Diacylglycerol Kinase δ Suppresses ER-to-Golgi Traffic via Its SAM and PH Domains

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We report here that the anterograde transport from the endoplasmic reticulum (ER) to the Golgi was markedly suppressed by diacylglycerol kinase δ (DGK δ) that uniquely possesses a pleckstrin homology (PH) and a sterile α motif (SAM) domain. A low-level expression of DGK δ in NIH3T3 cells caused redistribution into the ER of the marker proteins of the Golgi membranes and the vesicular-tubular clusters (VTCs). In this case DGK δ delayed the ER-to-Golgi traffic of vesicular stomatitis virus glycoprotein (VSV G) and also the reassembly of the Golgi apparatus after brefeldin A (BFA) treatment and washout. DGK δ was demonstrated to associate with the ER through its C-terminal SAM domain acting as an ER-targeting motif. Both of the SAM domain and the N-terminal PH domain of DGK δ were needed to exert its effects on ER-to-Golgi traffic. Kinase-dead mutants of DGK δ were also effective as the wild-type enzyme, suggesting that the catalytic activity of DGK was not involved in the present observation. Remarkably, the expression of DGK δ abrogated formation of COPII-coated structures labeled with Sec13p without affecting COPI structures. These findings indicate that DGK δ negatively regulates ER-to-Golgi traffic by selectively inhibiting the formation of ER export sites without significantly affecting retrograde transport.

INTRODUCTION

Diacylglycerol kinase (DGK) phosphorylates diacylglycerol to yield phosphatidic acid and is known in higher eukaryotes to be composed of a family of nine related genes (Topham and Prescott, 1999; van Blitterswijk and Houssa, 2000). All of the family members show unique structural features, suggesting different mechanisms of the enzyme regulation and distinct functions of DGK isozymes. DGK δ uniquely contains a set of folds consisting of a pleckstrin homology (PH) domain at the N-terminus and a sterile α motif (SAM) domain at the C terminus (see Figure 1; Sakane *et al.*, 1996). The functional significance of these domains remains unknown and needs to be defined in order to address the specific function of this DGK species. SAM domain

has been detected in a wide range of proteins involved in developmental regulation and signal transduction (Schultz *et al.*, 1997). Initially, SAM domain was reported to serve as the site of homotypic oligomerization (Stapleton *et al.*, 1999; Thanos *et al.*, 1999), although several SAM-containing proteins were later found to be monomeric (Chi *et al.*, 1999; Smalla *et al.*, 1999; Wang *et al.*, 2001). In view of the EphB2 binding to the SH2 domain upon tyrosine phosphorylation (Stein *et al.*, 1996), the function of SAM domains appears to be variable in different proteins. The PH domain is another structural module of a wide occurrence and has been found in more than a hundred different proteins involved in intracellular signaling, cytoskeletal organization, vesicular transport, and lipid metabolism (Rebecchi and Scarlata, 1998; Lemmon and Ferguson, 2000). This domain generally binds phosphoinositides, albeit with extremely varying affinities depending on the structural classification (Rebecchi and Scarlata, 1998; Lemmon and Ferguson, 2000). In the case of DGK δ , its PH domain showed a weak and promiscuous binding affinity to phosphoinositides in *in vitro* binding experiments (Kavran *et al.*, 1998), the physiological significance of which remains unknown.

In the early secretory pathway, the membrane components are continuously recycled among the constituent compartments (Cole *et al.*, 1998; Storrie *et al.*, 1998), resulting in the constitutive transport of cargo molecules to the downstream secretory pathway. The dynamics of anterograde and

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Abbreviations used: BFA, brefeldin A; BSA, bovine serum albumin; CFP, cyan fluorescent protein; DGK, diacylglycerol kinase; DMEM, Dulbecco's modified minimum essential medium; ER, endoplasmic reticulum; GFP, green fluorescent protein; GT, galactosyltransferase; PBS, phosphate-buffered saline; PH, pleckstrin homology; SAM, sterile α motif; VSV G, vesicular stomatitis virus glycoprotein; VTC, vesicular-tubular cluster; YFP, yellow fluorescent protein.

retrograde transports is tightly linked to attachment to the membranes of the two types of proteinaceous coat complexes, COPI and COPII (Kirchhausen, 2001). Recent studies have revealed that coat formations are strictly controlled processes in which multiple proteins are sequentially involved (Kirchhausen, 2001). Attachment of COPI to the donor membranes is triggered by recruitment of ARF1-GTP to membranes (Donaldson *et al.*, 1992; Helms and Rothman, 1992). The membrane association of ARF1 coupled to its activation is regulated positively by guanine nucleotide exchange factors (GEFs) and negatively by GTPase-activating proteins (GAPs; Puertollano *et al.*, 2001). On the other hand, the attachment of COPII coat, which occurs only in the ER membranes, is caused by recruitment of GTP-Sar1p (Kuge *et al.*, 1994). The activated Sar1p recruits the Sec23/24 complex, and budding of cargo-containing vesicles requires additional protein complex of Sec13/31 (Matsuoka *et al.*, 1998). The experiments using permeabilized cells in the presence and absence of cytosol have shown that GTP-Sar1p alone can determine the ER export sites and that the cytosol contains suppressive factors interfering with the formation of COPII coats (Aridor *et al.*, 2001). Only limited information of the cytosolic negative regulators has been available except for the GAP activity of Sec23p itself (Saito-Nakano and Nakano, 2000).

In the present work, we describe unexpected findings on the negative regulation by DGK δ of the COPII coat formation at the ER exit sites where anterograde transport is initiated. It is known that inhibition of each step of membrane transport in the early secretory pathway often results in redistribution of membrane proteins (e.g., Storrie *et al.*, 1998). We therefore monitored in single cells the disturbances of membrane protein dynamics caused by DGK δ at the early stage of its expression. We also examined the effects of DGK δ on the two types of anterograde transport: reassembly of Golgi membrane proteins from brefeldin A (BFA)-induced fusion with the ER and the transport of temperature-sensitive folding mutant (ts045) of vesicular stomatitis virus glycoprotein (VSV G). We found that DGK δ inhibited ER-to-Golgi traffic through interfering with the formation of COPII-coated structures. Interestingly, the blockage of anterograde transport occurred only when DGK δ equipped with an intact PH domain was targeted to the ER membranes via its SAM domain.

