Role of Diacylglycerol Kinase α in the Attenuation of Receptor Signaling

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Abstract. Diacylglycerol kinase (DGK) is suggested to attenuate diacylglycerol-induced cell responses through the phosphorylation of this second messenger to phosphatidic acid. Here, we show that DGKα, an isoform highly expressed in T lymphocytes, translocates from cytosol to the plasma membrane in response to two different receptors known to elicit T cell activation responses: an ectopically expressed muscarinic type I receptor and the endogenous T cell receptor. Translocation in response to receptor stimulation is rapid, transient, and requires calcium and tyrosine kinase activation. DGKα-mediated phosphatidic acid generation allows dissociation of the enzyme from the plasma membrane and return to the cytosol, as demonstrated using a pharmacological inhibitor and a catalytically inactive version of the enzyme. The NH2-terminal domain of the protein is shown to be responsible for receptor-induced translocation and phosphatidic acid–mediated membrane dissociation. After examining induction of the T cell activation marker CD69 in cells expressing a constitutively active form of the enzyme, we present evidence of the negative regulation that DGKα exerts on diacylglycerol-derived cell responses. This study is the first to describe DGKα as an integral component of the signaling cascades that link plasma membrane receptors to nuclear responses.

Key words: diacylglycerol kinase • lymphocytes • T cell activation • signal transduction • green fluorescent protein

Introduction

The magnitude and specificity of cellular responses are dictated by a delicate balance between positive and negative signals that are generated after receptor stimulation. Although the mechanisms that promote the intracellular generation of signals have been extensively analyzed, those leading to negative regulation of signal generation remain largely undefined. DAG kinase (DGK)[1] generates phosphatidic acid (PA) through the phosphorylation of DAG. Since DAG is a well known lipid second messenger that is rapidly generated in response to receptor stimulation, DGK activation may be related directly to the termination of DAG-derived signals. Nine different DGK isoforms have thus far been cloned and their cDNA characterized. Alignment of the different DGK sequences has allowed identification of motifs that may be important for DGK function and/or regulation (Topham and Prescott, 1999). All isoforms contain a highly conserved catalytic domain in the COOH-terminal region and a pair of cysteine-rich motifs similar to those present in PKC but lacking certain consensus residues present in phorbol ester–binding proteins (Hurley et al., 1997). DGKs also exhibit structurally different motifs, probably related to their regulation, which have allowed their classification into five different families (Van Blitterswijk and Houssa, 1999). Type I DGKs contain a pair of EF hand-like domains in the NH2-terminal half of the protein. Type II DGKs have a pleckstrin homology–like domain, whereas type IV DGKs have COOH-terminal ankyrin repeats and a region homologous to the phosphorylation site of the MARCKS protein. Type V DGK has three instead of two cysteine-rich domains and a Ras-binding domain. Type III DGKs are the only enzymes that have no domain with obvious regulatory functions. The presence of a variety of domains with known regulatory properties together with the differential tissue expression pattern of the isoforms suggests that regulation of this enzyme family must vary among cell types or in response to different stimuli. Although these enzymes are structurally well characterized, an important remaining question is that of their regulation by receptor-derived signals.

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[1]*Abbreviations used in this paper:* BAPTA, 1,2-bis(aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; DGK, DAG kinase; DiC8, 1,2-dioctanoyl *sn*-glycerol; GFP, green fluorescent protein; IL, interleukin; PA, phosphatidic acid; PBut, phosphatidylbutanol; PDBu, 1,2-dioleoylglycerol, phorbol-12,13-dibutyrate; PE, phycoerythrin; PI4,5P2, phosphatidylinositol 4,5-bisphosphate; PLD, phospholipase D; SAX-HPLC, strong ion exchange high performance liquid chromatography; TCR, T cell receptor.

DGKα belongs to the type I DGK family and is abundant in the cytosol of T lymphocytes (Sakane et al., 1991). Here, we have studied the role of this enzyme in the regulation of DAG-derived signals that control T cell activation events. T lymphocyte activation is triggered by stimulation of the antigen receptor in concert with costimulatory molecules through a complex signaling cascade, in which increases in membrane DAG and cytosolic calcium concentration are essential events (Kane et al., 2000). The intracellular increase in these two second messengers is sufficient to elicit several T cell responses, as has been shown by ectopic expression of other receptors that, through PLC-regulated hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$), can lead to antigen-independent T cell activation. A good example is the human muscarinic receptor (HM-1), which increases the intracellular calcium concentration and membrane DAG in neurons and effector cells through a G protein–coupled mechanism. This receptor is functional when stably transfected in the parental Jurkat T cell line (J-HM1-2.2) (Desai et al., 1990). Stimulation of J-HM1-2.2 cells by carbachol, an HM-1 agonist that has no effect on parental Jurkat cells, induces several T cell responses such as expression of the interleukin (IL)-2 receptor alpha chain, CD69, and Fas ligand (Desai et al., 1990; Izquierdo et al., 1996). This indicates that the stimulation of $PI4,5P_2$ hydrolysis elicited by this agonist can mimic antigen receptor–regulated events in T lymphocytes. Whereas some responses require a more complex set of receptor-derived signals, the expression of the CD69 activation marker is strictly dependent on the elevation of DAG in the plasma membrane (Cebrián et al., 1988). We have used this model in which receptor-induced DAG increase is directly related to transcriptionally regulated protein expression to study the role of DGKα as a modulator of receptor-derived cell responses. Our results indicate that, in response to carbachol stimulation of J-HM1-2.2 cells, DGKα translocates rapidly from the cytosol to the plasma membrane by a mechanism that requires the NH_2 terminus of the enzyme. This domain is also responsible for the dissociation of the enzyme from the plasma membrane which takes place after PA generation. Using a pharmacological inhibitor of the enzyme and plasmids encoding green fluorescent protein (GFP) fused to the wild-type or a truncated DGKα, we demonstrate for the first time the role of DGKα in the negative regulation of receptor-derived signals. DGKα translocation is also induced by T cell receptor (TCR) cross-linking, suggesting that membrane localization of DGKα is a general response to receptor stimulation. Plasma membrane translocation of DGKα cannot be induced by addition of DAG analogues but is mimicked by intracellular calcium elevation together with activation of tyrosine kinases, suggesting that receptor-induced enzyme translocation of DGKα requires at least these two signals.

Materials and Methods

Reagents

Carrier-free [^{32}P]orthophosphate and [γ-^{32}P]ATP were purchased from Amersham Pharmacia Biotech. Silica gel thin layer chromotography plates (60Å, LK6D) were from Whatman. R59949, 1,2-bis(aminophenoxy)ethane-*N,N,N´,N´*-tetraacetic acid (BAPTA), U73343, U73122,

and herbimycin were purchased from Calbiochem. 1,2-dioleoylglycerol, phorbol-12,13-dibutyrate (PDBu), carbachol, 1,2-dioctanoyl *sn*-glycerol (DiC8), ionomycin, orthovanadate, and poly-L-lysine were from Sigma-Aldrich. Radiolabeled standards for strong ion exchange high performance liquid chromatography (SAX-HPLC) were prepared as described previously (Jones et al., 1999). Anti-GFP monoclonal antibody was from CLONTECH Laboratories, Inc. HRP-conjugated goat anti–mouse IgG was from Dako, phycoerythrin (PE)-conjugated anti–human CD69 was from PharMingen, and Cy3-goat anti–rabbit IgG was from Jackson ImmunoResearch Laboratories.

Cell Culture

The J-HM1-2.2 cell line was generated by stable transfection of HM-1 in the human leukemic Jurkat T cell line (Desai et al., 1990). Cells were maintained in RPMI 1640 medium (Life Technologies). COS-7 cells (American Type Culture Collection) were cultured in DME. Media were supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM Hepes, and 100 μg/ml each of penicillin and streptomycin. Ba/F3 cells were maintained in complete RPMI medium supplemented with 10^{-5} M 2-mercaptoethanol and 5% WEHI-3B–conditioned medium as a source of IL-3.

Constructs and Transfection

The PEF-GFPC1 plasmid was generated by replacing the cytomegalovirus promoter in the GFP-C1 plasmid (CLONTECH Laboratories, Inc.) with the PEF-BOS promoter (donated by Dr. J.A. Garcia-Sanz, DIO/CNB, Madrid, Spain). Full-length DGKα and Δ(1–192)DGKα cDNAs subcloned into PSRE plasmids have been described previously (Sakane et al., 1996) and were given by Drs. F. Sakane and H. Kanoh (University of Sapporo, Sapporo, Japan). The cDNAs were excised from the PSRE plasmids and subcloned into PEF-GFP-C1 using the EcoR1 site. The kinase-dead version of GFP-DGK was generated replacing the Gly 432 in the catalytic domain by Ala, using the Quickchange Site-directed Mutagenesis kit (Stratagene). To target the GFP-DGKα to the membrane, the GFP-DGKα NH_2 terminus was modified by adding the pYes myristoylation sequence using specific primers flanked for restriction sites. The annealed DNA fragment was fused in frame via the respective restriction sites to the NH_2 terminus of GFP in the GFP-DGKα construct. For transfection, cells in logarithmic growth were transfected with 25 μg of the corresponding plasmid DNA by electroporation at 230 V/975 μF. Cells were analyzed between 24 and 48 h after transfection.

