Modulation of the Mammalian Target of Rapamycin Pathway by Diacylglycerol Kinase-produced Phosphatidic Acid*

Received for publication, October 29, 2004, and in revised form, December 28, 2004
Published, JBC Papers in Press, January 4, 2005, DOI 10.1074/jbc.M412296200

Antonia Ávila-Flores, Teresa Santos, Esther Rincón, and Isabel Mérida‡

From the Department of Immunology and Oncology, National Centre for Biotechnology, Consejo Superior de Investigaciones Científicas, Campus Cantoblanco, E-28049 Madrid, Spain

The protein known as mammalian target of rapamycin (mTOR) regulates cell growth by integrating different stimuli, such as available nutrients and mitogenic factors. The lipid messenger phosphatidic acid (PA) binds and positively regulates the mitogenic response of mTOR. PA generator enzymes are consequently potential regulators of mTOR. Here we explored the contribution to this pathway of the enzyme diacylglycerol kinase (DGK), which produces PA through phosphorylation of diacylglycerol. We found that overexpression of the DGKα, but not of the α isoform, in serum-deprived HEK293 cells induced mTOR-dependent phosphorylation of p70S6K (p70S6K). After serum addition, p70S6K phosphorylation was higher and more resistant to rapamycin treatment in cells overexpressing DGKα. The effect of this DGK isoform on p70S6K hyperphosphorylation required the mTOR PA binding region. Down-regulation of endogenous DGKβ by small interfering RNA in HEK293 cells diminished serum-induced p70S6K phosphorylation, highlighting the role of this isoform in the mTOR pathway. Our results confirm a role for PA in mTOR regulation and describe a novel pathway in which DGKα-derived PA acts as a mediator of mTOR signaling.

Diacylglycerol kinase (DGK) comprises an evolutionarily conserved family of lipid kinases that phosphorylate diacylglycerol (DAG) to produce phosphatidic acid (PA). These enzymes contain at least two N-terminal cysteine-rich domains and a conserved catalytic domain. The DGKs have additional functional domains that were used to classify them into five subgroups (I-V; for an extended review, see Refs. 1 and 2). Their structural diversity, as well as their distinct tissue expression and specific intracellular localization, may confer on each DGK isoform the ability to regulate distinct DAG and PA pools and thus to participate in different signaling complexes.

DAG is a second messenger whose levels increase transiently in response to hormones and growth factors. DAG is an allosteric activator of protein kinase C (PKC) (3) as well as non-kinase proteins such as GTPase-activating proteins and guanine nucleotide exchange factors for the Ras family of GTPases (4, 5). Termination of the DAG-derived signal must be rapid and requires DGK activity. DGKα and ζ are the best-studied isoforms; both function as DAG signaling terminators during T-cell receptor (TCR) activation.

Class I DGKα has two Ca2+ binding motifs (1) and is expressed in spleen, thymus, skeletal muscle, lung, testis, and peripheral T cells (6). During TCR-induced T-cell activation, the enzyme translocates to the plasma membrane, where it down-regulates DAG levels and thus diminishes Ras protein activation (6). DGKα membrane translocation is rapid and transient and requires both tyrosine kinase activity and an increase in intracellular Ca2+ levels. This isoform also diminishes the response elicited by an ectopically expressed muscarinic type I receptor (7).

DGKβ belongs to class IV and has nearly ubiquitous expression. It has a PDZ binding motif, four ankyrin repeats, and a myristoylated alanine-rich PKC substrate (MARCKS) homology domain, which overlaps with a bipartite nuclear localization signal (NLS) (8, 9). Like the α isoform, DGKβ acts as a negative regulator of TCR signaling; its overexpression thus attenuates activation of the extracellular signal-regulated kinase (ERK) pathway in both T lymphocytes after TCR binding (10) and gonadotrope cells stimulated with gonadotrophin-releasing hormone (11). DGKβ-deficient T cells are hyper-responsive to TCR stimulation; accordingly, mice lacking the enzyme have a more robust immune response than wild-type mice (12). DGKβ membrane translocation is a sustained event that requires phosphorylation of the MARCKS domain by PKC (13) and dissociation from the cytoskeleton induced by phosphorylation of the C-terminal region (14).

The DGK reaction is unique because it uses one messenger to create another. PA modulates the activity of a variety of enzymes such as phosphatidylinositol 4-phosphate 5-kinase (2), Raf kinase (15), PKCε and ζ (16, 17), sphingosine kinase (18), the tyrosine phosphatase SHP-1 (19), protein phosphatase-1 (20), and phospholipase C (21). PA also positively regulates the protein known as mammalian target of rapamycin (mTOR) (22), a master controller of cell growth.

