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Phosphatidylethanolamine and phosphatidylcholine are sources of diacylglycerol in *ras*-transformed NIH 3T3 fibroblasts

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Abstract

Ras-transformation of cells is accompanied by an increase of the level of diacylglycerol (DAG), which participates in the signal transduction pathways. DAG could be generated from phospholipids either by activation of phospholipase C or by a more complex pathway involving phospholipase D and phosphatidate phosphohydrolase. To clarify which phospholipids produce DAG and which pathways are involved, we examined the DAG generating enzyme activities, using phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) as substrates. The study showed that the breakdown of PC and more markedly of PE by phospholipases C and D was stimulated in membranes from *ras*-transformed cells. Phosphatidate phosphohydrolase activity was also elevated in oncogene-expressing cells. The increase in glycerol uptake was most pronounced in cells given PE, followed by PC. The fatty acid analysis revealed apparent similarities between the acyl chains of PE and DAG only in the transformed cells. These findings suggest that PE is a source of DAG in *ras*-fibroblasts but does not rule out the role of PC in DAG production, due to the activation of the PC-specific phospholipases C and D.

Keywords: Phosphatidylcholine; Phosphatidylethanolamine; Diacylglycerol; *Ras*; Fibroblasts

1. Introduction

Ras genes encode proteins which regulate key cellular signalling pathways involved in cell proliferation and differentiation [1–3]. The association of *ras* oncogenes with various human cancers has been well documented [1]. In *ras*-transfected cells some authors reported high levels of diacylglycerol (DAG) [4, 5]. Initially, phospholipase C (PLC)-induced degradation of phosphatidylinositol (PI) was presumed to produce DAG in *ras*-expressing cells [6]. However, Lacal et al. [4] were the first to suggest that DAG did not originate from PI in *ras*-cells based on the elevated phosphocholine and phosphoethanolamine levels. Attention has been focused mainly on phosphatidylcholine (PC) as a DAG source [7]. Other authors reported that

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phosphatidylcholine-specific phospholipase C (PC-PLC) was not activated in *ras*-transformed fibroblasts, thus ruling out the possibility for direct production of DAG from PC [8]. They suggested that DAG was accumulated as a result of the consecutive activation of phosphatidylcholine-specific phospholipase D (PC-PLD) and phosphatidate phosphohydrolase (PAP). Such controversies might be due to the possibility that either PLC or PLD could be activated alternatively under definite circumstances in cells. The aim of our experiments was to test this possibility in Ha-*ras* transformed NIH 3T3 fibroblasts.

Since the cell is a very complex system and some alternative pathways might interfere with one another, thus giving an unclear picture of the origin of their products, we investigated the enzyme activities in isolated plasma membranes and not in whole cells. Such an approach would provide a possibility to distinguish between the pathways specific only for plasma membranes from the ones typical for other subcellular fractions.

Our studies showed that both PC and phosphatidylethanolamine (PE) could produce DAG through the activated phospholipases C and D (and PAP) in *ras*-transformed cells. However, the marked similarity between the fatty acids of DAG and PE implied that the latter was a more potent DAG-donor in *ras*-expressing cells. This does not rule out the contribution of PC to the total increase of DAG due to the activation of PC-PLC and PC-PLD/PAP in the transformed cells.

2. Materials and methods

2.1. Materials

[^{3}H]Glycerol, L-3-phosphatidyl[*methyl*-^{14}C]choline, L-3-phosphatidyl[^{14}C] ethanolamine and L-3-phosphatidyl[U-^{14}C]inositol were purchased from Amersham International. Cell culture reagents were from GIBCO. The phospholipids and most of the other reagents were from Sigma.

2.2. Cell culture

NIH 3T3 fibroblasts transfected with activated human Ha-ras gene [9, 10] and the parental cell line were a kind gift from Professor Channing J. Der (The University of North Carolina at Chapel Hill, Chapel Hill, NC). Cells were cultured in Dulbecco's modified Eagles medium, supplemented with 9% (v/v) fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere. The transfected fibroblasts were continuously grown in a medium containing 400 μg/ml G 418 (Geneticin, GIBCO-BRL). For the experiments G 418 was replaced with antimycotic/antibiotic solution.

