ELEVATION OF A THREONINE PHOSPHOLIPID IN POLYOMA VIRUS TRANSFORMED HAMSTER EMBRYO FIBROBLASTS

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SUMMARY: Analysis of the lipids of normal hamster embryo fibroblasts and polyoma virus transformed fibroblasts shows a decrease in phosphatidylcholine and phosphatidylethanolamine and a marked increase in a threonine phospholipid after transformation. Transformed cells also react differently with fluorodinitrobenzene and trinitrobenzenesulfonate. Phosphatidylethanolamine of transformed cells reacts to a greater extent with both probes. Phosphatidylserine and the threonine phospholipid of both cells do not react with trinitrobenzenesulfonate. The threonine phospholipid is provisionally identified as phosphatidylthreonine.

INTRODUCTION: Virus transformed cells are reported to have altered lipid metabolism and lipid composition. These changes occur with cholesterol (1-4), phospholipids (5-15) or glycolipids (16-20). The significance of these changes is not well understood but is believed to be related in part to alteration of membrane fluidity and concomitant modulation of membrane transport properties or to modification of the surface recognition properties of the cell (16). A review of the lipids of normal and tumor cells grown in culture recently has been published by Howard and Howard (21).

In this paper we report a new type of lipid alteration in hamster embryo fibroblasts upon transformation by polyoma virus. Transformation leads to a reduction in phosphatidylethanolamine and phosphatidylcholine and an

Abbreviations: HEF = hamster embryo fibroblasts; HFT = hamster fibroblasts transformed; FDNB = 1-fluoro-2,4-dinitrobenzene; TNBS = 1,3,5-trinitrobenzenesulfonate; DNP = dinitrophenyl; PE = phosphatidylethanolamine; PS = phosphatidylserine; PC = phosphatidylcholine; PT = threonine phospholipid (tentatively identified as phosphatidylthreonine); TNP = trinitrophenyl.

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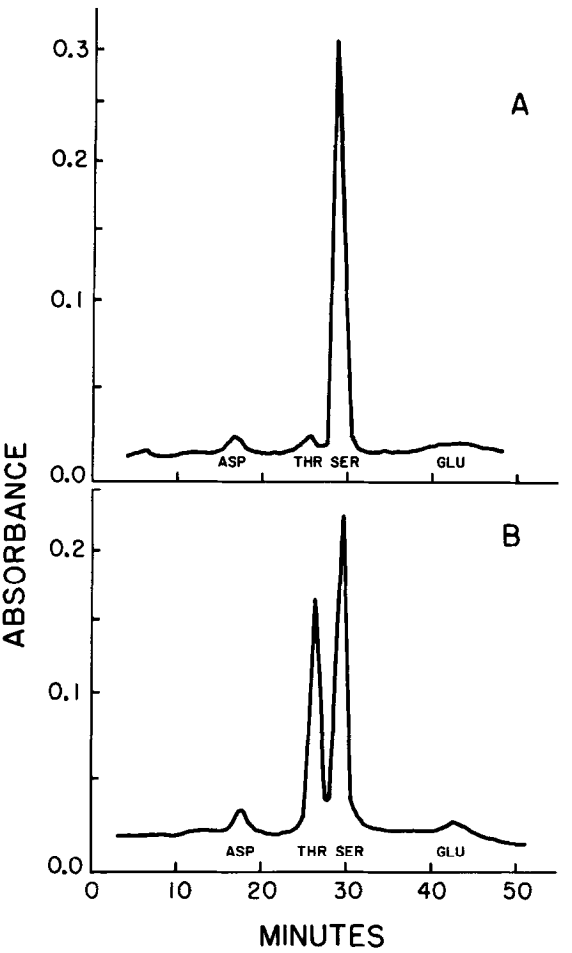

LEGENDS: Figure 1. Analysis of Serine and Threonine Obtained by HCl Hydrolysis of Phospholipids from HEF and HFT Cells.

