Identification of two cytosolic diacylglycerol kinase isoforms in rat brain, and in NIH-3T3 and *ras*-transformed fibroblasts

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Two major species of diacylglycerol kinase (type I and type II) were separated from brain cytosol and from NIH-3T3 or *ras*-transformed 3T3 cells by heparin-agarose chromatography. Multiple species of diacylglycerol kinase were also detected by non-denaturing isoelectric focusing. The two peaks of activity were of similar size, both co-eluted at ~ 95 kDa from a Superose f.p.l.c. column. Type II enzyme (pI 8.0) was more active when substrate was presented in a deoxycholate/phosphatidylserine undefined environment, as opposed to an octyl glucoside/phosphatidylserine micellar environment. Type II activity was also enhanced by the presence of phosphatidylcholine as cofactor. Type I enzyme (pI 4.0) was more active in the presence of either phosphatidylserine or phosphatidylinositol. Type I and II enzymes had different ATP affinities. Both enzymes showed a preference for diacylglycerol substrates with saturated acyl chains of 10–12 carbon atoms. The cytosolic enzyme activity was able to bind to diacylglycerol-enriched membranes in NIH-3T3 fibroblasts, and this translocation was unaffected in *ras*-transformed 3T3 cells. These results demonstrate the presence of multiple diacylglycerol kinases in brain cytosol and NIH-3T3 and *ras*-transformed 3T3 cells. The enzymes differ in cofactor, ATP and substrate requirements. These results can explain some of the contradictions between previous studies of cytosolic diacylglycerol kinase activity, and suggest the presence of a family of such kinases that are differentially regulated by phospholipid cofactors.

INTRODUCTION

Diacylglycerol (DAG) kinase converts DAG into phosphatidic acid. Both DAG and phosphatidic acid (PA) have been implicated as potential second messengers. Hormonal or growth-factor activation of phospholipase C can result in the breakdown of phosphatidylinositol biphosphate, which generates the lipid-soluble DAG [1]. DAG activates the phosphatidylserine (PS)- and Ca²⁺-sensitive protein kinase C [2]. The intracellular receptor for the tumour-promoting phorbol esters is protein kinase C [3]. Protein kinase C activation can be terminated by DAG kinase, which converts DAG into PA [4]. The DAG kinase inhibitor R59022 decreases PA formation and potentiates long-term protein kinase C activity in platelets [4]. A sustained increase in DAG has been suggested to be important for the maintenance of the transformed phenotype [5]. Several oncogene-transformed cell lines possess constitutively elevated DAG levels [6,7]. Kato *et al.* [7] have suggested that increased DAG in *ras*-transformed cells may be due to the inability of the DAG kinases in these cells to translocate to membranes. Therefore, DAG kinase is likely to prove a key element in the regulation of DAG metabolism during cell growth.

Another mechanism by which DAG can be produced is by activation of a phosphatidylcholine (PC)-specific phospholipase C. Interleukin-1 stimulates rapid DAG production from PC in the absence of PI turnover in human T-lymphocytes [8]. Besterman *et al.* [9] have shown that tumour-promoting phorbol esters, serum and platelet-derived growth factor stimulate, within seconds, the hydrolysis of PC in adipocytes. Other examples of PC-derived DAG have been found in mast cells [10], hepatocytes [11] and fibroblasts [12]. Agonist-generated DAG may also arise from synthesis *de novo*. In BC3H-1 myocytes, insulin has been

reported to cause a rapid but transient increase in DAG from phosphatidylinositol (PI) and a more sustained production derived from PA [13]. DAG has other effects besides activating protein kinase C. It is a strong chemoattractant for human polymorphonuclear leucocytes and T-cells [14]. DAGs with short acyl side chains mimic insulin by increasing uptake of glucose in rat adipocytes, but without activation of protein kinase C [15].

PA is a potent mitogen. When added to Swiss 3T3 and A431 cells it is as mitogenic as 10 % serum [16]. Micro-injection of anti-*ras* antibody into 3T3 cells inhibits proliferation and also inhibits the mitogenic effects of added PA [16]. PA inhibits the stimulation of *ras*-GTPase activity by its endogenous regulator GTPase-Activating Protein ('GAP') [17].

