Phosphatidic Acid Generation through Interleukin 2 (IL-2)-induced α-Diacylglycerol Kinase Activation Is an Essential Step in IL-2-mediated Lymphocyte Proliferation*

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Proliferation of T lymphocytes is triggered by the interaction of interleukin 2 (IL-2) with its high affinity specific receptor that is expressed on the cell surface following T lymphocyte activation. Significant advances have recently been made in identifying the multiple signals that follow IL-2 receptor occupancy, although the exact mechanism responsible for IL-2-induced proliferation remains an enigma. It has been shown previously that unique species of phosphatidic acid are rapidly produced in vivo following IL-2 binding. It was then suggested that, in contrast to other eukaryotic growth factor systems, phosphatidic acid was at least in part generated through IL-2-induced diacylglycerol (DG) kinase activation. In the present study we demonstrate IL-2-dependent activation of the α isoform of DG kinase. Confocal microscopy studies reveal that the enzyme is located in the cytosol and nuclei of resting T cells. Interleukin 2 stimulation induces translocation of the enzyme to the perinuclear region. Furthermore, our results indicate that inhibition of the α isoform of DG kinase has a profound effect on IL-2-induced T cell growth. Studies on cell cycle distribution demonstrate that the inhibition of IL-2-induced phosphatidic acid production induces arrest in late G1 phase of IL-2-dependent cells. Altogether, these results link previous observations of interleukin 2 and phosphatidic acid production to activation of an specific isoform of DG kinase and suggest that activation of this enzyme is part of a novel signaling cascade that utilizes phosphatidic acid as an effector molecule.

Activation of T lymphocytes by cell-bound antigens induces the expression of high affinity interleukin 2 (IL-2) receptors on the cell surface. The binding of IL-2 to its high affinity receptor triggers a complex signaling program that ultimately results in cell proliferation. Although the transmembrane pathways activated upon IL-2 binding have been the subject of intensive studies, the exact mechanism responsible for the IL-2-induced progression through the cell cycle remains largely undefined. The functional high affinity IL-2 receptor is a heterotrimeric complex composed of at least three distinct polypeptide chains designated IL-2Rα (p55), IL-2Rβ (p75), and IL-2Rγ (p64) (1–3). Although IL-2Rα is capable of low affinity binding to IL-2, high affinity binding and biological response to IL-2 require association of the three proteins. All the studies performed to date indicate that the cytoplasmic domains of the β and γ subunits are involved in transducing the IL-2 proliferative signal (4, 5). The cytoplasmic domains of both proteins are devoid of any known intrinsic catalytic activity; thus, the early responses to IL-2 stimulation must be therefore transmitted by receptor-associated cytoplasmic enzymes. In this regard, activation of Src family and Janus family tyrosine kinases occupy a central role in the initiation of the IL-2-induced proliferative signal (6, 7). As has been described for other receptors, tyrosine kinase activation would in turn induce association and activation of other signaling molecules such as phosphatidylinositol 3-kinase, p74 Raf kinase, or p21ras (8–10).

In our search for new molecules implicated in the transduction of the IL-2 proliferative signal, we have recently demonstrated that IL-2 induces the rapid activation of a DG kinase (DGK) in T lymphocytes following IL-2 binding to the high affinity receptor (11). DGK (ATP:1,2-diacylglycerol 3-phosphotransferase; EC 2.7.1.107) rapidly converts DG to phosphatidic acid (PA). This activity has been largely known as a regulator of the intracellular levels of DG, a second messenger that, in turn, regulates cellular events through activation of protein kinase C. In T cells, however, previous analysis indicates that proliferation upon IL-2 binding does not require activation of the classical and new protein kinase C family (12, 13). In fact, previous studies failed to reproducibly detect phosphatidylinositol 4,5-bisphosphate hydrolysis or increases in Ca2+ flux as components of the IL-2 proliferative response. Although IL-2 does not generate DG as a result of phosphatidylinositol hydrolysis by phospholipase C γ, accumulation of DG and PA through hydrolysis of GPI molecules following IL-2 binding has been reported (14, 15). Activation of DGK could therefore serve as a source of PA as an alternative mechanism to GPI-phospholipase D activation. Several findings indicate that, rather than being an intermediate in phosphatidylinositol turnover, the PA generated upon IL-2 binding may itself modulate signaling pathways responsible for the IL-2-induced lymphocyte proliferation. For instance, addition of exogenous PA to CTLL-2 cells is able to mimic the IL-2 effect in proliferation as well as c-Myc induction (11, 16). The consecutive activation of a GPI-PLC and a DGK would therefore constitute an essential step in the generation of the bioactive lipid PA.