The phospholipids of HEF and HFT cells were extracted by the method of Bligh and Dyer (22) and hydrolyzed as explained in the text. The aqueous phase of the hydrolysates was evaporated to dryness at 23°C under nitrogen and dissolved in 1.0 ml of 0.07 M citrate buffer pH 3.5. Half ml aliquots were analyzed on the Spinco Amino Acid Analyzer. The tracings are shown in the figure. Profiles A and B represent HEF and HFT cells respectively. Very small amounts of aspartic and glutamic acid were found in both samples.

increase in a threonine phospholipid which has the properties of phosphatidylthreonine.

METHODS AND REAGENTS: Hamster embryo fibroblasts (HEF) and transformed fibroblasts (HFT) (transformed by polyoma virus) were grown in cell culture in Eagle's minimal essential medium modified with Earle's salts (Auto-Pow Flow Labs) containing 5% bovine fetal serum. Cells (between 90-115 x 10^6

cells) were extracted three times with chloroform-methanol 1:1 and the extracts were rectified by the method of Bligh and Dyer (22) to remove non-lipid contaminants. Aliquots of lipids were analyzed directly by thin layer chromatography on silica gel plates (Merck & Darmstadt SG 5763) using a solvent mixture of chloroform:methanol:acetic acid:water (65:25:2:4 vol/vol). Other aliquots of the lipid extracts were hydrolyzed in 3N HCl at 100°C for 2 hours. The aqueous HCl phase was removed and evaporated to dryness under nitrogen at 23°C. The residues were analyzed for serine and threonine on the Spinco amino acid analyzer. Other aliquots of the HCl hydrolysate were brought up to 2 ml of 120mM $NaHCO_3$-40mM NaCl buffer pH 8.5 and treated with 2mM fluorodinitrobenzene for 2 hours at 37°C. The reaction medium was acidified with HCl extracted with ethyl acetate to obtain the dinitrophenyl derivatives of the amino acids (23). These derivatives were analyzed by thin layer chromatography on silica gel plates using chloroform:methanol:acetic acid:water (65:25:2:4 vol/vol) as solvent and compared to authentic standard DNP derivatives of serine, threonine and ethanolamine.

The labeling of washed trypsinized HEF* and HFT* cells with 2mM fluorodinitrobenzene (FDNB)* or 2mM trinitrobenzenesulfonate (TNBS)* was carried out in 120mM NaCl -40mM $NaHCO_3$ buffer pH 8.5 at 23°C for 1 hour. For these experiments suspensions of 30-40 x 10^6 cells were used per experiment. The cells were washed with buffer and the lipids extracted by the Bligh and Dyer technique (22). The intact lipids and the HCl hydrolysis products of the lipids were separated by thin layer chromatography as described above. The DNP and TNP derivatives of the amino-phospholipids and the DNP derivatives of serine and threonine were eluted with methanol and measured spectrophotometrically. The unreacted amino-phospholipids were eluted with 1N HCl in methanol and measured by analysis of their phosphate content after digestion with 70% perchloric acid. The percent of toal PE, PS or PT which reacted with FDNB or TNBS was then calculated. FDNB and TNBS were obtained from Pierce Chem. Co., Rockford, Ill.

RESULTS: Evidence for the occurrence of a threonine phospholipid which we provisionally identify as phosphatidylthreonine in transformed hamster embryo fibroblasts is shown in Figure 1. It can be seen that serine is the major amino acid of the phospholipid hydrolysate of normal cells (profile A) analyzed on the Spinco Amino Acid Analyzer. However, the profile for the transformed cells (profile B) shows both threonine and serine are present in nearly equal amounts. The quantitative data are given in Table 1. In normal cells, serine is the major component and threonine is barely detectable. In transformed cells, threonine occurs close to the same amount as serine. The phospholipid extract used for these studies were rectified by the Bligh and Dyer method (22) to remove free amino acids.