Two major pathways are found in cells to metabolize DAG, DAG lipase and DAG kinase. DAG kinase has been found in a variety of tissues and organisms, from *Escherichia coli* to mammals. DAG kinase is found in both the membrane and cytosol and has also been found to be associated with microtubules [18]. Kanoh *et al.* [19] have purified a mammalian DAG kinase from pig brain cytosol with an apparent molecular mass of 80 kDa. It requires Mg²⁺ and deoxycholate (DOC) for optimal activity. An enzyme has also been purified from rat liver with similar properties, but its molecular mass determined by gel filtration was reported to be 121 kDa [20].

The specific activity of the 80 kDa DAG kinase from pig brain is reportedly enhanced by addition of PC [19]. However, there is some confusion about phospholipid cofactor and substrate specificity for the enzyme. For instance, a rat brain DAG kinase partially purified by a phospholipid/cholesterol affinity column was shown to exhibit an absolute requirement for PS [21]. One explanation for the conflicting results is that distinct isoforms of

Abbreviations used: DAG, diacylglycerol; DOC, deoxycholate; OG, octyl glucoside; PA, phosphatidic acid; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol.

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cytosolic DAG kinase exist, with different phospholipid cofactor requirements.

Although Kanoh *et al.* [22] prepared a polyclonal antiserum against the 80 kDa protein, the antiserum could only precipitate 60 % of total cytosolic DAG kinase in pig brain. This antiserum was immunoreactive with an 80 kDa DAG kinase found in pig thymus, but not with DAG kinase found in platelets, kidney, heart or liver. Gel filtration of cytosolic enzymes from several tissues revealed three major peaks, corresponding to 280, 120 and 80 kDa. The 280 and 120 kDa species were non-immunoreactive with the anti-(80 kDa protein) antiserum [23]. These results support the hypothesis that more than one DAG kinase exists.

In this study we present chromatographic and kinetic evidence for two isoforms of DAG kinase found in brain cytosol which differ in phospholipid cofactor, ATP and substrate requirements.

MATERIALS AND METHODS

Materials

[γ - 32 P]ATP was obtained from New England Nuclear, Boston, MA, U.S.A. Ampholytes, PC, octyl glucoside (OG), trypsin and phospholipase C were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. 1,2-(*rac*)Dioleoylglycerol, PS and all the other DAG substrates were from Serdary Lipids (London, Ontario, Canada). All other chemicals and materials were purchased from standard suppliers. NIH-3T3 and K-*ras*-transformed cells were provided by R. Bassin (NIH), and H-*ras*-transformed NIH-3T3 cells were provided by P. Balduzzi (University of Rochester).

Preparation of brain cytosol

Fresh rat or pig brains were homogenized in buffer A (25 mM-Hepes, pH 7.4, 0.25 M-sucrose, 5 mM-dithiothreitol, 1 mM-EDTA, 0.02 % Na $_2$ S $_2$ O $_3$, 50 μ M-ATP, 1 mM-MgCl $_2$, 1 mM-EGTA, 20 mM-NaF), as described by Kanoh *et al.* [19]. The protease inhibitors aprotinin and leupeptin (10 μ g/ml each) and trypsin inhibitor (50 μ g/ml) were added to the buffer before use. Buffer was added at 1.5 ml/g of tissue. The homogenate was centrifuged for 20 min at 10000 *g*, and the supernatant was centrifuged for 60 min at 100000 *g* to obtain pure cytosol. The cytosolic supernatant had a protein concentration of 5–10 mg/ml. Protein was determined by the method of Bradford [24].

Cell cytosol preparation

Cytosol from normal and *ras*-transformed 3T3 cells was obtained by digitonin permeabilization as described previously [25], with some modifications. Cells were grown in Dulbecco's minimal essential medium with 10 % (v/v) calf serum at 37 °C until sub-confluent. The cells were washed three times with ice-cold phosphate-buffered saline (150 mM-NaCl/5 mM-NaH $_2$ PO $_4$ /0.5 mM-EDTA, pH 7.4) and harvested by trypsin treatment. The cells were centrifuged at 100 *g* for 10 min and the cell pellet was incubated with digitonin buffer (20 mM-Hepes, pH 7.4, 0.25 M-sucrose, 2.5 mM-EDTA, 10 mM-NaF, 0.025 % digitonin, 10 μ g of aprotinin and leupeptin each/ml) at 4 °C for 6–10 min. Cytosol was obtained by centrifugation (100 *g*, 10 min) and pelleting of the cell ghosts.

