Role of the Diacylglycerol Kinase α-Conserved Domains in Membrane Targeting in Intact T Cells*

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Diacylglycerol kinase (DGK) phosphorylates diacylglycerol to phosphatidic acid, modifying the cellular levels of these two lipid mediators. Ten DGK isoforms, grouped into five subtypes, are found in higher organisms. All contain a conserved C-terminal domain and at least two cysteine-rich motifs of unknown function. DGKα is a type I enzyme that acts as a negative modulator of diacylglycerol-based signals during T cell activation. Here we studied the functional role of the DGKα domains using mutational analysis to investigate membrane binding in intact cells. We show that the two atypical C1 domains are essential for plasma membrane targeting of the protein in intact cells but unnecessary for catalytic activity. We also identify the C-terminal sequence of the protein as essential for membrane binding in a phosphatidic acid-dependent manner. Finally we demonstrate that, in the absence of the calcium binding domain, receptor-dependent translocation of the truncated protein is regulated by phosphorylation of Tyr335. This functional study provides new insight into the role of the so-called conserved domains of this lipid kinase family and demonstrates the existence of additional domains that confer specific plasma membrane localization to this particular isoform.

The transient generation of lipids at the cell membrane by the concerted action of lipases, kinases, and phosphatases is a very effective mechanism for the localization and activation of signaling molecules. The majority of lipid-modifying enzymes are cytosolic proteins that must in turn translocate to the membrane to modify their substrates. Characterization of the mechanism that governs membrane translocation of these proteins is, thus, essential to assess precisely their role in the regulation of cell responses.

The diacylglycerol kinases (DGK) are a family of enzymes that phosphorylate diacylglycerol (DAG) to produce phosphatidic acid (PA); this modulates the levels of these two lipids, both of which have recognized second messenger functions. Early experiments showed that DGK activity occupies a central position in phospholipid synthesis; this enzyme attracted additional interest when it was shown to participate in intracellular signaling (1). DGK activity is found in organisms from bacteria to mammals, although the protein identified as a DGK in bacteria is an integral membrane protein (2) and has little structural homology with the DGK characterized in multicellular organisms (3). Ten DGK isoforms have been identified in mammals and grouped into five subtypes based on the presence of various domains in their primary sequences, which define distinct regulatory motifs.

Translocation from one subcellular compartment to another appears to be a general theme in the regulation of DGK proteins. Many of the early data regarding DGK activity regulation refer to changes in DGK activity or localization based on enzymatic studies without specifying the isoform concerned (4, 5). Characterization of the DGK isoforms has broadened the concept that these proteins regulate their actions by translocating from one cell compartment to another and by forming part of different protein complexes (6–11). The non-conserved regulatory domains appear to govern subtype-specific DGK translocation. The characterization of these domains has allowed an initial outline of the signaling pathways responsible for the subcellular relocalization of the various DGK isoforms (9, 12, 13).

In addition to their distinct regulatory motifs, all DGK family members have a conserved catalytic domain, proposed to harbor the ATP-binding site. Precise characterization of the DGKα ATP-binding site has remained elusive; all DGK have a conserved GGGDGXXG motif that is essential for activity that is hypothesized to fulfill this function (14). Members of this lipid kinase family share another conserved structure, a cysteine-rich motif (CRM), present at least twice in all DGK. The homology of DGK CRM domains to the phorbol ester/DAG binding, C1-type motifs of protein kinase C originally led to the idea that these motifs were responsible for DAG binding in DGK. Sequence analysis nonetheless indicates that, with the exception of the first C1 domains in DGKβ and DGKγ, the DGK C1 domains lack the key residues that define this motif as a canonical C1-like, phorbol ester binding domain (15). The participation of the conserved C1 domains in DGK enzyme activity, thus, remains a matter of debate. Some studies suggest that these conserved motifs are required (16), whereas others found them dispensable for DAG phosphorylation (17).

