Regulation of Diacylglycerol Kinase α by Phosphoinositide 3-Kinase Lipid Products*

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Diacylglycerol kinase α (DAGKα), like all type I DAGKs, has calcium regulatory motifs that act as negative regulators of enzyme activity and localization. Accordingly, DAGKα is activated by phospholipase C-coupled receptors in a calcium-dependent manner. One of the first functions attributed to DAGKs in lymphocytes was the regulation of interleukin 2-induced cell cycle entry. Interleukin-2 nonetheless exerts its action in the absence of cytosolic calcium increase. We have studied alternative receptor-derived signals to explain calcium-independent DAGKα activation, and show that DAGKα is stimulated by Src-like kinase-dependent phosphoinositide 3 kinase (PI3K) activation in lymphocytes. Our results demonstrate that, in vivo, the increase in cellular levels of PI3K products is sufficient to induce DAGKα activation, allowing DAGKα relocation to the intact lymphocyte plasma membrane. This activation is isoform-specific, because other type I DAGKs are not subject to this type of regulation. These studies are the first to describe a pathway in which, in the absence of receptor-regulated calcium increase, DAGKα activation and membrane localization is a direct consequence of PI3K activation.

Diacylglycerol kinases (DAGKs) are a family of enzymes that phosphorylate diacylglycerol (DAG) to generate phosphatidic acid (PA). Activation of these enzymes can modify the balance between the two lipids, both of which have important second messenger functions. Nine DAGK isoforms have been characterized in mammalian cells; all have a conserved catalytic domain and at least two cysteine-rich, C1-like domains of uncharacterized in mammalian cells; all have a conserved catalytic acid (PA). Activation of these enzymes can modify the balance of PI3K products is sufficient to induce DAGKα activation, allowing DAGKα relocation to the intact lymphocyte plasma membrane. This activation is isoform-specific, because other type I DAGKs are not subject to this type of regulation. These studies are the first to describe a pathway in which, in the absence of receptor-regulated calcium increase, DAGKα activation and membrane localization is a direct consequence of PI3K activation.

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The abbreviations used are: DAG, diacylglycerol kinase; DAG, diacylglycerol; PA, phosphatidic acid; PI, phosphoinositides; PI3K, phosphoinositide 3-kinase; HA, hemagglutinin; IL, interleukin; PH, pleckstrin homology; RFP, red fluorescent protein; PBS, phosphate-buffered saline; PI-3,4-P2, phosphoinositide 3,4-bisphosphate; PI-3,4,5-P3, phosphoinositol 3,4,5-triphosphate; GFP, green fluorescent protein; PKD-1, phosphoinositide-dependent protein kinase-1.

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DAGK activity was determined in the immunoprecipitated cell proliferation, together with the suggestion of Src kinase-mediated DAGK activation, prompted us to study in greater detail the possibility of DAGK regulation by a PI3K-dependent mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—CTLL-2 cells were obtained from the ATCC. ProB Ba/F3 lymphoid cells engineered to express human IL-2Rβ (Ba/F3β) were described previously (11). Cells were routinely maintained in RPMI 1640 medium (Invitrogen) supplemented with 2 mM l-glutamine, 5 × 10−5 M 2-mercaptoethanol, and either 20 units/ml recombinant IL-2 (generously provided by Hoffman-La Roche, Nutley, NJ) or 5% WEHI-3B cell supernatant as an IL-3 source. Before IL-2 stimulation, cells were washed and resuspended in serum- and cytokine-free medium for 5–6 h. Human leukemic Jurkat T cells were maintained in RPMI supplemented 2 mM l-glutamine. COS-7 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (BioWhittaker). All media were supplemented with 10% heat-inactivated fetal calf serum (Invitrogen).

