calbiochem (San Diego, CA) for phorbol 12-myristate 13-acetate (PMA) and monensin; Epicentre Technologies (Madison, WI) for brefeldin A (BFA); Jackson Immuno-Research (West Grove, PA) for cyanine (Cy3) conjugated anti-rabbit goat IgG; and Molecular Probes Inc (Eugene, OR) for BODIPY-conjugated wheat germ agglutinin.

[1] Present address: Tularik Inc., South San Francisco, CA 94080.
[2] Corresponding author: NCI, NIH, Bldg 37, Room 1E14, Bethesda, MD 20892. Fax: (301) 480-0471.

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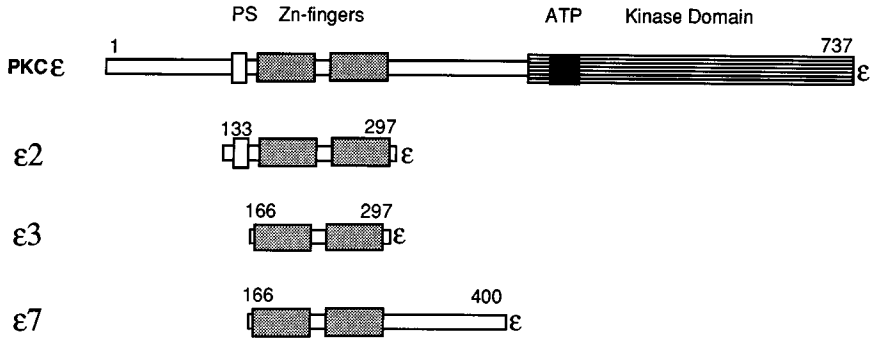


FIG. 1. Domain organization of PKCϵ and its various recombinant ϵ-epitope tagged derivatives. The numbers denote the N- and C-terminal amino acids of the various constructs. PS and ATP correspond to the pseudosubstrate region and the ATP binding site, respectively, while ϵ represents the 12 amino acid ϵ-epitope tag. PCR fragments were generated for the various recombinant proteins, cloned into the ϵ-epitope tagging vector (pϵMTH), and stably transfected into NIH 3T3 cells, as described (8).

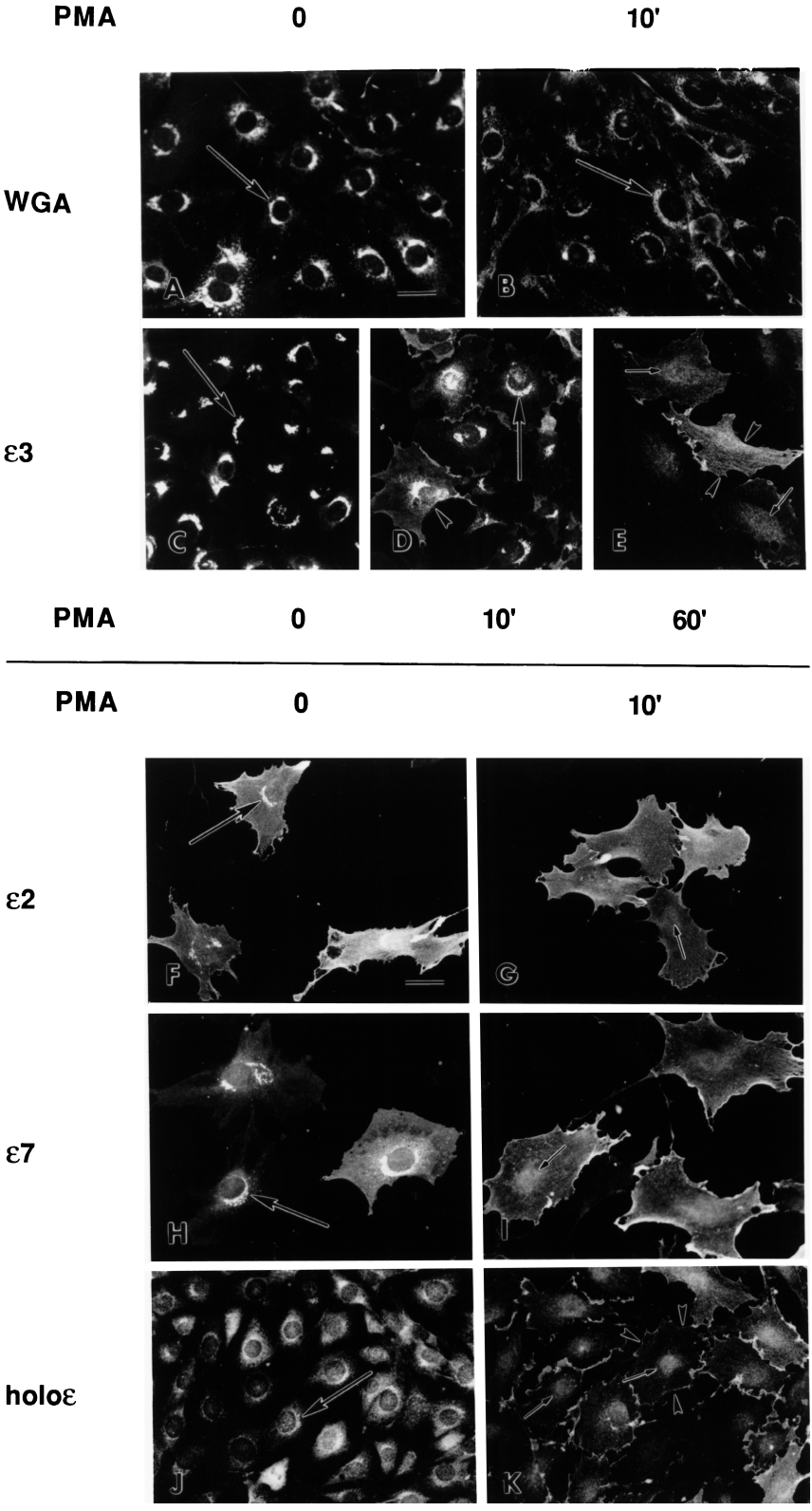
Cell lines. The various PKCϵ constructs were cloned into the ϵ epitope-tagging vector pϵMTH (10), and stable NIH 3T3 overexpressor cell lines were created and characterized as described (8). All cell lines were maintained in DMEM, supplemented with 10% fetal bovine serum and 200 μg/ml G418, and used in the experiments at low passage number.

