Expression of the Phospholipid-dependent *Escherichia coli* sn-1,2-Diacylglycerol Kinase in COS Cells Perturbs Cellular Lipid Composition*

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The *Escherichia coli* sn-1,2-diacylglycerol (DAG) kinase has been successfully expressed in COS cells. The *E. coli* dgkA locus which contains the coding sequences for DAG kinase was subcloned into an eukaryotic expression vector, pMT2. COS cells transfected with the vector pMT2dgk expressed the DAG kinase as shown by Western analysis. Immunofluorescence studies revealed that the *E. coli* DAG kinase was prominently but not exclusively located in the endoplasmic reticulum. In addition, mixed micellar assays in β-octyl glucoside revealed that membranes prepared from pMT2dgk-transfected COS cells contained over a 1500-fold increase in DAG kinase activity: 107 nmol/min/mg compared with only 0.067 nmol/min/mg for controls. DAG kinase activity from the *E. coli* enzyme was distinguished from endogenous COS cell activity based on differences in thermolability and the ability of the *E. coli* enzyme to use ceramide as a substrate. No ceramide kinase activity was detected in control COS cells, so the activity detected in pMT2dgk transfectants must have resulted from the expressed *E. coli* DAG kinase. The *Kₐ* values for DAG kinase derived from *E. coli* and COS cells were nearly identical. Finally, transfected COS cells were labeled with [32P]Pi to investigate possible perturbations in lipid composition induced by the action of the *E. coli* DAG kinase. Ceramide (generated by the action of sphingomyelinase) was also used to clearly implicate the *E. coli* enzyme. Levels of ceramide phosphate increased more than 150-fold in pMT2dgk-transfected cells relative to controls. The results of these studies show that the *E. coli* enzyme expressed in COS cells is active and perturbs lipid composition in the intact cell system: the absolute lipid cofactor requirement of *E. coli* DAG kinase can be satisfied in COS cells.

Diacylglycerol (DAG) is known to be a lipid second messenger in addition to serving as an important intermediate in glycerolipid synthesis and breakdown. As a second messenger, DAG functions by activating the Ca²⁺ and phospholipid-dependent protein kinase C (2, 9). A wide variety of responses (e.g. mitogenesis, hormone secretion, differentiation, etc.) are affected through DAG activation of protein kinase C. Signal-mediated events are easily studied by employing cell-permeable DAGs such as dioctanoylglycerol (dioC₄) (4, 5). When dioC₄ is added to cells, it is able to cross the cell membrane, activate protein kinase C, and thus elicit a cellular response. Studies which employ dioC₄ can identify physiologic responses which may be mediated by DAG. Another important tool in the analysis of DAG function is the method for mass quantitation of DAG levels (6). The amount of DAG in cells can be sensitively determined at various temporal stages of a response by quantitative phosphorylation of the DAG present in crude lipid extracts using [γ-³²P]ATP and the *Escherichia coli* DAG kinase. Studies which quantitate DAG levels can identify DAG as a potential mediator of a physiologic response. Use of cell-permeable DAGs along with DAG mass quantitation can provide strong correlational evidence that DAG does mediate a given physiologic response.

The development of additional approaches to investigate the role of DAG in signal transduction might prove useful. Expression of a DAG kinase in cells which respond to a DAG signal could cause DAG attenuation; the phosphorylated DAG, phosphatidic acid, does not activate protein kinase C. Using this strategy, DAG might be directly demonstrated to be the causative agent for various cellular responses. The only DAG kinase gene cloned is the *E. coli* dgkA gene (7, 8). The enzyme is 14 kDa, extremely hydrophobic, and displays an absolute dependence on lipid cofactor (9). Expression of the *E. coli* DAG kinase for studies of DAG effects would have an advantage in that the *E. coli* enzyme is unlikely to be subject to any posttranscriptional regulation in mammalian cells. The question then becomes whether the *E. coli* DAG kinase can be expressed in a functionally active state in mammalian cells. No precedent exists for the expression of a functionally active, integral membrane enzyme from *E. coli* in an eukaryotic system. Therefore, functional expression of DAG kinase would be a novel and significant finding. It might be expected, however that the *E. coli* DAG kinase could be properly assembled in the eukaryotic membrane. The insertion and folding are likely governed solely by molecular characteristics of the short and extremely hydrophobic primary structure of the enzyme (8). Still, for the production of functional DAG kinase, the folding and membrane insertion of the enzyme must be compatible with the eukaryotic translational machinery, a potentially large obstacle when considering the evolutionary disparity. In addition to protein folding and membrane assembly...
bly, the predominant concern actually pertained to the absolute lipid cofactor requirement exhibited by the enzyme (9). The lipids which are present in the membranes of mammalian cells differ significantly from those of E. coli. Unless the lipid cofactor requirement could be satisfied in the mammalian membranes, the expressed DAG kinase would not be active and therefore could not alter cellular DAG levels.