MATERIALS AND METHODS

Plasmid Construction

To construct expression vectors coding for the full-length DGK δ fused at its N-terminus to green fluorescent protein (GFP-DGK δ FL), *SalI* and *XbaI* sites were created by PCR at nucleotides 75 and 3594 of the DGK δ cDNA (Sakane *et al.*, 1996). The restricted fragment was ligated in-frame into the corresponding sites of pEGFP-C3 (Clontech, Tokyo, Japan). Similarly, cDNAs encoding several deletion mutants of DGK δ , such as those lacking SAM domain (DGK δ Δ SAM), PH domain (DGK δ Δ PH), or residues 294–318 in the catalytic region (DGK δ Δ 294–318), were amplified from pSRE-DGK δ (Sakane *et al.*, 1996) using the specific primers with *SalI* and *XbaI* sites at the ends. The amplified fragments were digested and subsequently subcloned into the *XhoI*-*XbaI* site of pEGFP-C3 to construct GFP-tagged chimera. The expression plasmids encoding cyan fluorescent protein (CFP)-fused DGK δ were also prepared using pECFP-C1 (Clontech). Point mutations were introduced into the PH

domain (DGK δ R28E) and the catalytic site (DGK δ G337D) as described in the QuickChange protocol (Stratagene, La Jolla, CA) using the sets of complementary oligonucleotides (DGK δ R28E: 5'-CATTCCAGCGAT-CAAAAGAGAGATACITTAAGCTTC-3' and 5'-GAAGCTTAAAGT-ATCTCTCTTTTGATCGCTGGAATG-3'; DGK δ G337D: 5'-GTGGCGG-GGATGACAGTGTGGCTGGG-3' and 5'-CCCAGCCAACACTGTCA-TCCCCGCCAC-3'). The SAM domain of DGK δ (3292–3532 base pairs) was also in-frame fused to pEGFP-C3 or to a protein A fusion vector, pRIT2 (Amersham Pharmacia Biotech, Tokyo, Japan). The constructs of DGK δ used in the present work are summarized in Figure 1.

To construct an expression vector for yellow fluorescent protein (YFP)-tagged KDEL receptor, the cDNA encoding ELP1, a human homolog of Erd2 (Hsu *et al.*, 1992), was obtained from the human liver QUICK-Clone cDNA (Clontech), and *SalI* and *ApaI* sites were created by PCR at the ends of the cDNA. The fragment was digested with *SalI* and *ApaI* and ligated in-frame into the corresponding sites of pEYFP-N1 (Clontech). Vectors for YFP-GT_{1–81} (GT_{1–81}; the amino-terminal 81 amino acids of human β -1,4-galactosyltransferase) and CFP-GT_{1–81} (pEYFP-Golgi and pECFP-Golgi, respectively) were purchased from Clontech. An expression vector for YFP-ERGIC53 (Itin *et al.*, 1996) was constructed as follows. The ERGIC53 cDNA was first obtained by reverse-transcribing HepG2 cell mRNA. *BsrGI* sites were then created at both ends by PCR, followed by *BsrGI* restriction, and ligated to pEYFP-ER (Clontech). An expression vector for hSec13-YFP was constructed by subcloning its cDNA, isolated from HepG2 cells, in-frame into pEYFP-N1 (Clontech) as described previously (Hammond and Glick, 2000). The authenticity of all cDNA constructs was verified by DNA sequencing.

Cell Culture, Transfection, and Time-lapse Analysis

NIH3T3, COS7, or normal rat kidney (NRK) cells (HSRRB, Osaka, Japan) were cultured in Dulbecco's modified minimum essential medium (DMEM) supplemented with 10% fetal calf serum at 37°C under 5% CO₂. In the experiments given in Figures 3, 8, and 9,

Figure 1. The schematic structures of DGK δ and its GFP-tagged mutants. The structure of the full-length DGK δ (DGK δ FL) is shown as previously designated (Sakane *et al.*, 1996). PH, Pleckstrin homology domain; C3, zinc finger structures; C4-a and C4-b, catalytic regions separated by an insertion of 317 residues; SAM, sterile α motif. GFP-DGK δ FL corresponds to the full-length DGK δ fused to GFP. Truncation mutants of DGK δ fused to GFP were, residues 101–1170 (GFP-DGK δ Δ PH), residues 1–1096 (GFP-DGK δ Δ SAM) and residues 1098–1170 (GFP-DGK δ SAM), respectively. In the case of GFP-DGK δ R28E and GFP-DGK δ G337D, Arg-28 and Gly-337 were changed to Glu and Asp, respectively. In GFP-DGK δ Δ (294–318), a region of residues 294–318 was deleted from the full-length DGK δ . In some experiments (given in Figures 5–7), DGK δ were fused to the cyan variant, CFP, instead of GFP.

plasmids were transfected into cells using LipofectAMINE PLUS (Life Technologies-BRL, Gaithersburg, MD) according to the manufacturer's instructions. VSV G (ts045) cDNA was kindly donated by Dr. T. Nakada (Tokyo University) and subcloned into pECFP-N1 (Clontech) essentially as described previously (Presley *et al.*, 1997). To achieve synchronized transport of VSV G (ts045)-CFP, NIH3T3 cells were incubated at 39.5°C for 24 h after introduction of expression vectors, and the anterograde transport of VSV G was then initiated in the medium prewarmed to 32°C. For detecting disturbance of membrane protein dynamics in single cells (see Figures 5–7 and 10), expression plasmids were introduced mechanically into the cells using siliconized glass microbeads. The original method using the beads (McNeil and Warder, 1987) was to deliver small molecules but was unsuitable for introducing large molecules such as plasmid DNA. However, we found that plasmid DNA could be efficiently introduced to cells when the beads were siliconized. This method was adopted because of its synchronized and rapid protein expression (see RESULTS), thus enabling us to achieve time-lapse analysis of cells from 30 min to 6 h of post-plasmid loading. Briefly, acid-washed beads ($\phi < 106 \mu\text{m}$; Sigma-Aldrich, Tokyo, Japan) were siliconized in a closed chamber by dimethyldichlorosilane (Sigma-Aldrich) for 1 h at room temperature and then heated to 180°C for 3 h. Before plasmid loading, the medium was removed, and 1–2 μl of DNA (3 $\mu\text{g}/\text{ml}$ DMEM) was placed onto the cells cultured on glass-based dishes (ATG, Tokyo, Japan). The cells were then covered with ca. 100 μl of dry glass beads, tapped vertically three times, and immediately rinsed with DMEM to remove the beads. The cells were further cultured in Phenol Red-free, CO_2 -independent MEM (Life Technologies-BRL) supplemented with 10% (wt/vol) fetal bovine serum on the stage of an inverted confocal laser microscope (LSM 510; Zeiss, Thornwood, NY) with a 100 $\times$ oil planapochromat lens (NA 1.4). The temperature on the microscope stage was maintained using an objective lens heater (Biophtechs, Butler, PA) in combination with a stage heater (Kitazato Supply, Fujinomiya, Japan). Images were captured and processed using Photoshop 5.0 (Adobe Systems, San Jose, CA). IPLab (Scanalytics, Fairfax, VA) was used to quantitate the signal.