Although increases in DGK activity following IL-2 binding were previously described, the identity of the enzyme responsible for this activity was not known. Several mammalian
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DGKs have been purified from various tissues so far, and some of them have already been cloned. At the present time the best characterized is the one having a mass of 80 kDa (αDGK) originally purified from porcine brain cytosol (17). This isoform, which is highly tissue-specific, is only present in brain and lymphoid tissue. This enzyme is extremely abundant in the cytosol of lymphocytes and comprises more than 0.2% of the total soluble protein in T cell-enriched pig splenocytes (18). The aa sequences described for this DGK isozyme from porcine, human, and rat tissue are more than 80% identical to each other. The primary structure contains two sets of EF-hand motifs and a cysteine-rich zinc finger-like sequence (19). Although lymphocytes contain very high levels of DGK α isoform, the role of this enzyme on the regulation of the immune response has never been addressed. In the present study we have investigated the activation of the αDGK isoform upon IL-2 binding on T lymphocytes. Immunoprecipitation studies using a specific antibody against αDGK demonstrate IL-2-dependent activation of this enzyme. By using inhibitors of this enzyme, we demonstrate that this isoform is responsible for the majority of PA generated in response to IL-2 through DGK activation. Confocal microscopy studies reveal that the enzyme, located in the cytosol and nuclei of resting cells, translocates to the perinuclear space in response to IL-2 binding. Using αDGK inhibitors we demonstrate the essential role of the PA generated through αDGK activation on T cell growth. Finally, cell cycle analysis demonstrates that inhibition of PA production prevents the cells from entering S-phase. These results further demonstrate that activation of the αDGK isoform and the subsequent accumulation of PA play a significant role in IL-2-dependent control of cell cycle progression in T lymphocytes.

EXPERIMENTAL PROCEDURES

Cells—The natural killer-like cell line YT was a gift from Dr. Kendall Smith (Dartmouth University). YT cells were maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum, 4 mM l-glutamine, 50 mM 2-mercaptoethanol, and 10 mM HEPES. CTL-L2 cells (obtained from the American Type Culture Collection) were maintained in the same medium supplemented with 10% (v/v) conditioned medium from concanavalin A-stimulated rat splenocytes. Before stimulation with IL-2, the cells were washed and resuspended in serum- and IL-2-free medium for 2 to 4 h. This incubation time was the minimum period necessary to arrest cell growth without loss of cell viability.

Reagents—Reconstituted human IL-2 was generously provided by Hoffmann-LaRoche Inc. (Nutley, NJ). DG kinase inhibitors I (R59022) and II (R59949) were from Calbiochem. Protein A-Sepharose, Nonidet P-40, protein inhibitors such as leupeptin, aprotinin, and PMSF, and phospholipids such as 1,2-DG (dioleoyl), 1,2-di-O-phosphatidic acid (diacylglycerol), and lysophosphatidylcholine (paclitaxol) were obtained from Sigma. 1,2-Dioctanoylgllycerol was obtained from Molecular Probes. [γ-32P]ATP, FITC-conjugated anti-rabbit monoclonal antibody, and ECL Western blotting detection system were from Amersham Corp. Silica Gel G-60 TLC plates were obtained from Whatman. All the organic solvents were from Merck. The preparation and characterization of rabbit antibody against αDGK has been described previously (17).

Measurement of DGK Activity—15 × 106 quiescent cells were stimulated with either buffer or 100 units/ml recombinant IL-2. After the time of period indicated in the figure legends, the cells were harvested, washed twice with ice-cold phosphate-buffered saline, and frozen at −70°C. The cells were thawed and lysed by nitrogen cavitation (10 min at 500 p.s.i., 4°C) in a buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 50 mM NaF, 2 mM Na3VO5, 1 mM PMSF. 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Chloroform was dried under nitrogen, resuspended in a small volume of 10 mM Tris, and sonicated before being added to the phosphorylation mix. Standard phosphorylation assays were carried out for 15 min at room temperature in a final volume of 50 μl. When inhibition studies were performed, DGK inhibitors were added to lysates or immunoprecipitates 10 min before the DG. At the end of phosphorylation assay, lipids were extracted by subsequent addition of 200 μl of CHCl3/MEOH (2:1), 50 μl of CHCl3, and 50 μl of 0.1 N HCl. After centrifugation at 500 × g, the organic layers were recovered, dried under a stream of nitrogen, redissolved in 20 μl of CHCl3/MEOH (2:1), and spotted on silica gel 60 plates along with dioleyl PA standard. Plates were developed with a solvent system of CHCl3/MeOH, 4 N NH4OH (9:7:2, v/v/v). Dried plates were subjected to autoradiography, and the bands corresponding to PA were quantified by scanning of the autoradiograms.