This paper reports the transient expression of E. coli DAG kinase in COS cells. Expression was demonstrated by identification of an appropriately sized protein in immunoblots. The protein was further identified by immunocytofluorescence so that a general assessment of intracellular location could be made. Enzymatic activity was increased in COS cell membranes toward both DAG and ceramide substrates (10); control COS cells did not contain any detectable ceramide kinase activity. Finally, radiolabeling studies showed perturbation of the lipid composition of COS cells. These data indicate that the E. coli DAG kinase is active in the "living" COS cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—L-a-Dioleoylphosphatidylcholine, L-a-dioleoylphosphatidylglycerol, and diC* were from Avanti Polar Lipids, Inc., Birmingham, AL. Sphingosine, Sphingolipids, and phatidylglycerol, and diC* were from Avanti Polar Lipids, Inc., Birmingham, AL. Sphingosine, Staphylococcus aureus sphingomyelinase, Kodak X-OMat film, hexamix anhydride, Limulus polyphemus hemocyanin, m-maleimidobenzo-N-hydroxysuccinimidom ester, fluoro resin isothiocyanate-conjugated goat anti-rabbit IgG, and 1,4-diazabicyclo[2.2.2]octane were from Sigma. Octyl-β-D-glucopyranoside (β-OG) was from Calbiochem and was purified by crystallization before use. Silica Gel 60 plates were from EM Science, Cherry Hill, NJ. Nitrocellulose membranes were from Schleicher & Schuell. [32P]ATP and [γ-32P]ATP were from Du Pont, New England Nuclear. Molecular biology reagents were obtained from Bethesda Research Laboratories. Cell culture reagents were from Gibco, except for DEAE-dextran which was from Pharmacia LKB Biotechnology Inc. Other reagents were of standard analytical grade.

**Expression Vector**—A plasmid vector was constructed for expression of DAG kinase in COS cells. This was done by subcloning into the EcoRI site of a eukaryotic expression vector, pMTr2 (11, 12). The dgkA locus used for subcloning was contained in a 564-bp fragment obtained by EcoRI digestion of pJW40 (8).

**Preparation of Membranes**—Typically, six plates of transfected COS cells were scraped into PBS and then pelleted in a clinical centrifuge. The cell pellet was suspended in 2 ml of TNE (50 mM Tris (pH 7.6), 100 mM NaCl, 2 mM EDTA, 5 mM β-mercaptoethanol, 0.1 mM phenylisothiocyanate (PITC), Dounce homogenized, and then sonicated three times for 15 s at 50 W with a Transon ultrasonicator fitted with the microprobe. Unbroken cells were removed by a 5,000 × g centrifugation for 10 min. The cell homogenate was then spun at 100,000 rpm for 15 min using a TL100.3 rotor in a Beckman TL100 ultracentrifuge. The final membrane pellet was suspended by homogenization in 0.4 ml of 10 mM phosphate buffer, pH 7.0, containing 20% glycerol.

Membranes were also prepared from E. coli for use as positive controls when measuring diacylglycerol kinase activity or detecting protein with antibodies. The bacterial strain employed was BL21DE3/pLYS/pPKRI (this work), in which diacylglycerol kinase is transcribed from the T7 promoter. This expression system produces extremely high levels of the protein of interest (14). Isolation procedures for E. coli membranes were followed as published previously (4). For the final membrane sample, the salt added in the phosphate/glycerol buffer (above) at 1 mg of protein/ml.