Our previous data indicate that DGK-generated PA is necessary for IL-2-regulated G1 to S-phase transition in IL-2-dependent cell lines (23, 24) and for CD4+/CD8+ cell survival during thymic development (25). DGK-derived PA is also essential for the stabilization and activity of the transcription factor HIF-1α during onset of the hypoxic response (26). These two processes, proliferation and hypoxic response, involve mTOR kinase activity (27–29). PA binds an mTOR region...
termed the FKB12-rapamycin binding domain (FRB) (22, 30). It is proposed that the complex formed by the immunophillin FKB12 and the drug rapamycin induces mTOR inhibition by competing with PA to bind the FRB region (22). The exact mechanism by which PA regulates mTOR activity has not yet been clarified, but PA might either recruit downstream effectors or remove a negative regulator (31).

mTOR plays a major role in regulating cell growth through the control of protein synthesis. In response to amino acids and growth factors, mTOR phosphorylates two translational regulatory proteins: eukaryotic initiation factor 4E-binding protein (4E-BP) and the 40S ribosomal protein S6 kinase (S6K). Through activation of S6, S6K enhances translation of mRNA with repressive 5’-TOP tracts; many of these mRNA encode components of the translational machinery (32).

In mammals, there are two homologous S6K proteins, S6K1 and S6K2. S6K1 consists of the p70S6K and p85S6K isoforms; the former is localized mainly in cytoplasm, whereas the latter is found in the nucleus due to the presence of an NLS. These isoforms are generated by use of different translational start codons of the same messenger; their sequences are thus identical, with the exception of a short region in the N terminus (33). S6K2 comprises two isoforms; both also have an NLS and are therefore nuclear enzymes (32, 34). p70S6K is the best-studied S6K; it is activated through a coordinated sequence of phosphorylations that begins at the auto-inhibitory region in the kinase C-terminal domain. The p70S6K hydrophobic domain is then phosphorylated, leading to phosphorylation of the T-loop by the phosphoinositide-dependent kinase (PDK) 1 (35). mTOR is proposed to phosphorylate the auto-inhibitory and the hydrophobic motif of p70S6K (36, 37).

Here we explored the role of DGK in the mTOR pathway. We determined DGK activity during mitogenic stimulation or hypoxic treatment in vitro and observed that this activity was down-regulated in serum-starved cells and increased after serum stimulation or hypoxia. Moreover, DGKα overexpression induced dose-dependent phosphorylation of the hydrophobic motif of p70S6K, an effect that requires the PA binding motif of mTOR. Our results suggest the existence of a pathway in which DGK-derived PA can modulate mTOR activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—The human embryonic kidney cell line HEK293 was cultured in Dulbecco’s medium containing 10% fetal bovine serum (37 °C, 5% CO2). For transfection, cells were plated in 6-well plates. When cells reached 40–50% confluence (24 h), transfection was carried out using the Jet-PEI reagent (PolyTransfection). The DGK constructs used have been described previously (14), with minor modifications. Briefly, the HEK293 cell pellet was resuspended in ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM MgCl2, 1 mM dithiothreitol, 50 mM NaF, 10 μg/ml each of leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate), sonicated, and centrifuged (800 × g, 10 min, 4 °C). The supernatant was assayed for total protein concentration. An aliquot of each sample was reserved to evaluate DGK expression levels by Western blot. A mock transfection was performed simultaneously using GFP as a control. The TLC was exposed for 3 h or overnight to detect overexpressed or endogenous DGK activity, respectively.

**Fractionation Analysis**—Subcellular fractionation was carried out as described previously (14), with minor modifications. Briefly, the HEK293 cell pellet was resuspended in ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 1 mM dithiothreitol, 50 mM NaF, 10 μg/ml each of leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate), sonicated, and centrifuged (800 × g, 10 min, 4 °C). The pellet corresponded to the crude nuclear fraction. Supernatant was centrifuged (100,000 × g, 1 h, 4 °C); the supernatant corresponded to the cytosolic fraction, and the pellet was resuspended thoroughly in 1% Triton X-100. Preparations were examined by confocal microscopy (TCS-NT; Leica).

**Small Interfering RNA**—The 21-nucleotide sequence for human DGKα (GenBank accession numbers L42300–L42310) was selected as the target sequence. A BLAST search showed no significant similarity to any other sequence in the database. siRNA was synthesized chemically (Ambion), and transfection was performed using Oligofectamine (Invitrogen) with 100 nM siRNA. The next day, cells were divided and plated into fresh medium. Two days later, cells were starved for 24 h and then serum-stimulated. A mock siRNA transfection was performed simultaneously using Oligofectamine (nucleotides 238–268) as a target.
DGK activity increases in response to mitogenic stimuli and hypoxia. A, total lysates of HEK293 cells were immunoblotted with specific anti-human DGKα or DGKζ antibodies. Jurkat T-cell lysates were used as a positive control. Bands corresponding to the DGK detected are indicated (arrowheads). DGKζ antibody recognized a 110-kDa doublet that corresponds to this isoform; the antibody also detected a nonspecific band that migrated at ~130 kDa. B, HEK293 cells were transiently transfected with GFP or GFP-DGKζ constructs and cultured with 10% fetal bovine serum (exponential growth); some were serum-starved for 20 h and serum-stimulated for 30 min or cultured in the presence of CoCl₂ to mimic hypoxic conditions. DGK activity was evaluated (see “Experimental Procedures”). PA production was separated by TLC and visualized by autoradiography. Lysates of transfected cells were immunoblotted with anti-GFP antibody to estimate transfected DGK expression levels. Results are representative of three independent experiments.