Translocation of DAG kinase in normal and transformed cells

Translocation experiments were performed as described by Kato *et al.* [7]. Briefly, cells were suspended in buffer B (50 mM-Tris, pH 7.4, 2.5 mM-EGTA, 10 μ g each of aprotinin and leupeptin/ml), and sonicated until the solution was clear. Cell lysates were then treated with or without 3 m-units of type XIII and XIV phospholipase C per 10 6 cells at 37 °C for 30 min. The

reaction was stopped with phenanthroline (final concn. 1 mM). The cell lysates were centrifuged at 100000 *g* for 15 min, and cytosol and membrane fractions collected. The cytosol was kept, and the membrane fraction was washed twice with 20 mM-Hepes, pH 7.4, then resuspended in solution B by sonication. DAG kinase activity in the cytosolic and membrane fraction was then determined either as described by Kato *et al.* [7] or by the mixed-micellar assay described below.

Enzyme assays

DOC/PS assay. DAG kinase activity was measured with modifications based on an assay developed by Kanoh *et al.* [19]. Enzyme samples were incubated with final concentrations of 10 mM-MgCl $_2$ and reaction buffer (50 mM-Tris, pH 7.4, 20 mM-NaF, 1 mM-DOC, 0.5 mM-dithiothreitol, 1 mM-dioleoylglycerol, 1 mM-PS). The reaction buffer was sonicated for 5 min at 0 °C, and protease inhibitors were added immediately before mixing samples. The total reaction volume was 100 μ l. After 5 min at 30 °C the reaction was started with 10 μ l of [γ - 32 P]ATP, to a final concentration of 1.5 mM (0.31 μ Ci/nmol). As described previously [25], the assay under these conditions was linear with respect to time (0–20 min) and protein concentration (0–1.5 mg/ml), and was saturated with respect to DAG and ATP concentrations. The reaction was stopped at 15 min by addition of 1 ml of 10 % trichloroacetic acid plus 100 μ g of BSA on ice. The solution was centrifuged (1000 *g*) for 3 min, and the pellet was rinsed with 1 ml of ice-cold water, centrifuged and the wash discarded. The pellet was extracted with 100 μ l of chloroform/methanol/HCl (200:100:1, by vol.). A 50 μ l volume was spotted on to a Baker Si:250 t.l.c. plate and developed in chloroform/methanol/acetic acid/acetone/water (40:13:12:15:3, by vol.). The radioactive spot corresponding to radioactive PA was scraped off the plate into a scintillation vial and counted for 32 P.

OG mixed-micellar assay. DAG kinase activity was measured by modifications of the mixed micellar assay developed by Walsh & Bell [26] and MacDonald *et al.* [27]. DAG (11.2 mol %) and PS (4.2 mol %) were mixed in reaction buffer containing 50 mM-OG, 50 mM-Hepes, pH 7.4, 5 mM-dithiothreitol and 10 mM-MgCl $_2$. Enzyme samples were added to this reaction mixture. After 5 min at 30 °C, the reaction was started with 5 μ l of [32 P]ATP (final concn. 1.0 mM, 0.2 μ Ci/nmol). The total reaction volume was 51 μ l. The reaction was stopped after 10 min in the same manner as the DOC/PS assay. The pellet after the first centrifugation was washed with 1 ml of trichloroacetic acid containing 1 mM non-radioactive ATP, better to remove the [32 P]ATP that had not reacted. The pellet was extracted with 100 μ l of chloroform/methanol/HCl, and 50 μ l of this extract was counted for radioactivity directly without t.l.c. Washing in this manner removed > 99 % of [32 P]ATP that had not reacted. Only radioactivity incorporated into PA remained, which was confirmed by t.l.c.

Sodium cholate/DAG kinase assay. DAG kinase was assayed by the method of Kato *et al.* [7] to determine translocation of DAG kinase from cytosol to membranes. Briefly, DAG kinase activity in both membrane and cytosolic fractions was measured in 50 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-EGTA, 10 mM-MgCl $_2$, 0.5 mM-2-mercaptoethanol, 20 mM-NaF, 1 mM-sodium cholate, 50 μ g of 1,2-dioleoyl diacylglycerol, 200 μ M-[γ - 32 P]ATP (1–2 μ Ci) and the enzyme sample. The reaction was started by addition of [32 P]ATP and allowed to continue for 20 min at 30 °C. PA formation was measured as in the DOC assay by t.l.c.