DGKα is a type I DGK expressed abundantly in the cytosol of T lymphocytes that translocates to the membrane during T cell...
activation (18). DGKα acts as a negative modulator of the signals that determine T lymphocyte activation (8). T cells from DGKα-deficient mice have enhanced responses (19), further confirming the relevance of this enzyme in regulating immune function. Structural-functional analyses showed that the regulatory N-terminal domain of the enzyme, containing a recoverin-like domain and a tandem of EF hand motifs, acts as a negative regulator of enzyme activation and receptor-induced membrane translocation (8, 20). Translocation of wild type (wt) DGKα is a very rapid and transient event, whereas a catalytically inactive mutant form remains at the membrane for a longer period of time (18). This suggests feedback regulation based on PA generation, which guarantees transient DGKα localization at the membrane. Deletion of the DGKα N-terminal domain does not alter enzyme activity but results in constitutive localization of the enzyme at the plasma membrane in intact T cells. This suggests that the Ca²⁺ regulatory domain, characteristic of type I enzymes, is responsible for this PA negative feedback.

Studies in primary T cells and T cell lines demonstrate that DGKα translocates to the plasma membrane after stimulation of endogenous T cell receptor (TCR) and/or ectopically expressed muscarinic type I receptor (8, 18). Analysis of other cell types also suggests that the plasma membrane is the main site of DGKα activity (13, 21). It is still not known, however, how DGKα is targeted specifically to this subcellular compartment. We used the DGKα construct that lacks the Ca²⁺ regulatory domain to further analyze the structural determinants responsible for its constitutive localization at the plasma membrane. Using mutational analysis and studies in intact cells, we show that the two DGKα C1 domains, although not necessary for catalytic activity, are critical for plasma membrane targeting. We identify the sequence C-terminal to a conserved Pro-rich region also necessary for membrane binding through a still unidentified mechanism. Finally, we confirm that Tyr335 phosphorylation is an absolute requirement for DGKα membrane localization in a receptor-dependent manner. With this study, we complete a detailed mapping of the diverse DGKα motifs and provide new insight into the functional role of the domains conserved in most members of this lipid kinase family.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The J-HM1–2.2 cell line was generated by stable transfection of the human muscarinic subtype-1 receptor in the Jurkat T cell line (22). J-HM1–2.2 cells were maintained in RPMI 1640 medium (Invitrogen); COS-7 and Jurkat cells were purchased from the ATCC and cultured in Dulbecco’s modified RPMI 1640 medium (Invitrogen); COS-7 and Jurkat cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer’s protocols. All experiments were performed 36–48 h after transfection.

**DGK Assay**—Transfected cells were frozen on dry ice and lysed by sonication in DGK assay buffer (50 mM Tris HCl, pH 7.4, 100 mM NaCl, 0.25 mM saccharose, 1 mM diothioretol, 1 mM EDTA, 5 mM EGTA, and the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, and aprotinin). Cell lysates were centrifuged (20,000 × g), and the supernatant was used for the DGK reaction as described (23). Protein (20 μg) from whole lysates or immunoprecipitates of tagged proteins was used as enzyme source. Standard phosphorylation assays were performed (10 min, 37 °C; final volume, 50 μl). The reaction was terminated by adding 100 μl of 1 mM HCl, and lipids were extracted with 20 μl of CHCl₃/MeOH (1:1, v/v). Organic layers were recovered, dried, dissolved in 20 μl of CHCl₃, and applied to silica gel TLC plates with dioleoyl-PA as a standard. Plates were developed with a CHCl₃, MeOH, 4 mM NH₄OH solvent system (9:7:2, v/v/v). Dried plates were autoradiographed, and bands corresponding to PA were quantified by autoradiogram scanning.

**Fractionation Studies**—T lymphocytes were harvested, washed in PBS, resuspended in TES buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.25 mM succors) with protein inhibitors, and lysed by 10 passages of the cell suspension through a 25-gauge needle. Nuclei were removed by centrifugation. Supernatants were centrifuged (100,000 × g, 1 h), and pellets were resuspended in TES buffer supplemented with 1% Nonidet P-40. Supernatants and pellets were resolved in SDS-PAGE and analyzed in Western blot with the indicated antibodies.