RESULTS

DGKζ Activity Increased in Response to Mitogenic Stimuli and Hypoxia—We predicted that the dual action of DGK might be integrated in mTOR activity. Mitogenic agents produce DAG, which is used by DGK, thus diminishing the mitogenic stimulation of mTOR, whereas conversion of DAG to PA positively stimulates mTOR. As stated above, DGKα and ζ are the best-studied isoforms. DGKα is proposed to participate in attenuation of the TCR response and IL-2-dependent T-cell proliferation (6, 7, 23–25); the ζ isoform has been assigned a role as a down-regulator of the TCR response (10–12). We explored the role of these two isoforms in mTOR regulation using HEK293 cells, a cell line widely used to dissect the mTOR pathway. DGKα is highly expressed in T cells and has limited tissue distribution (1), whereas DGKζ displays a broad expression pattern (9). In accordance with this observation, analysis of total extracts of HEK293 cells by immunoblot showed no DGKζ expression, whereas Jurkat T cells showed high DGKα levels (Fig. 1A, top panel). In HEK293 cells and the Jurkat T cell-line, anti-DGKζ antibody revealed high protein expression (Fig. 1A, bottom panel). In addition, this antibody recognized a nonspecific band of ~130 kDa.

We evaluated endogenous DGK activity in HEK293 cells under two conditions known to modify PA levels and mTOR activity, addition of a hypoxia mimetic agent (CoCl₂) and serum addition. DGK activity was measured using micelles formed with the detergent octylglucoside (41, 42). Activity diminished after serum deprivation and increased after serum addition to cells, and it was also elevated in response to CoCl₂ addition (Fig. 1B, left panel). Cells were then transfected with a construct encoding a chimeric GFP-DGKζ protein, and activity was assayed under the same conditions. The transfected DGK activity pattern was similar to that of the endogenous enzyme; DGKζ activity was down-regulated after starvation and increased after serum addition or during hypoxia-mimetic conditions (Fig. 1B, right panels).

Serum Stimulation Induced Phosphorylation of DGKζ MARCKS and C-terminal Domains—The previous experiments showed that DGKζ activity is serum-regulated; we therefore tested whether the enzyme was phosphorylated in response to serum addition. Reports from our group showed that in T lymphocytes, the DGKζ C-terminal domain has a restrictive role in membrane translocation (13). This region contains a MAPK consensus phosphorylation site (Fig. 2A) that is phosphorylated by ERK overexpression (14). We used an antibody raised against the p70S6K Ser421/Thr424 region that constitutes a canonical MAPK phosphorylation motif, to study MAPK-mediated, serum-dependent phosphorylation of

![Figure 1](image1.png)

![Figure 2](image2.png)
DGKζ. This antibody recognized GFP-DGKζ in a serum-dependent manner (Fig. 2B), but failed to recognize a DGKζ mutant in which the C-terminal region had been deleted, confirming that the serum-dependent phosphorylation takes place at the C-terminal end of the protein (Fig. 2B).

DGKζ also contains a MARCKS domain with a canonical PKC-dependent phosphorylation site (Fig. 2A). Phosphorylation at this site is required for plasma membrane translocation in T lymphocytes (13) and prevents nuclear localization in COS cells (8). We used a pan-PKC phosphorylation site antibody to detect serum-dependent DGKζ phosphorylation, but no phosphorylation was observed in total cell lysates (data not shown).

We tested whether DGKζ phosphorylation correlated with a specific subcellular localization. Analysis of different subcellular fractions showed that the main phosphorylation at the C-terminal end corresponded to cytosolic GFP-DGKζ, whereas cytoskeletal and membrane fractions showed a lesser degree of phosphorylation (Fig. 2C, middle panel). These data concur with observations in muscle cells, in which this motif appears to negatively regulate DGKζ association with the cytoskeleton (14). As for PKC-dependent phosphorylation, the antibody detected GFP-DGKζ only in the cytosolic fraction (Fig. 2C, bottom panel). These experiments suggest that the DGKζ pool recovered in the cytosolic fraction is phosphorylated at both the PKC and MAPK motifs after serum addition.