Immunoprecipitation and Immunoblotting—15 × 106 quiescent cells were incubated with either buffer or 100 units/ml recombinant IL-2. After the periods of time indicated in the figure legends, the cells were harvested, washed twice with ice-cold phosphate-buffered saline, and lysed in ice-cold lysis buffer (1% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 50 mM NaF, 2 mM Na3VO5, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After 15–30 min at 4°C, the whole cell lysate was centrifuged at 15000 rpm and the supernatant pre-cleared by treatment with 50 μl/sample protein A-Sepharose. The pre-cleared lysates were then subjected to immunoprecipitation with the αDGK antibody. The immune complexes were collected on protein A-Sepharose. After extensive washing immunoblots were ready for use for the DG kinase assay, performed as described above, or for immunoblotting. When immunoblotting was to be performed, proteins were eluted from protein A beads in Laemmli buffer, separated on reduced SDS-polyacylamide gels, and then electrophoretically transferred for 1 h at 250 mA to nitrocellulose (Bio-Rad) in buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol. The membranes were then blocked by incubation with Tris-buffered saline (0.15 M NaCl, 20 mM Tris-HCl, pH 7.4) containing 5% dried nonfat milk. The specific proteins were detected by enhanced chemiluminescence (ECL System) and autoradiography following incubation of the blot with primary antibody and horseradish peroxidase-linked secondary antibody.

Indirect Immunofluorescence—1 × 105 exponentially growing cells were washed, deprived of serum and IL-2 for 4 h, and then incubated either with buffer or with 100 units/ml IL-2 plus or minus R59022 (10 μg/ml final) for 10 min. Cells were harvested and centrifuged in a cryotube (Heraeus) for 10 min at 600 rpm over glass slides. Cells were fixed with methanol for 3 min at −20°C and permeabilized with 100 μg/ml lyso-phosphatidylcholine in phosphate-buffered saline for 2 min at room temperature. Slides were washed twice for 2 min at room temperature with 1% BSA in phosphate-buffered saline. Rabbit polyclonal anti-αDGK antibody diluted in PBS/BSA was added to the slides under a coverslip and incubated in a moist chamber for 1 h at room temperature. After washing once with PBS and twice with PBS/BSA, anti-rabbit FITC monoclonal antibody was added and incubated for 45 min in a moist chamber at room temperature. After washing three times with PBS/BSA and once with PBS, slides were mounted in Moviol (Sigma) with anti-fading. All confocal experiments were analyzed in a Zeiss laser scanning microscope. The images were acquired using software from Carl Zeiss.

DNA Synthesis—CTL-L2 cells exponentially growing on IL-2-supplemented medium were washed free of IL-2 and resuspended in RPMI, 10% fetal calf serum. After 15 h of starving, the IL-2-deprived cells were resuspended with either IL-2 (50 units/ml) or no IL-2 plus DGK inhibitors at the indicated concentrations. 10,000 cells were seeded in 96-well plates, and quadruplicate samples were pulsed at 2-h intervals with 1 μCi/well [3H]thymidine. Cellular DNA was subsequently harvested on glass-fiber filters, and 3H uptake was quantified by liquid scintillation counting.

Cell Cycle Analysis—Cells were harvested by centrifugation and washed with phosphate-buffered saline buffer. After centrifugation the cells were resuspended in permeabilization solution (0.1% sodium citrate, 0.05% Nonidet P-40). The samples were treated with 50 μg/ml RNase A for 30 min at room temperature, and then propidium iodide was added to a final concentration of 20 μg/ml. After 20 min, the fluorescence of the propidium iodine-stained DNA was quantitated on a per cell basis with an EPICS-XL flow cytometer (Coulter).

