Vol. 171, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1990 Pages 14-19

INCREASED INTRACELLULAR GLYCEROPHOSPHOINOSITOL IS A BIOCHEMICAL MARKER FOR TRANSFORMATION BY MEMBRANE-ASSOCIATED AND CYTOPLASMIC ONCOGENES

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Received June 27, 1990

Transformation of rodent fibroblasts by cytoplasmic (*mos*, *raf*) and membrane-associated (*ras*, *src*, *met*, *trk*), but not nuclear (*myc*, *fos*) oncogenes results specifically in a very significant elevation of intracellular levels of glycerophosphoinositol (GPI). This elevation is specifically associated with the transformed state of the cells and not merely with their active state of proliferation. The basal phospholipase A_2 (PLA_2) activity of the same cells is also significantly stimulated in vivo. Our results are consistent with the notion that the elevated levels of GPI result from deacylation of lysophosphatidylinositol released by the enhanced PLA_2 activity. GPI is a water-soluble, easily detectable metabolite which may constitute a convenient biochemical marker for malignant transformation by this particular group of oncogenes.

A number of reports have linked *ras*-mediated malignant transformation to alterations of phospholipid-dependent signalling pathways (1-8). In particular, microinjection of *ras* proteins into normal fibroblasts (1) or transformation of the same cells by transfected *ras* oncogenes results in a marked increase of phospholipase A_2 activity (3). However, the activation of PLA_2 is not limited to *ras* oncogenes, since transformation by other membrane and cytoplasmic oncogenes (but not nuclear oncogenes) leads also to increased basal PLA_2 activity (3). This increase is accompanied by elevated basal levels of unsterified arachidonic acid and the water soluble compound glycerophosphoinositol (GPI).

In this report we documented the specificity of the association between elevated GPI levels and transformation by cytoplasmic and membrane-associated oncogenes. We focused also on the mechanism of increased production of GPI and on the origin and nature of the increased PLA_2 activity. In an experimental approach similar to that used in classical metabolic studies of PLA_2 (9-12) we used a negative modulator (dexamethasone) of this enzyme in order to determine the biochemical origin of GPI and characterize the kinetics of its production. Furthermore, we used a specific DNA probe for mammalian PLA_2 (13) in an effort to ascertain whether the elevated levels of PLA_2 activity are due to changes at the level of mRNA expression. Our results suggest that a chronic activation of PLA_2, occurring specifically in cells transformed by cytoplasmic and membrane-associated oncogenes, is responsible for the accumulation of significant intracellular levels of glycerophosphoinositol. This water-soluble compound may be used as a convenient biochemical marker for transformation of fibroblasts by those oncogenes.

0006-291X/90 $1.50

MATERIALS AND METHODS

Tissue culture ^{3}H-inositol labeling, extraction and HPLC analysis. The sources of cell lines and regular growth conditions have been documented elsewhere (3,8,14). Subconfluent, actively growing normal and transformed cell lines were labeled homogeneously on 35 mm culture plates using L-myo (1,2-^{3}H [N]) inositol as described (3,8). Monolayer cultures labeled to equilibrium with ^{3}H-inositol and treated as appropriate in each case were washed 3 times with ice-cold PBS containing BSA (1 mg/ml) and the water-soluble ^{3}H-inositol derivatives extracted with TCA as described (3,8,15). After removal of TCA by repeated extraction with water-saturated diethyl ether, the samples were lyophilized, resuspended in ddH_2O (HPLC grade) and analyzed by HPLC using a Whatman Partisil 10 SAX (25 x 0.46 cm) HPLC column according to the procedure of Dean and Moyer (16) with slight modifications as described (8). Radioactivity in the HPLC effluent was quantified by liquid scintillation counting using an on-line radioactivity flow detector (Flo-One Beta IC, Radiomatic Instruments) as described (15).