Indirect Immunofluorescence

Cells cultured on coverslips were fixed for 10 min at room temperature with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.2% Triton X-100 for 1 min on ice. Alternatively, cells were fixed with methanol for 4 min at -20°C . The fixed and permeabilized cells were blocked in 0.1% bovine serum albumin (BSA) in PBS for 30 min and then incubated with the antibody solution in the blocking buffer for 45 min at 37°C. Cells were washed three times with PBS (for 5 min each) and then incubated with Alexa 594-conjugated anti-mouse IgG antibody (Molecular Probes, Eugene, OR) for 30 min at room temperature. The cells were again washed three times with PBS (5 min each), and the coverslips were mounted onto glass-slides using VECTASHIELD (Vector Laboratories, Burlingame, CA). Images were taken with an inverted confocal laser scanning microscopy (Zeiss LSM 510) with a 40 $\times$ oil plan-neofluar (NA1.3; Figure 5A) or a 100 $\times$ oil objective lens (other figures) and processed by Adobe Photoshop version 5.0.

Preparation of Protein A-DGK δ SAM Fusion Protein

Escherichia coli N4830–1 was transformed with pRIT2 vector (Amersham Pharmacia) encoding protein A-DGK δ SAM fusion protein. Protein expression was induced by incubating cells at 42°C for 6 h. The harvested cells were then washed twice with PBS, suspended in 0.1% Tween 20 in PBS, and lysed by sonication. The lysate was centrifuged at 12,000 $\times g$ for 20 min to remove cell debris, and the protein A fusion protein was purified using IgG-Sepharose (Amersham Pharmacia).

Preparation of ER-enriched Microsomes

ER-enriched microsomes were prepared essentially as described previously (Kappeler *et al.*, 1997). Briefly, mouse liver was homogenized with a Dounce homogenizer in 5 ml of homogenizing buffer/g tissues (10 mM Bes-KOH, pH 7.2/120 mM NaCl/5 mM KCl) supplemented with protease inhibitor cocktail (Roche, Tokyo, Japan). The homogenate was centrifuged at 10,000 $\times g$ for 10 min. The supernatant was then centrifuged at 300,000 $\times g$ for 30 min. The 300,000 $\times g$ pellet (microsomes) was suspended in the homogenizing buffer containing 35% (wt/vol) Nycodenz (Nycodenz Pharma, Oslo, Norway) and transferred to centrifuge tubes. A Nycodenz gradient of 29–13% in the homogenizing buffer was made on top of the microsomal suspension and centrifuged at 36,000 rpm at 4°C for 3 h in a Hitachi (Tokyo, Japan) RPS-40T rotor. Ten 1-ml fractions were collected from the bottom, and each fraction was subjected to Western blot analysis using antibodies to GM130 (Transduction laboratories, Lexington, KY), ERGIC53 (a gift of Dr. F. Tokunaga at Himeji Institute of Technology, Himeji, Japan), and calnexin (Stress-Gen Biotech, Sidney, British Columbia, Canada). Fractions enriched with calnexin were diluted with the homogenizing buffer and centrifuged at 300,000 $\times g$ for 30 min. The pellet was then resuspended with homogenization buffer. The ER-enriched microsomes thus prepared were further incubated with 1 M KCl in the buffer for 10 min on ice and then recovered by centrifuging at 300,000 $\times g$ for 30 min. The salt-washed microsomes were dialyzed against the binding buffer as described below and stored at -80°C .

Binding Assay

The salt-washed microsomes (100 μg of protein) were first preincubated at 4°C for 10 min with BSA (10 mg/ml) in the binding buffer containing 10 mM Bes-KOH (pH 7.2), 120 mM potassium acetate, 2 mM magnesium acetate, and 5 mM sodium acetate to block non-specific binding. After preincubation, the microsomes were further incubated with protein A-DGK δ SAM fusion protein in the same buffer for 10 min at 4°C. The microsomes were then recovered by centrifugation at 300,000 $\times g$ for 30 min and washed once with the binding buffer. The pellets were analyzed for the bound protein A fusion protein by Western blotting using horse radish peroxidase-conjugated IgG, and the signals were detected by SuperSignal (Pierce, Rockford, IL). In some experiments, the microsomes (100 μg of protein) were treated with trypsin (11 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) for 5 min at 37°C before the binding studies. The mixture was added with aprotinin (44 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) and was subjected to the binding assays.