Monoacylglycerol kinase activity was determined as in ref. [28], with 2-mono-olein as substrate.

Native vertical-slab isoelectric focusing

The isoelectric-focusing technique was adapted from Guilian *et al.* [29]. The slab gel was 0.75 mm thick and was run on a standard gel-electrophoresis apparatus. The broad pH gradient contained 2.4% ampholytes of pH range 3.5–10, and the narrow gradient contained a mixture of pH ranges 6–9 and 3.5–10 in the ratio 23:4 (v/v). Samples were mixed with the gel before polymerization. The power-supply setting for all gels was 500 V/h for 4 h. Slices (0.5 cm × 4 cm) were incubated in 1 ml of buffer A at 4 °C overnight, then assayed for DAG kinase activity. The pH gradient was determined by incubating parallel slices of the gel in 1 ml of water.

Heparin-agarose chromatography

Crude cytosol (5–10 mg of protein) was loaded on a 10 ml f.p.l.c. heparin-agarose column and eluted by a linear gradient of 0–1.0 M-NaCl in buffer A (total volume 70 ml) at a rate of 0.5 ml/min. Fractions (1 ml) were collected, quick-frozen in liquid nitrogen and stored at –80 °C. The samples were assayed for DAG kinase activity. The peak fractions were then utilized for substrate- and phospholipid-specificity assays.

RESULTS

Optimization of DAG kinase assay conditions

Several assay conditions have been used to determine DAG kinase activity. Kanoh *et al.* [19] and our laboratory [25] have used a DOC-containing mixture with PS and DAG to measure synthesis of PA. However, enzyme kinetic analyses are difficult to interpret under these conditions, since the DAG is not in solution. A mixed-micellar assay has been used by Walsh & Bell [26] and by MacDonald *et al.* [27] to determine DAG kinase activity. The advantage of this assay is the exposure of the enzyme to a homogeneous distribution of phospholipids in a micellar microenvironment [26]. In order to study DAG kinase activity in brain cytosol, we optimized the mixed-micellar assay conditions for our system. Rat brain cytosol was isolated as stated in the Materials and methods section and assayed by the mixed-micellar OG assay. The DAG and PS concentrations were kept constant initially at 8 mol% and 6 mol% respectively. The reaction was found to be linear up to 1.0 mg of cytosolic protein/ml and for 10 min under these conditions (results not shown). For subsequent substrate- and cofactor-requirement assays, 1–10 µg of protein was used per assay for 10 min at 30 °C.

Separation of DAG kinase from rat brain cytosol by heparin-agarose chromatography

We utilized heparin-agarose chromatography to purify DAG kinase partially from rat brain cytosol. Surprisingly, the DAG kinase activity was eluted in two distinct peaks (Fig. 1), at 0.4 M and 0.6 M salt respectively. These two forms are designated as type I and type II DAG kinase. Typically, type I was purified about 10-fold and type II about 100-fold, with a 5% yield for both enzymes. This elution pattern was very reproducible.

We also determined the amount of monoacylglycerol kinase activity in each of the heparin-agarose peaks. That activity was 10% of the DAG kinase activity in the peaks.

We utilized both the DOC/PS and micellar assays to determine if the two peaks of activity from the heparin-agarose column respond differently to the physical state of the substrate. The DOC/PS assay presents the substrate in an undefined environment rather than in a micelle. Remarkably, as shown in Table 1, the type II enzyme exhibits a much higher activity in the DOC/PS assay, whereas the type I enzyme shows no preference. This result explains some of the apparent contradictions in earlier work and indicates that the two peaks of DAG kinase activity

Fig. 1. Separation of multiple forms of DAG kinase by heparin-agarose chromatography

Rat brain cytosolic protein (7 mg) was loaded on to a 10 ml heparin-agarose column in the absence of salt and eluted with a gradient from 0.0 to 1.0 M-NaCl in buffer A at pH 7.4. Fractions were assayed for DAG kinase activity by the OG method.

Table 1. Activity of DAG kinase isoforms I and II utilizing different assay conditions

Heparin peak	Assay ...	DAG kinase activity (nmol/min per mg of cytosolic protein)	
		DOC/PS/DAG	OG/PS/DAG micelles
I		4.6 ± 0.7	2.8 ± 1.5
II		20.01 ± 0.09	1.7 ± 0.8

may represent distinct enzymes, rather than different forms of the same enzyme. This hypothesis was tested further, as described in the following section.