**Antipeptide Antibodies**—A 13-residue polypeptide corresponding to the amino terminus of diacylglycerol kinase (8), ANNTGFTFIRIK(3), was synthesized by Multiple Peptide Systems. A cysteine residue placed at the carboxyl terminus of the polypeptide allowed it to be conjugated to KLH. Photoreactive compound was synthesized as above and then transferred to nitrocellulose. Molecular weight markers were visualized with amido black before probing the blot with antiserum (10). Rabbit antiserum was used at a 1:100 dilution.

**Indirect Immunocytofluorescence**—Acid-washed coverslips were placed in the bottom of 100 × 20-mm dishes. COS cells were seeded into the dishes and transfected as usual. Following the 48-h recovery period, the dishes were rinsed with phosphate-buffered saline (150 mM NaCl, 10 mM P, pH 7.5), and then the coverslips were removed to a Petri dish containing ethanol/acetone and briefly rinsed with PBS containing 3% bovine serum albumin (BSA). Incubation with anti-DAG kinase was conducted overnight at 4 °C in polystyrene dishes. The coverslips were placed (cell side down) on a 200-μl portion of the primary antiserum in PBS with 3% BSA (dilution of 1:2500). Coverslips were washed 4 × 10 min in PBS with 3% BSA. Fluorescein-conjugated anti-rabbit IgG was used in the same manner at a dilution of 1:150 for 4 h at room temperature. The coverslips were once again washed 4 × 10 min with PBS containing 3% BSA and finally mounted on slides over 10 μl of mountant (PBS containing 90% glycerol and 2.5% bovine serum albumin). Photographs were taken using a Leitz Laborlux S microscope and Kodak TMX film with either phase contrast or incident fluorescent light.

**Diacylglycerol Kinase Assay**—The assay was conducted essentially as described previously (9), except that LiCl was substituted for NaCl in the final assay mixture. A small inhibition of DAG kinase activity was observed with NaCl which does not occur with LiCl (17). Briefly, 20 μl of enzyme sample was added to 70 μl of a detergent/buffer mixture. [γ-32P]ATP was added to start the reaction in a volume of 10 μl. Final concentrations of the reaction components were: 51 mM glycerol 2-phosphate, 60 mM imidazole (pH 6.8), 50 mM LiCl, 12.5 mM MgCl2, 1 mM EDTA, 0.03 mM diethylstilbestrol, 2.5 mM phenylmethylsulfonyl fluoride, 1 mM NaF, ATP (pH 6.8), and lipid substrate at indicated concentrations. [γ-32P]ATP was used routinely at ~100,000 cpm/nmol. The reaction was stopped by addition of 0.7 ml of 1% HClO4, and the products were extracted into CHCl3 as described previously (17). Product identities were verified by TLC using silica plates developed with CHCl3/pyridine/88% HCOOH (see below).

**Western Blot**—COS cells were transfected with a plasmid expressing the T7 promoter. The expression system produces extremely high levels of the protein of interest (14). Isolation procedures for E. coli membranes were followed as published previously (4). For the final membrane sample, the salt added in the phosphate/glycerol buffer (above) at 1 mg of protein/ml.

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be phosphate free. \[^{32}P\]Pi, was added to the 4 ml of phosphate-free media to give 2 mCi/culture dish. After 1 h, 10 \(\mu\)l of \(S. aureus\) sphingomyelinase in a 50% glycerol solution (25 units/0.66 ml), 8 \(\mu\)l of diocatanoyleglycerol at 29 mM in ethanol, or nothing was added to plates of cells for a 30-min incubation. Cells were scraped in 1 ml of methanol, and the plates were rinsed with an additional 1 ml of methanol. The suspensions in methanol were combined with 1 ml of CHCl\(_3\) in 13 \(\times\) 100-mm screw cap tubes (18). Sufficient 1% HClO\(_4\), was added to give a single phase as in the extraction procedure of Bligh and Dyer (19). Two phases were created with the addition of 1 ml of CHCl\(_3\) and 1% HClO\(_4\). The CHCl\(_3\) phase was washed twice with 2 ml of 1% HClO\(_4\)/MeOH (7:1).