RESULTS

IL-2-induced Production of PA Is Inhibited by the αDGK Inhibitor R59022—Previous studies have demonstrated that DGK activity was markedly increased following addition of IL-2 to lymphoid cells (11). This indicates that IL-2-dependent increases in PA can, at least in part, be attributed to DGK-dependent conversion of DG to PA. As a first step to demonstrate
The potential involvement of the αDGK isoform in the generation of PA by IL-2, we have utilized a specific inhibitor of this enzyme R59022. This inhibitor has been demonstrated to abolish activity of purified αDGK, whereas other DGK species do not (20). For these experiments we have used the human natural killer cell line YT, which expresses high levels of high affinity IL-2 receptor. Cells harvested at different times after addition of IL-2 were lysed by nitrogen cavitation. The assay was performed by quantifying the in vitro phosphorylation of exogenously added DG to yield [32P]PA as described under "Experimental Procedures." Fig. 1 shows the transient increase in phosphorylated PA obtained after addition of IL-2 (upper panel). The rate of PA formation, and thus DGK activation, was maximum at 5 min, approximately 6-fold higher in IL-2-treated cells than in control cells. When R59022 at a concentration of 25 μM final was added to the phosphorylation reaction, the rate of PA formation diminished by 70% of the initial value (lower panel). This inhibitor concentration has been shown previously to be enough to abolish αDGK activity. Similar results were obtained with another DGK inhibitor, R59949 (21), added at a final concentration of 10 μM (data not shown). The autoradiograms were scanned, and a quantification of four different assays results is presented.

IL-2-stimulated DGK Activity Is Immunoprecipitated by Anti-αDGK Antibodies—Although known to be very abundant in T lymphocytes, no agonist-induced activation of the αDGK isoform has been demonstrated. Using a polyclonal antibody, we immunoprecipitated the enzyme from detergent cell lysates of quiescent and IL-2-stimulated YT cells. To verify the specificity of the antibody in immunoprecipitating this DGK isoform, immunoblot analysis of the immunoprecipitates was performed using the same antibody. As shown in Fig. 2A, a protein with molecular weight corresponding to the αDGK is recognized by the anti-αDGK antibody in the immunoprecipitates. The next step was to demonstrate activation of the αDGK upon IL-2 binding. To this end, immunoprecipitated samples were resuspended in phosphorylation buffer and assayed for DGK activity as described under "Experimental Procedures." The samples were analyzed by TLC and autoradiography. As is shown in Fig. 2B, the ratio of phosphorylation of exogenously added DG is increased after 2 min of IL-2 treatment, reaching the maximum level of phosphorylation at 5 min and decrease thereafter. These results clearly indicate IL-2-induced activation of αDGK in YT cells. To further demonstrate that the IL-2-stimulated DGK activity present in the immunoprecipitates corresponds to the α isoform of the DGK, the R59022 inhibitor was added to the phosphorylation reaction. When the immunoprecipitates of αDGK were assayed for DGK activity in the presence of the inhibitor, the activity was completely abolished. Addition of R59949 to the immunoprecipitates had the same effect on the phosphorylation reaction (data not shown).

IL-2 Induces Translocation of αDGK to the Perinuclear Region—DGK activation has been detected previously on IL-2-stimulated T lymphocytes in both soluble and membrane-bound subcellular fraction (11). Whether both activities arose from the same enzyme species was at that time unknown. Previous studies have suggested that αDGK, located mainly on the cytosol, could translocate to the plasma membrane upon activation. Immunofluorescence analysis was performed to determine the distribution pattern of αDGK after IL-2 stimulation. YT cells were stained with a polyclonal antibody against αDGK and the primary antibody detected by using anti-rabbit antibody coupled to FITC. Confocal images from the middle of the cell were obtained. Three different fields under each condition are shown to guarantee the consistency of the results. In quiescent cells, staining with αDGK antibody resulted in a diffuse homogeneous pattern (Fig. 3A). This indicates that, aside from a cytosolic localization, a considerable portion of αDGK is present in the nucleus. After 10 min of IL-2 stimulation, the pattern observed was distinctly different (Fig. 3B). Staining disappeared from inner nucleus and also diminished in the cell periphery and tended to concentrate in the peri-
clear region. The localization of the signal suggested a translocation of both nuclear and cytoplasmic αDGK to the perinuclear region. Ten minutes of pretreatment with the αDGK inhibitor R59022 prior to IL-2 stimulation resulted in a granular pattern in the cytoplasmic region, with no effect in the nucleus (Fig. 3C). When the cells were incubated with R59022 alone, the pattern was the same that the one observed in control cells (data not shown). These results suggest that R59022 inhibits cytosolic αDGK, seizing it and not allowing the enzyme to reach its substrate.