**Plasmids and Transfection**—Murine DAGK cDNA fused to a hemagglutinin epitope tag (HA-DAGK) was subcloned into the pEF vector. The construct encoding an EF-hand deletion mutant of DAGK fused to green fluorescent protein (EGFP-ΔEFDAGK) has been described (5). The plasmid encoding p56Lck bearing a mutation of Tyr505 to Phe was a kind gift of Dr. A. Carrera. The plasmid encoding the catalytic subunit of PI3K type I fused to the CAAX domain has been described (16) and was a generous gift of Dr. J. Downward. The plasmid encoding DAGKβ fused to a FLAG tag was a gift of Dr. H. Rondo. The PH domain of Akt (a gift of Dr. P. Coffer) was subcloned into the pEF vector. The construct encoding an EF-hand deletion mutant of DAGK fused to an HA epitope tag (HA-DAGK) was monitored Lck-dependent DAGK activation. Transfection of a constitutive active form of p56Lck (p56	extsuperscript{Lck505}) in the BaF3 proB cell line greatly increased the enzymatic activity of immunoprecipitated DAGKα (Fig. 1A). In lymphocytes, activation of cytosolic Src-like tyrosine kinases (p56Lck and p56	extsuperscript{p95}) is a rapid result of IL-2 binding to its high affinity receptor (16). It has been proposed that activation of this family of tyrosine kinases is, in turn, closely related to this-2-induced activation of type I PI3K (11). We observed that p56Lck-mediated DAGKα activation was markedly reduced by treatment of the cells with the PI3K inhibitor LY294002. (Fig. 1A) suggesting that the p56Lck-induced DAGKα activation was in some way mediated by a PI3K-dependent mechanism.

A direct effect of PI3K on DAGKα activity was next determined by measuring enzyme activity in cells transiently transfected with a constitutively active, membrane-targeted PI3K mutant (p110 CAAX) (17). The increase in DAGKα activity under these conditions confirmed DAGKα activation by a PI3K-dependent mechanism (Fig. 1B). As is the case for other lipid kinases, DAGKα is found in the cytosol of lymphocytes and must relocalize to membranes to exert its functions (5). We analyzed the correlation between DAGKα activation by constitutively active forms of p56Lck and PI3K and the subcellular localization of the protein in intact cells (Fig. 1C). As previously reported for Jurkat T lymphocytes (5), DAGKα was found in the cytosol of Ba/F3 cells in the absence of additional signals. Co-transfection of constitutively active p56Lck and PI3K forms induced enzyme relocalization to the plasma membrane.

**RESULTS**

**Lck-dependent DAGKα Activation Is Mediated by a PI3K-dependent Mechanism**—Studies in non-lymphoid cells have suggested that Src kinase activation is required for hepatocyte growth factor induction of DAGKα (15). p56Lck is a Src-like tyrosine kinase known to mediate IL-2 receptor signals. We used a plasmid encoding murine DAGKα fused to a hemagglutinin epitope tag (HA-DAGKα) to monitor Lck-dependent DAGKα activation. Transfection of a constitutive active form of p56Lck (p56Lck505) in the BaF3 proB cell line greatly increased the enzymatic activity of immunoprecipitated DAGKα (Fig. 1A). In lymphocytes, activation of cytosolic Src-like tyrosine kinases (p56Lck and p56p95) is a rapid result of IL-2 binding to its high affinity receptor (16). It has been proposed that activation of this family of tyrosine kinases is, in turn, closely related to this-2-induced activation of type I PI3K (11). We observed that p56Lck-mediated DAGKα activation was markedly reduced by treatment of the cells with the PI3K inhibitor LY294002. (Fig. 1A) suggesting that the p56Lck-induced DAGKα activation was in some way mediated by a PI3K-dependent mechanism.

The role of DAGKα and PI3K in the regulation of IL-2-mediated cell proliferation, together with the suggestion of Src kinase-mediated DAGKα activation, prompted us to study in greater detail the possibility of DAGKα regulation by a PI3K-dependent mechanism.