Immunocytochemistry. The cells were grown on Permanox 8-well polystyrene chamber slides (Nunc Inc., Naperville, IL) in the presence of 75 μM zinc acetate to upregulate recombinant protein expression. The cells were treated as indicated, followed by a rapid wash with ice cold PBS. After fixation with 4% paraformaldehyde in PBS, the cells were washed again, and permeabilized by 1% Triton X-100 for 2 h at 4°C. After a 1 hour incubation with 2% low fat milk, the anti-e-tag antibody was applied at 1 μg/ml for 2 h at room temperature. Cy3-conjugated anti-rabbit goat IgG was added at 1 μg/ml for 2 h at room temperature, and immunoreactivity was visualized with a Leica fluorescent microscope.

RESULTS AND DISCUSSION

To better define the properties of agonist-induced translocation of PKCϵ from the Golgi to the plasma membrane, the ϵMTH epitope tagging vector was used to generate a series of stably transfected NIH 3T3 cell lines (8,10), each overexpressing a different truncated epitope-tagged version of PKCϵ (Figure 1). The ϵ3 protein encompasses only the zinc-finger domain. The ϵ2 and ϵ7 recombinant proteins contain, in addition to the zinc-finger domain, the pseudosubstrate and the hinge regions of PKCϵ, respectively. The overexpressed proteins each contain the ϵ-tag peptide at the C-terminus to allow ready detection (8, 10). The subcellular redistribution of these different epitope-tagged constructs of PKCϵ was initiated by the addition of 1 μM PMA, and was followed by immunocytochemistry. Exposure of ϵ3 overexpressor cells to PMA had no apparent effect on overall Golgi morphology, as determined by a fluorescent derivative of Golgi-decorating wheat germ agglutinin (Figure 2, panels A, B). Further, it was found that the PMA-induced translocation of Golgi-localized ϵ3 zinc-finger fragment to the plasma membrane was relatively slow, requiring about 60 min to complete (Figure 2, panels C–E). No change was observed in the Golgi localization of the ϵ3 zinc-finger protein with PMA treatment for 10 min. In contrast, the redistribution of holo PKCϵ, as well as of the ϵ2 and ϵ7 recombinant proteins, from both the cytosol and Golgi to the plasma membrane was rapid; i.e., complete within 10 min of exposure of the cells to PMA (Figure 2, panels F–K).