The further characterization of threonine and serine was accomplished by conversion to the dinitrophenyl derivatives, analysis by thin layer chromatography and comparison to authentic samples of dinitrophenylserine (DNPSer)

Table 1

Serine and Threonine Content of the HCl Hydrolysate of Lipids from Normal and Transformed Fibroblasts[a]

	Normal Cells	Transformed Cells
	nmoles	nmoles
Experiment 1		
Serine	94	59
Threonine	3	46
Experiment 2		
Serine	526	163
Threonine	13	151

[a] Lipid samples were hydrolyzed with 3 N HCl for 2 hours at 100°C. The hydrolysis products were analyzed on the Spinco Amino Acid Analyzer. Different amounts of lipids were used in experiments 1 and 2 and each experiment used a different batch of cells.

and dinitrophenylthreonine DNPThr). These compounds are completely separated by the layer chromatography on silica gel plates. The Rf values of DNPThr and DNPSer are 0.40 and 0.31 respectively in chloroform-methanol-acetic acid-water 65:25:2:4 v/v. It is noteworthy that the intact PS and the threonine-phospholipid have essentially the same mobility in chloroform:methanol:acetic acid:water. Since PS and PT differ by only a methylene group, they would be expected to have similar chromatographic mobilities.

The decrease in PE and PC content of transformed cells is shown in Table 2. HEF cells contain PS but very little PT whereas HFT cells contain nearly equal amounts of PS and PT. The total phospholipid content of transformed cells is decreased as compared to normal cells. Incubation of both cell types for 1 hour at 37°C in 120 mM $NaHCO_3$-40 mM NaCl buffer pH 8.5 shows

Table 2

Phospholipid Content of Normal and Transformed Hamster Embryo Fibroblasts

	Normal Cells	Transformed Cells
	nmoles P/10^6 cells[a]	
Total chloroform-methanol soluble P[b]	65.9 ± 1.1 (11)	61.3 ± 1.1 (14)
Total Bligh-Dyer lipid P[c]	55 ± 0.8 (11)	50 ± 1.6 (14)
Phosphatidylethanolamine	14.4 ± 0.3 (5)	11.8 ± 0.5 (7)
Phosphatidylserine + threonine phospholipid	9.5 ± 0.8 (5)[d]	8.6 ± 1.5 (7)[e]
Phosphatidylcholine	21.6 ± 1 (3)	13.2 ± 1.3 (2)

[a] Mean ± SD of 2-11 experiments done in duplicate or triplicate.

[b] This extract includes some non-lipid phosphate.

[c] This extract represents total lipid P free from most non-lipid P.

[d] Represents mainly phosphatidylserine based on the data in Figure 1.

[e] Represents about equal amounts of phosphatidylserine and threonine phospholipid, based on the data in Figure 1.

very little degradation of the phospholipids by lipases under these conditions.

Another change in the transformed cells is seen in Table 3 when cells are treated with TNBS and FDNB. PE of transformed cells reacts to a greater extent with FDNB and TNBS. The threonine phospholipid of transformed cells reacts to a greater extent with FDNB than does PS. Only 8% of this lipid reacts with FDNB. In normal cells 10% of PS reacts with FDNB. PS and the threonine phospholipid of both cells do not react with TNBS. It is also noteworthy that even with excess FDNB, the PE does not react to completion showing

Table 3

Extent of Reaction of Phosphatidylethanolamine, (PE) Phosphatidylserine, (PS) and Threonine Phospholipid (PT) of Normal and Transformed Fibroblasts with FDNB and TNBS[a]

	Percent of Total PE Which Reacts With FDNB or TNBS		Percent of Total PS or PT Which Reacts With FDNB or TNBS[b]	
	FDNB	TNBS	FDNB	TNBS
Normal Cells	64 ± 1	11.8 ± 0.8	8.2 ± 6	0
Transformed Cells	88 ± 14	18.8 ± 12	10.5 ± 3	0

[a] Cells were reacted with FDNB or TNBS as explained in the text. Values represent the mean ± SD of three experiments done in triplicate.