Two 0.5-ml portions of the CHCl\(_3\) extract from each sample were dried under nitrogen. To one set of tubes was added 0.5 ml of methylamine reagent (1 volume of water, 2 volumes of 33% methylamine in ethanol); the tubes were then capped and placed at 70°C for 90 min. This treatment results in the hydrolysis of all carboxyl ester bonds in the phospholipids, while amide bonds are preserved (20). The samples were then dialyzed to completion in a SpeedVac concentrator (Savant) under reduced pressure for 12 h. The dried lipid residue was suspended in 50 \(\mu\)l of CHCl\(_3\)/MeOH (4:1), and 20 \(\mu\)l of this was spotted on a Merck Silica Gel 60 plate which had been prerun in acetone. The plates were then developed in CHCl\(_3\)/pyridine/88% HCOOH (60:30:7). Once dry, the plates of cells for a 30-min incubation. Cells were scraped in 1 ml of methanol, and the plates were rinsed with an additional 1 ml of methanol. The suspensions in methanol were combined with 1 ml of CHCl\(_3\) in 13 \(\times\) 100-mm screw cap tubes (18). Sufficient 1% HClO\(_4\), was added to give a single phase as in the extraction procedure of Bligh and Dyer (19). Two phases were created with the addition of 1 ml of CHCl\(_3\) and 1% HClO\(_4\). The CHCl\(_3\) phase was washed twice with 2 ml of 1% HClO\(_4\)/MeOH (7:1).

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Other Techniques—sn-1,2-Dioleoylglycerol was prepared by phospholipase C cleavage of dioleoylphosphatidylcholine (21), purified by extraction into diethylether, and quantitated by the method of Stern and Shapiro (22). Proteins were determined by the method of Peterson (23). Phosphates were determined by the method of Ames and Dubin (24). Ceramide was prepared by combining a 10-fold molar excess of hexanoic anhydride with sphingosine at 25 mg/ml in CHCl\(_3\)/MeOH (1:1). The reaction ran quickly (complete within 1 min) at room temperature and was stopped by addition of equal volumes of 0.8 N NaOH and MeOH. CHCl\(_3\) was added to mimic a Bligh-Dyer extraction. Upon washing of the final CHCl\(_3\) phase with 1 M NaCl/MeOH (9:1), the pH reached a value of 7. The N-hexanoyl sphingosine was then purified over a silica gel column eluted with a gradient from CHCl\(_3\) to CHCl\(_3\)/MeOH/acetic acid (90:10:1). The concentration of N-hexanoyl sphingosine was determined by quantitative phosphorylation (25).

RESULTS AND DISCUSSION

Expression of E. coli DAG Kinase in COS Cells—Although a variety of prokaryotic proteins have been expressed in cultured cells, none of these possessed the extreme hydrophobicity or small size (M, 14,000) of DAG kinase (8). These structural features might aid in successful protein production or alternatively might target DAG kinase for rapid degradation. The first goal, then, was to establish whether the E. coli DAG kinase could effectively be transcribed and translated in the heterologous system. Immunoblotting was employed to investigate this question.

Protein samples of the arbitrary pH of 8.0 were extracted from COS membranes. When the membrane extracts were dried to completion, aggregates of the extremely hydrophobic protein are presumed to form. The protein aggregates may not be completely solubilized in SDS buffer and may enter the gel only partially. Additionally, the aggregates might not dissociate upon electrophoresis. Such aggregation might explain the diffuse labeling at M, 61,000 and also faintly present at M, >100,000 in the E. coli sample. There was also a diffuse labeling at M, 66,000, a slightly larger species than the aggregated matter. The M, 66,000 species was, however, recognized by preimmune serum (data not shown) and is not related to the presence of DAG kinase. It is evident from this data that DAG kinase is present in COS cells transfectected with pMT2dgk.

Detection of E. coli DAG Kinase in COS Cells by Immunofluorescence—The extreme hydrophobicity of the E. coli DAG kinase virtually ensured a localization to some cellular membrane. Functional intracellular targeting signals for eukaryotic organelles are not likely for the E. coli DAG kinase. Not only is the evolutionary distance extreme, but the diminutive size of the enzyme virtually eliminates any possibility of operational targeting signals. The location of DAG kinase within COS cells could not be definitively assigned on the basis of primary structure nor could the slim possibility of a coincidental targeting signal be eliminated.