DGKζ Overexpression Increased p70S6K Phosphorylation in the Hydrophobic Motif—Serum addition, which is known to stimulate mTOR, also modulated DGKζ; thus, we studied the effect of DGKζ overexpression on mTOR activity by examining mTOR-dependent phosphorylation of Thr389 in p70S6K translocated into quiescent or serum-stimulated cells. Thr389 is found in the linker or hydrophobic motif of p70S6K and phosphorylated in vitro by mTOR, and it is the major rapamycin-sensitive site (36, 43).

In cells overexpressing DGKζ, both basal and serum-induced phosphorylation of transfected p70S6K were greater than that seen in control cells (Fig. 3A, middle panel, solid arrowhead). In cells overexpressing DGKζ, we also detected phosphorylation of a faster migrating band (Fig. 3A, middle panel, open arrowhead), which probably corresponds to endogenous p70S6K. To evaluate expression levels of transfected and endogenous p70S6K, we used a commercial antibody generated against the p70S6K C terminus, a region also present in the pS6K isoform; it can thus be considered an anti-S6K1 antibody. This antibody recognized two bands, the slow-migrating band of transfected EE-p70S6K and a fast-migrating endogenous p70S6K band. Because transfected EE-p70S6K migrates with an apparent mass greater than the predicted mass of 70 kDa and nearer to 80 kDa, antibody recognition of EE-p70S6K may have masked pS6K isoform recognition.

DGKζ-induced p70S6K phosphorylation is mTOR-mediated because it was inhibited by rapamycin. DGKζ-induced phosphorylation was reduced by addition of the DGK inhibitor R5949 (Fig. 3A). This inhibitor, albeit much more effective for class I DGK, diminished the in vitro activity of endogenous and overexpressed enzymes when added to intact cells (Fig. 3B).

DGKζ-induced p70S6K phosphorylation was dependent on protein levels. At higher DGKζ concentrations, we observed intense p70S6K phosphorylation even in the absence of serum (Fig. 4A, middle panel, solid arrowhead). As before, phospho-Thr389 antibody also recognized a faster-migrating band, whose pattern was similar to that of transfected EE-p70S6K and corresponded to endogenous p70S6K (Fig. 4A, middle panel, open arrowhead). The antibody also detected an intermediate migrating band, whose pattern differed from that of the other bands. Because the p70S6K antibody did not recognize this band, it is not pS6K, but it may correspond to S6K2 isoforms or another abundant kinase with a hydrophobic domain similar to that of the S6K family.

The precise role of PA on mTOR modulation is not well known, although it has been suggested that PA competes with the FKBP12-rapamycin complex for mTOR binding (22). Some cells lines that overexpress phospholipase D (PLD) are reported to have greater rapamycin resistance (44); this prompted us to determine whether DGKζ overexpression also conferred rapamycin resistance. Although endogenous p70S6K phosphorylation was more resistant to rapamycin action in DGKζ-overexpressing cells, it remained susceptible (Fig. 4B, open arrowheads), suggesting that DGKζ-dependent p70S6K phosphorylation acts via mTOR.

The phospho-Thr389 antibody again recognized a slow-migrating doublet, whose response pattern to serum addition was similar to that of p70S6K. However, phosphorylation of these bands was resistant at the rapamycin doses tested, and DGKζ overexpression promoted only partial phosphorylation in the absence of serum. Reprobing the membranes with the p70S6K1 antibody confirmed that the fast-migrating band recognized by the phospho-Thr389 antibody was p70S6K and suggested that part of the phosphorylated slow-migrating bands corresponded to pS6K. Nonetheless, we cannot rule out that these bands may be S6K2 isoforms or a kinase with a hydrophobic domain similar to that of S6K.

p70S6K Is Phosphorylated in Different Compartments by DGKζ Overexpression—Most DGKs are cytosolic enzymes, and translocation from cytosol to other subcellular compartments

![Fig. 3. DGKζ-dependent phosphorylation of the p70S6K hydrophobic motif.](image-url)
appears to be an activation mechanism for this enzyme family. Analysis of different cell fractions with anti-DGKζ antibody showed serum-dependent translocation of both endogenous DGKζ and GFP-DGKζ to the membrane (Fig. 5A, top panel). Endogenous DGKζ was mainly cytosolic, and it migrated as several bands. These bands have been described in other cell lines to correspond to distinct enzyme phosphorylation states (14). After serum stimulation, a small fraction translocated to membrane (Fig. 5, top panel, open arrowhead). GFP-DGKζ behaved similarly, although there was a small but detectable amount of the enzyme in the membrane fraction under starvation conditions. In serum-treated cells, DGKζ translocation to the membrane was increased, whereas the enzyme was reduced in cytoskeletal and cytosolic fractions (Fig. 5, top panel, solid arrowhead). In the presence of GFP-DGKζ, the endogenous enzyme translocated more efficiently to the membrane, suggesting enzyme heterodimerization.

The DGKζ nuclear pool showed no marked changes after serum addition, although it is important to consider that the fractionation method used did not render completely pure nuclei (Fig. 5B). In addition, weakly bound proteins in the nucleus may exit this compartment during sample processing. The DGKζ antibody recognized a higher molecular mass protein in the nuclear fraction that did not translocate after serum addition.