RNA expression studies. Total RNA was extracted from monolayers of normal and transformed cells grown as indicated above. Cells were disrupted with RNAzol (Cinna/Biotecx Laboratories International, Inc., Friendswood, TX), and all subsequent steps were performed according to the manufacturer's instructions. Serial dilutions (starting with 5 µg of total RNA) of denatured RNA from each of the cell lines were applied onto nitrocellulose membranes using a Minifold II slot blot apparatus (Schleicher & Schuell). After fixing, the membranes were prehybridized and hybridized under high stringency conditions (50% formamide, 5x SSPE, 1x Denhardt's solution at 42°C) using 8 x 10^6 cpm of a ^{32}P-nick-translated probes. Probes used in hybridization included a 450bp Eco RI fragment from a porcine phospholipase A_2 cDNA clone (13) and a 2 kbp Hind III fragment of plasmid B2000 (17) containing chicken ß-actin. The hybridized filters were washed 3 times (5 min each) with 2x SSC/0.1% SDS at room temperature, and 2 times (15 min each) with 0.1x SSC/0.1% SDS at 45°C before drying and exposure at -70° C to Kodak XAR-5 film using Dupont Cronex intensifying screens.

RESULTS AND DISCUSSION

Levels of intracellular glycerophosphoinositol (GPI) in normal and transformed rodent fibroblasts. We detected a striking difference between the levels of GPI in normal NIH 3T3 or Rat1 cells and in their derivative cell lines transformed by cytoplasmic and membrane-associated oncogenes, although not by nuclear oncogenes (Fig. 1). When compared to normal NIH 3T3 and Rat-1 cells, all cell lines transformed by cytoplasmic (*mos*, *raf*) or membrane-associated oncogenes (*src*, *met*, *trk*, *ras*) showed strikingly increased levels (200% to 600%) of GPI (Fig. 1). In sharp contrast, transformation of Rat-1 cells by nuclear oncogenes *myc* or *fos* did not produce such increase (Fig. 1), suggesting that the change in levels of GPI is limited to transformation by oncogenes whose products localize to the plasma membrane or the cytoplasm. Similar studies with nuclear oncogenes are not possible in NIH 3T3 cells as they are not readily transformed by *fos* or *myc* (18). The cellular localization of the product of the *vav* oncogene (14) has not yet been described. Given the elevated levels of GPI in K49-3 cells (Fig.1) we predict a non-nuclear localization for this oncogene product.

The specific correlation between the transformed state and elevated levels of GPI is underscored by the significant reduction in GPI levels observed in normal revertant C11 cells (19) compared to their transformed progenitor DT cell line (Fig. 1). In addition, the absence of increased levels of GPI in *myc*- and *fos*-transformed cells shows that elevated GPI is an indicator of the transformed state of the cells and not merely a result of active proliferation. It should be noted that under the conditions of these experiments, only actively growing normal and transformed cells were studied.

The water-soluble nature of GPI facilitates its separation and detection in either HPLC systems like those used here (15,16) or in simpler TLC separation systems (20). These properties make it attractive for use as a biochemical marker of transformation by cytoplasmic and membrane oncogenes. This is, to our knowledge, the first report establishing a specific relationship between the transformed state of

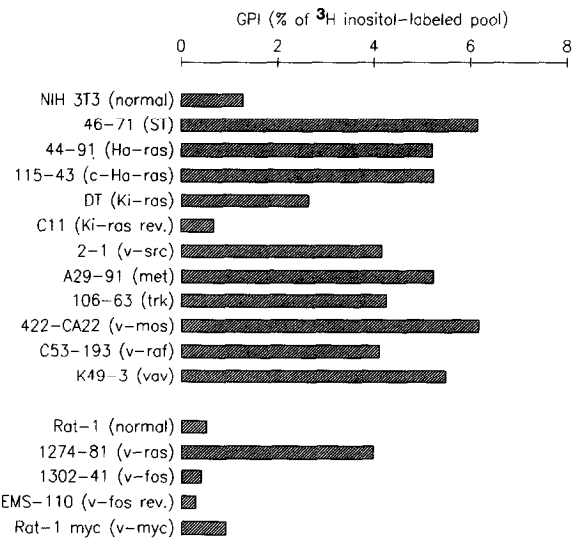

Figure 1. **[^{3}H]Ins-labeled glycerophosphoinositol from normal and transformed NIH 3T3 and Rat-1 cells.** Subconfluent (actively growing) cultures of normal and transformed cell lines labeled to equilibrium for 72 h with ^{3}H-inositol were extracted and analysed by HPLC as described in Methods. Except for GPI, no significant differences were found between normal and transformed cell lines in any inositol metabolite (3,8). Radioactivity in the GPI peak is represented here as percentage of the total ^{3}H-inositol-labeled pool. For comparison purposes, the radioactivity in the GPI peak of normal NIH 3T3 cells was approximately 13000 cpm. Results presented (except for K49-3 cells) are an average of at least 4 separate experiments, where all experimental conditions and controls were done in triplicate. Results for K49-3 cells are from a single experiment (also assayed in triplicate). Standard deviation of the experiments was always less than or equal to 15% of the values presented.