2.3. Lipid analysis

Lipid extraction was performed with chloroform/methanol according to the method of Bligh and Dyer [11]. The organic phase obtained after the extraction was concentrated and analysed by TLC. The phospholipid fractions were separated on silica gel G 60 plates (Merck) in a solvent system containing chloroform/methanol/isopropanol/triethylamine/0.25% KCl (30:9:25:18:6 v/v) [12]. The neutral lipids were fractionated on silica gel G 60 plates in hexane/diethyl ether/acetic acid (50:50:1 v/v). The location of the radioactive metabolites was monitored by autoradiography, the corresponding spots were scraped into scintillation vials and the radioactivity was measured in a Rackbeta II 1215 (LKB) scintillation counter. When testing non-radioactive phospholipids, the separate fractions were visualized with iodine vapours. The spots were scraped and quantitated by determination of the inorganic phosphorus.

2.4. Determination of the steady-state level of DAG

The cells were incubated for 48 h in a medium containing 5 μCi/ml [^{3}H]glycerol. After incubation the cells were washed twice with PBS, scraped with a rubber policeman and collected. After extraction [11] the organic phase was evaporated to dryness, dissolved in chloroform and the DAG fraction was separated by TLC in a

solvent system for neutral lipids with diolein as standard. The corresponding spots were scraped and counted in a scintillation counter. The DAG content was determined according to the procedure of Preiss et al. [13].

2.5. Uptake studies

Subconfluent control and *ras*-transformed NIH 3T3 fibroblasts were seeded in DMEM, containing 9% calf serum, at a density of 2×10^5 cells in triplicates in 30 mm culture dishes. After 6 h the medium was changed with a fresh one and the radioactive glycerol (2 μCi/ml medium) was added. At the indicated time the medium was aspirated, the fibroblasts were washed gently three times with PBS and scraped with a rubber policeman. The cell suspension thus obtained was extracted, the corresponding phospholipids were separated by TLC and counted.

2.6. Isolation of plasma membranes

The plasma membrane fraction was obtained according to the procedure described by Evans [14] with modifications. Briefly, subconfluent control and *ras*-transformed NIH 3T3 fibroblasts were washed three times with cold PBS, scraped with a rubber policeman and disrupted by Dounce homogenizer in 10 mM Tris–HCl (pH 7.5). The suspension was first pelleted at $1000 \times g$ for 5 min, loaded on a discontinuous sucrose gradient and centrifuged in a Sorvall OTD-50 ultracentrifuge. The plasma membrane fraction was collected at a density of approximately 45% (w/w) sucrose. The purity of the plasma membrane fraction was monitored by the specific activity of 5′-nucleotidase, glucose-6-phosphatase, succinate dehydrogenase and acid phosphatase.

2.7. Enzyme assays

Phospholipase C was assayed according to Homma et al. [15] with modifications. Incubations were performed at 37°C in a total volume of 0.1 ml containing 50 mM Tris–HCl (pH 6.8), 100 mM NaCl, 0.5% w/w sodium cholate, 2 mM $CaCl_2$ and 20 μg membrane protein. The phospholipid substrate (100 μM phosphatidyl [^{14}C]inositol or L-3-phosphatidyl[*N*-methyl-^{14}C]choline or L-3-phosphatidyl [^{14}C]ethanolamine) was added in the form of a sonicated suspension in aqueous solution. The reaction was carried out for 10 min and was terminated by 2 ml chloroform/methanol (2:1 v/v) and 0.5 ml 1 M HCl. The aqueous phase containing phosphocholine or phosphoethanolamine was concentrated, the radioactive products were separated as described by Yavin [16] and counted. Incubations without membrane protein were carried out simultaneously. The specific activity was calculated as nmol phosphocholine or phosphoethanolamine per min per mg protein.