We determined the phosphorylation state of p70S6K in the different subcellular compartments. In cells overexpressing DGKζ, serum-dependent phosphorylation of both transfected and endogenous p70S6K at the hydrophobic motif was greatly increased in the cytosolic and membrane fractions (Fig. 5A, middle panel, solid and open arrowheads). Strikingly, DGKζ overexpression also increased phosphorylation of a nuclear p70S6K pool, even in quiescence, to an extent similar to that observed in control cells after serum addition. The effect of DGKζ correlates with an increase in the nuclear p70S6K pool, suggesting a certain enrichment of this fraction. This might be due to an accumulation of p70S6K in either active perinuclear ribosomes or nucleoli.

**DGKζ-dependent p70S6K Phosphorylation Required the mTOR PA Binding Region**—The results suggested that DGKζ-mediated PA generation increases p70S6K phosphorylation due to direct action on mTOR kinase. An alternative hypothesis is that of PA-dependent phosphatase inhibition, which would result in p70S6K hyperphosphorylation. To distinguish between these two possibilities, we examined DGKζ-mediated p70S6K phosphorylation in cells expressing either wild-type mTOR or a mutant (3ATOR) with diminished PA binding capacity (22). DGKζ-induced phosphorylation of p70S6K in Thr389 was enhanced in cells expressing wild-type TOR kinase, but not in cells expressing 3ATOR (Fig. 6). These results confirmed that DGKζ-generated PA acts directly on the FRB region of mTOR.

**mTOR-dependent p70S6K Phosphorylation at the Hydrophobic Motif Is Specific for DGKζ Isoform**—DGKζ overexpression resulted in hyperphosphorylation of the p70S6K hydrophobic motif; we thus analyzed whether this effect could be exerted by other DGK isoforms. HEK293 cells were transfected with plasmids encoding tagged DGKα or DGKζ. When we examined the subcellular localization of these isoforms, DGKα was found in the cytosol, whereas DGKζ displayed a punctate pattern in cytosol and nucleus (Fig. 7A). To assess the role of these DGKs on p70S6K phosphorylation, we examined serum-dependent mTOR-mediated p70S6K phosphorylation after transfection using increasing cDNA concentrations of the plasmids. DGKζ overexpression increased p70S6K phosphorylation, whereas DGKα diminished phosphorylation at the Thr389 residue (Fig. 7B). These results suggest that the distinct subcellular distribution of these DGK isoforms and/or the differences in their domain composition are determinants of their capacity to modulate mTOR activity.

**DGKζ siRNA Reduced p70S6K Phosphorylation in HEK293 Cells**—To assess the role of DGKζ in the mTOR pathway, we attenuated endogenous DGKζ protein levels in siRNA experiments. We selected a region between the catalytic domain and the first ankyrin repeat to knock down DGKζ expression. Interference was maximal at 4 days post-transfection, producing a 60% decrease in endogenous DGKζ levels (Fig. 8). Serum stimulation induced a marked increase in p70S6K and p85S6K phosphorylation at Thr389 in mock siRNA-transfected HEK293 cells, whereas knocked-down DGKζ cells showed reduced S6K phosphorylation. The same effect was detected, to a lesser extent, in the phosphorylation of Ser421/Thr424 of S6K, another mTOR putative phosphorylation region in S6K. This effect is more evident in the p85S6K isoform (Fig. 8).

mTOR is regulated by nutrients and by the phosphatidylinositol 3-kinase/Akt pathway (45), whose activation leads to mTOR phosphorylation in Ser2448 (46). We thus evaluated modulation of the phosphatidylinositol 3-kinase/Akt pathway using anti-phospho-mTOR (Ser2448) and anti-phospho-AKT (Ser473) antibodies. We found no great difference in phosphorylation of these proteins between control and DGKζ knocked-down cells (Fig. 8), suggesting that DGKζ-promoted p70S6K phosphorylation lies downstream of the phosphatidylinositol 3-kinase/Akt pathway.

Because DGKζ generates PA at DAG expenses, we evaluated the effect on DAG-dependent responses of a reduction in DGKζ.
protein. We examined the activation state of PKC family members using a pan-phospho-PKC antibody (pan-PDK1 site) that recognizes phosphorylation by PDK1 in the PKC activation loop. PKC is phosphorylated by PDK1 when it is membrane-bound, and PDK1-dependent phosphorylation is required to render PKC catalytically competent (3). Because DGK\(\alpha\)/H9256 produces PA by phosphorylating DAG, the absence of DGK\(\alpha\)/H9256 causes DAG accumulation. This elevation in DAG levels must lead to receptor-independent membrane translocation of DAG-sensitive PKC isoforms (classical and novel), even under starvation conditions, thus favoring PDK1-dependent phosphorylation.