αDGK Inhibition Prevents IL-2-induced DNA Synthesis—Earlier findings have suggested that PA could mediate the growth-promoting effects of IL-2. The previous experiments using αDGK immunoprecipitates indicated that both R59022 and R59949 inhibit IL-2-induced αDGK activation. To determine if the PA accumulated in response to IL-2 through αDGK activation was responsible to the same extent of IL-2-dependent cell growth. We questioned whether this inhibition was related to the blocking of IL-2-induced G1-phase progression. CTLL-2 cells were stained with propidium iodine, and cell cycle distributions were analyzed by flow cytometry. When exponentially growing CTLL2 cells were washed free of IL-2 and cultured for 12 h in medium without IL-2 (basal medium), a marked increase in the percentage of G1-phase cells was observed (Fig. 5A). Upon addition of IL-2, a typical histogram from CTLL2 cell cultures under exponential growth is shown in Fig. 5B. The addition of either R59022 or R59949 prevented the IL-2 effect, as determined by propidium iodine staining (Fig. 5, C and D). As a control of late G1 arrest, starving CTLL2 cells were restimulated with IL-2 plus rapamycin (Fig. 5E). In Fig. 6, it is shown how the growth factor-deprived CTLL2 cells entered S-phase in a synchronous fashion after 10–12 h of restimulation with IL-2. However, the addition of IL-2 plus either R59022 or R59949 to the IL-2-starved CTLL2 cells completely inhibited IL-2-induced S-phase entry, as determined by [3H]thymidine incorporation (see Fig. 6).

DISCUSSION
This study shows what our understanding is the first evidence of agonist activation of αDGK. Following three different
approaches: immunoprecipitation, inhibition studies, and confocal microscopy analysis, the data presented in this study indicate for the first time that IL-2 activates \( \alpha \)DGK. Indirect evidence, such as the lack of selectivity for different DG substrates as well as phosphatidylyserine-mediated activation of this DGK, also indicates that \( \alpha \)DGK is the isoform activated by IL-2 (data not shown). Moreover, our studies indicate that IL-2 activation of this isoform of DGK is the main source of PA production through this mechanism. Finally, another important observation derived from the present work is the finding that, in the IL-2 signaling system, PA and not DG is the lipid second messenger with mitogenic activity; PA generation is a key event for the correct proliferative function of this cytokine.

The results presented here demonstrate that the formation of \( [32P]PA \) and thus DGK activation takes place over a similar time course after IL-2 addition in both total cell lysates and specific immunoprecipitates of \( \alpha \)DGK. While DGK activity is completely abolished when R59022 is added to the immunoprecipitates, there is some activity still present if the assay is performed on cell lysates. These data suggest that another DGK isoform could also be activated by IL-2. In this regard we have previously reported the presence of DGK activity in the membrane fraction that is enhanced in response to IL-2 (11). This subtype of DGK could be similar to the arachidonoyl-DGK identified by Glomset and others as a membrane-bound DGK (23).

Initial studies demonstrated that \( \alpha \)DGK is mostly present in the cytosolic fraction, being the content in the particulate fraction very scarce (24). By analogy with other DG-binding proteins, translocation from the cytosol to the plasma membrane has been postulated. To determine if IL-2 induces translocation of \( \alpha \)DGK, confocal studies were performed. The confocal immunofluorescence assays indicate that, in resting cells, \( \alpha \)DGK is present not only in the cytosol but also in the nucleus and is perhaps partly associated with the cytoskeleton. This is not

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**Fig. 4.** Effect of DGK inhibitors on IL-2-induced CTLL-2 proliferation. Proliferation of IL-2-dependent CTLL-2 cells in the presence of increasing concentrations of \( \alpha \)DGK inhibitors was determined by \( [3H] \)thymidine incorporation into DNA. Cells were incubated for 20 h at 37 °C in flat-bottomed 96-well plates either in the absence of IL-2 (control) or with 50 units/ml IL-2 (IL-2). The \( \alpha \)DGK inhibitors were added at the concentrations indicated. \( [3H] \)Thymidine was added for the final 4 h, and cells were harvested on glass-fiber filters. Results of three experiments performed in quadruplicate are presented. Histograms show \( [3H] \)thymidine incorporation as percentage of maximal proliferation (x axis) plotted versus cell treatment (y axis).

**Fig. 5.** Cell cycle distribution analysis CTLL2 cells were cultured for 12 h in basal medium without IL-2. The IL-2-deprived cells were exposed to the indicated stimuli, and cell cycle distributions were determined by propidium iodide staining and flow cytometry. Quiescent cells (A) were cultured under exponential growth conditions in the presence of saturating concentrations of IL-2 alone (B) or plus R59022 (C) or R59949 (D) or rapamycin (E). Histograms show relative DNA content (x axis) plotted versus cell number (y axis). Insets indicate the percentage of cells in each phase of the cell cycle.