RESULTS

ER Localization of DGK δ

Previously, we reported that most of DGK δ expressed in COS7 cells was recovered in the total particulate fraction (Sakane *et al.*, 1996) despite the apparent lack of membrane-binding sequence motifs. We therefore attempted first to define the intracellular distribution of DGK δ and also to characterize the mechanisms underlying its membrane association. The analysis of cellular DGK δ protein in the subcellular fractions of NIH3T3 cells showed that DGK δ was markedly enriched in the microsomal fraction (Figure 2A) compared with the cytosol. To examine the nature of the enzyme interactions with microsomes, we next treated the membranes with chemicals that selectively disrupt interactions on the surface without extracting proteins from phospholipid bilayers. Treatments with alkaline buffer and high salt concentration, as well as incubation with 6 M urea, all abolished the membrane association of DGK δ (Figure 2B, lanes 3–8). This suggests that DGK δ associates with the

Figure 2. Association of endogenous DGK δ with microsomes. (A) Homogenates of NIH3T3 cells were subjected to subcellular fractionation as described in MATERIALS AND METHODS. Ten micrograms of proteins of the cytosolic (lane 1) and microsomal (lane 2) fractions was analyzed by SDS-PAGE (10%) followed by Western blot analysis using anti-DGK δ antibody (Sakane *et al.*, 1996). (B) Microsomes (10 μ g) were incubated for 30 min on ice with homogenization buffer alone (lanes 1 and 2) or the buffer containing 1 M KCl (lanes 3 and 4), or 6 M urea (lanes 7 and 8). The microsomes were also treated with the buffer added with 0.2 M sodium carbonate (pH 11.0; lanes 5 and 6). After treatments, the microsomes were separated into supernatant (S) and pellet (P) fractions by centrifuging at $300,000 \times g$ for 30 min. Microsomes (10 μ g) were also subjected to Triton X-114 phase separation, and the insoluble aggregates (P, lane 9), aqueous phase (Aq, lane 11), and detergent phase (D, lane 10) were obtained. Samples were separated by SDS-PAGE (7.5%) and analyzed by Western blotting using anti-DGK δ antibody.

microsomal surface through ionic protein/protein interactions. Consistent with this notion, the microsomal DGK δ partitioned into the aqueous phase in the phase separation experiments using Triton X-114 (Bordier, 1981), although a significant fraction was not solubilized (Figure 2B, lane 9). We also noted that a part of the microsomal DGK δ remained insoluble when treated with Triton X-100. These data indicate that a considerable part of the cellular DGK δ is associated with the microsomal surface, although a minor portion exists in the cytosol and in the detergent-insoluble cytoskeletal elements.

We next expressed in NIH3T3 cells GFP-tagged DGK δ in order to further characterize its intracellular localization in intact cells. In the experiments given in Figure 3, the cells at 24 h posttransfection were immunostained with antibody against BiP, an ER marker. Although the localization profiles of the full-length DGK δ were rather heterogeneous, a portion of the GFP-DGK δ signal was consistently overlapped with BiP. Because of the cytoplasmic signal, the structure of the ER as detected by the BiP staining (Figure 3b) was not clearly demarcated by GFP-DGK δ , except for the nuclear rim (Figure 3a, inset). The N-terminus tagging of DGK δ with GFP had little influence on the enzyme localization because immunostaining of nontagged DGK δ expressed in NIH3T3 cells showed a staining pattern indistinguishable from that given in Figure 3a. The heterogeneous distribution of DGK δ indicates two possibilities; 1) the enzyme contains multiple localization signals resulting in its association with different intracellular sites, or 2) DGK δ has no particular localization signal. To distinguish between these possibilities, we made a series of truncated DGK δ mutants fused N-terminally to

Figure 3. The SAM domain of DGK δ serves as an ER-targeting signal. NIH3T3 cells were transfected with the expression plasmids for GFP-DGK δ FL (a–c), GFP-DGK δ ΔSAM (d–f), or GFP-DGK δ SAM (g–i). At 24 h after transfection, the cells were fixed with methanol at -20°C and immunostained with anti-BiP antibody (b, e, and h). Note that GFP-DGK δ FL (a) was partially colocalized with BiP (b), particularly at the nuclear rim (insets). In contrast, DGK δ ΔSAM (d) showed little overlap with BiP (e). Distribution of GFP-DGK δ SAM (g) was nearly identical to BiP (h). Bars, 10 μ m. DGK δ FL, the full-length DGK δ ; DGK δ ΔSAM, DGK δ lacking SAM domain; DGK δ SAM, the SAM domain at the C terminus of DGK δ .

GFP (Figure 1). When their distributions were examined in transfected cells, we found that deletion of the C-terminal SAM domain abrogated its signal at the ER (Figure 3, d–f). Other deletion mutants with intact SAM domain showed cellular localizations indistinguishable from those of the full-length enzyme. If the SAM domain possesses an ER-targeting signal, then GFP fused to the SAM domain alone should localize in the ER. We thus expressed the GFP-tagged SAM domain and found that the localization pattern was nearly identical to that of BiP (Figure 3, g–i). These data suggest that the SAM domain is responsible for targeting DGK δ to the ER.

To confirm that the DGK δ SAM domain acts as an ER-anchoring sequence, we intended to see whether the SAM domain could bind to the ER *in vitro*. For this purpose, we expressed and purified DGK δ SAM fused to protein A at its N-terminus. As the source of the ER membranes, we used mouse liver microsomes highly enriched with the ER on a Nycodenz gradient. The ER preparation was further washed with 1 M KCl to remove endogenous DGK δ . The ER fraction thus prepared was incubated with protein A fused to DGK δ SAM, and then the ER membranes were recovered through a sucrose cushion. As shown in lanes 2 and 4 of Figure 4, DGK δ SAM fused to Protein A, but not unfused Protein A, was bound to the recovered ER membranes. Because SAM domains are generally known to interact with other proteins

(Schultz *et al.*, 1997), we tested whether the binding of DGK δ SAM to the ER depended on protein–protein interactions. As seen in lane 1 of Figure 4, the binding of DGK δ SAM to the ER was markedly reduced when the membranes were pretreated with trypsin. Taken together with the effects of the SAM domain on the intracellular localization (Figure 3), it becomes now clear that DGK δ binds to the ER membrane protein(s) through its SAM domain acting as an ER-targeting signal.

Redistribution of Golgi Markers Caused by DGK δ Expression

We next examined the possibility that the ER-resident DGK δ might be involved in the regulation of vesicular traffics. In the early secretory pathway, membrane components are rapidly exchanged among the organelle compartments, resulting in the achievements of their specific distributions (Cole *et al.*, 1998; Storrie *et al.*, 1998). Alteration of each process of the pathway should cause a shift in the apparent distribution of the membrane components at a given time. Hence, we monitored disturbance of membrane protein distribution in a single cell upon expression of DGK δ . Initially, we examined the effects of DGK δ overexpression on an endogenous Golgi membrane protein, GS15. After expressing DGK δ fused to GFP for 36 h using Fugene 6, the cells were fixed, and the distribution of GS15 was observed at a low magnification. As shown in Figure 5A, whereas GS15 signals in control cells were confined to the ribbon-like juxtannuclear structures of Golgi apparatus, the Golgi protein in all cells expressing DGK δ -GFP was redistributed to the ER-like perinuclear structures. We next attempted to keep the expression level of the various constructs as low as possible to minimize the spurious effects of overexpression.