Phospholipase D activity was measured according to the procedure described by Brown et al. [17] with modifications. The incubation mixture contained 50 mM Tris–HCl (pH 7.5), 8 mM KCl, 1 mM dithiothreitol, 3 mM $MgCl_2$ and 2 mM $CaCl_2$, 100 μM L-3-phosphatidyl[*N*-methyl-^{14}C-]choline 1,2-dipalmitoyl or L-3-phosphatidyl[^{14}C] ethanolamine 1,2-dioleoyl in a total volume of 0.1 ml. The incubation was performed at 37°C for 20 min and the reaction was terminated by addition of 10% trichloroacetic acid. Radiolabeled choline/ethanolamine was separated according to Yavin [16]. The specific activity was calculated as nmol choline or ethanolamine per min per mg protein.

Phosphatidate phosphohydrolase (PAP) was assayed in an incubation mixture containing 100 mM Tris–HCl, (pH 7.5), 1 mM dithiothreitol, 5 mM $MgCl_2$ and 100 μM labeled phosphatidic acid (PA) in a total volume of 0.2 ml. PA was prepared by PLD-induced degradation of labeled phosphatidylcholine [18]. The reaction was initiated by addition of 100 μg membrane protein. Incubations were performed for 20 min at 37°C and terminated by chloroform/methanol 2:1 (v/v). The organic phase was concentrated and run on silica gel G 60 TLC plates in a solvent system for neutral lipids as described above. The spots corresponding to DAG were scraped into scintillation vials and counted.

2.8. Fatty acid analysis

Control and transformed preconfluent fibroblasts were washed three times with PBS and harvested. The tested lipids were separated by TLC, scraped from the plates, saponified with 0.5 N methanolic KOH and methylated with boron trifluoride–methanol complex (Merck). The fatty acid methyl esters were extracted with hexane and separated by gas chromatography on a capillary column of the bonded phase Supelcowax 10 (diameter 0.32 mm, 30 m long (Supelco) fitted on a Girdell gas chromatograph. Quantification was referred to an internal standard of heptadecanoic methyl ester.

2.9. Other procedures

Protein was determined by the method of Lowry et al. [19]. Statistical analysis was performed using Student's *t*-test.

3. Results

The phospholipid analysis indicated that all phospholipids were decreased in *ras*-fibroblasts except PE (Table 1). The DAG level was elevated from 0.65 ± 0.05 nmol/100 nmol of total phospholipid in control cells to 0.96 ± 0.09 in *ras*-cells. The total radioactivity in the DAG fraction after a 48 h-incubation with radiolabeled glycerol was 15600 cpm/10^6 cells in control fibroblasts and 23800 cpm/10^6 cells in their transformed counterparts. The uptake of radioactive glycerol was increased most markedly in PE at all time points, followed by PC (Fig. 1A and B). A slight elevation was observed in PI radioactivity during the first hour of incubation with glycerol (Fig. 1C).

Further we examined the enzyme activities generating DAG, including PLC and PLD/PAP. PC, PE and PI were used as substrates not only because they were the most abundant membrane phospholipids, but also because all three of them could serve as DAG donors through PLC-triggered breakdown. PI–PLC was not activated in *ras*-transformed cells (Table 2). In contrast, PC–PLC was activated by 69% and PE–PLC about 3.5-fold in membranes from transformed cells (Table 2).