To partially characterize intracellular localization, COS cells were transfected with pMT2dgk and analyzed by immunofluorescence. The upper two panels of Fig. 2 unambiguously demonstrate presence of antigen in a small portion (<10%) of the COS cell population. Although non-DAG kinase-expressing cells serve as negative controls in the experiment shown, studies were also conducted using preimmune serum or cells transfected with the parent vector, pMT2. No
fluorescent labeling was detected in any of the controls. Transfection efficiencies as determined by immunofluorescence were typically ~10%. Others have reported significantly greater transfection efficiencies (26), indicating that the methods of transfection employed could be further optimized or, alternatively, the transfection efficiencies could be affected by the protein being expressed. The lower two panels of Fig. 2 show fluorescent staining in an individual cell. There is heavy reticular staining over the entire cell area. The major portion of enzyme appeared in the endoplasmic reticulum. No heavy reticular staining over the entire cell area. The major portion of enzyme appeared in the endoplasmic reticulum. No subcellular compartment appeared to accumulate DAG kinase, so it is unlikely to undergo specific targeting.

In Vitro Demonstration of E. coli DAG Kinase Activity Expressed in COS Cells—Functional expression of the enzyme could be determined by measuring activity increases in membranes from pMT2dgk transfectants compared with controls. Additionally, DAG kinase activity derived from the E. coli enzyme could be distinguished from the background activity endogenous to COS cells on the basis of differences in thermostability. While the membrane-bound E. coli enzyme is extremely stable to heat treatment (27, 28), DAG kinase activities from eukaryotic sources are sensitive (29, 30). Specific activities for phosphorylation of sn-1,2-dioleoylglycerol are given in Table I. Estimations of specific activities were made using 2 mM dioleoylglycerol in the assays, a concentration over 7-fold higher than the apparent K_m. Activities were investigated in membranes because of the extreme hydrophobic nature of the E. coli DAG kinase. Indeed, preliminary studies showed that greater than 90% of the activity in COS cells transfected with pMT2dgk was associated with the membrane fraction. In contrast to this, COS cells transfected with the parent vector (pMT2) exhibited an almost even distribution of activity between the cytosolic and membrane fractions (data not shown (29)). The specific activity of membranes from pMT2dgk-transfected COS cells was 0.2 μmol/min/mg, a value comparable to that found in wild-type E. coli (31). This level of activity is nearly 1600-fold higher than in control cells transfected with parent vector. After treatment at 65 °C for 10 min, the ratio of activities increased even further. Approximately two-thirds of the eukaryotic activity was lost, while the activities attributable to the E. coli enzyme declined by only 7%. The DAG kinase activity obtained from pMT2dgk-transfected COS cells was, in fact, similar to that from E. coli in that it was extremely heat-stable.

Identification of the E. coli DAG Kinase Activity Based on Utilization of Ceramide as a Substrate—The previous data indicate that the E. coli DAG kinase can be expressed in COS cells. By capitalizing on the ability of the E. coli DAG kinase to phosphorylate ceramide (10), stronger evidence was obtained. Membranes prepared from pMT2dgk-transfected COS cells contained ceramide kinase activity when assayed in mixed micelles, but membranes prepared from pMT2-transfected COS cells did not. No ceramide kinase activity from control COS cells could be detected (data not shown). The activity may not be present in COS cells, may exist at levels too low to detect, or may not be active in the β-OH mixed micelles. Thus, ceramide kinase activity obtained from COS cells must result from the presence of the expressed E. coli DAG kinase. Fig. 3 shows dependence of kinase activity with N-hexanoylphosphoglycerine for membranes prepared from E. coli which over-produce DAG kinase and for COS cells transfected with pMT2dgk. The apparent K_m value obtained for ceramide was approximately 2.5 mM in both cases. These data argue strongly that the E. coli DAG kinase was expressed in COS cells.