In Vivo Generation of PA

When J-HM1-2.2 cells reached a density of ∼10^6 cells/ml, they were washed twice in phosphate-free RPMI medium supplemented with 2 mM glutamine and further incubated for 90 min. Metabolic labeling was performed using 150 μCi/ml [^{32}P]orthophosphate for 1 h. Cells were then challenged as indicated in the figure legends. When R59949 (10 μM) was used, a 10-min preincubation was used before addition of carbachol. After the indicated times, total cellular lipids were extracted and separated by SAX-HPLC (Jones et al., 1999) or by TLC with a solvent system of 1-propanol/2 M acetic acid (2:1 vol/vol) in the presence of phospholipid standards. After drying at room temperature, phospholipid migration on the TLC plate was identified using iodine staining, and associated radioactivity was monitored in a Bio-Rad Laboratories PhosphorImager. When phospholipase D (PLD) activity was determined by the transphosphatidylation reaction, experiments were performed as described above except that cells were preincubated for 15 min in the presence of 1% 1-butanol before carbachol stimulation. After total lipid extraction, PA and phosphatidylbutanol (PBut) were separated by TLC with a solvent system of ethyl acetate/isooctane/acetic acid (9:5:2 vol/vol/vol).

Determination of DAG Levels

DAG generation was quantified according to a modification of the DAG kinase assay (Preiss et al., 1986) as has been described previously (Flores et al., 1998).

Measurement of DGK Activity

COS-1 cells were transfected with the indicated cDNA, harvested after 36–48 h, washed twice with ice-cold PBS, and frozen at −70°C. The cells were thawed and lysed by nitrogen cavitation (10 min at 500 psi, 4°C) in a buffer

containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 50 mM NaF, 2 mM Na_3VO_5, 1 mM PMSF, and 10 μg/ml each of leupeptin and aprotinin. Lysates were then centrifuged (800 *g*) and the supernatant was used to determine DGK activity, as described previously (Flores et al., 1996). At the end of the phosphorylation assay, radiolabeled lipids were extracted, dried under a nitrogen stream, dissolved in 20 μl of chloroform/methanol (2:1 vol/vol), and applied to silica gel plates, with DiC18 PA as a standard. Plates were developed with a solvent system consisting of chloroform/methanol/4 M ammonia (9:7:2 vol/vol/vol). Dried plates were autoradiographed and the bands corresponding to PA were quantified by phosphorimaging.

Analysis of CD69 Cell Surface Expression

Cells were preincubated with vehicle or the DGK inhibitor R59949 for 10 min, then stimulated with carbachol or PDBu as indicated. CD69 expression on the cell surface was analyzed using a PE-conjugated anti–human CD69 monoclonal antibody. Immunofluorescence intensity of the cells was determined by flow cytometry (EPICS-XL; Beckman Coulter). The CD69 expression level in transfected cells was analyzed 48 h after transfection, gating for GFP-positive and -negative cells.

Immunoblotting

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 Na_3VO_4, 1% Nonidet P-40, 1 mM PMSF, 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 expression of the GFP-coupled constructs was analyzed using anti-GFP antibody and the ECL detection kit (Amersham Pharmacia Biotech).

Immunofluorescence Microscopy

Cells were harvested 36 h after electroporation with the corresponding plasmids, washed, and allowed to attach to poly-L-lysine–coated coverslips (1 h, room temperature). Cells were treated as indicated (in corresponding figure legends). Cells were fixed with 4% (wt/vol) paraformaldehyde in PBS, washed twice with PBS, and analyzed by confocal microscopy (TCS-NT; Leica). For visualization of endogenous DGKα, cells were stained with anti-DGKα antibody and with Cy3-goat anti–rabbit IgG. Samples were fixed and permeabilized with 2% BSA plus 0.1% Triton X-100 in PBS. To visualize the nuclei, cells were incubated with TOPRO-3 or Sylver Green (1 μg/ml, 10 min) (Molecular Probes). For TCR and anti-CD28 stimulation, slides were precoated with antibodies at a final concentration of 10 μg/ml. After coating, slides were washed with PBS, and the cells were seeded and centrifuged (80 *g*, 20 s). At the end of the times indicated, cells were fixed with 4% (wt/vol) paraformaldehyde in PBS, washed twice with PBS, and analyzed by confocal microscopy (TCS-NT; Leica).