**Western Blot**—To determine expression of transfected proteins, cells were lysed in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each aprotinin and leupeptin) for 30 min on ice. After centrifugation (20,000 × g, 10 min, 4 °C), supernatants were analyzed by SDS-PAGE. Electrophoresed samples were transferred to nitrocellulose, and protein expression was analyzed using the indicated antibodies and ECL (Amersham Biosciences). Anti-hemagglutinin monoclonal antibody was from Covance, anti-GFP monoclonal antibody was from Roche Applied Science, horseradish peroxidase-conjugated goat antimum IgG from Dako.

**Analysis of CD69 Cell Surface Expression**—Transfected cells were stimulated with anti-CD3/anti-CD28 antibodies. CD69 expression on the cell surface was analyzed 6 h after stimulation gating for GFP-positive cells) using a phosphatidylethanolamine-conjugated anti-human CD69 monoclonal antibody (Pharminingen). Immunofluorescence intensity of the cells was determined by flow cytometry (FACSCalibur, BD Biosciences).

**Immunofluorescence Microscopy**—T cells were harvested 36 h after electroporation with plasmids, washed, and allowed to attach to poly-l-lysine-coated coverslips (1 h, room temper-
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ature). In the case of TCR stimulation, Jurkat cells were harvested 24 h post-transfection, washed once, and resuspended in HEPES-balanced solution. Cells were transferred to chambered coverslips coated with anti-CD3/CD28 monoclonal antibody (final concentration 5 μg/ml, 4°C, overnight). All subsequent incubations and washes used a 0.5% bovine serum albumin, 0.1% Triton X-100 in PBS buffer; antibodies were incubated (37 °C, 1 h) in a humidified chamber. Nuclei were labeled with Topro-3 (Molecular Probes). Fluorescence was analyzed on a Leica confocal microscope (TCS-NT) with the associated software. For inhibitor treatment, the DGK inhibitor R59949 (10 μM; Calbiochem) was added after the first frame, and images were recorded every 11 s.

Stimulation with Antibody-coated Microspheres—Antibodies were adsorbed to microspheres by mixing 1 μg of antibody (1:1 CD3:CD28) in PBS with 0.5 × 10⁶ microspheres in a final volume of 1 ml and incubated (1.5 h at room temperature) with continuous mixing; 1.5 ml of 1% bovine serum albumin in PBS was added, and mixing was continued (30 min). Microspheres were washed three times with PBS and suspended in PBS for the addition to cells. For stimulation, cells were mixed with antibody-coated microspheres at a 2:1 cell/bead proportion and plated on poly-DL-lysine-coated chamber slides. Images were captured each 15 s by confocal microscopy and processed using ImageJ software.

RESULTS

Identification of the DGKα Minimal Catalytic Core—DGKα has a highly conserved catalytic domain and a variable N-terminal domain shown to exert a negative regulatory role for enzyme activity and subcellular localization. The exact identity of the “minimal catalytic domain” needed to phosphorylate DAG nonetheless remains unclear. To study enzymatic activity of the catalytic domain, we engineered a protein lacking the regulatory N terminus and both C1 domains (Δ327DGKα) (Fig. 1A). Expression of the truncated protein was confirmed by Western blot, and enzymatic activity was determined in cell lysates and immunoprecipitates (IP). In a lower panel, analysis of protein expression with an anti-hemagglutinin antibody. The experiment shown is representative of three performed with similar results.

C1 Domains Are Essential for DGKα Membrane Targeting—In addition to the C-terminal conserved domain, all DGK family members have at least two C1 domains, which belong to the general HX₁₀₋₁₂CX₁₂₋₁₄CX₂₋₄HX₅₋₇CX₅₋₇C motif found in more than 200 distinct signaling proteins (15). Structural analysis showed that in addition to Cys and His, other residues are required for phorbol ester and DAG binding (24). Based on the presence of these additional residues, C1 domains can be classified as typical or atypical (25). Sequence analysis indicated that, with the exception of the first C1 domains in DGKβ and DGKγ, C1 DGK domains lack one, two, or all three residues (Pro, Gly, and Gln) that define a C1 as a typical phorbol ester binding (and probably DAG binding) domain (Fig. 2A).