The differential rates of translocation of the various recombinant proteins suggested that different mechanisms may be involved in these processes. Since the zinc-finger domain localizes exclusively to the Golgi, we attempted to gain additional information on the possible mechanisms involved by using treatments known to modulate Golgi vesicle traffic. Incubation of cells at 20°C is known to block exocytotic vesicle traffic from the trans-Golgi network towards the plasma membrane (11–13). This treatment completely abolished the PMA-induced translocation of the ϵ3 protein, while redistribution of the other recombinant proteins remained unaffected (Figure 3).


PMA
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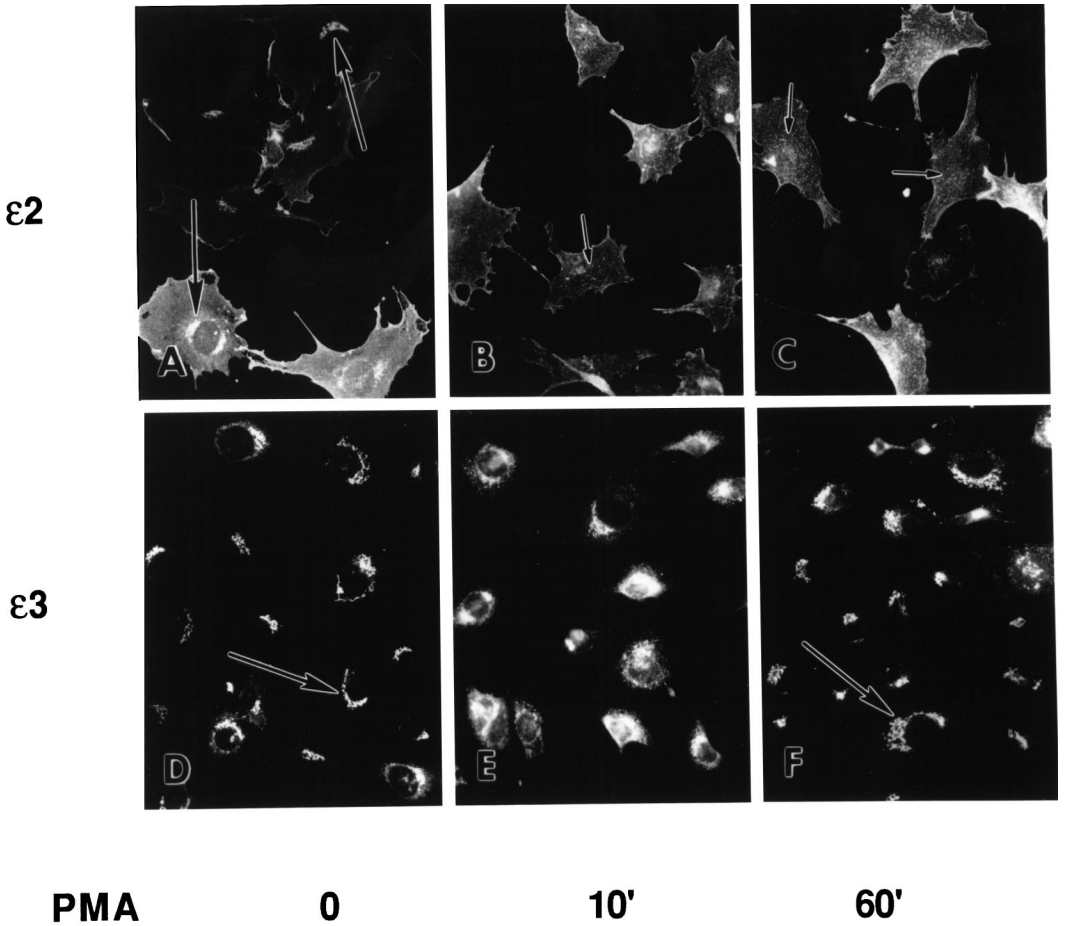

FIG. 3. Analysis of the translocation properties of the various PKCϵ mutant proteins at 20°C. The overexpressor cells were preincubated at 20°C for 30 min to block vesicle-mediated distribution from the trans Golgi network to the plasma membrane, exposed to 1 μM PMA at 20°C for the indicated time, then fixed and stained as described in the legend for Figure 2. Panels A, B and C: ϵ2 cells; panels D, E and F: ϵ3 cells, respectively. Panels A and D: untreated cells. Panels B and E: cells after 10 min PMA treatment. Panels C and F: cells after 1 hour PMA treatment. The arrows designate the Golgi.