Figure 4. Binding in vitro of DGK δ SAM to ER-enriched microsomes. ER-enriched microsomes purified by Nycodenz gradients were further treated with 1 M KCl to remove endogenous DGK δ . The salt-washed microsomes were preincubated with (lanes 1 and 3) or without trypsin (lanes 2 and 4) for 30 min on ice, and then aprotinin was added to stop the digestion. The pretreated microsomes were next incubated with protein A-DGK δ SAM (lanes 1 and 2) or protein A (lanes 3 and 4) in the binding buffer containing BSA (10 mg/ml) for 10 min on ice. Microsomes were then recovered by centrifugation and analyzed by SDS-PAGE (12.5%) followed by Western blotting using horseradish peroxidase-conjugated rabbit IgG. The blots were stripped and reprobed with anti-BiP antibody to confirm an equal loading of the microsomal fractions.

Figure 5. Redistribution of Golgi membrane proteins caused by DGK δ expression. (A) COS7 cells were transfected with the expression plasmids for GFP-DGK δ FL (a–d) or GFP (e and f) using Fugene 6. At 36 h after transfection, the cells were fixed with paraformaldehyde and immunostained with anti-GS15 antibody (Transduction Laboratories, Lexington, KY) and Alexa 594-conjugated anti-mouse antibody (b, d, and f). Arrows show cells expressing GFP-DGK δ FL or GFP. Bars, 50 μ m. (B) pEYFP-GT_{1–81} and expression vectors for CFP (a–d), CFP-DGK δ FL (e–h), or CFP-DGK δ SAM (i–l) were introduced into COS7 cells using siliconized glass microbeads as described in MATERIALS AND METHODS. Confocal images were taken after 1 h at 30-min intervals. Shown are the images at 2 h (a, c, e, g, i, and k) and 4 h (b, d, f, h, j, and l) of plasmid loading. Bars, 10 μ m. (C) On the basis of the assumption that the juxtannuclear aggregates with intense signal of YFP-GT_{1–81} represented the Golgi region, we selected such areas in the LSM images by setting threshold fluorescence levels using IPLab software (Scanalytics, VA). Distribution of YFP-GT_{1–81} was calculated by dividing fluorescent intensities at the Golgi by those of whole cell signal at 4 h after plasmid loading. Mean values $\pm$ SEM of four cells are presented. (D) Expression levels of DGK δ and distribution of YFP-GT_{1–81}. Either of the vectors for CFP (open triangles) or CFP-DGK δ FL (open rhombuses) was cotransduced into COS7 cells with the vector for YFP-GT_{1–81} using microbeads. At 4 h after loading, the cells were fixed with methanol and immunostained with anti-DGK δ antibody or preimmune IgG followed by secondary anti-rabbit antibody conjugated to Alexa 594 (Molecular Probes, Eugene, OR). The YFP and Alexa594 signals of the LSM images were quantitated by IPLab. In quantitating the Alexa594 signal, the average signal obtained by the preimmune IgG incubation was set to zero. Distribution of YFP-GT_{1–81} was calculated as in C. The relative expression levels of DGK δ were obtained by dividing the whole-cell Alexa 594 signal in CFP-DGK δ FL-expressing cells by the averaged endogenous DGK signal in control CFP-expressing cells and were compared with distribution of YFP-GT_{1–81} in the same cell.

Figure 5 (cont).

At the same time, we attempted to evaluate the effects of DGK δ at a very early phase of its expression so that we could minimize various adaptive responses of the cells. For this purpose, we directly introduced into COS7 cells expression plasmids using siliconized glass microbeads. Throughout these experiments, we usually obtained detectable protein expression as early as 1 h of bead loading.

To monitor possible perturbation of membrane dynamics, cells were introduced with expression plasmids of specific membrane markers tagged with YFP. In the initial experiments (Figure 5B), we introduced plasmids of CFP-DGK δ and of YFP fused to the N-terminal 81 amino acids of galactosyltransferase (YFP-GT₁₋₈₁), thus engineered to possess *medial-trans* Golgi localization signal (Yamaguchi and Fukuda, 1995). YFP-GT₁₋₈₁ usually became detectable as early as 1 h postloading, whereas CFP-DGK δ was barely detected during the first 2 h (Figure 5B, e). At 2 h postloading, YFP-GT₁₋₈₁ (Figure 5B, g) was mostly detected at the juxtannuclear region, and little ER network was labeled. However, CFP-DGK δ became detectable after 4 h of incubation (Figure 5B, f), when the YFP signal at the juxtannuclear Golgi region in the same cell was diminished and the ER network structure was highlighted (Figure 5B, h). In contrast, when a plasmid for CFP was loaded (Figure 5B, a–d), no redistribution of YFP-GT₁₋₈₁ to the ER was observed (Figure 5B, d). These findings demonstrated that DGK δ caused redistribution of the Golgi proteins into the ER at a very early phase of its expression. In these experiments, we confirmed that redistribution of YFP-GT₁₋₈₁ occurred similarly when nontagged full-length DGK δ was expressed instead of CFP-fusion protein. We also confirmed that the expression of the full-length DGK γ (Kai *et al.*, 1994) fused N-terminally to GFP failed to affect the distribution of the Golgi marker during the experimental periods. If the observed effects of DGK δ were caused by its association with the ER membranes via the SAM domain as shown in Figures 3 and 4, deletion of this domain should abolish the redistribution of Golgi markers. Hence the cells were coloaded with plasmids coding for CFP-DGK δ Δ SAM and YFP-GT₁₋₈₁ (Figure 5B, i–l) and cultured on a microscope stage for up to 4 h. As expected, the intense YFP signal at the Golgi region was unchanged (Figure 5B, l) even after expression of CFP-DGK δ Δ SAM (Figure 5B, j), and little signal was detected at the ER network (Figure 5B, i). Quantitation of the YFP signal intensity at the Golgi region revealed that nearly 60% of Golgi membrane protein was redistributed to the reticular network within 4 h of DGK δ expression, whereas CFP or CFP-DGK δ Δ SAM caused little change in its distribution (Figure 5C). To assess how many fold overexpression is required for the phenotype, we measured the levels of DGK δ by immunostaining using anti DGK δ antibody. As shown in Figure 5D, increased ER localization of YFP-GT₁₋₈₁ was detectable at a few-fold enhancement of DGK δ over the endogenous level. It becomes thus clear that relatively low-level expression of DGK δ is sufficient to cause apparent redistribution of the Golgi proteins (Figure 5D).