To study the role of C1 domains in DGKα cell membrane localization, we used a Jurkat T cell line variant in which TCR-like responses, including DGKα translocation, are triggered by carbachol stimulation of an ectopically expressed muscarinic type I receptor (8, 26). We engineered point mutations of the conserved His in the first and the second C1 in the full-length GFP-DGKα sequence. Membrane translocation was then examined by confocal microscopy after carbachol stimulation of the cells. Mutation of the conserved His residues in either C1 prevented protein translocation in response to receptor stimulation (Fig. 2B). Alignment of DGK C1 sequences showed a conserved Trp after the first His, with the exception of the first and second C1 of DGKθ, which had the Phe generally found in C1 domains in other proteins (Fig. 2A). Because the Trp after the first His appears to be characteristic of DGK, we analyzed the role of this residue in DGKα. A conservative mutation of this Trp to Phe did not alter receptor-regulated translocation. Mutation to a non-conservative Gly abolished enzyme translocation after receptor stimulation, indicating the need for an aromatic residue (Fig. 2B). These results point to the DGKα C1 region as essential for membrane localization of the protein through lipid and/or protein interactions.

Deletion of the N-terminal Ca²⁺ binding domain results in a protein (GFP-DGKαΔEF) constitutively located at the plasma membrane when expressed in Jurkat cells (8). We next analyzed whether the C1 domains were also required for constitutive membrane localization of this mutant. As for the wt protein, mutation of Trp to Phe had no effect on enzyme localization, whereas mutation to Gly induced complete protein dissociation from the membrane (Fig. 2B, bottom). These results concur with those described for the full-length construct and show that

![Diagram](image_url)
failure of the enzyme to translocate after receptor stimulation was not due to a delay in translocation kinetics. Finally, a construct expressing the C1 domains alone fused to enhanced GFP did not localize to the plasma membrane (not shown), suggesting that this region, although necessary, is not sufficient for DGKα plasma membrane localization.

The previous experiments suggest that the C1 region, whereas necessary, is not sufficient for plasma membrane localization. We next decided to perform additional mutations/truncation on the GFP-DGKα/H9251 construct to investigate the nature of the requirements for its constitutive membrane localization. A summary of the different mutants used in the next experiments is shown in Fig. 3.

Deletion of the DGKα C-terminal Region Prevents Constitutive Membrane Association of GFP-DGKα/H9251EF—Functional studies with the DGKβ isoform have identified an alternative spliced isoform that lacks the last 35 amino acids. Deletion of this C-terminal region in vivo (27). Sequence analysis of the C-terminal sequence of type I enzymes indicated conservation of a Pro-rich sequence followed by an amino acid stretch with no obvious conservation among distinct subtypes (Fig. 4A). To analyze the relevance of the C-terminal sequence in DGKα stability at the membrane, we generated a new construct in which the last 13 amino acids of the GFP-DGKα/H9251/H9004EF construct were deleted (GFP-DGKα/H9251/H9004CT). When expressed in Jurkat T cells, we found that the protein showed the appropriate molecular weight, but DGK activity was much lower than that detected for GFP-DGKα/H9251/H9004EF when assayed in vitro (Fig. 4B). Confocal analysis in intact cells established that deletion of the C-terminal sequence disrupted constitutive membrane localization of the GFP-DGKα construct at the membrane (Fig. 4C). This suggests that, as previously shown for DGKβ, this region might represent an important site for membrane interaction. Fractionation analysis confirmed that deletion of the C-terminal domain affects cytosol/membrane distribution (Fig. 4D).
We have previously demonstrated that the GFP-DGKαΔEF construct acts as a constitutive active DGKα mutant, decreasing the DAG levels generated during stimulation of the TCR. As a result, cells expressing this mutant show decreased membrane expression of the activation marker CD69 in response to TCR triggering (8). To further confirm that deletion of the C terminus affected membrane localization of DGKα and consequently enzyme function, we determined CD69 expression after TCR stimulation in cells expressing GFP-DGKαΔEF. As previously shown for the wild type protein bearing the same mutation (8), the GFP-DGKαΔEF mutant, decreas-
ing enzyme function, we determined CD69 expression after TCR stimulation in cells expressing GFP-DGKαΔEF or GFP-DGKαΔEFΔCT. As is shown, the attenuation on CD69 expression observed in GFP-DGKαΔEF expressing cells is lost in cells expressing GFP-DGKαΔEFΔCT (Fig. 4E).