![Image](https://via.placeholder.com/150)

**FIG. 5.** DGK\(\alpha\) translocates to the membrane after serum stimulation and promotes p70S6K phosphorylation in all cell fractions. A, HEK293 cells were transiently cotransfected with GFP or GFP-DGK\(\alpha\) and EE-p70S6K and then stimulated as indicated. Nuclear (N), cytosolic (C), membrane (M), and cytoskeletal (Cb) fractions were prepared. The subcellular localization of DGK\(\alpha\) (top panel), p70S6K phosphorylated in the hydrophobic motif (middle panel), and total p70S6K (bottom panel) was determined by immunoblot of the fractions with appropriate antibodies. Endogenous DGK\(\alpha\) and endogenous p70S6K, open arrowheads; GFP-DGK\(\alpha\) and EE-p70S6K, solid arrowheads. Similar results were obtained in three independent experiments. B, the blots shown in A were re-blotted with antibodies against a nuclear/cytosolic (ck2) protein and typical nuclear (histones), cytoskeletal (vimentin), and membrane (human transferrin receptor; hTfR) proteins to assess the efficiency of the fractionation procedure.

![Image](https://via.placeholder.com/150)

**FIG. 6.** DGK\(\alpha\)-dependent p70S6K phosphorylation requires the mTOR PA-binding region. HEK293 cells were cotransfected with GFP-DGK\(\alpha\), EE-p70S6K, and FLAG-tagged wild-type mTOR (TORwt) or a mutant (TOR 3A) version that has diminished PA binding capacity. p70S6K phosphorylation was evaluated using phospho-antibodies against Thr389 of p70S6K. Expression levels of the overexpressed proteins were measured using appropriate antibodies. p70S6K phosphorylation at Thr389, quantified and normalized as described in the Fig. 3 legend, is shown at the bottom of this figure; the phosphorylation ratio in control cells in the absence of serum was taken as 1.00, and the values obtained at the different conditions were expressed relative to this. In the table, the ratio of DGK\(\alpha\)-induced phosphorylation of p70S6K in Thr389 is expressed independently and relative to the values obtained in the absence of TOR expression or cotransfection with either wild-type (wt) or mutant (3A) TOR. Similar results were obtained in three independent experiments.

![Image](https://via.placeholder.com/150)

**FIG. 7.** Overexpression of DGK\(\alpha\), but not DGK\(\epsilon\), promoted phosphorylation of the p70S6K hydrophobic domain. A, immunofluorescence localization of overexpressed DGK. HEK293 cells were transfected with DGK\(\alpha\) or DGK\(\epsilon\), fixed, and stained with a mouse anti-HA or a rabbit anti-DGK\(\alpha\) antibody. DGK\(\epsilon\) showed a uniform pattern in cytosol, whereas DGK\(\alpha\) showed a punctuate pattern in cytosol and nucleus. B, HEK293 cells were cotransfected with EE-p70S6K and increasing amounts of plasmid encoding GFP, HA-DGK\(\epsilon\), or GFP-DGK\(\alpha\). p70S6K phosphorylation in Thr389 was evaluated after starvation or serum addition. DGK expression levels were evaluated using anti-GFP or anti-HA antibodies. p70S6K phosphorylation at Thr389, quantified and normalized as described in the Fig. 3 legend, is shown at the bottom of the figure; the phosphorylation ratio in nontransfected cells in the presence of serum was taken as 1.00, and the values obtained at the different DGK concentrations in the presence of serum were expressed relative to this. Similar results were obtained in three independent experiments.
DGKζ siRNA diminished p70S6K phosphorylation in HEK293 cells. HEK293 cells were transfected with a control siRNA (−) or DGKζ siRNA (+). After 3 days, cells were starved and serum-stimulated. The phosphorylation state of p70S6K and of other proteins of the mTOR pathway was evaluated using the antibodies indicated. The quantitation of the bands indicated with arrowheads was performed as described under “Experimental Procedures” and is shown immediately below each blot. The value of expression for DGKζ in cells transfected with control siRNA was taken as 1.00, and expression of this protein in cells transfected with DGKζ siRNA is expressed relative to this. Values for the corresponding phosphorylation sites in p70S6K were normalized to p70S6K protein levels. Values for TOR, Akt, and PKC phosphorylation were normalized to tubulin values. When detectable, values in quiescent control cells were taken as 1.00. If they were not detectable, the values were normalized for those obtained after serum stimulation of control cells. Similar results were obtained in at least three independent experiments.

**DISCUSSION**

The lipid PA has mitogenic properties due, at least in part, to its capacity to modulate the mTOR pathway. Stimulation with serum or factors such as IL-2 induces an increase in cellular PA levels (22, 23), which is required for activation of the mTOR targets p70S6K and 4E-BP1 (22). Three different enzymes generate PA: PLD, lysophosphatidic acid acyltransferase, and DGK. PLD is regarded as the main contributor of PA to mTOR signaling (22, 47). Nonetheless, other PA-generating enzymes can also contribute to mTOR activation (48); lysophosphatidic acid acyltransferase is thus reported to be elevated in some tumors, and its overexpression leads to cell transformation (49). Here we demonstrate the contribution of DGKζ to mTOR-dependent p70S6K phosphorylation, suggesting a role for this enzyme in the regulation of mTOR-dependent pathways.