Considering the constitutive recycling of Golgi membrane components to the ER (Cole *et al.*, 1998; Storrie *et al.*, 1998), the above data indicate that DGK δ may affect membrane dynamics in the early secretory pathway. Because the kinetics of recycling of Golgi membrane proteins was generally known to be slow (Cole *et al.*, 1998; Storrie *et al.*, 1998; Zaai

et al., 1999), we used in the next experiments the two membrane proteins that rapidly recycle via COPI mediated retrograde transport: KDEL receptor (Hsu *et al.*, 1992) and ERGIC53 (Hauri *et al.*, 2000). To visualize the KDEL receptor, which is involved in the retrieval of ER-resident proteins from the *cis*-Golgi network to the ER, YFP was fused to the C terminus of the KDEL receptor. When KDEL receptor-YFP was expressed, intense labeling of the Golgi region was observed with a faint ER signal (Figure 6A, c and d). In the cells coexpressing CFP and KDEL receptor, the ratio of the signals between the ER and Golgi remained unchanged throughout the experiments (Figure 6A, c and d, and 6B). In contrast, even at 2 h when CFP-DGK δ was expressed to a very limited extent, the signal of KDEL receptor-YFP at the Golgi area was significantly reduced with its redistribution to the reticular network of the ER (Figure 6A, g). More redistribution was observed at 4 h (Figure 6A, h, and 6B). This phenotype was not observed when DGK δ lacking SAM domain was introduced (Figure 6A, k and l). We also used another well-characterized recycling protein, ERGIC53 (Hauri *et al.*, 2000), a marker of vesicular-tubular clusters (VTCs). Because this type I membrane protein contains a cytoplasmic tail that interacts with COPI (Tisdale *et al.*, 1997) and COPII coats (Kappeler *et al.*, 1997), we inserted YFP immediately after the signal sequence cleavage site so that the cytoplasmic tail was unchanged. YFP-tagged ERGIC53 showed a distribution pattern identical to that of untagged ERGIC53 when expressed in NIH3T3 cells. Different from the localization profile of the KDEL receptor (Figure 6A), a portion of ERGIC53 was found in punctate structures adjacent to the ER network (Figure 7, c and d). However, upon expression of DGK δ , such clusters were nearly abolished and the ER network became highlighted (Figure 7, g and h). Consistent with the observations described above, a DGK δ mutant lacking the C-terminal SAM domain had little influence on the dynamics of YFP-ERGIC53 (Figure 7, k and l).

Inhibition of ER-to-Golgi Traffic by DGK δ

The results described so far suggested that the ER-resident DGK δ disturbed membrane dynamics of the early secretory pathway by affecting the rate-limiting steps operating on the ER membranes. To determine if the ER exit was regulated by DGK δ , we next studied its effects on the two types of anterograde transport: reformation of the Golgi after BFA treatment and transport of VSV G. A fungus metabolite, BFA, causes rapid redistribution of Golgi membrane proteins to the ER and blocks the ER-to-Golgi transport (Fujiwara *et al.*, 1988). Removal of BFA reinitiates rapid anterograde membrane transport from the ER, resulting in the reformation of Golgi apparatus (Fujiwara *et al.*, 1988). We treated NIH3T3 cells with BFA and observed the time-dependent reassembly of CFP-GT₁₋₈₁ upon washout of BFA. As shown in Figure 8b, the Golgi apparatus was completely reformed within 30 min after removal of BFA from the cells. However, the expression of DGK δ markedly retarded the reformation of the Golgi apparatus in all cells examined. This retardation was not seen when the SAM domain was omitted (Figure 8h), in accordance with the results obtained for Golgi marker redistribution (Figure 5). Furthermore, we found that in addition to the SAM domain, the N-terminal PH domain was also required for the effects of DGK δ as shown for the PH domain-deleted mutant (Figure 8, e and f).

Figure 6. Redistribution of KDEL receptor-YFP caused by DGK δ expression. (A) The expression vector coding for KDEL receptor (KDEL-R)-YFP was coloaded to COS7 cells using microbeads with those encoding CFP (a–d), CFP-DGK δ FL (e–h), or CFP-DGK δ ΔSAM (i–l). LSM images were recorded as in Figure 5. Bars, 10 μ m. (B) The intense signal of KDEL-R-YFP at the Golgi areas was quantitated as in Figure 5C.

Figure 7. Redistribution of YFP-ERGIC53 caused by DGK δ expression. The expression vector coding for YFP-ERGIC53 was introduced into COS7 cells using microbeads together with those encoding CFP (a–d), CFP-DGK δ FL (e–h), or CFP-DGK δ ΔSAM (i–l). Confocal images were taken as in Figure 5. Bars, 10 μ m.

In the next experiments analyzing the constitutive transport from the ER to the Golgi, we used a temperature-sensitive folding mutant of VSV G, ts045, tagged to CFP. This type I membrane protein is the most extensively studied cargo protein, and it has been shown that the cytoplasmic tail directly interacts with Sar1p to trigger the COPII coat assembly (Aridor *et al.*, 2001). At the restrictive temperature, VSV G-CFP was mostly arrested in the ER (Figure 9, b, f, j, and n), and a rapid transport to the juxtanuclear region occurred maximally at 9 min after a temperature shift to 32°C (Figure 9, c and d). When GFP-DGK δ was coexpressed, the transport of VSV G-CFP to the Golgi region was barely detected at 9 min (Figure 9h). In contrast, the expression of the C terminus truncation mutant of DGK δ , GFP-DGK δ ΔSAM, failed to significantly affect the exit from the ER (Figure 9p). We found that deletion of the N-terminal 100 amino acids corresponding to the PH domain also caused an apparent lack of the phenotype for both types of anterograde transports. Interestingly, the expression of GFP-

DGK δ ΔPH had little effect, if any, on the kinetics of the ER-to-Golgi transport of CFP-GT_{1–81} (Figure 8f) and VSV G-CFP (Figure 9l). In a separate experiment, we confirmed that the deletion of PH domain did not significantly affect the ER-localization of the enzyme molecule. The results therefore showed that the PH domain was also required for the inhibition of both types of anterograde transports in addition to targeting DGK δ to the ER via its SAM domain.