Western Blot—Immunoprecipitated proteins or total cell lysates were resolved in SDS-PAGE and transferred to nitrocellulose membranes for Western blot analysis. Proteins were visualized with anti-HA antibody (12B10, anti-phospho-Akt (Ser-473; Cell Signaling Technology, Beverly, MA), or anti-Akt (Upstate Biotechnology, Lake Placid, NY).

**Lipid Effect on DAGK Activity**—HA-DAGKα-expressing Ba/F3 cells were serum-starved (5 h), lysed in Nonidet P-40 buffer, and HA-DAGKα was immunoprecipitated with anti-HA antibody. Pollets were washed twice with lysis buffer, once with 0.5 M LiCl, and three times with 10 mM Hepes. The indicated lipids were added as a 10-fold concentrate (1 mM in 5 μl) prepared by sonication in 10 mM Tris-HCl, pH 7.4, 5 min before C8-DAG addition. The phosphorylation reaction was carried out as described (7).

**Immunofluorescence Microscopy**—Cells were harvested 24 h after electroporation encoding plasmids, washed, and allowed to attach to poly-l-lysine-coated coverslips (1 h, room temperature). Samples were washed with PBS, fixed with 4% paraformaldehyde in PBS (4 °C, 20 min), permeabilized in PBS, 2% bovine serum albumin, 0.2% Triton X-100, and blocked with 2% bovine serum albumin, 0.1% Triton X-100 in PBS buffer; antibodies were incubated (37 °C, 1 h) in a humidified chamber. Nuclei were labeled with Topro-3 (Molecular Probes, Eugene, OR). Fluorescence was analyzed on a Leica confocal microscope (TCS-NT) with associated software.

**Dephosphorylation**—For dephosphorylation, proteins were subjected to in vitro dephosphorylation using exogenous recombinant protein phosphatase 1 (PP1) or 2A (PP2A) for 60 min at 30 °C in the presence of 1 μg of PP1 or 2A.

**RESULTS**

**Lck-dependent DAGKα Activation Is Mediated by a PI3K-dependent Mechanism**—Studies in non-lymphoid cells have suggested that Src kinase activation is required for hepatocyte growth factor induction of DAGKα (15). p56Lck is a Src-like tyrosine kinase known to mediate IL-2 receptor signals. We used a plasmid encoding murine DAGKα fused to a hemagglutinin epitope tag (HA-DAGKα) to monitor Lck-dependent DAGKα activation. Transfection of a constitutive active form of p56Lck (p56Lck505) in the BaF3 proB cell line greatly increased the enzymatic activity of immunoprecipitated DAGKα (Fig. 1A). In lymphocytes, activation of cytosolic Src-like tyrosine kinases (p56Lck and p56p95) is a rapid result of IL-2 binding to its high affinity receptor (16). It has been proposed that activation of this family of tyrosine kinases is, in turn, closely related to IL-2-induced activation of type I PI3K (11). We observed that p56Lck-mediated DAGKα activation was markedly reduced by treatment of the cells with the PI3K inhibitor LY294002. (Fig. 1A) suggesting that the p56Lck-induced DAGKα activation was in some way mediated by a PI3K-dependent mechanism.

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**DAGKα Is Activated by PI3K Lipid Products**—The previous experiments suggested a mechanism that regulates DAGKα in response to PI3K activation. In vivo, PI3K activation following receptor triggering catalyzes PI-4,5-P₂ phosphorylation, generating the intracellular messenger PI-3,4,5-P₅, which is rapidly converted to PI-3-P₂ by the SH2-containing inositol phosphatase lipid phosphatases. Generation of these two lipids, phosphorylated at the 3' position of the inositol ring, induces membrane relocalization and activation of several signaling proteins that bear specific lipid-binding domains (18). By dephosphorylating at the D3 position, the phosphatase PTEN terminates PI3K-dependent signals. PI3K-dependent regulation of DAGKα may thus be a direct consequence of the increase in 3'-phosphorylated lipid products or may be mediated by a PI3K-regulated protein.
We analyzed the effect of 3'-phosphorylated lipid addition on DAGKα activity. When immunoprecipitated, HA-DAGKα produced a single band in silver-stained SDS-PAGE gels (not shown). The immunoprecipitated protein was thus used as a source of purified protein to determine enzyme activity after addition of lipid vesicles. Addition of purified PI-4,5-P2 caused no apparent effect on enzyme activity, whereas vesicles containing PI-3,4,5-P3 or PI-3,4-P2 induced a marked increase in DAGKα activity (Fig. 2A). The results indicate direct DAGKα regulation by the lipid products of PI3K, excluding nonspecific regulation by negatively charged lipids.