[b] With normal cells PS was the major component which reacts with FDNB. However, with transformed cells, PT was the major component which reacts with FDNB. This was determined by thin layer chromatographic analysis of the HCl hydrolysis products of the phospholipids as explained in the text.

that 36% of this lipid is not accessible in normal cells compared to 12% in transformed cells. The inaccessible fraction of PE, PS and the threonine phospholipid is probably tightly bound to membrane protein.

DISCUSSION: The present work demonstrates a change in phospholipid type in addition to phospholipid content of transformed cells. Changes in phospholipid, cholesterol and glycolipid content of transformed cells have been found by others (1-21). The significance of these changes is not well understood but is presumed to be related to critical changes in the properties of the cell membrane that contain these lipids.

Assuming that our identification of the threonine phospholipid as phosphatidylthreonine is correct, the following reactions show the possible

ways in which phosphatidylthreonine may be formed in the transformed cells:

(1) PS + threonine $\rightleftharpoons$ PT + serine

(2) PE + threonine $\rightleftharpoons$ PT + ethanolamine

(3) PC + threonine $\rightleftharpoons$ PT + choline

(4) CDP-diacylglycerol + threonine $\rightleftharpoons$ PT + CMP

(5) CDP-threonine + diacyglycerol $\rightleftharpoons$ PT + CMP

Reactions 1-3 represent base exchange transformations which have been demonstrated for serine in both prokaryote and eukaryote cells (24-30). This type of reaction to our knowledge has not been demonstrated for threonine. Reaction 4 has been demonstrated for serine primarily in prokaryote cells (32, 33). A similar reaction in eukaryote cells has been reported but not yet proven (34). Reaction 5 has been demonstrated for CDP-choline and CDP-ethanolamine but not for CDP-serine (35,36). Base exchange between serine and PC (Reaction 3) has been postulated recently in mammalian cells (30,31). Any of the 5 reactions listed above can lead to the synthesis of PT. Since we see a decrease in PC and PE, since reaction 4 has not been shown to occur unequivocally in eukaryote cells and since reaction 5 is hypothetical, we favor reactions 2 and 3 as being the most likely ones involved in the synthesis of PT. One of the base exchange enzymes appears to have been altered so that it can now recognize threonine as well as serine.

Our results with chemical probes demonstrate differences in the membranes of transformed cells. The extent of labeling of the aminophospholipids by FDNB and TNBS is greater in transformed cells indicating a greater accessability of those lipids. We also find in transformed cells that contain both PS and the threonine phospholipid, primarily the latter reacts with FDNB suggesting a different topological arrangement of this unique phospholipid compared to PS. It appears that PS is more tightly bound to protein or is masked in some other way. Furthermore neither PS or the threonine phospholipid of intact cells reacts with TNBS yet 12-19% of the PE reacts with this probe. These results suggest that 12-19% of the PE and essentially none

of the PS or the threonine phospholipid occurs on the outer surface of the plasma membrane in these cells.

Threonine has been reported to occur in phospholipids of hen egg (37) and tuna muscle (38). These studies as well as ours suggest the existence of phosphatidylthreonine as a new type of phospholipid. The threonine may exist as an O-acyl ester of phosphatidylglycerol but this is unlikely since this type of lipid would have a lower mobility in our chromatography system than PS or PT. The complete characterization of PT will require its isolation and conversion to glycerophosphorylthreonine by mild alkaline hydrolysis. We have considered the possibility that the threonine phospholipid may be phosphatidylserylthreonine. This type of lipid is unlikely since it would have a considerably lower mobility in our chromatographic system and furthermore would yield primarily serylthreonine on HCl hydrolysis for 2 hours.

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