These data provide evidence that at least two mechanisms may be involved in PMA-induced translocation of holo PKCϵ and of PKCϵ fragments from the Golgi to the plasma membrane: a slower, vesicle traffic dependent process, and a faster, vesicle traffic-independent mechanism. The latter requires the presence of either the pseudosubstrate or hinge regions of PKCϵ and is likely to represent the physiologically relevant pathway for rapid agonist-induced redistribution of PKCϵ to the plasma membrane.

FIG. 2. Immunocytochemical analysis of the translocation properties of the various PKCϵ mutant proteins at 37°C. The NIH 3T3 overexpressor cells were incubated in the presence and absence of 1 μM PMA for the indicated times, and then fixed with paraformaldehyde and solubilized by exposure to Triton X-100 detergent. Panels A and B: ϵ3 cells, stained with BODIPY-conjugated wheat germ agglutinin. Panels C-K: cells were incubated with the anti ϵ-tag antibody, and immunofluorescent staining was employed using Cy3 conjugated anti-rabbit antibody as described in Materials and Methods. Panels C, D, and E: ϵ3 cells; panels F and G: ϵ2 cells; panels H and I: ϵ7 overexpressor cells, panels J and K: holo PKCϵ cells, respectively. Panels A, C, F, H, and J: untreated cells. Panels B, D, G, I, and K: cells after 10 min PMA treatment. Panel E: ϵ3 cells after 1 hour PMA treatment. The arrowheads indicate the plasma membrane, while the long arrows designate the Golgi. The bars represent 20 mm.

While the blockade of translocation of the ϵ3 protein by incubation of cells at 20°C suggests that this process is vesicle dependent, it does not define the exact localization of the ϵ3 zinc finger fragment prior to addition of PMA. To address this question, studies were carried out to determine the effects of brefeldin A (BFA) and monensin on the subcellular localization of ϵ3. Exposure of cells to BFA is known to disrupt Golgi structure, and the contents of the Golgi cisternae then are mixed with the endoplasmic reticulum (14,15). The effect of BFA on the TGN-endosome system varies depending on the cell type (13), but in most cell types the content of the TGN is mixed with the endosomes (12,14–16). At the same time vesicle traffic between the Golgi cisternae and the TGN is completely blocked (14,17). Monensin also has a pleiotropic effect on Golgi. While it may induce the swelling of many Golgi compartments, it has been shown to specifically inhibit vesicle traffic between the medium- and trans-Golgi cisternae (18,19). Treatments with these two drugs have been successfully used to dissect enzymatic processes characteristic to the trans-Golgi cisternae and the TGN (20).

BFA pretreatment of ϵ3 protein overexpressor cells resulted in disruption of the ϵ3 perinuclear staining (Figure 4A), while the PMA-induced translocation of ϵ3 to the plasma membrane was not inhibited (Figure 4B). Monensin pretreatment, as expected, caused some enlargement of the immunoreactive perinuclear region (Figure 4C), while again translocation of the ϵ3 protein in response to PMA treatment was not affected (Figure 4D). The lack of inhibition of ϵ3 protein translocation by these treatments, together with the observed inhibition by incubation at 20°C, strongly suggests the TGN as the specific localization site for the PKCϵ zinc-finger fragment prior