PI-3,4,5-P3 and PI-3,4-P2 are synthesized by class I PI3K in response to receptor stimulation. In addition to type I PI3K lipid products, generation of other phosphoinositides is less finely regulated and appears to be implicated in secretion and vesicular trafficking (19). PI-3-P can be generated by PI phosphorylation by class III PI3K or by sequential hydrolysis of PI-3,4,5-P3 by 4- and 5-phosphatases. Fab1-like kinases in turn phosphorylate PI-3-P to PI-3,5-P2, which increases in an IL-2-dependent manner in hematopoietic cells (20). Addition of purified PI-3-P and PI-3,5-P2 did not affect DAGKα activity (Fig. 2B), suggesting specific regulation of DAGKα by class I PI3K lipid products.

**DAGKα N-terminal Domain Is a Negative Modulator of Enzyme Interaction with PI3K Products in Intact Cells**—The DAGKα N-terminal domain contains two Ca2+ regulatory elements that act as negative regulatory domains (4, 5). Deletion of the Ca2+ regulatory elements in the DAGKα N-terminal domain induces an active enzyme form (ΔEF-DAGKα) that is constitutively located at the plasma membrane in the Jurkat leukemic T cell line (5). Jurkat cells are characterized by defective PTEN expression, which results in constitutive membrane localization of the tyrosine kinase Itk via interaction through its PH domain (21). Because the previous experiments suggested direct DAGKα interaction with type I PI3K lipid products, we examined whether constitutive ΔEF-DAGKα localization to the plasma membrane was a direct result of the elevated PI-3,4,5-P3 and PI-3,4-P2 levels in Jurkat cells. As a control for endogenous 3'-phosphorylated lipid levels in these

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**Fig. 1.** Active pp56<sup>lck</sup> or PI3K forms induce DAGKα activation and membrane localization. A, Ba/F3 cells were stably transfected with DAGKα cDNA fused to a hemagglutinin epitope tag (HA-DAGKα). Cells were transiently transfected with empty plasmid (−) or a plasmid encoding a constitutive active form of pp56<sup>lck</sup> (Lck505). After 24 h post-transfection, cells were washed and serum-starved for 5 h. During the last 2 h, cells were incubated with LY294002 at the indicated concentrations. DAGKα activity was determined in the immunopellets as described (“Experimental Procedures”). A tenth of the immunoprecipitated protein was analyzed by SDS-PAGE and Western blot with anti-HA antibody. One representative experiment is shown of four performed with similar results. B, cells expressing HA-DAGKα were transiently transfected with a constitutively active form of PI3K (p110 CAAX). After 24 h, cells were washed, serum-starved, and DAGKα activity was determined in the pellets. One representative experiment of three is shown. C, Ba/F3 cells were transiently cotransfected with HA-DAGKα and empty plasmid, p110 CAAX, or pp56<sup>lck</sup><sup>505</sup>. After 24 h, cells were serum-starved, fixed, and subcellular DAGKα localization was determined by immunostaining with an anti-HA antibody. Nuclei were visualized using Topro-3.
cells, we examined the subcellular localization of a construct expressing the Akt PH domain fused to RFP (RFP-AktPH). As shown for Itk (21), the RFP-AktPH construct was found constitutively associated to the plasma membrane of Jurkat cells in the absence of stimulation (Fig. 3). As we have described (25), ΔEFDAGKα also exhibits plasma membrane localization in these cells. Expression of wild type PTEN caused rapid RFP-AktPH redistribution from the plasma membrane to cytosol; the same was observed for GFP-ΔEFDAGKα (Fig. 3). Two important conclusions can be derived from these studies. The first is that DAGKα interaction with 3'-phosphorylated phosphoinositides takes place in intact cells; the second, that the N-terminal regulatory motif, which governs DAGKα membrane translocation in response to Ca++ elevation, prevents DAGKα binding to 3'-phosphorylated lipids in the absence of stimulus.