Results

DGK Activity Regulates Expression of T Cell Activation Markers

Carbachol stimulation of the Jurkat-derived J-HM1-2.2 cell line, which ectopically expresses the muscarinic type 1 receptor, has been shown previously to induce a rapid elevation of inositol polyphosphates (Desai et al., 1990). We confirmed that carbachol addition to this cell line also induces the rapid and transient elevation of DAG, which reaches maximal values 2–5 min after stimulation (data not shown). These data suggest the existence of a mechanism responsible for the removal of the generated DAG. One enzyme that may perform this process is DGK, which in doing so causes generation of PA. After carbachol stimulation of J-HM1-2.2 cells, a rapid increase in the cellular PA content could be detected, which by 2 min was more than twice that of control values (Fig. 1 a). To assess
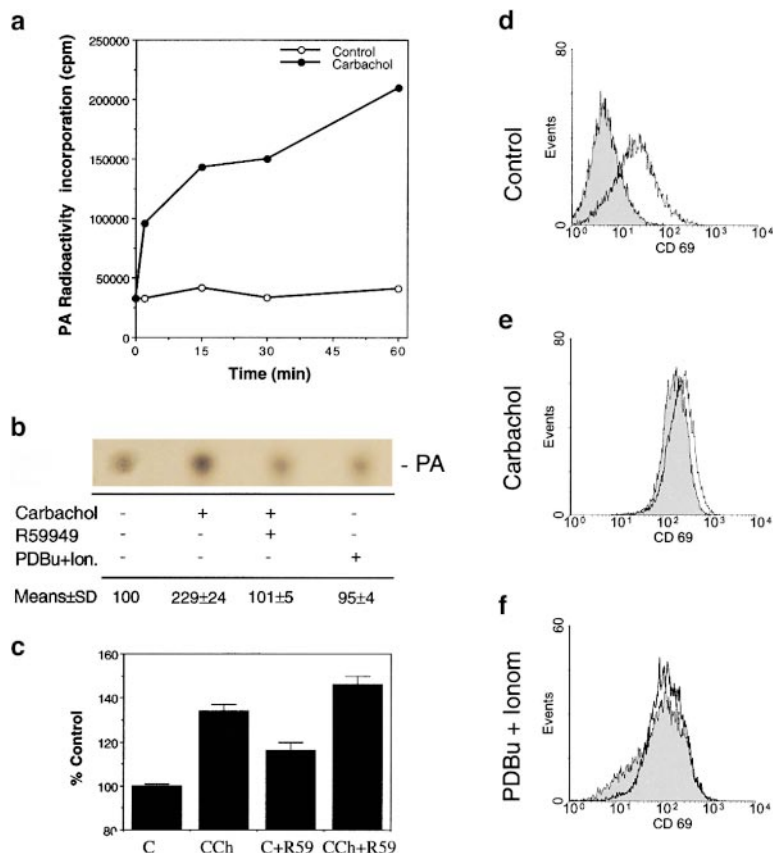
Figure 1. Carbachol induces DGK activation in J-HM1-2.2 cells. (a) Generation of PA after carbachol addition. Cells were metabolically radiolabeled with [^{32}P]orthophosphate and stimulated with vehicle or carbachol (50 nM). At the times indicated, cells were collected, total cellular lipids were extracted, and the PA content was determined by SAX-HPLC analysis of deacylated samples (see Materials and Methods). The results are shown of a single experiment representative of three independent experiments. (b) Effect of DGK inhibition on PA generation. Cells were metabolically radiolabeled with [^{32}P]orthophosphate and stimulated as indicated. After 2 min, total lipids were extracted, and the PA generated was determined by TLC and quantified as described in Materials and Methods. The top of the figure represents a typical experiment, showing the autoradiogram of the TLC plate corresponding to the area of PA standard migration. The quantification of the PA generated in three independent experiments is shown. (c) Effect of DGK inhibition on DAG generation. Cells were stimulated as indicated: control (C), carbachol (CCh), and R59949 (R59). After 2 min, total lipids were extracted and the level of DAG was quantified as described in Materials and Methods. The quantification of the DAG measured in three independent experiments is shown. (d–f) Effect of DGK inhibition on CD69 expression. J-HM1-2.2 cells were stimulated with vehicle (d), carbachol (e), or PDBu + ionomycin (f); after 6 h, cells were collected and CD69 expression was determined. Filled areas represent CD69 expression by cells after treatment, and empty areas represent cells pretreated with the DGK inhibitor R59949 (10 μM, 10 min) before further stimulation.
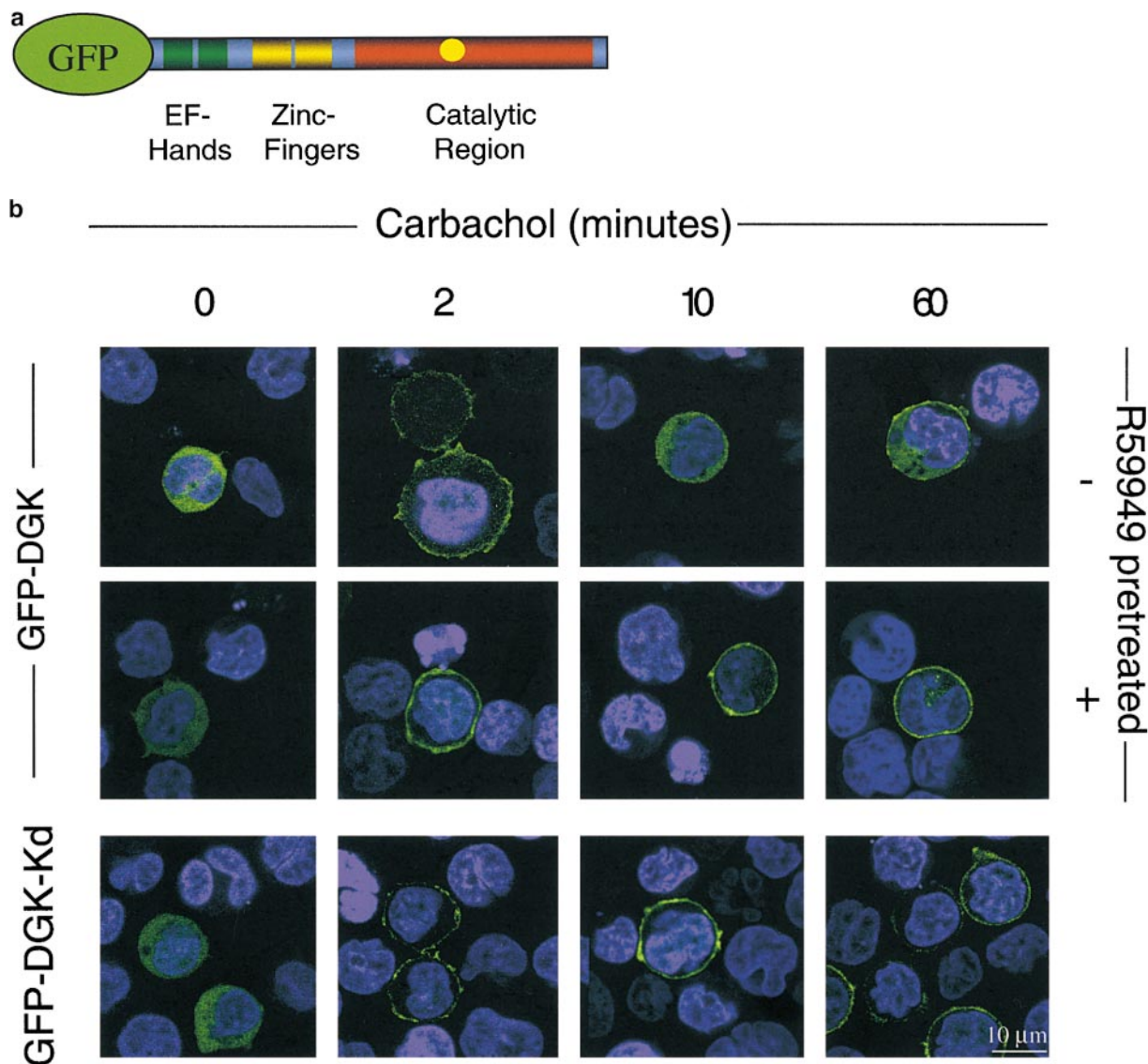
Figure 2. Carbachol-mediated plasma membrane translocation of GFP-tagged DGKα. (a) The drawing shows the GFP-DGKα construct used in this study; the conserved cluster in the catalytic domain in which Gly[432] was mutated is marked as a yellow dot. The conserved motifs of the enzyme are indicated. (b) Analysis of carbachol-mediated plasma membrane translocation of GFP-tagged DGKα. J-HM1-2.2 cells were transfected with either GFP-DGKα (top and middle) or a catalytically inactive form of the enzyme (GFP-DGKα kD). After 48 h, cells were treated with vehicle or carbachol for the times indicated. In the middle panel, cells were pretreated with R59949 (10 μM, 10 min) before carbachol stimulation. Cells were fixed and GFP fluorescence was visualized by confocal microscopy. TOPRO-3 was used to visualize nuclei.

whether the increase in carbachol-stimulated PA production was due to activation of a PLD, identical experiments were performed in the presence of the primary alcohol 1-butanol. PLD is the only enzyme that catalyzes a transphosphatidylation reaction, diverting PA production to PBut. No significant PBut accumulation was detected after 2 min of carbachol stimulation. At later time points, only a small percentage of PA (∼20% of total) was converted to PBut (data not shown). This indicates that the majority of PA is generated via DGK. DGK-regulated PA generation in response to carbachol was demonstrated further using the specific type I DGK inhibitor R59949 (Jiang et al., 2000). The rapid PA elevation detected 2 min after carbachol addition was blocked completely by preincubating cells with R59949 before carbachol stimulation (Fig. 1 b). In con-

trast, PDBu and calcium ionophore treatment did not affect the endogenous PA level, indicating that receptor-regulated DGK activation is not a downstream effect of PKC activation. Accordingly, pharmacological inhibition of DGK activity induced an elevation of the DAG level both in control and carbachol-stimulated cells (Fig. 1 c).

Since the rapid PA increase appeared to be mediated by DGK, the DGK requirement for DAG transcriptionally regulated events was determined. In J-HM1-2.2 cells, carbachol is a potent inducer of the early activation marker CD69 (Desai et al., 1990). Whereas the requirements for other T cell–regulated responses are more stringent, CD69 expression is mimicked by PDBu or DiC8 treatment of cells (Cebrián et al., 1988). This indicates that transcriptional regulation of CD69 expression is directly
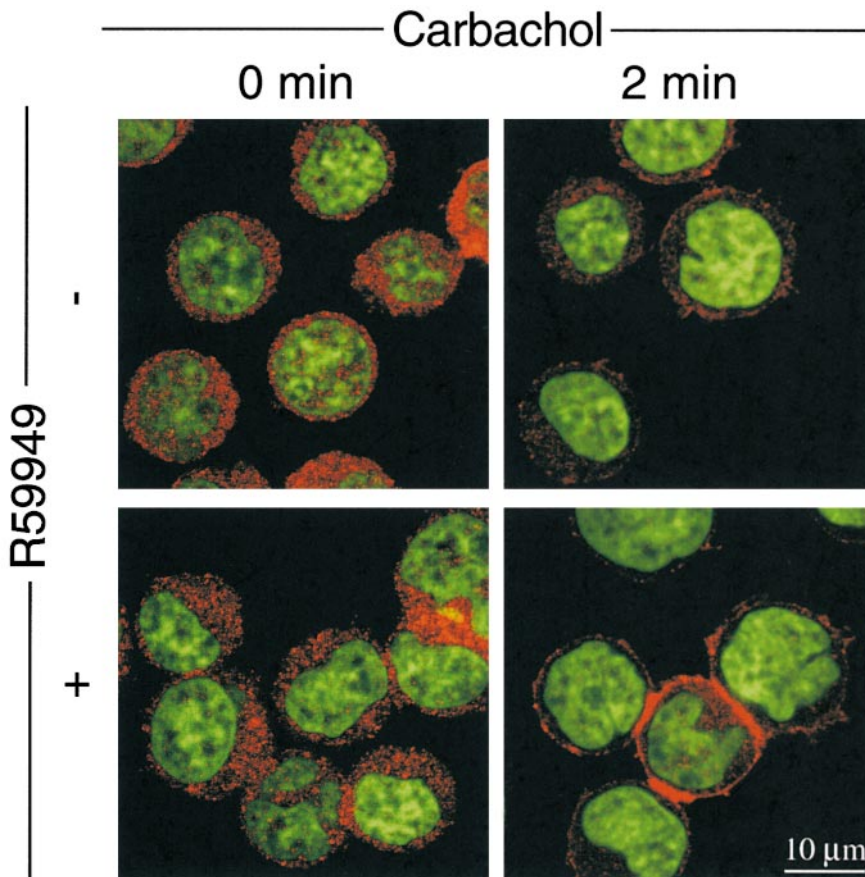
Figure 3. Carbachol-mediated plasma membrane translocation of endogenous DGKα. J-HM1-2.2 cells were treated with vehicle or carbachol for 2 min as in the legend to Fig. 2. In the bottom panel, cells were pretreated with R59949 (10 μM, 10 min) before carbachol stimulation. Cells were fixed and fluorescence was visualized by confocal microscopy. Nuclei were visualized by staining with Sylver green.