Substrate and co-factor specificities of DAG kinase isoforms

We utilized the two peaks of enzyme activity from the heparin-agarose column to determine substrate (DAG) and lipid cofactor requirements for each peak with the OG micellar assay. Slight differences were seen in substrate requirements for each peak. Type I reaches a plateau at ~11 mol% DAG and type II at 8 mol% (Fig. 2a). PS effects are similar for both peaks (Fig. 2b), reaching a plateau at 4.2 mol%. In both cases, positive co-operativity appears to occur with respect to substrate concentration (Hill coefficient for PS = 1.24 for type I), an effect observed previously for the *E. coli* DAG kinase. We examined the cofactor requirement more closely. As shown in Table 2, type II has a preference for PC as a cofactor. Type I can utilize both PS and PI, but has a lower preference for PC. This difference in cofactor specificity may be important in the regulation of the two forms of the enzyme.

Kanoh & Akesson [30] showed that the *K_m* for ATP of the cytosolic DAG kinase was 160 µM. Quite different ATP requirements for the two peaks were seen with the micellar assay (Figs. 3a and 3b). The *K_m* for type I is 105 µM and for type II,

Fig. 2. Substrate and cofactor requirements for DAG kinase by using the OG-micelle assay method

(a) Partially purified type I and type II enzymes were incubated for 10 min at 30 °C with various amounts of DAG. (b) DAG was kept constant at 11.2 mol % for type I and 8.8 mol % for type II, and PS was varied. Activities are expressed as nmol/min per mg of protein in the active fractions from the heparin-agarose column. Values are averages $\pm$ range of duplicate samples for both (a) and (b).

Table 2. Specific cofactor requirements of type I and type II DAG kinase determined by the OG micelle method

Samples from each peak were incubated for 10 min at 30 °C in the presence of 11 mol % of DAG and with the indicated phospholipids at the optimal concentration found for PS (4.2 mol %). The data are expressed as percentage activity relative to that with DAG alone without cofactor present (control). The control activities were 1.2 nmol/min per mg for type I enzyme and 0.317 nmol/min per mg for type II. Results are means for three experiments.

Cofactor	Activity (% of control)	
	Type I	Type II
PC	244	268
PI	375	203
PS	397	179

506 μ M. The difference in ATP affinity strongly suggests that the two peaks represent different enzymes. Lin *et al.* [31] partially purified a very labile DAG kinase from bovine brain with an ATP requirement of approx. 500 μ M, which could be similar to the type II enzyme found in the present study, although the method of assay used was not the OG micelle assay.

One function for the expression of multiple isoforms of DAG kinase might be to phosphorylate different DAG species. To determine if the two heparin-agarose peaks have different substrate specificities, DAG substrates with 8–20-carbon acyl chains and various degrees of unsaturation were utilized to assay

Fig. 3. ATP requirements for type I and type II enzymes

Samples of the enzymes were incubated with 11 mol % DAG and 4.2 mol % PS for 10 min at 30 °C. The reaction was initiated with various ATP concentrations. Lineweaver-Burk plots of the results are shown for type I (a) and type II (b) enzymes. The K_m was 105 μ M for type I enzyme and 506 μ M for type II. The values represent averages of duplicate samples, and the line was best-fit to the data by a linear least-squares regression analysis. The OG-micelle assay method was used to assay for activity.

Table 3. DAG substrate specificities of type I and type II enzymes

Enzyme samples were incubated with the various DAG substrates indicated at the optimal concentration (11 mol %) found for dioleoyl DAG with the OG micelle assay. The PS concentration present was 4.2 mol %. Data are expressed as percentage of activity with dioleoyl DAG ($C_{18:1 \text{ rac}}$). The activities were 1.21 nmol/min per mg for type I and 1.12 nmol/min per mg for type II. Results are means for three experiments.