**Fig. 6.** S-phase entry after IL-2 stimulation. Growth factor deprived cells were restimulated with 50 units/ml IL-2 minus or plus \( \alpha \)DGK inhibitors at time 0. Quadruplicate samples were subsequently pulsed at 2-h intervals with \( [3H] \)thymidine. Data represent mean \( [3H] \)thymidine incorporation into DNA, plotted at the end of each 2-h pulse-labeling interval. Coefficients of variation were less than 10% of mean values. IL-2, closed circles; IL-2 + R59022, closed triangles; IL-2 + R59949, closed squares.
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surprising since DGK activity has been previously described in both the nuclei (25) and associated to the cytoskeleton (26–28). Our results here show that, upon IL-2 stimulation of the cells, the enzyme does not translocate to the plasma membrane and is instead accumulated mostly in the perinuclear space. In view of these results, it must be postulated that at least some if not most of the PA being generated in response to IL-2 binding is produced in inner compartments and not in the plasma membrane.

Previous studies by Sakane and co-workers (29) suggested that the translocation of αDGK to membranes occurs in a Ca2+-dependent manner. Furthermore, the existence of EF-hand motifs in the αDGK sequence suggested that the enzyme was functionally linked to Ca2+ signals (30). IL-2 fails to induce Ca2+-mobilization, but we have shown here that αDGK is successfully translocated in an IL-2-dependent manner. In this regard other groups have described the Ca2+-independent activation and translocation of this DGK isoform (31, 32). Interestingly, Kanoh and co-workers (33) have reported that an αDGK mutant lacking EF-hands lost Ca2+-binding activity, but could be fully activated by phosphatidylserine or deoxycholate in the absence of Ca2+. Recently another DGK isoform containing EF-hands in its sequence has been cloned, and its activity resulted to be Ca2+-independent (33). All these experiments suggest that a Ca2+-independent mechanism may be responsible for the IL-2-induced αDGK translocation, and therefore some other unidentified roles could be attributed to the EF-hand motif. As for other possible mechanisms responsible for the enzyme translocation, Besterman and co-workers (32) have reported that the presence of DG and phosphatidylserine is enough to induce translocation of the DGK enzyme from cytosol to membranes. The same mechanism where substrate concentration is responsible for the translocation has been also described for protein kinase C-depleted 3T3 cells when cell-permeable dioctanoylglycerol is added (34).

If IL-2 induces translocation of the enzyme, could the increase on activity be due to an increase on the level of protein? In the immunoprecipitation studies, the content of αDGK in the immunoprecipitates is the same in resting and activated cells. This implies that the increase in the DGK activity is not related to an increase in the αDGK protein level and suggests that the activation occurs independently and previous to translocation. In fact, maximum translocation measured by microscopy confocal analysis is obtained about 10 min after IL-2 addition, several minutes after the maximum activation is obtained in the in vitro studies. It could be hypothesized that a covariant modification occurs. In this context, Schap and co-workers (35) have reported that αDGK can be phosphorylated by Ser/Thr kinases and in Tyr by epidermal growth factor receptor. However, we have not been able to demonstrate any increase on tyrosine phosphorylation of the enzyme in response to IL-2 treatment (data not shown). Experiments are currently under way to study if any other covariant modification of the αDGK takes place in response to IL-2.

The main biological function of DGK has largely been considered to modulate the levels of DG that, in turn, regulate the activation of classical and new PKCs. Our experiments support a new role for this enzymatic activity in the generation of PA, a lipid with a potential central role in IL-2-induced proliferation. When DGK is inhibited by using specific inhibitors in IL-2-stimulated cells, IL-2-induced proliferation is impaired. Cell cycle analysis demonstrates that, upon DGK inhibition, the cells become arrested in the late G1 phase. These results indicate that, in contrast to what has been described for other systems, it is not DG but the αDGK product PA that is the effector molecule in this system. We must therefore consider the novel hypothesis where accumulation of certain αDGK-derived PA species following IL-2 stimulation constitutes an essential step for the T cells to reach the restriction point and enter S-phase, progressing afterward through the cell cycle.