related to the rapid elevation of plasma membrane DAG that occurs after receptor stimulation. The effect of pharmacological inhibition of DGK activity on CD69 induction by carbachol was subsequently studied. Pretreatment of J-HM1-2.2 cells with R59949 induced an increase in CD69 expression, even in the absence of carbachol (Fig. 1 d). R59949 inhibition of DGK activity also increased CD69 cell surface expression compared with that induced by carbachol alone (Fig. 1 e). On the contrary, DGK inhibition had no effect on PDBu-induced CD69 expression (Fig. 1 f), suggesting that DGK activity regulates receptor-induced signals but lies upstream of PKC activation.

Localization of GFP-tagged DGKα in Response to Carbachol

Carbachol stimulates PA generation, and inhibition of DGK activity results in increased DAG generation and CD69 expression. These findings point to a model in which DGK participates in the early signaling generated by the receptor. Jurkat cells express high DGKα levels, which have been shown to be mostly cytosolic by subcellular fractionation (Yamada et al., 1989). The recruitment of cytosolic enzymes from cytosol to the plasma membrane is usually a prerequisite for participation of intracellular molecules in signal transduction after receptor stimulation. If DGK is the enzyme responsible for the rapid clearance of the DAG generated in the plasma membrane, it must translocate after carbachol stimulation. To study the effect of carbachol on subcellular DGKα redistribution, we generated a plasmid encoding the cDNA for DGKα fused to GFP (Fig. 2 a). The GFP-tagged DGKα fluores-

cence in unstimulated cells corresponded to a cytosolic enzyme, as described for endogenous DGKα (Flores et al., 1999) (Fig. 2 b, top). Carbachol addition resulted in the rapid and transient enzyme translocation from cytosol to the plasma membrane, which was detected as early as 2 min after stimulation; by 10 min after stimulation, the majority of the enzyme had returned to the cytosol. At later times, even at 1 h after receptor stimulation, a fraction of the enzyme was again found in the plasma membrane. Pretreatment of unstimulated cells with the DGK inhibitor R59949 did not affect the cytosolic localization of GFP-DGKα (Fig. 2 b, middle); nonetheless, R59949 completely prevented dissociation of the enzyme from the plasma membrane after carbachol addition. When cells were pretreated with the inhibitor, carbachol-induced DGK translocation was complete and lasted for ≥1 h after receptor stimulation (Fig. 2 b, middle).

Inhibition of DGK activity prevented enzyme dissociation from the plasma membrane. To determine whether this effect was directly related to enzyme activity, we examined the membrane localization of a catalytically inactive DGKα fused to GFP. Rapid translocation of the inactive mutant was detected in response to carbachol (Fig. 2 b, bottom). Nevertheless, as was the case after inhibitor treatment, the kinase-dead DGKα remained at the membrane at all the times examined.

GFP-DGKα Behaves Similarly to Endogenous DGKα

To determine whether GFP-tagged DGKα responded to carbachol stimulation as did the endogenous enzyme, we analyzed the cells using a specific antibody shown to recog-
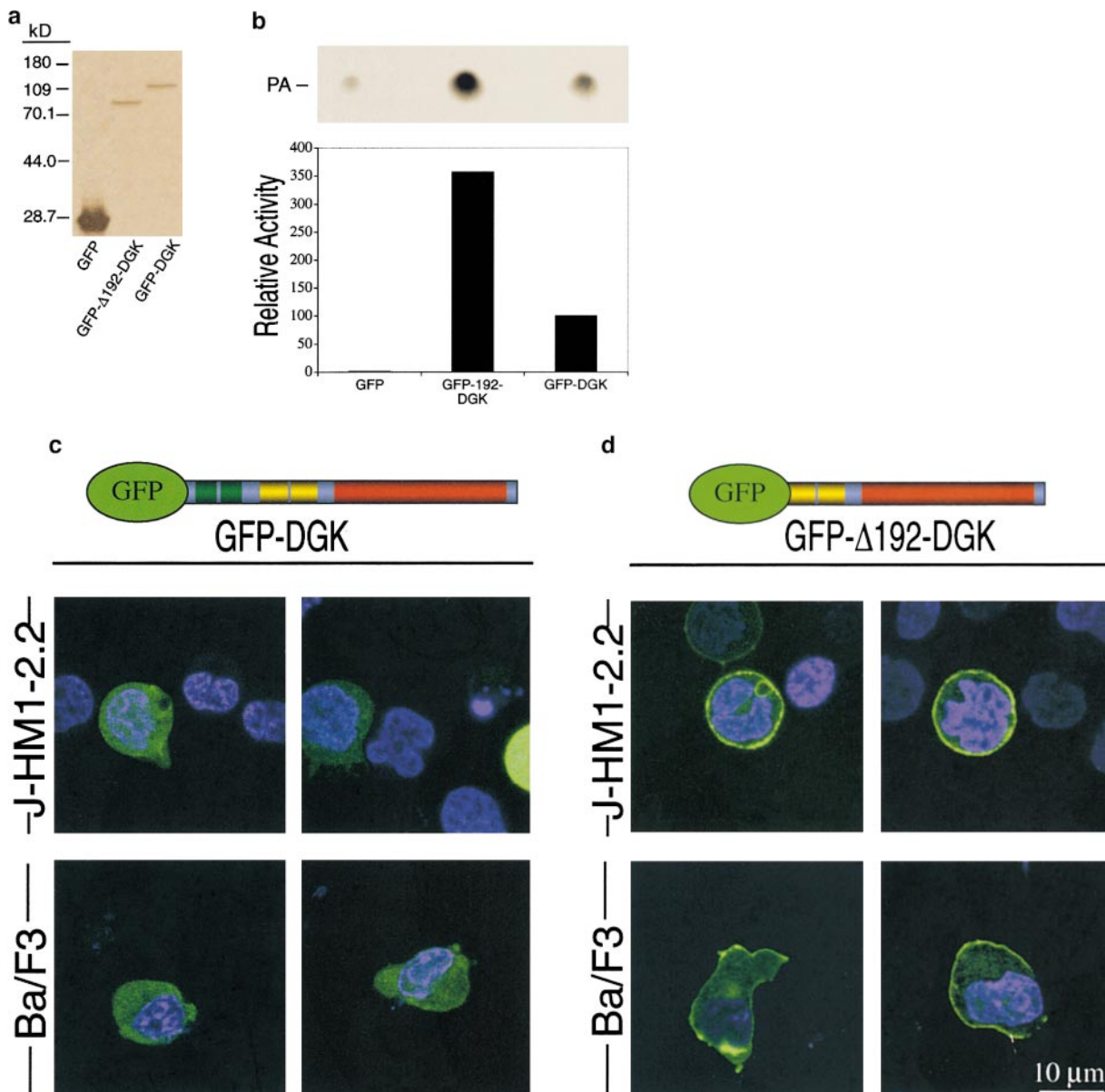
Figure 4. Deletion of the NH2-terminal domain of DGKα increases its enzymatic activity and allows constitutive localization in the plasma membrane. (a) COS-7 cells were transfected with vectors encoding GFP, GFP-DGKα, or GFP–Δ(1-192)DGKα. At 48 h after transfection, expressed proteins were analyzed by Western blotting with an anti-GFP antibody. (b) Deletion of the EF hand motif induces increased enzymatic activity. DGK activity of the expressed proteins was measured in total lysates of COS-7 cells 48 h after transfection. Radiolabeled PA generated during the reaction was analyzed by TLC and quantified as described in Materials and Methods. The top panel shows an autoradiogram of the TLC plate, and quantification of radioactivity incorporated into PA is shown (bottom). Three independent experiments were performed with similar results. (c) Subcellular distribution of wild-type GFP-DGKα. J-HM1-2.2 (top) and Ba/F3 (bottom) cells were transfected with the GFP-DGKα plasmid. (d) Deletion of the EF hand motif induces constitutive localization to the plasma membrane. J-HM1-2.2 (top) and Ba/F3 (bottom) cells were transfected with the GFP–Δ(1-192)DGKα plasmid. GFP fluorescence was determined by confocal microscopy of cells 48 h after transfection. Nuclei were visualized by staining with TOPRO-3.

nize endogenous DGKα in T lymphocytes (Yamada et al., 1989; Flores et al., 1996). After carbachol addition, there was partial translocation of the cytosolic enzyme to the plasma membrane within 2 min (Fig. 3). Again, R59949 pretreatment of cells did not alter cytosolic localization of the enzyme, but it did greatly enhance DGKα redistribution to the plasma membrane. These results confirm that transfected GFP-tagged DGKα behaves as does the endogenous enzyme, providing a useful tool to examine protein translocation in response to receptor stimulation.