fibroblastic cell lines and elevated levels of GPI. We are aware of a previous report (21) in cells transformed by avian sarcoma virus UR2 (carrying the tyrosine-kinase *v-ros* oncogene) where very elevated levels of GPI were shown. However, in that case the elevated GPI was unnoticed in terms of its relation to the transformed state of the cells under study. It will be of interest to determine whether the relationship between elevated GPI and malignant state is also true of other transformed cell lineages and *in vivo* tumors.

Effect of dexamethasone on glycerophosphoinositol levels in normal and transformed NIH 3T3 and Rat-1 fibroblasts.

Transformation of rodent fibroblasts by cytoplasmic and membrane oncogenes results in concomitant elevation of the cellular levels of free arachidonic acid and glycerophosphoinositol. While arachidonic acid is clearly a direct product of hydrolysis of phospholipids by the activated PLA_2 present in the transformed cells (3), we wished to characterize further the molecular origin of the enhanced levels of GPI.

It has been previously shown that glucocorticoid treatment results in preferential inhibition of PLA_2, with little or no effect on the generation of PLC products (10-12). Thus, treatment of BALB/3T3 (10), Swiss 3T3 (9,11) cells or C62B glioma cells (12) with dexamethasone has been reported to result in a significant and concomitant drop in intracellular levels of a variety of products (dependent on PLA2 activity) including (i) arachidonic acid (AA) and its metabolic derivatives (prostaglandins), (ii) the lysophophatidyl derivatives of inositol- and choline-phospholipids, and (iii) glycerophosphoinositol or

glycerophosphocholine. While arachidonic acid and lysophosphatidylinositol are direct products of hydrolysis of phospholipids by PLA_2, those results indicate that, in those cell types, GPI is the product of subsequent lysophospholipase (phospholipase B-like) on lysophophatidylinositol released by the PLA_2 activity (9-12).

In order to determine here whether the elevated GPI levels found in our transformed NIH 3T3 cell lines were generated through a similar mechanism, we compared the effects of dexamethasone (a negative modulator of PLA_2 activity) on the levels of GPI in normal and transformed NIH3T3 cells. In the experiment described in Table 1, normal NIH 3T3 and Ha-*ras* transformed 44-91 cells were labeled homogeneously with ^{3}H-inositol in the presence or absence of dexamethasone before analysis of their inositol metabolites (Table 1). As shown previously, the basal levels of GPI were much higher in transformed than in normal NIH 3T3 cells. Short term (4 h) exposure to dexamethasone produced a slight decrease (10-15%) of the GPI levels in both normal and transformed cells. However, prolonged dexamethasone treatment (96 h) resulted in a dramatic decrease (by more than 60%) of the GPI levels measured in both normal and transformed cells (Table 1). The long term dexamethasone treatment did not produce any visible phenotypical changes or alterations of growth of both normal and transformed cells. The basal levels of free inositol remained virtually constant even after the prolonged dexamethasone treatments (Table 1), an indication that inositol metabolism was not impaired under those conditions. These results strongly suggest that the high levels of GPI observed in transformed cells result from further deacylation of the lysophosphatidylinositol released by the enhanced PLA_2 activity. The similar percentage of inhibition of GPI levels by dexamethasone in both normal and transformed cells (Table 1) also suggests that the mechanism of GPI generation through a phospholipase B-like activity is similar in normal and transformed cells. Therefore, these data support the notion that the very different basal levels of GPI observed between normal and transformed cells are due to differences in basal PLA_2 activity, i.e., chronic stimulation of this activity in the transformed cells.