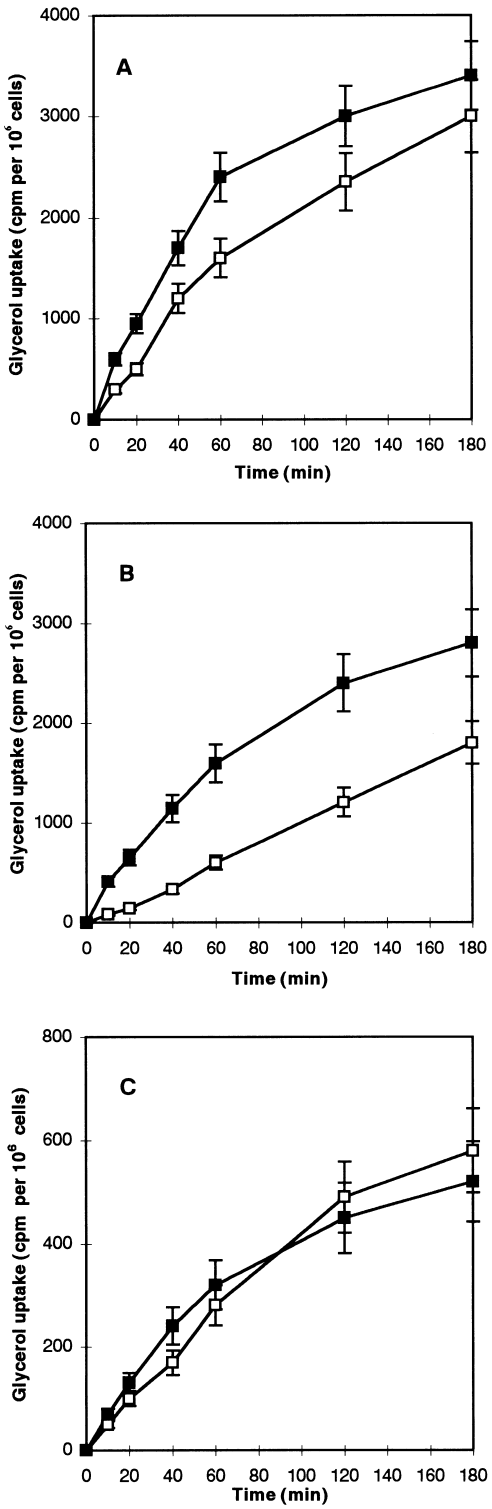
Similarly, both PC- and PE–PLD activities were elevated in *ras*-cells (Table 2), the latter being much more pronounced (about 6-fold). PAP was activated as well in the transformed cells (Table 2). PI–PLD activity was not detectable.

The fatty acid composition of the major membrane phospholipids was also investigated, because such data would illustrate the similarities/or differences between the acyl chains of DAG and the corresponding DAG donors (Table 3). A simple comparison revealed an apparent resemblance between the acyl chains of PE and DAG in the *ras*-transformed cells. However, no similarities were observed between the acyl chains of PE and DAG in non-transfected fibroblasts. In addition, there were no evi-

Table 1
Phospholipid composition of control and *ras*-transformed NIH 3T3 fibroblasts

Phospholipids	Control		*ras*	
	nmol/mg protein	%	nmol/mg protein	%
Phosphatidylcholine	59.64 ± 2.17	50.22	55.25 ± 2.57^a	50.13
Phosphatidylethanolamine	28.71 ± 1.85	24.17	32.02 ± 2.17^b	29.05
Phosphatidylinositol	9.05 ± 0.89	7.62	7.85 ± 0.77^a	7.12
Phosphatidylserine	6.55 ± 0.74	5.52	4.16 ± 0.35^c	3.77
Phosphatidylglycerol	4.27 ± 0.43	3.59	2.78 ± 0.37^c	2.52
Sphingomyelin	10.53 ± 1.12	8.87	8.15 ± 0.92^c	7.39

Values are means $\pm$ S.D. from two replicates in two experiments.[a] $P < 0.05$.[b] $P < 0.01$.[c] $P < 0.001$.



dent similarities between the fatty acids of PC, PI and DAG in the transformed cells (Table 3). These data also demonstrated that *ras*-transformation was accompanied by a general augmentation of the saturated and monoenoic fatty acids and a decrease of the polyunsaturated ones.