The DGKα NH2-terminal Domain Contains a Negative Regulatory Site

The DGKα NH2-terminal domain contains a pair of EF hand motifs found in a large number of functionally diverse Ca^{2+}-binding proteins (Sakane et al., 1990). To study the role of this domain in carbachol-induced DGKα translocation, we generated a plasmid encoding the GFP protein fused to a DGKα truncation mutant, in which the first 192 NH2-terminal amino acids, including the EF hand do-
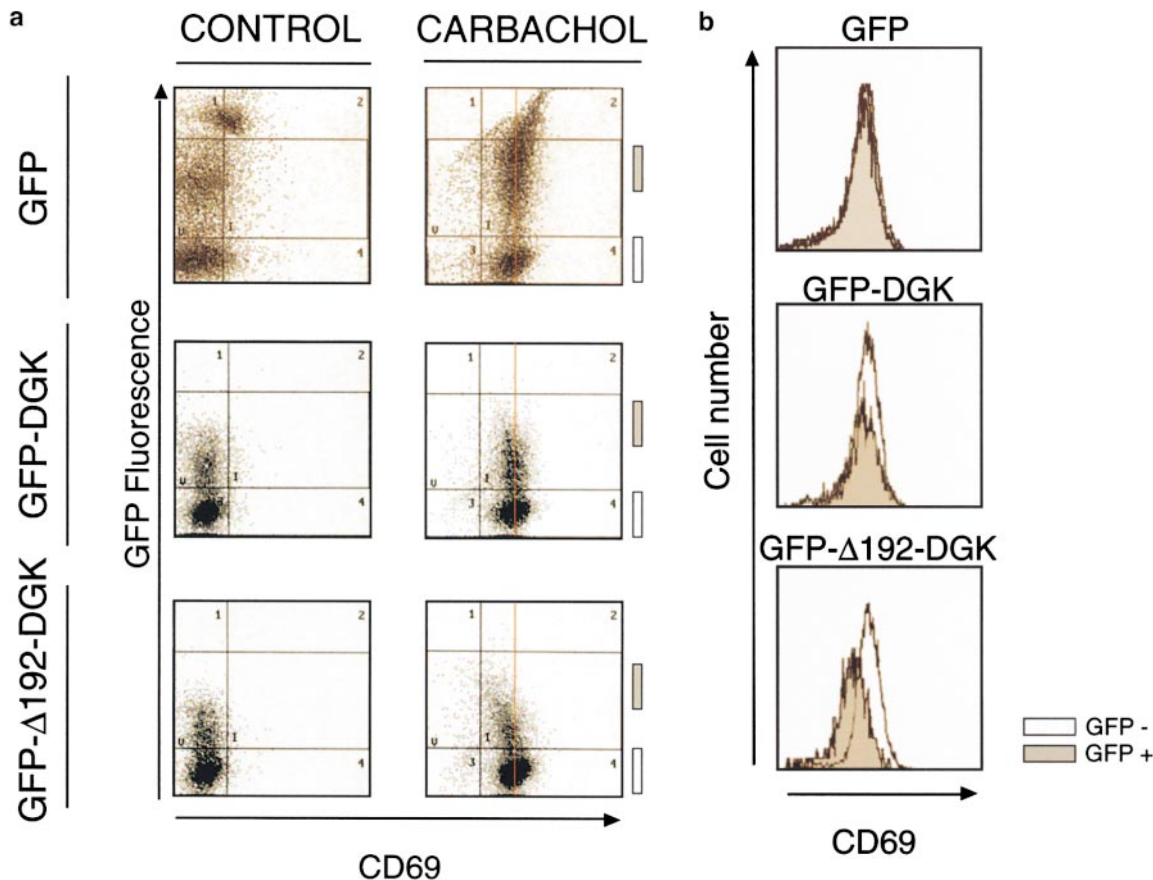
Figure 5. Constitutive membrane localization of GFP–Δ(1-192)DGKα correlates with reduced cell surface CD69 expression. (a) J-HM1-2.2 cells were transfected with plasmids encoding GFP, GFP-DGKα, or GFP–Δ(1-192)DGKα plasmids. At 48 h after transfection, cells were left untreated or stimulated with carbachol. CD69 expression was determined by flow cytometry 6 h after carbachol addition to the cells. Histograms show GFP fluorescence (y-axis) plotted versus CD69 expression of control (left) and carbachol-treated (right) cells. (b) Analysis of CD69 expression in GFP-negative versus GFP-positive cells. The CD69 level was determined in the different cell populations after transfection by gating for GFP-negative and GFP-positive cells. CD69 expression in untransfected cells is represented by empty areas and in GFP-positive cells by filled areas. The results shown are from a single experiment representative of three performed with similar results.

main, are deleted. DGKα bearing a deletion of the EF hand domain is described to have higher activity than the wild-type enzyme (Sakane et al., 1996). Fusion of the GFP tag to this truncated construct did not alter its characteristics. Transfection of both constructs in COS-7 cells generated proteins of the expected molecular weights, both of which were expressed to a similar extent (Fig. 4 a); nonetheless, the enzyme bearing the NH_2-terminal truncation was far more active than its wild-type counterpart (Fig. 4 b). When J-HM1-2.2 cells were transfected with the deletion mutant, a striking difference was found in the subcellular localization of the protein compared with that of the wild-type enzyme (Fig. 4 c, top). GFP–Δ(1-192)DGKα was constitutively associated with the plasma membrane, even in the absence of carbachol (Fig. 4 d, top). This suggests that the NH_2-terminal domain, deleted in this construct, exerts a negative regulatory role, maintaining the enzyme in the cytosol in the absence of receptor signaling and also allowing its dissociation from the plasma membrane after PA generation. Constitutive membrane localization of the GFP–Δ(1-192)DGKα construct is not exclusive of T cells, since a similar distribution was detected after transfection

of the pre-B murine cell line Ba/F3 (Fig. 4, c and d, bottom). To further evaluate the effect of expression of the different constructs on DGK activity, cells were sorted after transfection to enrich the GFP-positive population. PA formation was measured both in basal conditions and after 2 min of carbachol addition. We consistently found a threefold increase in PA levels in cells expressing wild-type DGKα compared with control cells. When the same experiment was performed in cells expressing the GFP–Δ(1-192)DGKα, PA elevation was more than fivefold compared with that measured in GFP-transfected cells (data not shown).