Perturbation of Lipid Composition in Cells Expressing DAG Kinase—The data demonstrate E. coli DAG kinase expressed in COS cells is active in vitro. Also, the enzyme obtained from COS cells has kinetic parameters similar to DAG kinase isolated from E. coli. These facts imply that the enzyme is appropriately folded and assembled within the eukaryotic membrane. An alternative explanation is that DAG kinase simply partitions into the COS cell membranes and only later attains an active conformation when it is reconstituted into β-OH mixed micelles. Even if DAG kinase is properly folded in the membranes of COS cells, it may not be active if the requirement for lipid cofactor cannot be satisfied. Therefore, activity of the enzyme was investigated in intact COS cells by labeling the cells with [32P]Pi, extracting the lipids into CHCl_3, and analyzing the labeled lipids by TLC. If the E. coli DAG kinase was active in COS cells, those cells would be expected to have increased levels of [32P]-labeled PA and ceramide phosphate relative to control cells transfected with pMT2. The outcome from a typical experiment is shown in Fig. 4. Introduction of the DAG kinase into COS cells produced an obvious perturbation to the levels of [32P]-labeled PA and ceramide phosphate. Cells expressing E. coli DAG kinase had [32P]-labeled PA levels nearly 2-fold greater than control cells

**TABLE I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>E. coli</th>
<th>pMT2dgk</th>
<th>pMT2</th>
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<tr>
<td>°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>24,400</td>
<td>107</td>
<td>0.087</td>
</tr>
<tr>
<td>65</td>
<td>22,600</td>
<td>100</td>
<td>0.020</td>
</tr>
</tbody>
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Maximal velocities for each of the indicated membrane preparations were estimated in 10-min assays with 2 mM sn-1,2-dioleoylglycerol. For the E. coli sample, 10 ng of protein were used, and for the samples from COS cells 2 μg of protein were used. Estimations of specific activities were given in Table I. Specific activities were sensitive (29, 30). Specific activities from eukaryotic sources are sensitive (29, 30). Specific activities for phosphorylation of sn-1,2-dioleoylglycerol are given in Table I. Specific activities were made using 2 mM dioleoylglycerol in the assays, a concentration over 7-fold higher than the apparent K_m. Activities were investigated in membranes because of the extreme hydrophobic nature of the E. coli DAG kinase. Indeed, preliminary studies showed that greater than 90% of the activity in COS cells transfected with pMT2dgk was associated with the membrane fraction. In contrast to this, COS cells transfected with the parent vector (pMT2) exhibited an almost even distribution of activity between the cytosolic and membrane fractions (data not shown (29)). The specific activity of membranes from pMT2dgk-transfected COS cells was 0.2 μmol/min/mg, a value comparable to that found in wild-type E. coli (31). This level of activity is nearly 1600-fold higher than in control cells transfected with parent vector. After treatment at 65 °C for 10 min, the ratio of activities increased even further. Approximately two-thirds of the eukaryotic activity was lost, while the activities attributable to the E. coli enzyme declined by only 7%. The DAG kinase activity obtained from pMT2dgk-transfected COS cells was, in fact, similar to that from E. coli in that it was extremely heat-stable.
Expression of DAG Kinase

The difficulty in quantitation referred to above was circumvented by capitalizing on the utilization of ceramide as a substrate for the E. coli DAG kinase. Enzymatic production of ceramide phosphate could be monitored easily after base hydrolysis of lipid extracts. Furthermore, it was the levels of ceramide phosphate which appeared to be the most dramatically affected by expression of DAG kinase. For this experiment, lipid extracts were hydrolyzed with methylamine before TLC analysis to eliminate the glycerolipids possessing diester linkages. Ceramide phosphates identified on the silica plates were now easily quantified, and the results are given in Table II. Control cells have virtually no label incorporated into ceramide phosphate, and this is not affected by addition of diC8. However, in cells treated with sphingomyelinase (Fig. 4), a small amount of ceramide phosphate is detectable. (Apparently COS cells do possess some activity capable of phosphorlyating ceramide.) In those cells transfected with the E. coli DAG kinase, however, a substantial amount of radiolabeled ceramide phosphate was produced. This ceramide phosphate must arise from direct phosphorylation of ceramide by the E. coli DAG kinase. It appears that the E. coli enzyme is active in the heterologous environment of COS cells.

Concluding Discussion—Three important observations resulted from this work: (a) the E. coli enzyme is produced in an active conformation in mammalian cells, (b) the enzyme is active in the altered lipid environment of the mammalian cell membrane, and (c) expression of the enzyme causes perturbations in lipid composition.