Serum stimulation leads to PLD activation, which correlates with increased mTOR signaling (22, 47). Our experiments here show serum-dependent modulation of DGKζ and DGKζ-dependent p70S6K phosphorylation in an mTOR-modulated residue. These results suggest that PLD and DGKζ act in parallel pathways as PA generators (Fig. 9). Serum is, in fact, a mixture of mitogenic agents that act through G protein-coupled receptors or tyrosine kinase-coupled receptors; PLD activity increases in response to stimulation of both receptor types. Butanol (a widely used PLD inhibitor) nevertheless blocks mTOR signaling elicited by G protein-coupled receptors, such as those for lysophosphatidic acid (50) or phenylephrine (41), but does not block mTOR signaling elicited by tyrosine kinase-coupled receptors, such as that for platelet-derived growth factor (41, 50). It is thus possible that some mitogens, while promoting PLD activity, modulate mTOR signaling either by a PA-independent mechanism or, more likely, by activating alternative routes to generate PA.

Lipids such as DAG and PA are generated in membrane domains, where an intimate connection between distinct lipid metabolic pathways is maintained, to produce appropriate spatio-temporal responses. PLD and DGK may operate in parallel metabolic pathways as PA generators (Fig. 9). Serum is, in fact, a mixture of mitogenic factors in serum promote DGK translocation and activation. Plasma membrane DGK translocation attenuates the response elicited by some receptors, probably G protein-coupled receptors, whereas translocation to internal membranes produces PA able to bind the mTOR FRB region. mTOR in turns phosphorylates the p70S6K hydrophobic motif. Other PA generator enzymes, such as PLD, also modulate the mTOR pathway.

**Fig. 8.** DGKζ siRNA diminished p70S6K phosphorylation in HEK293 cells. HEK293 cells were transfected with a control siRNA (−) or DGKζ siRNA (+). After 3 days, cells were starved and serum-stimulated. The phosphorylation state of p70S6K and of other proteins of the mTOR pathway was evaluated using the antibodies indicated.

**Fig. 9.** mTOR pathway modulation by DGKζ. Mitogenic factors in serum promote DGK translocation and activation. Plasma membrane DGK translocation attenuates the response elicited by some receptors, probably G protein-coupled receptors, whereas translocation to internal membranes produces PA able to bind the mTOR FRB region. mTOR in turns phosphorylates the p70S6K hydrophobic motif. Other PA generator enzymes, such as PLD, also modulate the mTOR pathway.
strate. In agreement with this hypothesis, we found that part of the DGKζ-driven phosphorylation of p70S6K can be blocked with n-butanol (data not shown), suggesting that PLD can in fact provide a share of the DGKζ substrate.

Our results indicate that in HEK293 cells, the positive effect of DGK-dependent PA generation on the mTOR pathway is isoform-specific because DGKζ and DGKα exerted opposite effects on p70S6K phosphorylation at Thr389. The difference in effects of these isoforms might be a consequence of their distinct modular architecture, which would confer on each isoform a unique regulatory response to stimuli and/or different in vivo substrate specificity. In HEK293 cells, which have no endogenous DGKα, overexpression of this kinase has a negative effect on mTOR-dependent phosphorylation, probably due to phosphorylation of a DAG pool that participates in the mitogenic pathway. In this case, DGKα renders a PA that is ineffective or does not have access to mTOR. On the contrary, PA produced by DGKζ, which has a broad subcellular distribution, increases p70S6K phosphorylation. This suggests that the unique combination of structural domains in DGKζ allows this isoform to gain access to DAG pools with no mitogenic effect on mTOR and to generate PA that is an effective modulator of mTOR activity. Another explanation for the lack of positive DGKα action on mTOR would be a difference in the nature of the PA generated. Other PA-modulated targets are reported to respond specifically to different PA species; for example, the MAPK cascade is elicited more efficiently by saturated PA than by unsaturated PA (58). Whether mTOR has such a preference is not known; nonetheless, our results showed that DGKζ-derived PA is capable of modulating mTOR.

The DGK isoform specificity suggested by these experiments concurs with the work of Pettit and Wakelam (59), who reported that DGKζ overexpression in porcine aortic endothelial cells specifically diminishes polyunsaturated DAG levels, causing redistribution of DAG-responsive PKC isoforms from membrane to cytosol. On the contrary, DGKζ overexpression caused an overall reduction in DAG levels, but it had no effect on PKC distribution. Moreover, DGKζ increased PKCε activity, possibly due to the reported PA requirement for PKCε (16). We observed that DGKζ overexpression in HEK293 cells provoked an increase in the amount of cytosolic protein recognized by a pan-phospho-PKC site antibody (data not shown) and that a decrease in endogenous DGKζ correlated with a lack of response to serum by some PKC isoforms (probably those that require PA for their activation).