Several observations have previously suggested that PA may act as a second messenger. Specific species of PA that contain polyunsaturated fatty acids have been shown to be increased in ras-transformed fibroblasts (36). PA has also been shown to induce invasion of tumor cells in vitro (37). When added exogenously to cells, PA exhibits growth factor-like activity (11, 16, 38–40) and can induce actin polymerization (41). Moreover, intracellular accumulation of PA, rather than an increase in DG, correlates well with mitogenesis in growth factor-stimulated fibroblasts (42), similarly to what has been described here. Therefore, it appears that specific PA species may be associated with proliferation and a high level of this bioactive lipid could be present in tumor cells. Cellular targets of PA action have not been identified to date, although activation of cellular kinases has been suggested (43). Limatola and co-workers (44), using a cell-free assay system, have demonstrated a strong PA-dependent activation of the DG-insensitive ζ isotype of PKC. Interestingly, PA-induced activation of PKCζ is inhibited by Ca2+. This would imply that, in intact stimulated cells, PKCζ can only be activated by PA when Ca2+ remains at basal levels as it happens upon IL-2 binding. On the other hand, Gomez and co-workers (45) have recently reported, using antisense techniques, that the ζ isotype of PKC could be implicated in the control of IL-2 mediated proliferation. Nevertheless, further studies will be necessary to establish the exact role of ζPKC on IL-2 signaling processes and its activation mechanism by PA. Future work must also contemplate the identification of other possible targets of the PA action as well as the characterization of the fatty acid composition of the effector PA species generated in response to IL-2.