It is interesting that the expressed enzyme is active in the mammalian cell system. No precedent appears to exist for the expression of a prokaryotic integral membrane-bound enzyme with an absolute requirement for lipid cofactor in mammalian cells. For this case, the extreme evolutionary distance is consequential to functional enzyme expression. DAG kinase may, however, be unusual in that it is small and extremely hydrophobic (8). Proteins with similar characteristics have been shown to spontaneously insert into membranes (32, 33). DAG kinase must not only insert into the membrane, but it must also attain a catalytically active conformation. Translocation off the mammalian ribosomes must allow for proper enzyme folding into a membrane of substantially different composition.

It could not be known that proper assembly of DAG kinase could occur in the heterologous COS system. For the enzyme to be functional in these intact cells is of even greater interest. In COS cells, the lipid cofactor requirement of DAG kinase is satisfied in membranes rich with phosphatidylcholine, a lipid which is not present in E. coli. Although phosphatidylcholine can function as an activator of the enzyme, it is not nearly as effective as cardiolipin or phosphatidylglycerol (17), lipids which are present in E. coli. The absolute lipid cofactor

transfected with pMT2. The change in 32P-labeled ceramide phosphate levels was even more substantial, appearing to increase nearly 10-fold from 335 to 3147 cpm/nmol of Pi. No differences in the mass of DAG or ceramide present in pMT2-compared with pMT2dgk-transfected COS cells were detected using previously described methods (data not shown (6)). This may be due to intrinsic characteristics of the transient expression system. Only a portion of COS cells actually express the transfected enzyme (see above). Consequently, only a portion of the total DAG or ceramide in the COS cell population would be affected by the expressed DAG kinase. Additionally, the phosphorylated species may exist in smaller quantities than the corresponding DAG or ceramides. For instance, a minute percentage decrease in ceramide levels due to kinase action may result in a manyfold increase in levels of ceramide phosphate if the ceramide:ceramide phosphate ratio is very large.

The activity of E. coli DAG kinase in COS cells was further investigated by utilizing the cell-permeable DAG, diC8. When diC8 was added to the culture medium, there was a dramatic appearance of diocanoylphosphatidic acid in cells expressing DAG kinase (Fig. 4). Also, one other phospholipid was produced which had a slightly higher Rf than the endogenous phosphatidic acid. The identity of the new lipid is not known, but its production appears to be dependent on the action of protein kinase C.2 Control experiments exclude the possibility of ethanol carrier inducing the phosphorylation event (data not shown). The appearance of this phospholipid is not affected by DAG kinase expression (Fig. 4). Diocanoylphosphatidic acid could not be separated from a comigrating species of ceramide phosphate, so quantitations of this species are not reported. Qualitatively though, it is clear that diC8 is rapidly phosphorylated, and this implies a direct action of the  

FIG. 4. Alterations of labeled lipids in COS cells transfected with pMT2dgk. Radiolabeled lipid extracts from approximately 0.5 x 106 COS cells were analyzed by TLC. Cells prelabeled with [32P]Pi were treated for 30 min with nothing (none), 0.1 unit of S. aureus sphingomyelinase (SMase), or 58 μM diocanoylglycerol (diC8). The right panel differs in that before TLC analysis, the lipids were treated with methylamine to hydrolyze ester linkages. Standards indicated at left are: endogenous phosphatidic acid (end-PA); diocanoylphosphatidic acid (diC8-PA); ceramide phosphate (cer-Pi); and lysophosphatidic acid (lyso).

Table II

Incorporation of radiolabel into ceramide phosphate

Regions corresponding to ceramide phosphate in the TLC of 4 were scraped from the silica plate, and radioactivity was determined by scintillation spectrometry. The values obtained were standardized to total lipid phosphate.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Incorporation after treatment with:</th>
</tr>
</thead>
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<tr>
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<td>No treatment</td>
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<td>pMT2dgk</td>
<td>2407</td>
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</table>

*SMase, sphingomyelinase.

†J. K. Ramer and R. M. Bell, unpublished data.
Expression of DAG Kinase


Acknowledgements—We are sincerely appreciative of the advice and technical assistance provided by Dr. Allen Eckhardt and are grateful for the thoughtful discussions with Dr. Tommy S. Tillman.

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