Membrane Localization of DGKα Attenuates CD69 Expression

To examine the effect of DGK activity on carbachol-induced CD69 expression, J-HM1-2.2 cells were transfected with control GFP, GFP-DGKα, or GFP–Δ(1-192)DGKα. GFP expression allowed us to determine for each transfected cDNA CD69 surface expression in cells expressing high levels of the transfected proteins, compared with GFP-negative cells. Carbachol-induced CD69 expression was not
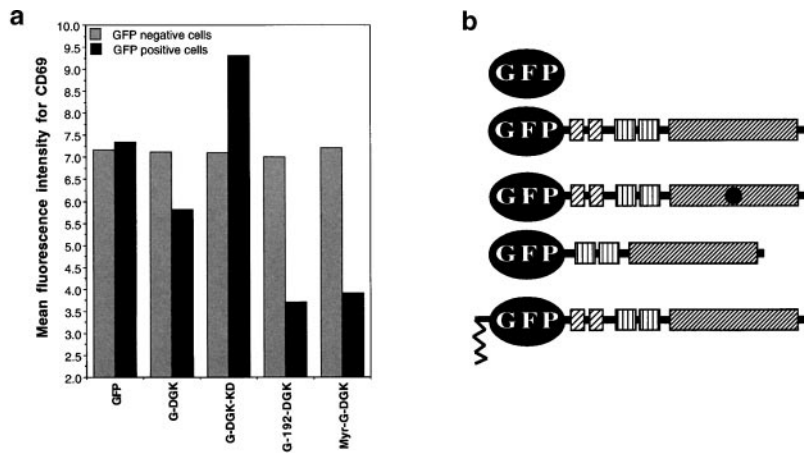
Figure 6. Membrane localization of DGKα correlates with reduced cell surface CD69 expression, whereas lack of enzyme activity enhances CD69 expression. (a) J-HM1-2.2 cells were transfected with plasmids encoding GFP, GFP-DGKα, GFP-DGKα–Kd, GFP–Δ(1-192)DGKα, or Myr-GFP-DGKα plasmids. At 48 h after transfection, cells were left untreated or stimulated with carbachol. CD69 expression was determined by flow cytometry 6 h after carbachol addition to the cells. Histograms show carbachol-induced CD69 expression in GFP negative (gray bars) and GFP positive (black bars) after transfection of the different constructs. Results shown are representative of a single experiment of two performed with similar results. (b) The cartoon shows the different GFP-DGKα constructs used in these experiments.

altered in GFP-expressing cells compared with GFP-negative cells (Fig. 5 a, top). A slight decrease on CD69 expression in the GFP population was detected when cells were transfected with the GFP-DGKα construct (Fig. 5 a, middle). This indicates that the regulation exerted by carbachol on DGKα translocation to the plasma membrane remained effective. Nevertheless, when cells were transfected with the GFP–Δ(1-192)DGKα construct, which has higher enzymatic activity and is constitutively located in the plasma membrane, CD69 expression was significantly reduced compared with that of untransfected cells (Fig. 5 a, bottom). Moreover, there was an inverse correlation between GFP–Δ(1-192)DGKα expression levels and CD69 cell surface protein levels (Fig. 5 b). These results indicate that constitutive plasma membrane localization of DGKα attenuates CD69 expression in response to this ligand.

To further demonstrate that membrane localization of DGKα mediated the reduction of CD69 expression, a myristoylation signal sequence was added to the NH_2-terminal end of the GFP-DGKα construct to induce constitutive association of this protein to the plasma membrane. After transfection of this construct, cells were stimulated with carbachol and CD69 expression in GFP-negative and -positive gated cells was assessed. As is shown in Fig 6 a, constitutive plasma membrane localization of DGKα severely reduces CD69 expression compared with the wild-type enzyme and behaves similarly to the truncated GFP-DGKαΔ192 mutant. A comparison of CD69 levels in cells expressing DGKα versus those in cells expressing membrane-associated enzyme further confirms that membrane localization of DGKα has a dramatic effect on the downregulation of this activation marker. Finally, to demon-
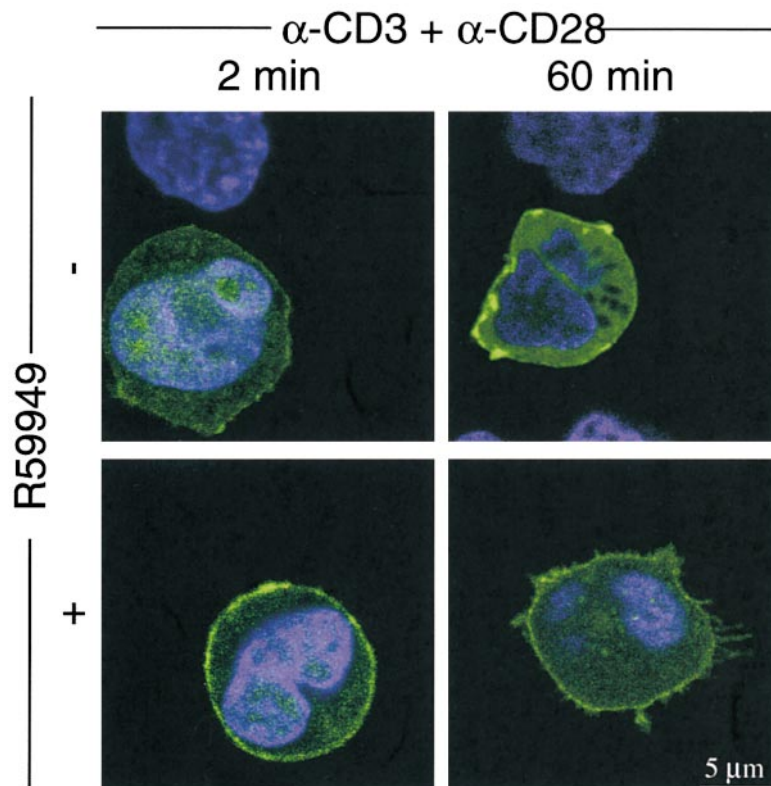

Figure 7. Effect of TCR/CD28 cross-linking on the subcellular redistribution of GFP-tagged DGKα. J-HM1-2.2 cells were transfected with GFP-DGKα and after 48 h, were seeded onto slides precoated with anti-CD3 and anti-CD28 antibodies and centrifuged for 20 s at 500 rpm. After incubation for the times indicated, cells were fixed and GFP fluorescence was visualized by confocal microscopy. In the bottom panel, cells were pretreated with R59949 for 10 min before layering onto the slides. TOPRO-3 was used to visualize nuclei.
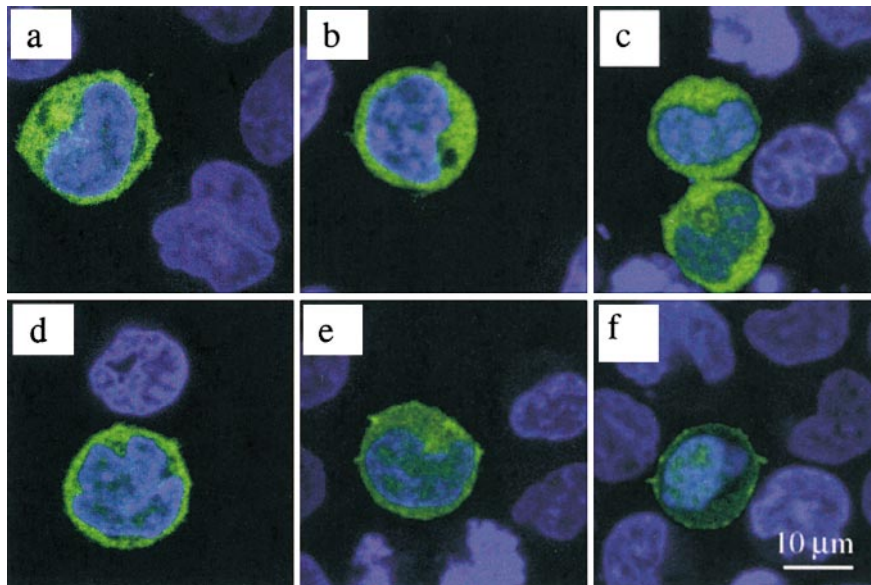
Figure 8. Plasma membrane translocation of GFP-tagged DGKα requires tyrosine kinase activation and intracellular calcium increases. J-HM1-2.2 cells were transfected with GFP-DGKα. After 48 h, the subcellular distribution of the enzyme was determined by confocal microscopy analysis of the cells after 2 min of the following treatments: (a) DiC8 (100 μg/ml); (b) ionomycin (1 μM) + calcium (2 mM) in the extracellular buffer; (c) DiC8 + ionomycin/calcium; (d) H_2O_2 (2 mM) + sodium orthovanadate (1 mM); (e) H_2O_2, sodium orthovanadate, and DiC8; and (f) H_2O_2, sodium orthovanadate, and ionomycin/calcium.

strate the relevance of DGK activity in the control of DAG-regulated responses, expression of CD69 was examined in cells expressing the GFP-tagged, kinase-dead version of the enzyme. As shown in Fig. 6 a, CD69 expression after carbachol treatment was higher in GFP-positive cells compared with the GFP-negative ones. The effect on CD69 expression after transfection of the kinase-dead mutant was similar to that observed after pharmacological inhibition of DGK activity (Fig. 1 e). These results suggest that overexpression of the kinase-dead mutant has a dominant negative effect over the wild-type enzyme.

GFP-DGKα Translocates to the Membrane after TCR/CD28 Cross-linking

We analyzed whether, in addition to carbachol, a more physiological stimulus could also induce DGKα translocation. For this, we examined redistribution of GFP-tagged DGKα in J-HM1-2.2 cells after anti-CD3 and anti-CD28 antibody cross-linking, which resembles the physiological mechanism of T cell activation (Grakoui et al., 1999). After TCR and CD28 triggering, there was partial translocation of the enzyme. As observed previously, pretreatment of cells with the DGK inhibitor caused complete subcellular redistribution of the enzyme from cytosol to the plasma membrane (Fig. 7). These results indicate that DGKα translocation after receptor triggering is a general response to both G protein– and tyrosine kinase–coupled receptors, confirming the role of DGKα in the regulation of DAG-based signals.