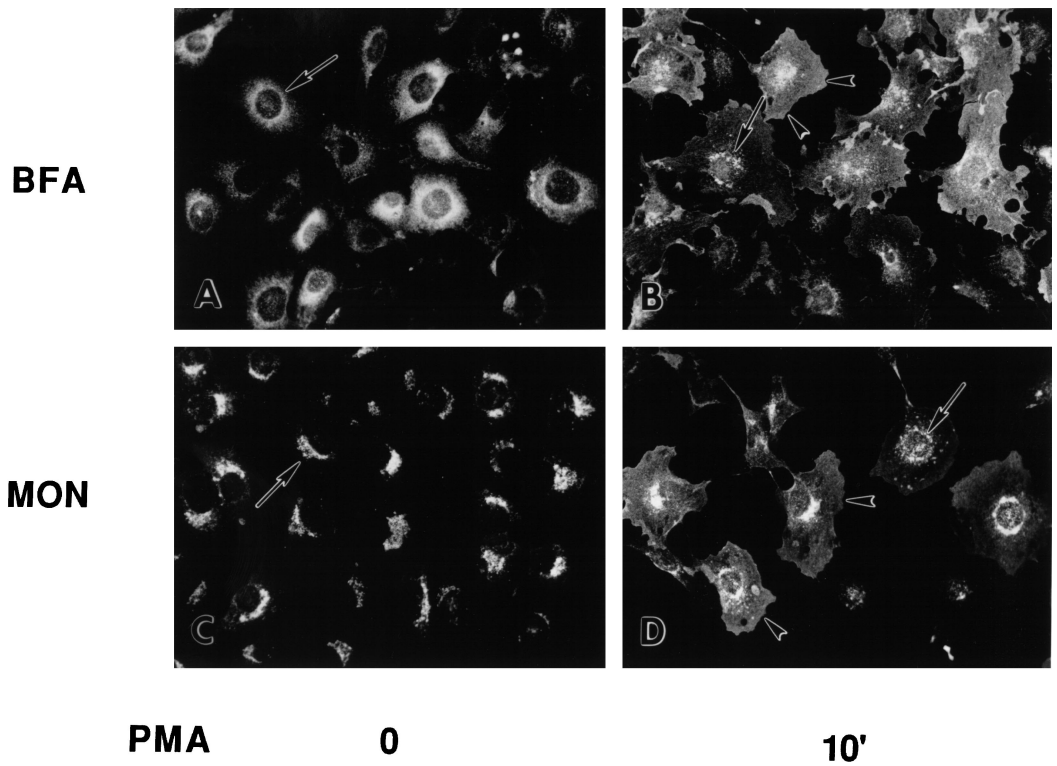

FIG. 4. Effect of treatment of cells with brefeldin A and monensin on the localization and translocation of the ϵ3 recombinant protein. The ϵ3 overexpressor cells were pretreated for 30 min with 2 μg/ml BFA (panels A and B) or 5 μM monensin (panels C and D), then incubated in the presence and absence of 1 μM PMA for 10 min as indicated. The cells then were fixed and stained as described in the legend to Figure 2. The arrows indicate the Golgi, while the arrowheads mark the plasma membrane.

to the PMA-mediated translocation. This is in agreement with our previous finding that sulfation reactions, which in most cell types are known to take place exclusively in the TGN (20, and references therein), are effected by the overexpression of the ϵ3 protein (7).

In summary, a general model for the regulation of PKC often includes the translocation (tight association) of PKC to membranes in response to activator (particularly PMA) binding to the enzyme (1). However, the results presented in this communication indicate that PMA binding to the zinc finger region instead appears to release holo PKCϵ, as well as the ϵ2 and ϵ7 recombinant proteins, from association with the Golgi to allow redistribution to the plasma membrane. This is a rapid (<10 min) vesicle-independent translocation, since it is not blocked by incubation of the cells at 20°C. The presence of the pseudosubstrate and/or hinge regions of PKCϵ appear to be required for targeting of these fragments to the plasma membrane. In comparison, PMA treatment induced a slow (30–60 min) translocation of the ϵ3 zinc finger fragment from the Golgi to the plasma membrane. The ϵ3 fragment is specifically localized to the TGN, and PMA-induced translocation of this fragment from the TGN to the plasma membrane is vesicle dependent. This TGN localization appears to be specific for the PKCϵ zinc finger domain fragment since the similarly ϵ epitope-tagged PKCγ and PKCδ zinc finger domains were found in the cytosol, with no detectable localization to the Golgi apparatus (unpublished results). Further, this ϵ3 protein, even though it lacks catalytic function, nonetheless was found to exhibit potential regulatory capabilities by inhibiting both sulfate uptake and the secretion of sulfated glycosaminoglycans (7). Thus, it is of interest that this ϵ3 zinc finger domain also responds differently than either holo PKCϵ or the other PKCϵ recombinant fragments relative to the noted vesicle-dependent translocation from the TGN to the plasma membrane.

Studies now are in progress to identify Golgi domain(s) involved in PKCϵ-Golgi interactions, to determine the role of PMA in altering PKCϵ association with these Golgi binding site(s), and to define the regulatory role(s) of holo PKCϵ, and of PKCϵ domain fragments, in modulating Golgi functions.

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