In the last few years, a great number of studies have emerged describing the role of the sphingomyelin cycle in signal transduction (46). Ceramide, the product of this pathway, has been implicated as a mediator of programmed cell death (47–49), cell cycle arrest (50), and differentiation (51). All of these mechanisms imply a stop in cell cycle progression. Thus, ceramide could be considered an antimitogenic lipid. Interestingly, ceramide inhibits the activity of a DGK isoform (52). It also inhibits the activation of phosphatidylycholine-phospholipase D by several agonists (53). Therefore, the generation of ceramide would inhibit the production of PA either by DGK or phosphatidylycholine-phospholipase D activation. Furthermore, it has been reported that an inverse relationship exists between the cellular concentrations of ceramide and the proliferative capacity of human T-lymphocytes after IL-2 stimulation (47). Thus, we can envisage the exciting hypothesis that PA (a mitogenic lipid) and ceramide (an antimitogenic lipid) act as biosensors of the cellular state determining, together with other factors, if the cells are going to die, proliferate, or remain quiescent.
Munakata, H., Nakamura, M., and Sugamura, K. (1992) Science 257, 379–382
4. Nakamura, Y., Russell, S. M., Mess, S. A., Friedman, M., Erdos, M., Francois, C., Jacques, Y., Adelstein, S., and Leonard, W. J. (1994) Nature 370, 330–333
5. Nelson, B. H., Lord, J. D., and Greenberg, P. D. (1994) Nature 369, 333–336
6. Minami, Y., Kono, T., Yamada, K., Kobayashi, N., Kawahara, A., Perlmutter, R. M., and Taniguchi, T. (1993) EMBO J. 12, 759–768
7. Miyazaki, T., Liu, Z.-J., Kawahara, A., Minami, Y., Yamada, K., Tsujimoto, Y., Barsoumian, C., App, H., Greene, M., Dobashi, K., Reed, J. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 91–100
8. Merida, I., Diez, E., and Gaulton, G. N. (1991) J. Biol. Chem. 266, 2537–2544
9. Graves, J. D., Downward, J., Izquierdo-Pastor, M., Rayter, S., Warne, P. H., and Cantrell, D. A. (1992) J. Immunol. 148, 2417–2422
10. Turner, B., Rapp, U., App, H., Greene, M., Dobashi, K., and Reed, J. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 1227–1231
11. Merida, I., Williamson, P., Smith, K., and Gaulton, G. N. (1993) DNA Cell Biol. 12, 473–479
12. Mills, G. B., Girard, P., Grinstein, S., and Gelfand, E. W. (1988) Cell 55, 91–100
13. Vale, V. E., Wong, J. G. P., Datof, B. M., Sinskey, A. J., and Rao, A. (1980) Cell 55, 101–112
14. Merida, I., Pratt, J. C., and Gaulton, G. N. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 9421–9425
15. Eardley, D. D., and Koshland, M. E. (1991) Science 251, 369–376
16. Goto, K., Funayama, M., and Kondo, H. (1994) J. Biol. Chem. 269, 1883–1889
17. MacDonald, M. L., Mack, K. F., Williams, B. W., King, W. C., and Glomset, J. A. (1988) J. Biol. Chem. 263, 1584–1592
18. Yamada, K., Sakane, F., and Kanoh, H. (1989) FEBS Lett. 244, 402–406
19. Payrastre, B., Nieuwenhuijse, J., Breton, M., Verkleij, A. J., and Van Bergen En Henegouwen, P. M. P. (1992) J. Biol. Chem. 267, 5078–5084
20. Payrastre, B., Van Bergen En Henegouwen, P. M. P., Breton, M., Den Hartigh, J. C., Plantavid, M., Verkleij, A. J., and Boorsma, J. (1991) J. Cell Biol. 115, 121–127
21. Grondin, P., Plantavid, M., Sultan, C., Breton, M., Maou, G., and Chap, H. (1991) J. Biol. Chem. 266, 15705–15709
22. Goto, K., Funayama, M., and Kondo, H. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 13042–13046
23. Sakane, F., Yamada, K., Imai, S., and Kanoh, H. (1991) J. Biol. Chem. 266, 7096–7100
24. Sakane, F., Imai, S., Yamada, K., and Kanoh, H. (1991) Biochem. Biophys. Res. Commun. 181, 1015–1021
25. Schaap, D., van der Wal, J., van Blitterswijk, W. J., and van der Bend, R. L. (1990) FEBS Lett. 275, 151–158
26. Besterman, J. M., Pollen, R. S., E. L. Booker, J., and Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 9378–9382
27. Kail, M., Sakane, F., Imai, S., Wada, I., and Kanoh, H. (1994) J. Biol. Chem. 269, 18492–18498
28. Maroney, A. C., and Macara, I. G. (1989) J. Biol. Chem. 264, 2537–2544
29. Schaap, D., van der Wal, J., van Blitterswijk, W. J., van der Bend, R. L., and Ploegh, H. L. (1993) Biochem. J. 299, 875–881
30. Martin, A., Gomez-Murzo, A., Waggoner, D. W., Stone, J. C., and Brindley, D. N. (1993) J. Biol. Chem. 268, 23924–23932
31. Imamura, R., Horay, T., Mukai, M., Shinkai, K., Sawada, K., and Akedo, H. (1993) Biochem. Biophys. Res. Commun. 193, 497–503
32. Knous, T. C., Jaff, F. E., and Abbdou, H. E. (1990) J. Biol. Chem. 265, 14457–14463
33. Moolenaar, W. H., Krujer, W., Tilly, B. C., Verlaan, I., Bierman, A. J., and de Laat, S. W. (1986) Nature 323, 171–173
34. Yu, C., Tsai, M., and Stacey, D. W. (1988) Cell 52, 63–71
35. Ha, K. S., and Exton, J. H. (1993) J. Biol. Chem. 268, 1789–1796
36. Fukami, K., and Takenawa, T. (1992) J. Biol. Chem. 267, 10988–10993
37. Bocckino, S. B., Wilson, P. B., and Exton, J. H. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 6210–6213
38. Limatola, C., Shaap, D., Moolenaar, W. H., and van Blitterswijk, W. J. (1994) Biochem. J. 304, 1001–1008
39. Gomez, J., Pillon, C., Garcia, A., de Aragon, A. M., Silve, A., and Rebollo, A. (1995) Exp. Cell Res. 215, 105–113
40. Hannun, Y. A. (1994) J. Biol. Chem. 269, 3125–3128
41. Borchardt, R. A., Lee, W. T., Kalen, A., Buckley, R. H., Peters, C., Schiff, S., and Bell, R. M. (1994) Biochim. Biophys. Acta 1212, 327–336
42. Guiblin, E., Bissoulette, R., A. Mahboubi, Martin, S., Nishioka, W., Brunner, T., Baier, G., Baier-Bitterlich, G., Byrd, C., Lang, F., Kolesnik, R., Altman, A., and Green, D. (1995) Immunity 2, 341–351
43. Obeid, L. M., Limardic, C. M., Karolak, L. A., and Hannun, Y. A. (1993) Science 259, 1769–1771
44. Javed, S., Liu, B., Bielawska, A. E., Lee, J. Y., Nazaire, F., Pushkareva, M. Y., Obeid, L. N., and Hannun, Y. A. (1995). J. Biol. Chem. 270, 2047–2052
45. Mitchell, R. H., and Wakelam, M. J. O. (1994) Curr. Biol. 4, 370
46. Younes, A., Kahn, D. W., Besterman, J. M., Bittman, R., Byun, H., and Kolesnik, R. N. (1992) J. Biol. Chem. 267, 105–113
47. Gomez-Murzo, A., Martin, A., O’Brien, L., and Brindley, D. N. (1994) J. Biol. Chem. 269, 8937–8943