4. Discussion

Our studies showed that PC and more markedly PE could generate DAG in *ras*-transformed NIH 3T3 fibroblasts. The analysis of the phospholipid composition indicated that PE was the only phospholipid which was increased in the transformed cells (Table 1) in spite of the activated PE-degradation (Table 2). Possibly, this was due to the more intensive PE synthesis compared to the other tested phospholipids, as illustrated by the rate of glycerol uptake (Fig. 1). Both PC- and PE–PLC activities were augmented in the transformed cells, the latter being much more pronounced. The activation of PI–PLC was not statistically significant. What is more, PIP_2–PLC activity was also unchanged in the transformed cells (data not shown). These results implied that besides PC, which is the major structural phospholipid fraction, PE could also produce DAG in *ras*-transformed fibroblasts. The existence of a DAG source different from PI was suggested for the first time by Lacal et al. [4]. It should also be noted that some authors failed to observe PC–PLC activity in mammalian cells [8, 20]. One possible reason is that most such experiments have been performed with whole cells in which the newly formed intermediates could rapidly be transformed to others. For

Fig. 1. Time course of incorporation of radiolabeled glycerol into phosphatidylcholine (A), phosphatidylethanolamine (B) and phosphatidylinositol (C) in control (□) and *ras*-transformed (■) NIH 3T3 fibroblasts. Subconfluent control and transformed cells were incubated with 2 μCi/ml medium glycerol. At the indicated time, the medium was aspirated, the cells were washed, collected and the phospholipids were analyzed as described in Section 2. Values of uptake are given as mean cpm/10^6 cells ± S.D. from 3 replicates in one experiment.

Table 2
Specific activity of membrane-bound enzymes in control and *ras*-transformed cells (nmol·min^{-1}·mg protein^{-1})

Enzyme	Substrate	Control	*ras*
Phospholipase C	PC	1.23 ± 0.31	2.08 ± 0.44^{b}
	PE	0.74 ± 0.05	2.63 ± 0.23^{c}
	PI	0.28 ± 0.06	0.34 ± 0.03
Phospholipase D	PC	0.78 ± 0.06	2.19 ± 0.22^{c}
	PE	0.39 ± 0.04	2.35 ± 0.29^{c}
Phosphatidate-phosphohydrolase	PA	1.67 ± 0.64	4.39 ± 0.97^{c}

Values are means $\pm$ S.D. from three replicates in two experiments.PC, phosphatidylcholine; PE,

example, phosphocholine could be a product of either PC–PLC, which is a membrane-bound enzyme or choline kinase, which is a cytosolic enzyme [21]. To distinguish between alternative pathways generating similar products, we performed investigations on purified isolated membranes thus eliminating the enzyme activities typical for the cytosol or other subcellular fractions. The lack of choline/ethanolamine kinase in the isolated plasma membranes was verified experimentally in our preliminary studies. Thus the measured phosphocholine/phosphoethanolamine could not be a product of additional phosphorylation of the present choline/ethanolamine, and could be used for determination of PLC activity.

As mentioned above, an alternative pathway including the consecutive activation of PLD and PAP could also be responsible for DAG accumulation. PLD-induced breakdown of PC and PE produces phosphatidic acid (PA) and choline/ethanolamine, respectively, whereas PAP hydrolyzes PA to DAG and phosphate. The produced PA has been reported to act as a mitogen in fibroblasts and other cell lines [22]. At this point

Table 3
Fatty acid composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) in control and ras-transformed NIH 3T3 fibroblasts (in mol%)

Fatty acids	PC		PE		PI		DAG	
	control	*ras*	control	*ras*	control	*ras*	control	*ras*
C16:0	37.89	27.58^{c}	10.39	18.72^{c}	9.87	16.62^{c}	40.36	19.72^{c}
C16:1	11.34	14.21^{b}	3.78	4.83^{b}	1.59	3.71^{c}	6.82	4.95^{c}
C18:0	11.55	7.57^{c}	39.15	28.22^{c}	40.89	34.43^{c}	21.27	27.38^{c}
C18:1	29.53	43.31^{c}	25.51	32.07^{c}	21.54	23.63^{c}	13.59	31.54^{c}
C18:2	4.74	4.18^{a}	3.51	5.62^{c}	1.95	4.22^{c}	1.81	5.78^{c}
C20:1	1.59	0.73^{a}	4.71	4.11^{a}	8.53	7.22^{b}	2.28	3.70^{c}
C20:3	1.46	1.06^{a}	1.23	1.97^{a}	2.05	1.65^{a}	1.31	1.98^{a}
C20:4	1.90	1.36^{a}	11.72	4.46^{c}	13.58	8.52^{c}	12.56	4.95^{c}
SAT	49.44	35.15	49.54	46.94	50.76	51.05	61.63	47.10
MONO	42.46	58.25	34.00	41.01	31.66	34.56	22.69	40.19
PUFA	8.10	6.60	16.46	12.05	17.58	14.40	15.68	12.71