**Regulation by PI3K Lipid Products Is DAGKα Isoform-specific**—Membrane translocation seems a general mechanism for regulation of the three subtype I DAGKs (6). DAGKα and -γ translocate from cytosol to the membrane in response to purinergic receptor stimulation, although the kinetics of the former are more rapid than those of the latter. DAGKγ translocation, which has a classical 12-O-tetradecanoylphorbol-13-acetate/DAG-binding C1α domain, is induced by 12-O-tetradecanoylphorbol-13-acetate treatment of cells. DAGKα, in which the C1α domain is atypical, does not respond to 12-O-tetradecanoylphorbol-13-acetate. Regulation of DAGKβ translocation appears more complex because, in COS cells, this isoform is found at the plasma membrane in the absence of stimulus and relocates to the cytosol only after serum starvation (22).

We examined whether PI3K-dependent regulation can also be extended to other type I subtype isoforms. Because DAGKγ membrane localization appears to be exclusively DAG-dependent (6), we studied DAGKβ subcellular redistribution in lymphoid cells. As described for COS cells (22), DAGKβ is found at the BaF/3 cell plasma membrane in the absence of additional stimuli (Fig. 4A). At difference from the case of COS cells, the enzyme remained membrane-bound after an extended starvation period (Fig. 4B). The distinct subcellular localization of DAGKα and -β in unstimulated cells suggests isofrom-specific regulation for membrane targeting. We next analyzed DAGKβ activation by phosphorylated PI derivatives. 3'-Phosphorylated lipid addition to immunoprecipitated DAGKβ did not affect enzyme activity; whereas the protein was activated following PI-4,5-P2 addition (Fig. 4B). This indicates that DAGKα regulation by 3'-phosphorylated PI is specific to this isoform, and suggests isoform-specific regulation of type I DAGKs by different phosphorylated PI forms.

**IL-2-dependent DAGKα Activation Requires Functional PI3K**—Our experiments define a DAGKα regulation mechanism based on PI3K activation. To confirm that this signaling pathway is engaged following agonist stimulation of cells, we examined IL-2-dependent activation of endogenous DAGKα. We generated a polyclonal antibody that immunoprecipitates murine DAGKα (Fig. 5A) and used this antibody to examine IL-2-dependent activation of endogenous DAGKα in BaF/3 cells engineered to express the human IL-2Rβ (BaF/3β). These cells proliferate in response to IL-2 and have been used extensively to study IL-2-dependent signals (10). IL-2 addition to BaF/3β cells increased enzyme activity measured in DAGKα immunoprecipitates (Fig. 5B). In these experiments, immunoprecipitated protein could not be quantified accurately because of limita-
tions of the antibody for Western blot analysis. We thus used the HA-DAGKα construct to monitor DAGKα activation after IL-2 stimulation. As for the endogenous enzyme, IL-2 addition to transfected BaF/3 cells induced a rapid increase in HA-DAGKα activity confirming IL-2-dependent regulation (Fig. 5C). IL-2-dependent DAGKα activation was completely abolished when cells were serum-starved and, where indicated, incubated with the PI3K inhibitor LY294002 prior to IL-2 stimulation. DAGK activity was determined in the anti-HA pellets. A tenth of the immunoprecipitated protein was analyzed in SDS-PAGE and Western blot with anti-HA antibody. E, CTLL-2 cells were pretreated with the indicated concentrations of LY294002 prior to IL-2 stimulation. Activity was determined in DAGKα immunopellets as described under "Experimental Procedures." Prior to immunoprecipitation, cell lysate (20 μg) was resolved in SDS-PAGE and Western blot with antibodies to phospho-Ser473-Akt or Akt.