Substrate	Activity (% of that with $C_{18:1 \text{ rac}}$)	
	Type I	Type II
$C_{8:0}$	149	114
$C_{10:0}$	157	141
$C_{12:0}$	107	227
$C_{14:0}$	200	262
$C_{16:0}$	198	231
$C_{18:1 \text{ rac}}$	100	100
$C_{18:1 \text{ sn}}$	18	19
$C_{16:0, 18:1}$	125	101
$C_{16:0, 18:2}$	116	86
$C_{16:0, 20:4}$	181	116

enzyme activity. However, as shown in Table 3, type I enzyme shows little substrate specificity, although it will not efficiently utilize substrates with straight acyl chains beyond 12 carbon atoms long. Type II shows a slight dependency on the acyl chain length, reaching a peak of activity at 12 carbon atoms. Type II enzyme is also unable to utilize the unsaturated-acyl-chain DAGs as well as the type I enzyme. It is noteworthy that neither enzyme

Fig. 4. Separation of DAG kinase by gel-filtration chromatography

Rat brain cytosolic protein (100 μ g) was loaded on a 12 ml Superose-12 gel-filtration f.p.l.c. column. The enzyme was eluted with buffer A containing 200 mM-NaCl at a rate of 0.5 ml/min, and 100 μ l samples were collected after the void volume (6.55 ml) of the column. The fractions were assayed for DAG kinase activity by the OG micellar assay (○) and the DOC/PS assay (□). The molecular-mass markers (▲) were: β -amylase (205 kDa), BSA (66 kDa), egg-white albumin (45 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.3 kDa).

shows a preference for the *sn* or *rac* forms of 1,2-dioleoyl DAG, unlike a membrane-bound form of DAG kinase, which appears to be specific for the *sn* species of 1-stearoyl-2-arachidonoyl-glycerol [27].

Molecular mass of the DAG kinase isoforms

The different activities with the two types of DAG kinase assay raised the possibility that the heparin-agarose peak corresponding to the type II enzyme actually comprises two enzymes, one of which is only detectable with the DOC/PS assay system. As an approach to test this possibility, rat brain cytosol was size-fractionated on a Superose-12 f.p.l.c. column, and then fractions were assayed by each method. The enzyme activity detected by the mixed-micellar assay separated as a single peak from 75–120 kDa (Fig. 4), with a mode at 95 kDa. The same peak was observed when assayed by the DOC method, suggesting that if the activities are different they are mediated by DAG kinases of very similar size. Parallel thermal-inactivation curves were also observed when type II enzyme was assayed by the two methods, again indicating that the two assays are measuring the same or very similar enzymes in the type II DAG kinase peak (results not shown). Attempts to determine the size of the individual peaks from the heparin-agarose column by gel filtration were unsuccessful, possibly owing to the low protein concentration contained in the peak.

Interestingly, the peak eluted from the Superose gel-filtration column could be resolved into type I and type II enzyme when applied to the heparin-agarose column and assayed by the mixed-micellar method, indicating that the two forms of DAG kinase are also of similar size and neither is lost during gel filtration (results not shown).

Separation of DAG kinase isoforms by isoelectric focusing

We also attempted to separate isoforms of DAG kinase by isoelectric focusing. By using a broad pH range (3.5–10), two major peaks of activity were separated, with isoelectric points centred at approx. 8.4 and 6.5 (Fig. 5). Focusing occurred within 4 h at 500 V/h. Type I enzyme from the heparin-agarose column focused at about pH 4.0, and type II at about pH 8.0, corresponding to the two major peaks seen with pig brain cytosol (Fig. 5). Staining the gels, by the procedure of Guilian *et al.* [29],

Fig. 5. Separation of multiple forms of pig brain DAG kinase by non-denaturing isoelectric-focusing gel in the pH 3–10 range

Cytosol (8 mg of total protein) was mixed with the gel before polymerization, focused, sliced and incubated overnight in buffer A, at 4 °C. DAG kinase activity was assayed in the presence of 1 mM-DAG (●) by the DOC/PS method. The pH was determined from gel slices incubated in water (○). The solid black bars indicate the pI values of type I and type II DAG kinase from the heparin-agarose column.

revealed sharp protein bands. A narrow pH range (6–9) consistently resolved the two major cytosolic peaks into six bands, with pI values of 6.9, 7.15, 7.5, 8.15, 8.5 and 8.7 (results not shown). The same pattern was seen with rat brain cytosol (results not shown).

To determine if these peaks of activity were due to proteolytic cleavage, fresh pig brain was homogenized in the presence or absence of protease inhibitors. The sample without protease inhibitors was incubated at 30 °C for 4 h, and the sample with protease inhibitors was left on ice at 4 °C. Both preparations were then focused using the pH range 6–9. No significant alteration of peak proportions nor the appearance of different peaks was observed (results not shown). It remains unclear, however, whether the multiple species observed by this method represent distinct isoforms of the enzyme, or are modified versions of the type I and type II DAG kinases.