PA Generation Stabilizes GFP-DGKα in the Absence of the N-terminal Region—The GFP-DGKαΔEF deletion mutant shows higher activity in vitro than the wt enzyme and is no longer stimulated by Ca2+ (8, 28). We reasoned that lack of plasma membrane localization of GFP-DGKαΔEFΔCT could be related to its diminished enzymatic activity. To further confirm the requirement for enzyme activity in the constitutive plasma membrane localization of the GFP-DGKαΔEF mutant, we generated a kinase-dead version of this construct (DGKαΔEFKD) by mutation of Gly433 to Ala in the conserved GGDG motif that was first identified in the Drosophila melanogaster DGK1 (14) and which is essential for catalysis (8, 12). As previously shown for the wild type protein bearing the same mutation (8), the GFP-DGKαΔEFKD did not phosphorylate DAG in vitro (not shown). Again, plasma membrane localization was observed for the GFP-DGKαΔEF mutant (Fig. 5A, left). As predicted, this localization was lost for the GFP-DGKαΔEFKD (Fig. 5A, right), confirming that DGKα activity is needed for plasma membrane stabilization in the absence of the Ca2+ regulatory motifs.

Lack of enzyme activity does not prevent receptor-dep-
endent translocation to the membrane (8). We next analyzed whether GFP-DGKαΔEFKD could relocate to the plasma membrane in response to external stimuli. The construct was ectopically expressed in Jurkat T cells, and localization was examined after triggering of the TCR and the co-stimulatory molecule CD28. Plating the cells onto anti-CD3/anti-CD28-coated plates induced the characteristic spreading of the cells that was accompanied by rapid relocation of GFP-DGKαΔEFKD to the plasma membrane (Fig. 5B). Subcellular fractionation of unstimulated cells showed some redistribution of the GFP-DGKαΔEFKD with the membrane fraction. This probably reflects partial localization of this mutant at internal membr-
anes, at organelles difficult to identify by confocal analysis of intact cells. Fractionation of stimulated cells further confirmed the complete relocation of the mutant to the membrane in response to TCR triggering (Fig. 5C). These experiments demon-
strate that, whereas lack of enzyme activity impairs stabiliza-
tion of GFP-DGKαΔEFKD at the plasma membrane, receptor-
derived signals are able to relocate the truncated enzyme to the site of substrate generation. The C-terminal region was again indispensable for TCR-dependent translocation since GFP-DGKαΔEFΔCT, which lacks this region, remained in the cytosol under the same conditions (Fig. 5D).

Phosphorylation of Tyr335 Is Required for TCR-dependent Membrane Translocation—The previous experiments demon-
strate that the C1 domains together with the C-terminal region are essential for DGKα binding to the plasma membrane. The GFP-DGKαΔEFKD mutant translocates to the membrane in response to TCR triggering, although it does not contain Ca2+ regulatory elements. This suggests that, in addition to Ca2+ generation, supplementary signals are needed to promote receptor-dependent DGKα translocation to the membrane.