Membrane dynamics in the early secretory pathway is controlled by the formation and detachment of COPI and COPII coats on the membranes (Kirchhausen, 2001). The presence of several regulating factors (Kirchhausen, 2001) has been suggested in the process of coated vesicle formation. It now seems possible that DGK δ is one of the negative regulators of anterograde transport. To analyze the membrane dynamics at the early stage of anterograde transport, we tagged with YFP Sec13p, a component of the COPII coat (Tang *et al.*, 1997). When expressed in NRK cells, punctate structures of Sec13p were clearly observed adjacent to the

Figure 8. DGK δ inhibited anterograde transport through its SAM and PH domains. The ER-to-Golgi anterograde transport to reassemble Golgi apparatus was observed after washing-out NIH3T3 cells pre-treated with BFA. The cells were transfected using LipofectAMINE PLUS with expression vectors coding for CFP-GT₁₋₈₁ and GFP (a and b), CFP-GT₁₋₈₁ and GFP-DGK δ FL (c and d), or CFP-GT₁₋₈₁ and GFP-DGK δ Δ SAM (e and f), or CFP-GT₁₋₈₁ and GFP-DGK δ Δ PH (g and h). At 24 h posttransfection, cells were treated with BFA (10 μ g/ml) in DMEM containing 10% fetal calf serum for 60 min at 37°C. The cells were then washed twice with the BFA-free medium. The cells were further incubated with BFA-free medium for 30 min at 37°C. The confocal images of single cells coexpressing CFP-GT₁₋₈₁ (b, d, f, and g) and various DGK δ constructs (a, c, e, and g) are shown. Bars, 10 μ m.

ER network (Figure 10b). Previously, Sec13p-tagged with GFP was used to record movement of COPII vesicles (Hammond and Glick, 2000). We confirmed that the punctate structure disappeared when treated with H89, a protein kinase inhibitor, in accord with previous report (Lee and Linstedt, 2000). It is therefore clear that these Sec13p structures represent VTCs. Remarkably, the expression of DGK δ almost completely abolished the VTC structures of Sec13p and instead Sec13p was evenly distributed in the cytoplasm (Figure 10e). In contrast, the profiles of COPI-coated vesicles, as revealed by anti- β COP antibody staining, were unchanged upon expression of CFP-DGK δ (Figure 10f). This finding indicated that retrograde transport was not affected by DGK δ , consistent with the notion that DGK δ selectively inhibited anterograde transport. From these results, we concluded that DGK δ could regulate negatively anterograde transport by preventing formation of COPII-coated transitional ER structures.

In the final experiments, we introduced into NIH3T3 cells two types of DGK δ constructs mutated at the catalytic domain, i.e., a kinase-dead mutant, DGK δ G337D, and that deleted with 25 amino acids at 294–318, which corresponded to the internal truncation detected previously in a catalytically inactive form of DGK γ (Kai *et al.*, 1994). These mutants expressed as GFP-fusions caused redistribution of CFP-GT₁₋₈₁ to the ER similarly as did the full-length DGK δ (Figure 11). We also noted that addition into the culture media of phosphatidic acid or diacylglycerol did not affect the vesicular transport in NIH3T3 cells. We considered from these results that the catalytic activity of DGK δ was not significantly involved in the negative control of ER-to-Golgi traffic.

DISCUSSION

In the present work, we have demonstrated that DGK δ can be involved in the negative regulation of the early secretory pathway by inhibiting ER exit. This conclusion is based on three lines of observations obtained from single cells expressing DGK δ : 1) redistribution of Golgi-related membrane proteins, 2) reduced anterograde transport rate, and 3) inhibition of transitional ER structure formation. The negative effects of DGK δ were selectively observed in the COPII-dependent pathway, and no effect was discernible in COPI-dependent transport from the Golgi to the ER or clathrin-mediated endocytosis (our unpublished data). Furthermore, we found that both of the two domains uniquely present in the DGK δ were essential for this function. The SAM domain was shown to act as an ER-targeting motif, whereas the targeted molecule further required the PH domain for expressing the function. Such a selective mode of inhibitory effects and the requirements of definite structural motifs strongly suggest that DGK δ constitutes one of the negative regulators of the COPII-dependent pathway.

Interestingly, the effects of DGK δ closely resemble those recently described for H89, an isoquinolinesulfonamide protein kinase inhibitor (Aridor and Balch, 2000; Lee and Linstedt, 2000), which was described to cause loss of transitional ER structures labeled with Sec13p without affecting constitutive Golgi-to-ER retrograde transport (Lee and Linstedt, 2000). Balch and colleagues later showed that the H89-sensitive step in the COPII-dependent pathway is the

Figure 9. DGK δ slowed the ER-to-Golgi transport of VSV G(ts045). Kinetics of ER-to-Golgi transport was measured in NIH3T3 cells expressing temperature-sensitive folding mutant, VSV G(ts045) fused to CFP (b–d, f–h, j–l, and n–p). The cells coexpressed GFP (a–d), GFP-DGK δ FL (e–h), GFP-DGK δ ΔPH (i–l), and GFP-DGK δ ΔSAM (m–p). Cells transfected with the expression vectors were cultured first at nonpermissive temperature (39.5°C) for 24 h (=0 min; a, b, e, f, i, j, m, and n), and then the incubation temperature was shifted to permissive temperature (32°C). Thereafter, the confocal images were recorded at 1-min intervals. Images taken at 0 (a, b, e, f, i, j, m, and n), 3 (c, g, k, and o), and 9 min (d, h, l, and p) are shown. Arrowheads indicate the Golgi areas. Bars, 10 μ m.