Tyrosine Kinase Activation Is Required for GFP-DGKα Translocation

Translocation of GFP-DGKα occurs very rapidly in response to two alternative receptors known to activate either PLCγ or PLCβ, suggesting DAG- and/or calcium-dependent regulation of DGKα. PDBu and ionomycin treatment of GFP-DGKα–transfected cells did not induce enzyme translocation (not shown), thereby excluding PKC- and/or calcium-dependent mechanisms. Unchanged

subcellular localization of the transfected GFP-DGKα correlated with no increase in endogenous PA (Fig. 1 b). This further confirmed that endogenous DGKα is not activated/translocated by PKC-dependent signals. We then evaluated whether GFP-DGKα translocation, although independent of PKC activation, was directly mediated through PLC-mediated generation of second messengers. Extracellular addition of DiC8, a DAG analogue with short fatty acid chains, did not induce GFP-DGKα translocation to the plasma membrane (Fig. 8 a). No translocation was detected after ionomycin treatment, either alone (Fig. 8 b) or in combination with DiC8 (Fig. 8 c). The same results were obtained when the cells were pretreated with the DGK inhibitor R59949 to stabilize the plasma membrane localization of the enzyme, which would exclude the existence of very rapid and/or transient translocation after calcium and/or DAG increase (data not shown). GFP-DGKα translocation apparently required a receptor-derived signal independent of those generated after PLC activation. Cell surface receptors that lack intrinsic tyrosine kinase activity, such as the antigen TCR, are known to initiate their actions by recruiting nonreceptor tyrosine kinases (Kane et al., 2000); recent data suggest that G protein–coupled receptors function in an analogous manner (Luttrell et al., 1999). Moreover, DGKα has been shown to be a direct substrate of the EGF receptor in vivo (Schaap et al., 1993). We therefore studied the role of tyrosine kinase activation in the translocation of GFP-DGKα. Induction of tyrosine kinase activity in the transfected cells had no apparent effect either alone (Fig. 8 d) or combined with extracellular addition of DiC8 (Fig. 8 e). Nonetheless, activation of tyrosine kinases together with an increase in intracellular calcium concentration clearly induced redistribution of the enzyme from the cytosol to the plasma membrane (Fig. 8 f). In agreement with these data, carbachol-induced translocation of GFP-DGKα (Fig. 9 a) was prevented by pretreatment of transfected cells with the PLC inhibitor U-73122 (Fig. 9 b) or with the calcium chelator BAPTA (Fig. 9 c), indicating that calcium signals, although not sufficient, are necessary for
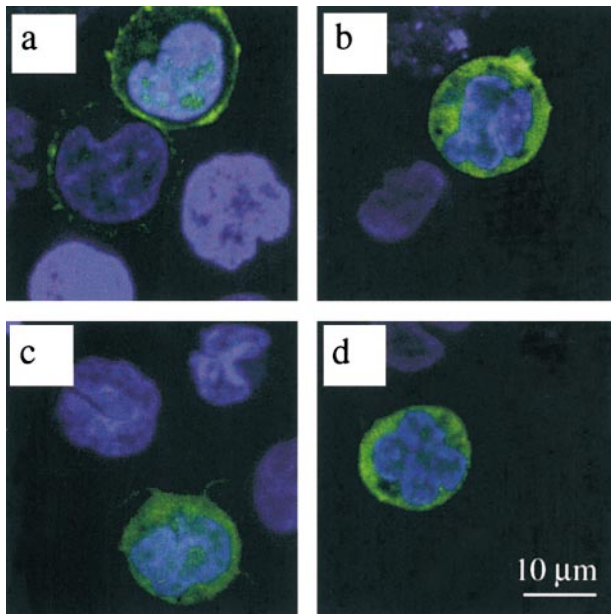
Figure 9. Pharmacological inhibition of calcium generation and tyrosine kinase activation prevents carbachol-induced translocation of DGKα. Cells were carbachol-stimulated for 2 min after pretreatments with vehicle (a), PLC inhibitor U73122 (10 μM) (b), BAPTA (50 mM) (c), and herbimycin (1 μg/ml) (d). TOPRO-3 was used to visualize nuclei.

DGKα recruitment to the plasma membrane. Finally, enzyme translocation in response to carbachol was blocked by pretreatment of the cells with the tyrosine kinase inhibitor herbimycin A (Fig. 9 d). This inhibitor is known to interfere with early T cell signaling events by inhibiting lck and ZAP-70 functions, although it does not prevent the carbachol-induced calcium increase (Graber et al., 1992). Calcium generation together with activation of tyrosine kinases are required to provide the signals necessary for receptor-induced translocation of DGKα.

Discussion

DAG phosphorylation by DGK to generate PA has long been considered an intermediate step in the resynthesis of phosphatidylinositol, with no specific role in the transduction of receptor-derived signals. Nonetheless, several recent studies have suggested a role for DGK in the regulation of cell responses elicited by external stimuli. DGKs are encoded by a large gene family conserved throughout phylogeny. This suggests that, as shown for other enzymes involved in signaling such as PKC, PLC, or phosphatidylinositide kinases, this is an extended family that shares common enzymatic activity. The simplest explanation for the existence of multiple DGK isoforms is that of redundancy in their actions. Notwithstanding, the differences in the regulatory domains of the various subtypes, together with the dissimilar expression pattern in multiple tissues for each isoform, indicate that the various DGK isoforms serve distinct although related functions. DGKα was the first enzyme of this family to be purified and have its cDNA cloned (Sakane et al., 1990). The first hint of a role for DGKα in the control of T cell responses was suggested

by the activation of this enzyme in response to IL-2, the main T cell growth factor, and the demonstration that this activity is necessary for coordinated transition of T cells from late G1 to the S phase of the cell cycle (Flores et al., 1996, 1999). These results indicated that activation of DGKα correlated with receptor-regulated signals and suggested a functional role for this enzyme in T cell response control. Binding of IL-2 to its high affinity plasma membrane receptor does not, however, cause an appreciable increase in DAG levels. As we have shown, PA is generated rapidly in response to IL-2 through the phosphorylation of a preexisting DAG pool in the cells (Jones et al., 1999).

T lymphocytes express high DGKα levels in cytosol, and the possibility that this enzyme has a role in the DAG downregulation generated in response to receptor stimulation remained to be investigated. In this study, we have demonstrated that DGKα translocates to the plasma membrane in response to two alternative receptor pathways known to promote T cell activation responses. DGKα translocation is induced through stimulation of an ectopically expressed, G protein–coupled muscarinic type 1 receptor that exerts its actions through activation of PLCβ. Our results demonstrate that DGKα translocates to the plasma membrane in response to receptor stimulation and that membrane localization of DGKα is directly related to the attenuation of transcriptionally regulated DAG-derived responses, such as CD69 cell surface expression. Moreover, translocation takes place in response to a more physiological stimulus such as TCR cross-linking, where DAG is also generated from PI4,5P$_2$ through activation of the PLCγ isoform. All together, these results indicate that receptor-stimulated DGKα activation is a general feature responsible for regulation of the cellular DAG level generated through PI4,5P$_2$ hydrolysis, a role that has been suggested but never directly demonstrated.