Phosphorylation of the Tyr335 residue, found in the hinge region between the C1 domains and the conserved catalytic region of DGKα, has been proposed to be necessary for stimulus-dependent translocation to the membrane of ectopically expressed DGKα (21). We, therefore, tested whether this resi-
due was also important for either constitutive localization of GFP-DGKαΔEF or receptor-dependent translocation of GFP-DGKαΔEFKD. To this end new mutants were generated where Tyr335 was mutated to Phe both in the GFP-DGKαΔEF and the GFP-DGKαΔEF mutants. Replacement of Tyr335 by Phe did not impair enzyme activity or constitutive membrane localization of the GFP-DGKαΔEF mutant (Fig. 6A). This suggests that, once the protein is located at the membrane, phosphorylation of this Tyr is no longer required for membrane stabilization. On the contrary, the GFP-DGKαΔEFKDY335F mutant was unable to translocate to the membrane after TCR triggering (Fig. 6B) showing that whereas phosphorylation of this residue is not needed for enzyme stabilization at the membrane, it is essential for receptor-dependent DGKα translocation. To further evaluate if translocation to the membrane was exclusively related to TCR dependent signals, Jurkat T cells were stimulated by incuba-
tion with anti-CD3/C28-coated microspheres. As was observed, the GFP-DGKαΔEFKD mutant relocated to the membrane with apparently exclusion from the T cell synapse (Fig. 6C). Again Tyr335 mutation to Phe fully prevented relocal-
ization of the protein, confirming the role of this residue for TCR-dependent regulation of DGKα.

FIGURE 3. The schematic represents the construct used along this study. The localization of the conserved GGDG region, the Tyr335 residue, and the C-terminal domain are indicated.
DISCUSSION

The DGK enzymes have recently emerged as an important family of lipid kinases that participate in a number of biological processes. Most of these enzymes are found in the cytosol and must relocate to the membrane, where their substrates are found.

Like the other two type I DGK, DGKα contains Ca\(^{2+}\) regulatory elements and is activated in vitro in response to Ca\(^{2+}\) and anionic lipids (28). Studies in intact lymphocytes have shown that the N-terminal region acts as negative regulatory domain preventing membrane localization of DGKα in the absence of receptor-triggered signals (8). Removal of the Ca\(^{2+}\) regulatory elements generates a truncated form of DGKα that presents constitutive localization at the plasma membrane (8). Based on these previous observations, we have performed additional analyses to explore in depth the requisites for DGKα binding to membranes in intact cells.

Our studies reveal that impairment of enzyme activity by mutation of the conserved Gly\(^{433}\) in the catalytic region disrupts constitutive membrane localization of the truncated mutant. Mutation of the DGKα C1 domains and truncation of the C-terminal region also prevent constitutive membrane binding. Whereas mutants lacking enzyme activity relocated to the membrane in response to receptor stimulation, intact C1 domains and the C-terminal region were absolutely necessary for membrane localization, suggesting independent functions.

DGKα contains two C1 domains that, according to the classification by Hurley and Misra (25), are atypical and do not bind DAG. This is a common feature for the C1 domains of all DGK family members, with the exception of the first C1 in DGKβ and DGKγ. As expected phorbol 12-myristate,13-acetate cannot bind to the DGKα C1 domains (17) and does not induce enzyme translocation when added to intact cells (8, 13). Our results indicate that the C1 domains are not necessary for DGKα kinase activity and suggest that previous reports of very low (17) or no C-terminal domain activity (29) were probably due to differences in assay conditions. These studies also concur with reports showing that a mutant devoid of the C1 domain retains activity and can be activated by phosphatidylserine (PS) (20).

We show that, although they are atypical, the DGKα C1 domains represent an essential module in plasma membrane interaction through protein and/or lipid binding. Our experiments agree with previous reports using the DGKz and DGKθ isoforms (9, 30) and confirm that the essential role of the C1 domains for membrane localization is a general characteristic of the DGK family. The precise function of C1 domains in DGK membrane binding is not known, although they have been identified as sites of interactions with lipids (31) and proteins (7), as shown for atypical C1 domains in other proteins such as Raf (32).
Our analysis reveals that DGKα C-terminal domain plays a role in membrane binding similar to that reported for DGKβ (27). Ectopically expressed GFP-DGKβ localizes at the plasma membrane of exponentially growing HEK293 cells, whereas it is cytosolic in quiescent cells (27). Phorbol 12-myristate,13-acetate treatment of cells induces membrane translocation of GFP-DGKβ by virtue of its typical C1 domain. Characterization of a spliced form with a truncated C terminus has shown that this region prevents phorbol 12-myristate,13-acetate-induced membrane translocation in the absence of the Ca2+ regulatory domain. However, other possibilities such as existence of auxiliary proteins that will bind only to the active DGKα must also be considered.