SAT, saturated; MONO, monounsaturated and PUFA, polyunsaturated fatty acids. Values are means $\pm$ S.D. from two replicates in two experiments.$^{a}P < 0.05$.$^{b}P < 0.01$.$^{c}P < 0.001$.

it is not yet clear whether PA is itself a triggering signal for cell transformation or whether in order to act as such, it has to be converted to DAG [22]. PAP is the enzyme maintaining the equilibrium between DAG and PA, thus possibly participating in the signal transduction regulation. PAP inhibition would lead to PA accumulation, whereas its activation would increase the production of DAG.

Del Peso et al. [23] demonstrated that phosphocholine production was dependent on choline phosphorylation by choline kinase in *ras*-transformed cells. Phosphocholine has been presumed to act as a positive regulator in tumor development and growth [24]. In addition, Kiss and Crilly [25] suggested that the aggressive growth of some cancers requires high levels of phosphoethanolamine. Thus, the activation of PLD becomes increasingly important because it is responsible for production of choline/ethanolamine which can be phosphorylated to phosphocholine/phosphoethanolamine. Choline and ethanolamine kinase activities were reported to reside on the same enzyme [21]. So the marked activation of PC/PE–PLD which we observed, together with the stimulated choline/ethanolamine-kinase [26], indicates that these enzymes represent a pathway essential for phosphocholine/phosphoethanolamine production in *ras*-cells. Apparently, PLD activity, which has been reported to depend on various factors, including ADP-ribosylation factor, Rho proteins and PIP_2 [17, 27], is of particular interest in *ras*-expressing cells. Interestingly, Del Peso et al. [23] reported that PLD in *ras*-cells was not PKC-dependent. The reasons for the substantial activation of PLD and especially PE–PLD, as well as the relation of this activity and its products to signal transduction need further clarification.

However, the direct way for DAG production including PC/PE–PLC also seems of special interest. In spite of the lower degree of PC–PLC activation compared to PE–PLC, it should be noted that the content of PC was about twice as high that of PE (Table 1). Thus, the contribution of PC to DAG elevation in *ras*-cells should not be underestimated. Accordingly, Price et al. [5] and Lacal [28] reported data confirming the role of PC as a DAG source.

Our data suggest that both pathways including PLC and PLD/PAP could be responsible for DAG elevation in *ras*-cells. It is also possible that DAG generated by either of the two pathways carry out different functions in cells as reported by Pettitt et al. [29].

One possible approach to examine in more detail the origin of DAG is to analyse the fatty acid composition of DAG and its potential phospholipid donors. Such studies could produce reliable evidence which would help to discriminate between the DAG-generating phospholipid molecules. Our results showed that the proportions between the saturated, monounsaturated and polyunsaturated fatty acids in PE and DAG were quite similar in the transformed cells, this making PE a very probable DAG donor. In contrast, no apparent DAG source could be distinguished by comparing the acyl chains of DAG and the tested phospholipids in non-transformed fibroblasts.

In conclusion, the degree of activation of PE-PLC and PE-PLD/PAP together with the acyl chain analysis indicated that besides PC, PE could also generate DAG in *ras*-transformed fibroblasts. The marked increase in glycerol uptake together with the activated PE breakdown demonstrated that PE metabolism was stimulated in *ras*-transformed cells this making it a source of important cellular intermediates like DAG, PA and phosphethanolamine. Thus, in spite of being major structural components of cellular membranes, PC and PE apparently play active metabolic and maybe regulatory role in the processes of oncogenic transformation of cells.

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