DAG kinase in NIH-3T3 and *ras*-transformed cells

We next wanted to determine if the same heparin-agarose peaks could be observed in normal and transformed cells as in brain cytosol. Cytosol from normal and *ras*-transformed NIH-3T3 fibroblasts was isolated as described in the Materials and methods section. For this, 3–10 mg of protein was applied to a 3 ml heparin-agarose column and eluted with a linear NaCl (0–1 M) gradient. Two peaks of activity were seen in both the normal and *ras*-transformed cells. The elution profile corresponded to the peaks obtained from rat brain cytosol (Fig. 1). These results demonstrate that type I and type II DAG kinase can be co-expressed in the same cell type. They also indicate that there are no significant changes in overall cytosolic activity or isoform expression in *ras*-transformed cells.

Translocation of DAG kinase from cytosol to membrane in normal and *ras*-transformed cells

Besterman *et al.* [21] and Coco-Maroney & Macara [25] have shown that DAG kinase will translocate from the cytosol to the membrane when the membrane is treated with phospholipase C to generate endogenous DAG. DAG levels are higher in transformed than in normal cells [6]. Kato *et al.* [7] showed that a possible reason for the higher levels of DAG was an inability of the DAG kinase to translocate to the membrane in transformed cells. We tested this possibility. Cells were grown and the membrane and cytosolic fractions isolated as indicated in the

Table 4. Phospholipase C-induced translocation of DAG kinase from cytosol to membranes in normal and *ras*-transformed 3T3 cells

Cytosolic and membrane fractions were assayed as described in the Materials and methods section. The sodium cholate assay was performed exactly as described by Kato *et al.* [7]. Treatment with phospholipase C was for 30 min at 37 °C as described in the Materials and methods section. Each fraction was assayed in triplicate, and the s.d. of the ratio was calculated by using the formula $\Delta Z = [(\Delta A)^2 + (\Delta B)^2]^{1/2}$, where ΔZ is the error of the ratio, and ΔA and ΔB are the errors in the membrane and cytosolic assays respectively. Results are means $\pm$ s.d.

Cells	Phospholipase C	Ratio of DAG kinase activities (membrane/cytosol)
(a) OG Micelle assay		
NIH 3T3	—	4.1 $\pm$ 0.26
	+	6.5 $\pm$ 0.4
Ha1- <i>ras</i> 3T3	—	2.5 $\pm$ 0.36
	+	9.0 $\pm$ 0.68
(b) Sodium cholate assay [7]		
NIH 3T3	—	2.1 $\pm$ 0.25
	+	3.4 $\pm$ 0.4
Ha1- <i>ras</i> 3T3	—	1.5 $\pm$ 0.05
	+	2.4 $\pm$ 0.18

Materials and methods section. The cells were treated with or without phospholipase C, and the DAG kinase activities in each fraction were determined by both the OG and the sodium cholate method. The results are shown in Table 4. The membrane/cytosolic DAG kinase activity ratio increases in both normal and *ras*-transformed cells after phospholipase C treatment, indicating that translocation to the membrane is not affected in these *ras*-transformed cells.

DISCUSSION

Our data show the presence in rat and pig brain cytosol of two distinct enzymes that phosphorylate DAG. The two major species are also found in non-transformed and *ras*-transformed 3T3 fibroblasts. Both the DAG kinases are water-soluble. We have previously shown that DAG kinase can translocate to DAG-enriched red-blood-cell membranes [25]. The DAG kinase isoenzymes are therefore members of the ambiquitous class of proteins that can exist in either a free or a membrane-bound state, which includes protein kinase C [32], phosphatidate phosphatase and CTP:phosphocholine cytidyltransferase [33].