Here, we show that the plasma membrane localization of DGKα is tightly regulated by receptor-derived signals, where tyrosine kinase activation after receptor occupancy seems to be essential for receptor-induced translocation. Moreover, membrane localization is regulated by enzyme activity, suggesting that PA generation releases the enzyme from the membrane back to the cytosol. Experiments with the myristoylated form of the enzyme indicate that, although enzymatic activity regulates subcellular localization, the contrary is also true since targeting of the enzyme to the membrane appears to be sufficient to maintain the protein in an active conformation. Those results further confirm the close correlation between membrane localization and enzyme activity and indicate that the more important aspect when studying the physiological regulation of this enzyme by different stimuli should be that of its subcellular localization. This study also allows us to draw some important conclusions on the role of the different DGKα domains in the control of its subcellular distribution in response to receptor activation. We demonstrate the negative regulatory role of the DGKα NH$_2$-terminal domain not only by examining enzymatic activity but also by analyzing the subcellular localization of the truncated protein. Deletion of the NH$_2$-terminal domain increases enzymatic activity and induces constitutive localization of the enzyme to the plasma membrane in both Jurkat and Ba/F3 cells, suggesting that DGKα association

with the plasma membrane does not require receptor-generated signals, including DAG generation. A model can be envisaged in which the DGKα NH₂-terminal domain is necessary to prevent membrane interaction, maintaining the enzyme in a cytosolic/inactive conformation unless modified by receptor-derived signals to an active/membrane-bound conformation. The type I DGK NH₂-terminal domain is characterized by two EF hand motifs, a helix-loop-helix structure, found in a large number of functionally unrelated calcium-regulated proteins (Ikura, 1996). In many of these proteins, calcium binding induces a conformational change in the EF hand motif, leading to activation or inactivation of target proteins. DGKα purified from thymus cytosol binds calcium with an apparent K_d of 0.3 μM (Sakane et al., 1991). The simplest model for DGKα activation is that in which increases in cytosolic calcium that follow receptor ligation induce a "closed-to-open" conformational transition of the enzyme, allowing interaction of the COOH-terminal domain with the membrane. Our experiments nonetheless indicate that cytosolic calcium elevation, although necessary, is not sufficient even in combination with DAG to induce enzyme translocation. Our results suggest the existence of a more complex model in which activation of tyrosine kinases, triggered by receptor occupancy, would also participate in DGKα activation/translocation. These data concur with previous studies in which it was shown that DGKα was tyrosine phosphorylated after EGF stimulation (Schaap et al., 1993). The physiological function of this phosphorylation was not determined, although the authors suggested that tyrosine phosphorylation may play a role in DGKα association with the receptor. We do not detect tyrosine phosphorylation of the enzyme either in response to receptor stimulation or after truncation of the NH₂-terminal domain, suggesting a more complex mechanism of enzyme regulation, possibly through interaction with some adaptor-like protein. Activation of src-family tyrosine kinases has very recently been described as necessary for hepatocyte growth factor–induced DGKα activation in endothelial cells (Cutrupi et al., 2000). Although the subcellular localization of the enzyme is not examined in those studies, the authors report a direct correlation between activation of src-family kinases and DGKα activation by an undefined mechanism. As is the case in our studies, hepatocyte growth factor–induced DGKα activation does not induce DGKα phosphorylation on tyrosine residues.

Plasma membrane translocation of GFP-DGKα in response to receptor stimulation is rapid and reversible. Nevertheless, a considerable amount of enzyme is still plasma membrane bound even at 1 h after receptor stimulation. This indicates that receptor-mediated translocation is not sustained, as has been found for the in vivo translocation of certain PKC isoforms in response to physiological stimuli (Sakai et al., 1997). Carbachol-induced translocation of GFP-DGKα is no longer reversible in the presence of the DGK inhibitor R59949. Similar results are obtained with a catalytically inactive form of the enzyme, suggesting that local PA generation is necessary for enzyme release from the plasma membrane. This implies the existence of a feedback mechanism in which the enzymatic reaction product releases the enzyme from the membrane. The truncated enzyme form, which has higher activity than

the wild-type, is insensitive to this feedback regulatory mechanism since it is constitutively located in the plasma membrane. This indicates that the deleted NH₂-terminal domain, which allows translocation in response to receptor-derived signals, is also responsible for enzyme dissociation from the plasma membrane in response to high local PA generation. In this regard, in vitro experiments demonstrate that the NH₂-terminal domain of DGKα is responsible for the regulation of enzyme activity by PA and other acidic lipids (our unpublished results). Generation of PA is suggested to have a significant role in protein redistribution from cytosol to the membrane, as proposed for Rac release from RhoGDI (Tolias et al., 1998) or association of the NADPH oxidase complex (Erickson et al., 1999). In this study, we propose a novel role for PA in the regulation of DGKα membrane localization.

This study demonstrates that subcellular translocation is an important mechanism in the control of DGKα. Plasma membrane localization DGKα is tightly regulated not only by receptor-derived signals but also by its own enzymatic activity. Studies by us and other authors have indicated the existence of different subcellular localizations for this and other isoforms, suggesting that DGKs may phosphorylate different DAG pools in distinct subcellular compartments (Flores et al., 1996; Houssa et al., 1999; Topham et al., 1998; Shirai et al., 2000). DGKζ, for instance, is found in the nucleus and translocates to the cytosol through a PKC-dependent mechanism (Topham and Prescott, 1999). This translocation is proposed to be necessary for the regulated elevation of nuclear DAG during cell division.

DAG generated after receptor activation is responsible for the binding and subsequent activation of classical and novel PKCs and other proteins with phorbol ester–sensitive domains such as unc-13 and chimerins (Oancea et al., 1998). Cell fractionation studies also indicate the existence of local DAG generation in the nucleus and other internal membranes (Divecha et al., 1991; Nishizuka, 1992). The regulated targeting of different DGKs to the plasma membrane and other intracellular sites must be considered a novel mechanism to regulate the spatiotemporal differences in DAG signaling for different receptors and/or cell types. The genetic analysis of DGK deficiency in *Drosophila* and *Caenorhabditis elegans* is currently helping to elucidate the role of this enzyme as an essential component of distinct receptor-regulated events. The most severe *Drosophila* retinal degeneration mutant is generated by mutations in the *rdg-A* gene, which encodes an eye-specific DGK isoform essential for maintaining subrrabdhomeric structures in the retina (Masai et al., 1993). A recently discovered DGK isoform (DGKι) expressed in human brain and retina has 49% identity with *rdgA* and maps to a known locus of inherited retinitis pigmentosa (Ding et al., 1998). The *C. elegans dgk-1* gene codes for a protein-regulating acetylcholine release at neuromuscular junctions, attenuating the effects of serotonin on synaptic transmission (Nurrish et al., 1999). A general model can be conceived in which distinct DGK isoforms coupled to different receptors provide a mechanism of DAG consumption. This would act in concert with pathways that lead to the generation of this second messenger, guaranteeing the strict control of DAG-regulated responses such as CD69 cell surface expression in the model we studied (Fig. 10).
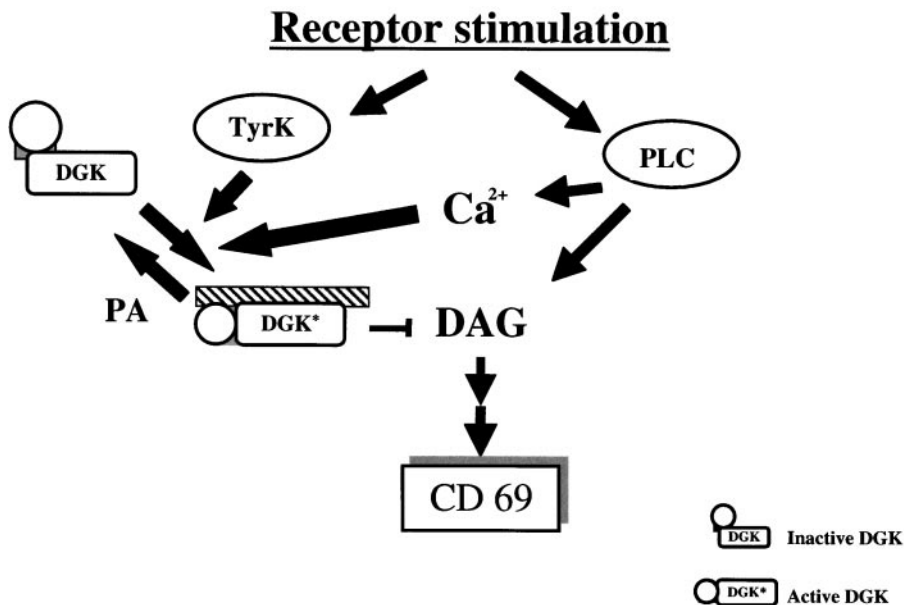
Receptor stimulation

Figure 10. A model for receptor regulation of DAG generation and consumption. After receptor stimulation, DAG is generated through PLC activation. Receptor-dependent signals including activation of tyrosine kinases together with a PLC-dependent intracellular calcium increase are responsible for the translocation of the cytosolic/inactive DGK to the plasma membrane. Generation of PA induces the release of the enzyme from the plasma membrane to a cytosolic localization. Membrane localization of the active enzyme (DGK*) regulates the intensity of the DAG-derived responses (such as induction of CD69 in this model) by converting DAG to PA.

The magnitude, duration, and frequency of activation of a signaling pathway exert a major influence on the cellular response, which in turn demands that attention be paid to the various control mechanisms that attenuate these signals. Our studies indicate that DGKα is another player in the generation of the complex scaffold of signaling proteins that locate to the plasma membrane in response to receptor activation. A better knowledge of the role of DGKα in the termination and propagation of the signals generated after triggering of plasma membrane receptors will be the object of future studies.

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218

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