Figure 10. Expression of DGK δ inhibited formation of COPII-coated structures in the ER network whereas COPI-coated structures remained unchanged. Expression vectors coding for Sec13-YFP plus CFP (a–c) or Sec13-YFP plus CFP-DGK δ FL (d–e) were transfected into NRK cells cultured on collagen-coated glass dishes. The confocal images of Sec13p were taken at 12 h posttransfection (b and e). To visualize COPI-coated vesicles, cells (c and f) were fixed with paraformaldehyde and immunostained with anti- β COP antibody. Bars, 10 μ m.

recruitment of Sar1p rather than the late assembly step (Aridor and Balch, 2000). Based on analysis using a panel of protein kinase inhibitors, both reports concluded that the target molecule of H89 in COPII coat recruitment was neither protein kinases A nor D but probably unidentified serine-threonine kinase. Considering the consequences of DGK δ expression, we think it likely that DGK δ may regulate an unknown machinery including the putative protein kinase required for COPII coat recruitment. Classical members of the DGK family phosphorylate diacylglycerol to produce phosphatidic acid so that the action of protein kinase C, which requires diacylglycerol for activation, is attenuated (Sakane and Kanoh, 1997). Recently, phospholipid metabolism has been implicated in the control of membrane traffic particularly at the distal stage of secretory pathway (De Camilli *et al.*, 1996; Siddhanta and Shields, 1998; Roth, 1999). Phosphatidic acid generated by the action of phospholipase D has been shown to regulate protein traffic from the Golgi complex, although the significance of lipids in the control of ER-to-Golgi traffic remains largely unknown. Initially, we assumed that the effects of the expressed DGK δ were mediated by the formation of phosphatidic acid in restricted areas at the ER surface. However, the results of expression of DGK δ mutated at its catalytic region have led us to consider at the present stage of investigation that the alteration of

lipid metabolism at the ER is not involved in the observed effects of DGK δ . Furthermore, neither the PH domain nor SAM domain appears to be directly involved in the DGK catalytic action (Sakane *et al.*, 1996). We also analyzed cellular lipids in COS7 cells overexpressing DGK δ for 3 d and failed to detect significant increase of phosphatidic acid. DGK δ that contains a large insert in the catalytic domain exhibits a very low molecular activity compared with the other isozymes (Sakane *et al.*, 1996), and the present work revealed unexpectedly a novel function specifically ascribed to this DGK isozyme. This may be consistent with the reports that suggest an involvement of a novel protein kinase sensitive to H89, rather than diacylglycerol-dependent protein kinase C or D for the transitional ER formation (Lee and Linstedt, 2000; Aridor *et al.*, 2001).

Intracellular distribution of tagged DGK δ was highly heterogeneous, but a portion of the molecules was consistently associated with the ER. Given that the SAM domain brings the molecule to the ER surface as revealed in the present work, why did a considerable part of DGK δ still associate with non-ER compartments? Deleterious effect of the enzyme overexpression seems unlikely, because we made time-lapse analysis of DGK δ at its very early stage of expression. Two explanations are possible. One is that the SAM domain in DGK δ may be covalently modified or steri-

Figure 11. Kinase-dead mutants of DGK δ also induced redistribution of CFP-GT₁₋₈₁. Expression vectors for GFP-DGK δ Δ 294-318 (a and b) or GFP-DGK δ G337D (c and d) were coloaded into COS7 cells with a vector coding for CFP-GT₁₋₈₁ using microbeads. Shown are the GFP (a and c) or CFP images (b and d) of cells at 4 h postloading. Bars, 10 μ m.

cally hindered in order to prevent its ER association. For example, the SAM domain of EphB1 was tyrosine phosphorylated so that the interaction of EphB1 with a low-molecular-weight phosphotyrosine phosphatase was regulated by the tyrosine phosphorylation cascade (Stein *et al.*, 1996). The tyrosine residue phosphorylated in EphB1 is conserved at Tyr-1118 of the DGK δ SAM domain; thus it may be phosphorylated and prevented from association with the ER, although tyrosine phosphorylation of DGK δ is the subject of future investigation. The second possibility is the presence of another localization signal(s) in the DGK δ molecule. The PH domain at the N-terminus is a likely candidate. The PH domain was previously shown to bind to a number of polyphosphoinositides and phosphatidylserine with relatively low affinity (Kavran *et al.*, 1998). The DGK δ PH domain appears to attribute to the cell surface association because deletion of this domain abolished the signal at the plasma membrane (see Figures 8e and 9i). The deletion of the SAM domain, on the other hand, always enhanced the enzyme localization at the plasma membranes, presumably representing binding through the PH domain (see, e.g., Figures 3d and 5B, j). Thus we suggest that DGK δ localization may be dynamically determined by a balance between actions of the two domains. We are currently trying to find the regulatory mechanisms of the membrane association of DGK δ .

In addition to the SAM domain, the presence of a PH domain distinguishes this isoform from the other members of DGK. The present work demonstrated that in addition to being targeted to the ER, the PH domain was needed for

DGK δ to exert its negative effects upon the formation of the ER exit sites. Thus it is conceivable that the effects of DGK δ may be exerted through its PH domain sequestering the target lipid(s) involved in the control of ER-to-Golgi transport, because the presence of phosphatidylinositol(3,4)P₂ in the membranes is required for binding the COPII coat protein complex (Yoshihisa *et al.*, 1993) and because association of an isoform of phosphatidylinositol-4-kinase with the ER is reported (Wong *et al.*, 1997). A similar case has been reported for the GFP-fused PH domain of phospholipase D₁, which was recruited to the cell surface and inhibited exocytosis in chromaffin cells (Holz *et al.*, 2000). However, the possibility of DGK δ PH domain interacting with key phospholipids needs to be further explored because DGK δ with a mutation of Arg-28 to Glu in the PH domain, which is supposedly required for water-mediated contact with the 1-phosphate of phosphoinositides (Lietzke *et al.*, 2000), inhibited anterograde transport of CFP-GT₁₋₈₁ upon BFA washout as effectively as wild-type enzyme. Apparently further work is needed to characterize the role of DGK δ PH domain acting in the COPII coat assembly. Previously, ionic interactions of PH domains with other proteins, presumably via a positively charged binding pocket, have been reported (Pitcher *et al.*, 1996; Burks *et al.*, 1998). It seems, therefore, possible that the DGK δ PH domain interacts with regulatory protein(s) rather than phospholipids required for COPII coat attachment.

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