**Fig. 5. IL-2-induced DAGKα activation depends on PI3K activation.** A, immunoprecipitation of DAGKα by the anti-DAGKα rabbit polyclonal antibody. COS cells were transfected with empty vector or HA-DAGKα, and lysates were immunoprecipitated with anti-HA or anti-DAGKα antibodies. Immunopellets were washed and analyzed by Western blot with anti-HA antibody. The band corresponding to the 80-kDa DAGKα is shown. B, IL-2-dependent activation of endogenous DAGKα was determined by immunoprecipitation from BaF/3 cells lysates using the anti-DAGKα antibody. The lipid kinase assay was performed as described under "Experimental Procedures." An autoradiogram of the TLC shows the radioactivity co-migrating with the C8-PA standard. C, BaF/3β cells were transiently transfected with HA-DAGKα. After 24 h, cells were serum-starved and IL-2 stimulated. The tagged enzyme was immunoprecipitated and activity was determined. A tenth of the immunoprecipitated protein was analyzed in SDS-PAGE and Western blot with anti-HA antibody. D, BaF/3β cells were HA-DAGKα transfected. After 24 h, cells were serum-starved and, where indicated, incubated with the PI3K inhibitor LY294002 prior to IL-2 stimulation. DAGK activity was determined in the anti-HA pellets. A tenth of the immunoprecipitated protein was analyzed in SDS-PAGE and Western blot with anti-HA antibody. E, CTLL-2 cells were transiently transfected with HA-DAGKα. After 24 h, cells were serum-starved and IL-2 stimulated. The tagged enzyme was immunoprecipitated and activity was determined. A tenth of the immunoprecipitated protein was analyzed in SDS-PAGE and Western blot with anti-HA antibody.

As previously shown for hepatocyte growth factor induction of DAGKα in non-lymphoid cells (15), IL-2-dependent DAGKα activation in CTLL2 cells was greatly diminished following PP2 treatment of the cells (Fig. 6A). Inhibition of Src-like kinases also prevented IL-2-induced activation of exogenous DAGKα in BaF/3β cells (Fig. 6B). The PP2 doses shown to prevent DAGKα activation also inhibited IL-2-induced Akt phosphorylation (Fig. 6C). In summary, these results confirm the existence of a signaling pathway in which Src-like kinases regulate DAGKα activation via a PI3K-dependent mechanism.

**Fig. 6. IL-2-induced DAGKα activation requires Src-like kinase-dependent PI3K activation.** A, CTLL-2 cells were pretreated with the indicated concentrations of PP2 prior to IL-2 stimulation. Activity was determined in DAGKα immunopellets as described under "Experimental Procedures." B, BaF/3β cells were HA-DAGKα transfected. After 24 h, cells were serum-starved and, where indicated, incubated with the Src kinase inhibitor PP2. C, BaF/3β cells were serum-starved and, where indicated, incubated with the Src kinase inhibitor PP2. After IL-2 stimulation, cell lysate (20 μg) was resolved in SDS-PAGE and Western blot with antibodies to phospho-Ser473-Akt or Akt.

**DISCUSSION**

Class I PI3K activation by receptor and cytosolic tyrosine kinases is essential for regulation of cell proliferation, survival,
metabolism, cytoskeletal reorganization, and membrane trafficking (18). This lipid kinase can participate in such a large array of cellular functions by activating multiple target molecules, which bear 3’-phosphoinositide-binding domains. Here we describe a novel pathway in which Src kinase-dependent PI3K activation induces DAGKα membrane localization and activation, highlighting the role of this enzyme as another direct effector of PI3K action.