Although no substrate specificity has been shown for DGKα, studies of this isoform in T lymphocytes showed differential regulation in response to distinct stimuli. TCR activation causes DGKα translocation to the plasma membrane, where it phosphorylates phospholipase C-generated DAG and acts as a negative modulator of DAG-dependent responses (6, 7). IL-2, a mitogenic factor for T cells that does not stimulate phospholipase C activity, induces DGKα translocation to a perinuclear region (23), with concomitant PA production derived not from receptor-generated DAG but from a pre-existing pool of diradylglycerol 1-0-alk-1'-enyl-2-acylglycerol (60). Taken together, the data allow us to hypothesize that the net effect of a specific DGK isoform on mTOR is dictated by the sum of factors such as mitogen concentration, the nature of the substrate, and/or the type and spatio-temporal accessibility of the PA. These factors would vary depending on cell type, mitogen, and growth conditions, as well as the presence of different DGK isoforms.

We analyzed whether there was a correlation between a specific DGKζ localization and p70S6K phosphorylation. Our data indicate that serum induces an increase in both DGKζ activity and its translocation to the membrane fraction. Nonetheless, DGKζ promotes p70S6K phosphorylation in any subcellular compartment, including the nucleus. When we examined endogenous p70S6K phosphorylation in serum-deprived cells, we noted that DGKζ overexpression induced phosphorylation of a slow-migrating band, probably nuclear p85S6K. mTOR and S6K family proteins are found in the nucleus, a surprising location for proteins of the translational machinery; nonetheless, nuclear mTOR and S6K are essential for cell growth and survival (34, 61). DGKζ has an NLS (8), and confocal microscopy analysis of whole cells shows a large amount of the enzyme in the nucleus. Additional studies are needed to determine whether DGKζ promotes S6K phosphorylation in this compartment or whether phosphorylation of any of the four S6K isoforms can augment their nuclear transport or retention.

These studies describe a novel function for DGKζ as a PA-generating enzyme. We and others have observed that, in DGKζ-overexpressing T lymphocytes, agonist stimulation of endogenous TCR or of an ectopically expressed muscarinic receptor induced a decrease in ERK signaling (10, 13) due to DGKζ down-regulation of DAG. In these cells, serum stimulation provoked p70S6K Thr389 hyperphosphorylation. This again suggests that, as for DGKα, the role of DGKζ as a DAG attenuator or PA generator depends on the type of stimuli. It remains to be determined whether specific subcellular localization would favor the attenuation of receptor-elicited DAG signals versus PA-dependent mTOR activation.

siRNA experiments further confirm that DGKζ acts in more than one pathway; these studies also show attenuation of mTOR-dependent S6K phosphorylation as well as constitutive PKC activation. Analysis of DGKζ-deficient mice shows that the lack of this enzyme does not induce a strong phenotype because mice are viable, fertile, and appear normal. Peripheral T cells develop normally in these mice but respond more efficiently to TCR stimulation. T-cell proliferation is increased, and knockout mice mount a stronger immune response to choriomeningitis virus infection. After TCR binding, DGKζ deficiency did not result in complete inhibition of PA production. This indicates that, at least during attenuation of the TCR response, the role of DGKζ overlaps that of other isoforms, probably DGKα (12). In these mice, the effect of the decrease in PA production is not known, but the PA required for T-cell proliferation could be provided by activation of another DGK isoform, or the PA deficiency could be counteracted by the robust TCR response.

Previous reports identified the PA produced during DGK activation as a mitogenic agent (23, 62). The present work, however, is the first to provide direct evidence of a DGK positive role on mTOR signaling. Because the mTOR pathway is critical for regulating normal and tumor growth, determining the exact mechanisms that control the activation and possible interrelation of the different PA generators enzymes will be of valuable interest to conceive effective therapeutic strategies.

Acknowledgments—We are grateful to Dr. J. Chen for the gift of reagents, Dr. M. Topham for the anti-DGKζ antibody, and Dr. W. J. van Blitterswijk for the anti-DGKζ antibody. We also thank Dr. I. Mérida’s group members for critical discussion and C. Mark for editorial assistance. The Department of Immunology and Oncology was founded and is supported by the Spanish Council for Scientific Research and by Pfizer.

REFERENCES

1. van Blitterswijk, W. J., and Houssa, B. (2000) Cell Signal. 12, 595–605
2. Luo, B., Prescott, S. M., and Topham, M. K. (2004) Cell Signal. 16, 891–897
3. A. Avila-Florés, T. Santos, E. Rincón, and I. Mérida, unpublished observations.