Yamada & Kanoh [23] have identified several DAG kinases from bovine thymus cytosol with widely different molecular masses. However, we have been unable to detect DAG kinases from rat brain cytosol with different molecular masses. Both type I and type II enzymes isolated after heparin-agarose chromatography appear to be of similar size (~95 kDa). Rat and pig brain cytosol could also be resolved into two peaks by isoelectric focusing at a broad pH range, and these two peaks could each be consistently resolved into three components by using a more narrow pH range, indicating that at least six isoenzymes may exist. Type I enzyme from the heparin-agarose column has pI 4.0 and type II has pI 8.0 (see Fig. 5). These pI values are consistent with the elution pattern from heparin-agarose, that is, proteins which are eluted at low salt concentration have more acidic pI than those eluted at high salt. Type I and type II enzymes possess distinct phospholipid cofactor specificities, type I and II being activated by PS but type II preferentially by PC. These effects indicate a potential mode for

the differential regulation of the DAG kinases by alteration of the lipid environment. Although multiple species of protein kinase C and phospholipase C have also been isolated [34–37], no similar differential control by phospholipids has yet been reported for these enzymes, to our knowledge. Both enzymes isolated from the heparin-agarose column also possessed different affinities for ATP. The type II enzyme appears to be similar to an activity partially purified from bovine brain by Lin *et al.* [31]. They showed that the bovine brain enzyme had a preference for PC as cofactor and an ATP requirement of approx. 500 μ M; however, they used the DOC method to assay DAG kinase activity, which might have different ATP and phospholipid requirements compared with the OG micelle method that we use.

MacDonald *et al.* [27] have identified a thermolabile membrane-bound DAG kinase that exhibits substrate specificity for the 2-arachidonoyl species of DAG, but no ability to distinguish substrates with different acyl side chains has previously been reported for the cytosolic activity [38,31]. Our data also indicate that neither enzyme is highly specific for any particular acyl group, but there are small, reproducible differences in substrate utilization by the two enzymes. Moreover, the enzymes respond very differently to the physical state of the substrate, because the type II DAG kinase is more active when DAG is presented in a non-defined environment (the DOC/PS assay) rather than in a micelle, whereas the type I enzyme shows no preference. This finding might explain some of the confusing discrepancies between results of different workers in earlier reports, where in some cases the DAG kinase was activated by PS only [21] or by PC and/or DOC [19]. The PS-activated enzyme [21] and the 280 kDa species reported by Yamada & Kanoh [23] might be related to our type I DAG kinase, and the PC-activated species reported by Kanoh *et al.* [19] and Lin *et al.* [31] might be related to the type II enzyme from rat brain cytosol, although the assay used in each case is different. These findings also point to the complexity of regulation of DAG metabolism. It is possible that more stringent specificities will only be detected when these two major forms are purified to homogeneity.

The question arises as to why a tissue would require multiple forms of DAG kinase. Both the substrates and products of DAG kinase activity are important mediators of cellular events. Different species of these lipids may possess different second-messenger functions, which could require independent means of regulation. For example, specific forms of 1,2-DAG have been reported to mimic the activation of the glucose transporter in adipocytes by insulin [15]. Those species that activate protein kinase C were ineffective in the stimulation of glucose transport, and vice versa, suggesting that separate pools of DAG with different acyl side-groups have independent functions. Different DAG kinases might be activated, depending on hormonal treatments. Rider & Baquet [39] showed that DAG kinase activity in rat liver plasma membrane was increased after treatment with vasopressin and phenylephrine. The activity was greater when the enzyme was assayed in the presence of PS rather than DOC, and with 1-stearoyl-2-arachidonoyl-*sn*-glycerol or dioleoyl-*sn*-glycerol as substrates, which could indicate that a specific DAG kinase isoform is activated after such treatment.

The role of DAG in maintaining the transformed phenotype is unclear. Higher levels of DAG are found in some oncogene-transformed cells [6,40]. Kato *et al.* [7] reported that one possible reason for higher levels of DAG in transformed cells was a lesion in the ability of DAG kinase to translocate to the membrane, which would result in decreased catalysis of DAG to PA. We were unable to confirm these results by either our methods or the methods described by Kato *et al.* [7]. Thus changes in DAG kinase translocation probably have little to do with the higher levels of DAG seen in *ras*-transformed cells.

Although the effects of the acyl side chains on the functions of PA have not been carefully studied to date, the mitogenic response elicited by PA, and the inhibition of *ras* 'GAP' activity in 3T3 fibroblasts, appear to depend strongly on the length of the acyl chains in the phospholipid [16,17]. It is therefore apparent that DAG kinase isoenzymes may play a pivotal role in controlling the abundance of different DAG and PA species, which act as second messengers for a variety of hormones and growth factors. It will be of great interest to determine how the activities of different DAG kinase isoforms are regulated by the cell.

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