TABLE 1
Effect of dexamethasone on levels of glycerophosphoinositol in normal and transformed NIH 3T3 cells

Cell line	NIH 3T3				44-91 (Ha-*ras*)			
Incubation	4 hours		96 hours		4 hours		96 hours	
Dexamethasone	-	+	-	+	-	+	-	+
Ins	1185169	1133678	1160404	1309281	1361442	1181269	1175679	1218815
GPI	13243	11452	16322	7282	76499	60271	92847	34933

Subconfluent cultures grown and labeled with ^{3}H-inositol for 96 hours as for Fig.1 were treated in the presence (+) or absence (-) of 1 μM dexamethasone for the last 4h or the whole 96 h labeling period before extraction and HPLC analysis of inositol metabolites. Actual radioactivity (cpm) associated with HPLC peaks for ^{3}H-inositol-labeled glycerophosphoinositol (GPI) and free ^{3}H-inositol (Ins) is presented here. Values are the mean of triplicate experiments run in parallel. S.D. was < 5% of the values presented in all cases.

Expression of PLA_2 mRNA in normal and transformed cells.

The molecular mechanism underlying the elevated levels of PLA_2 activity detected in cells transformed by cytoplasmic and membrane-associated oncogenes is unknown. The recent availability of probes specific for some mammalian PLA_2 allowed us to start addressing the question of whether the mechanism underlying the elevated levels of PLA_2 involves regulatory alterations at the level of transcription or at a later stage.

Clones for intracellular, "pancreatic" group I PLA_2 and secreted human group II PLA_2 isozymes have been recently obtained (13, 22-24). The analysis of their sequences reveals important sequence differences but shows also that group I and II enzymes share significant homologies in different regions of the molecule that warrant some degree of cross-reactivity in hybridization studies (22,23). Since the elevated AA and GPI measured in transformed cells are intracellular, it is likely that the responsible PLA_2 enzyme is also intracellular and therefore pancreatic PLA2 is best suited for hybridization studies of RNA extracted from the normal and transformed NIH3T3 cells.

The porcine probe used here has been shown to detect single copy PLA_2 genes in a variety of mammalian backgrounds including rodents and humans (13). In addition, this "pancreatic" PLA_2 has also been found in many other tissues including gastric mucosa, lung and spleen (22-24). Hybridization of serial dilutions (not shown) of RNA extracted from a variety of rodent cell lines with this PLA_2 specific probe showed no significant differences in the expression of phospholipase A_2 RNA between normal and oncogene-transformed cells. Parallel hybridization of the same amounts of RNA from each cell line with probes for PLA_2 and ß-actin showed that all normal and transformed cells analyzed possess similar relative levels of PLA_2 RNA (Fig. 2). These observations suggest that there is not overexpression of intracellular PLA_2 mRNA in cells transformed by cytoplasmic and membrane-associated oncogenes and that posttranscriptional events are likely to be responsible for the observed elevated PLA_2 activity.

In summary, the elevated GPI levels in cells transformed by cytoplasmic and membrane oncogenes are the result of permanent activation of PLA_2. However, the increased basal PLA_2 activity probably does not correlate with increased levels of specific RNA for the enzyme. Therefore, the increased activity presumably does not reflect increased synthesis of the enzymatic protein but, rather, activation at the biochemical level resulting probably from altered metabolic regulation. A very noticeable consequence of the constitutive activation of PLA_2 is the accumulation of very high levels of GPI, an easily detected

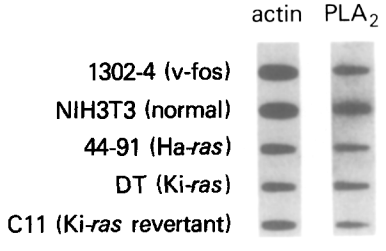

Figure 2. **Steady-state levels of phospholipase A_2 RNA in normal and transformed NIH 3T3 and Rat-1 fibroblasts.** Slot blot analysis of total RNA derived from normal and transformed cell lines. Hybridization was performed under high stringency conditions with ^{32}P-nick-translated probes including PLA_2 (450bp Eco RI fragment from a cDNA clone [13]) and chicken ß-actin (2 kbp Hind III fragment of plasmid B2000 [17]). Hybridization of these probes with pBR322 DNA (not shown) was used as a negative control.

compound which could serve as a biochemical marker for transformation of fibroblasts by a particular subset of oncogenes (cytoplasmic and membrane-associated).

ACKNOWLEDGMENTS. We thank Jeff Seilhamer for the porcine PLA_2 cDNA plasmid, Michael Beaven for help with HPLC separation of inositol metabolites and useful suggestions, and Malcolm A. Martin for support. T.A. was the recipient of a fellowship from the Education Council of the Canary Islands, Spain.

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