In intact cells, DGKα bearing a deletion of the N-terminal region is found exclusively at the plasma membrane, which is greatly enriched in PS (39). Exposure of additional sites for PS recognition as a result of N-terminal domain truncation would be required to prevent DGKα binding in plasma membrane in the absence of the Ca2+ regulatory domain. However, other possibilities such as existence of auxiliary proteins that will bind only to the active DGKα must also be considered.

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PS-dependent enzyme activation (36), confirming that PS recognition sites are found in the catalytic region. There is limited structural information on the stereospecific recognition of the PS head group by its effector proteins. Crystallization of the protein kinase Ca C2 domain bound to a PS molecule revealed direct interaction of an Asn in the side chain with the Ser in the PS head group, whereas a Thr in the side chain interacts with other PS polar moieties (40). The DGKα C-terminal domain contains polar residues (Asn, Ser, and Thr) that are conserved among DGKα orthologues, which could provide the residues required for PS interaction. It is, thus, tempting to speculate that the lack of membrane stabilization of the C-terminal-truncated mutant is due to the deletion of a region that allows PS and/or PA-mediated binding.

Lack of kinase activity in the DGKαΔ EF mutant eliminates constitutive plasma membrane localization, although TCR triggering can relocate this mutant to the membrane confirming that kinase activity is not required for receptor-dependent DGKα translocation. Tyr phosphorylation of DGKα is proposed to be an important mechanism for its receptor-dependent membrane localization (8). Tyr335 in the human sequence modulates vitamin E-dependent membrane relocalization of DGKα (21). Our results here confirm the importance of this residue in receptor-dependent translocation, suggesting that its phosphorylation induces a DGKα conformation that favors membrane binding. Again, either mutation of the C1 domain or truncation of the C-terminal region impairs membrane localization in response to external stimuli, further confirming that these two regions represent important membrane binding domains.

This structural-functional analysis of the DGKα conserved motifs allows us to draw important conclusions regarding enzyme structure. DGKα activity requires its location at the membrane, where its substrate lies. This is the role of the C1 domains, which is necessary for membrane localization in all DGK family members. In contrast, the C-terminal domain appears to be essential only for type I enzymes. The simplest explanation for the lack of membrane localization of the GFP-DGKαΔ EFKD mutant is that PA generation contributes to membrane binding in the absence of the N-terminal domain. Alternatively, the mutation might alter the conformation of the catalytic domain, diminishing enzyme affinity for the membrane. This would be compensated by Tyr335 phosphorylation, which induces a conformation that promotes C1 and C-terminal-mediated membrane interactions.

Based on our functional analysis of enzyme domains and other available data, we propose a detailed model of DGKα activation after receptor engagement (Fig. 7). In the absence of receptor triggering, the DGKα N-terminal Ca2⁺ regulatory domain interacts with the C-termi-
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The functional half of the protein, masking all regions required for membrane binding. This closed conformation maintains the enzyme inactive in the cytosol. After receptor stimulation, a Ca\textsuperscript{2+}-dependent conformational change takes place favoring Tyr\textsuperscript{355} phosphorylation. This in turn induces an open, active conformation that allows enzyme interaction with the membrane. The C1 domains and the C-terminal region represent binding sites for membrane PS, facilitating DGKα localization to the plasma membrane, where it phosphorylates DAG. Finally, the elevated PA concentration and dephosphorylation of DGKα restore the “closed” conformation, and the enzyme returns to the cytosol.

This model of DGKα membrane targeting and activation would provide exquisite control of DAG membrane levels, responsible in turn for activation/localization of DAG-regulated proteins. Our study provides new insights into the mechanisms by which membrane recruitment of this important enzyme is regulated and establishes a basis for additional systematic mechanistic and functional studies.

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