PI3K is activated rapidly following growth factor stimulation, and recent studies have demonstrated direct PI3K regulation by growth factor-induced activation of Src tyrosine kinase (23). In lymphocytes, activation of cytosolic Src-like tyrosine kinases (p56\(^{Lck}\) and p55\(^{Fyn}\)) is a rapid result of IL-2 binding to its high affinity receptor (16). Src tyrosine kinase activation is, in turn, closely related to IL-2-induced activation of type I PI3K. Studies in resting T cells have shown that, in the absence of Jak3 expression, IL-2 activates PI3K through a p56\(^{Lck}\)-mediated mechanism (24). In activated T cells, full PI3K activation by IL-2 also requires p56\(^{Lck}\) (11, 25). The data presented here confirm, using several approaches, that PI3K lies upstream of DAGK activation and that Src kinase-dependent DAGK activation is mediated by generation of PI3K lipid products. Although several studies linked tyrosine kinases to receptor-induced DAGK activation (5, 15), none reported receptor-induced DAGK phosphorylation on tyrosine residues. Concuring with these results, we do not detect IL-2-dependent tyrosine phosphorylation of endogenous or ectopically expressed DAGKα. Nonetheless, the existence of such a mechanism cannot be disregarded. Additional experiments are currently under way to analyze the existence of rapid, transient DAGK phosphorylation on tyrosine residues as a consequence of receptor-regulated tyrosine kinase activation. Notwithstanding, and in accordance with the results presented here, it would be of interest to examine the role of PI3K-dependent signaling in DAGK regulatory after triggering of other receptors.

Addition of 3’-phosphorylated lipids activates immunoprecipitated DAGKα, suggesting a direct role for lipid PI3K products in regulating enzyme activity. In vivo elevation of 3’-phosphorylated lipids translates the protein to the membrane in intact cells, suggesting a correlation between DAGKα activation and membrane localization. In agreement with previous studies (7, 15), we show that DAGKα activation can be measured in immunoprecipitates following cell stimulation with IL-2 or co-transfection with active forms of p56\(^{Lck}\) or PI3K. Assays in immunoprecipitates are performed in the absence of PI-3,4,5-P\(_3\) or PI-3,4-P\(_2\), suggesting that, once activated, DAGKα is locked into an active conformation that is maintained throughout the immunoprecipitation process. This is reminiscent of the model proposed for Akt and other AGC kinases, in which both lipid binding and phosphorylation by the phosphoinositide-dependent protein kinase-1 (PKD-1) are required for full activation (26). PKD-1 is a key regulator of PI3K-dependent activation of several substrates which, in some cases, phosphorylates its substrates after direct interaction through the so called hydrophobic motif (27). DAGKα has a partial hydrophobic motif (Phe-X-X-Phe) at the C terminus, suggestive of PKD-1 interaction. Nonetheless, we did not detect interaction between DAGKα and PKD-1, even after co-transfection with active PI3K (not shown), excluding direct DAGKα-PKD1 interaction. DAGKα phosphorylation was not observed following IL-2 stimulation after in vivo (\(^{32}\)P) labeling of the cells (not shown) apparently excluding any PI3K-dependent phosphorylation. PI3K-dependent DAGKα activation thus appears to differ from that described for PKD-1-regulated PI3K effectors.

The primary DAGKα sequence contains distinct conserved domains, none of which corresponds to any of the 3’-phosphorylated binding domains found in PI3K-regulated proteins. This renders it difficult to assign the PI3K-dependent regulation described here to a specific protein domain. The DAGKα N-terminal region contains both a recoverin homology domain and a pair of Ca\(^{2+}\)-binding EF-hands shown to act jointly as an autoinhibitory region (3–5). We demonstrate that the constitutive association of the ΔEF DAGKα mutant is reverted by PTEN overexpression, suggesting that membrane association of this truncated mutant is due, at least in part, to the high constitutive levels of 3’-phosphorylated lipids in this cell line. These experiments rule out the N-terminal region as the domain responsible for lipid interaction, and extend the previously reported role of this region as a negative modulator of enzyme activity to that of membrane binding.

Like all DAGKs, DAGKα also contains a pair of C1-like domains of unknown function. Based on their primary sequence, the two DAGKα C1 domains can be classified as atypical, similar to those found in proteins such as atypical protein kinase C isoforms, the proto-oncogenes raf-1 and Vav, and in proteins implicated in small G-protein-dependent signaling, including ROCK, Citron, and Lck (28). There is no clear function assigned to this type of domain, although they are suggested to participate in membrane binding through protein-protein and/or protein-lipid interactions. In the case of DAGKα, the disruption of either of the two C1 domains abolishes protein membrane association.2 Similar results are obtained with other isoforms such as DAGKπ (29), suggesting that these domains are critical in protein-membrane interaction. We tried to investigate the translocation of GFP-tagged DAGKα into the membrane in response to PI3K elevation, but this construct is not correctly expressed in mammalian cells. This suggests that, as shown for the atypical C1 domains in Raf-1 or Vav, this conserved region does not act as a membrane targeting module on its own.

Studies in other type I DAGK isoforms have shown that the C1 domains, albeit necessary, are not exclusively responsible for membrane localization of DAGK family members. The other two type I DAGK isoforms, β and γ, each contain at least one classical phorbol ester-binding C1 domain, and phorbol ester-dependent membrane translocation has been demonstrated for these two isoforms (6, 22). The characterization of alternative splicing forms of DAGKβ, however, indicates that sequences located at the C-terminal domain are also responsible for membrane localization. Accordingly, the deletion of the 35 last residues in the DAGKβ sequence abolishes its ability to relocate to the membrane after phorbol ester treatment of serum-starved COS cells (22). The data presented here suggest that DAGKβ, which is constitutively associated to the plasma membrane in lymphoid cells, is positively regulated by PI-4,5-P\(_2\). This indicates lipid-based regulation for this isoform in addition to DAG/12-O-tetradecanoylphorbol-13-acetate-dependent regulation. Further studies should address the potential role of both the C1 domains and the C-terminal sequences in lipid-based regulation of DAGK isoforms.

Membrane translocation is an effective process for regulating DAGK activity and subtype-specific functions. Experiments using GFP-fused chimeric proteins have shown subtype-specific translocation of the three type I DAGKs (∝, β, and γ) (6) and also DAGKπ (29). Our experiments here demonstrate that the same isoform is regulated by distinct mechanisms in response to stimulation of different receptors. The existence of more than one mechanism for DAGKα activation would thus...

\(^2\) M. A. Sanjuán, D. R. Jones, S. Carrasco, and I. Mérida, manuscript submitted for publication.
define distinct functional roles for this enzyme in receptor-regulated responses. In response to the phospholipase C-derived Ca\(^{2+}\) elevation that follows TCR triggering, DAGK\(\alpha\) acts as a negative regulator in DAG-mediated responses (5). As a result of IL-2-dependent PI3K activation, DAGK\(\alpha\) participates as a positive modulator of PA-regulated signals (8). The precise role of PA in IL-2-dependent cell proliferation has not been fully elucidated, although recent studies linked PA to the regulation of several molecules known to regulate cell proliferation including c-Raf (30), phosphatases (31), and mTOR (32). IL-2-mediated activation of PI3K is required for induction of IL-2-dependent cell cycle entry (33), and is known to mediate mRNA translation through regulation of p70S6 kinase (13). In lymphocytes, PI3K and DAGK\(\alpha\) thus emerge as two lipid kinases essential for IL-2-regulated cell proliferation. This is the first report of a functional link between these two enzymes, and future studies should help to characterize the role of DAGK\(\alpha\)-mediated PA production in the regulation of PI3K-dependent events such as transductional regulation, cell cycling, and cytoskeletal reorganization. Moreover, the activation of this SrcK/PI3K/DAGK\(\alpha\) pathway in response to other agonists known to act in the absence of phospholipase C activation remains to be examined.

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REFERENCES
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