FURTHER INVESTIGATIONS OF THE EXPRESSION OF SV40 -INDUCED SURFACE ANTIGENS SUBSEQUENT TO ENZYMATIC TREATMENT OF TRANSFORMED CELLS*

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1. Introduction

During investigations of the surface antigens present on SV40-transformed cells, we demonstrated that virus-induced, host-coded surface antigens appear which are unique for each transformation event [1,2]. Sera obtained from rabbits hyperimmunized with autochthonous SV40-transformed cells or raised in inbred or outbred hamsters bearing tumors induced with SV40-transformed syngeneic or autochthonous cells, respectively, were used to stain intact cells in suspension which were examined by fluorescence microscopy [1,2]. With certain transformed outbred hamster kidney cell lines, these antigens were detected directly on the cell surface [1]. However, with a cloned inbred hamster kidney cell line and a rabbit kidney cell line, these surface antigens were detected only after the cells had been pre-treated with phospholipase C [2]. Pre-treatment of the cells with phospholipase A or D, neuraminidase, hyaluronidase, or trypsin did not result in exposure of these antigens [2]. The only other enzyme known to be present in the commercial phospholipase C preparations utilized was caseinase, an enzyme thought to be inconsequential in our experiments. These results suggested that hydrolysis of phospholipid molecules by pretreatment with phospholipase C resulted in the uncovering of a cryptic antigenic determinant or in the appearance of the antigen subsequent to reorganization of the membrane structure.

The calcium dependence for phospholipase C activity has been reported by several authors [3–5], although limited hydrolysis by this enzyme may occur in its absence [6,7]. Since calcium was absent during the phospholipase C pre-treatment of the cells, we performed experiments to determine the extent of hydrolysis of phospholipids, as measured by the release of phosphoryl choline and the generation of diglycerides, after exposure of the transformed cells to phospholipase C in the presence or in the absence of calcium. In addition, the effect of various calcium concentrations on exposure of the surface antigen by this preparation of phospholipase C was determined.

* Part 3. of Analysis of surface antigens on SV40-transformed cells. Previous parts (see ref. [1,2]). Address correspondence to Jeffrey J. Collins.

2. Materials and methods

One of the cell lines utilized in our previous experiments [2], and SV40-transformed rabbit kidney cell line (TRK-73), was maintained in Eagle's minimal essential medium with a 4-fold excess of vitamins and amino acids (MEM × 4), 10% unheated fetal calf serum, and antibiotics as described [2]. The cultures were labeled with 0.4–4 μCi/ml [^{3}H]choline (specific activity 1 Ci/mM) and 0.22 μCi/ml [^{14}C]glycerol (specific activity 8 mCi/mM) for 3 days, during which time the cells achieved confluence. Labeling medium consisted of MEM × 4, 10% dialyzed fetal calf serum, 2% fetal calf serum, 10% tryptose phosphate broth and antibiotics. The monolayer cultures were washed twice with phosphate-buffered saline, (PBS), pH 7.2, and the cells were removed from the surface with either 0.02% ethylene diaminetetra acetic acid (EDTA) in PBS or 0.02% ethylene glycol diaminetetra acetic acid (EGTA) in PBS ; the resulting cell suspensions will be referred to as EDTA cells or EGTA cells, respectively. EDTA and EGTA were compared in the present experiments since more consistent surface staining had been obtained with EGTA than with EDTA cells [2]. We therefore wished to determine whether the type of chelating agent used affected the phospholipase C hydrolysis. The cells were sedimented, washed 3 times with PBS, adjusted to a concentration of 7×10^6 cells/ml of a solution containing 20 μg/ml *Clostridium perfringens* phospholipase C (Worthington; activity = 2 units/mg upon hydrolysis of egg lecithin) in PBS or in saline containing 10 mM $CaCl_2$ in 0.1 M Tris buffer, pH 7.5. Controls consisted of cells incubated in PBS. Incubation was carried out at room temperature for 20 min with frequent, gentle agitation [2]. The reaction was terminated by the addition of two volumes PBS and the suspension was processed immediately.

The release of phosphoryl choline from the TRK-73 cells treated with phospholipase C with and without calcium was measured by counting the ^{3}H label in the filtered supernatant (0.22 μ Millipore filter) after centrifugation of the cell suspension at 1000 rpm in an International centrifuge. Extraction of aliquots of the supernatant fluid with diethyl ether resulted in a very small percentage distribution of label in the organic phase, indicating the absence of intact phospholipids. The predominant labeled component appearing in the supernatant after incubation was identified as phosphoryl choline after thin layer chromatography on microcrystalline cellulose in butanol:acetic acid:water (4:1:1) with phosphoryl choline and choline as reference standards. This procedure revealed that some [^{3}H]choline was present together with phosphoryl choline in the supernatant fluid. The degree of such contamination (50% ^{3}H cpm in control and 20% ^{3}H cpm in treated cell supernatants) was calculated by subtraction of the label precipitated with ammonium reineckate following the precedure of McCaman et al. [8]; utilizing this procedure, 96–98% of a known amount of either [^{14}C]-or [^{3}H]choline was precipitated.

3. Results and discussion

As shown in table 1, treatment with phospholipase C, either in the presence or in the absence of calcium, caused an increase of labeled phosphoryl choline in the incubation medium. In order to determine whether diglycerides, which are known to remain associated with membranes [10–12], were generated after treatment with phospholipase C, the lipids were extracted from control and phospholipase C-treated cells by the method of Folch et al. [13] and separated by thin layer chromatography in silica gel H with hexane: diethyl-ether:acetic acid (85:15:1). The areas in the chromatogram corresponding to diglycerides, after identification by autoradiography with no-screen X-ray film (Kodak), were scraped into scintillation vials, suspended in 1 ml H_2O and counted in Aquasol. Table 1 also shows that, in comparison to controls, the diglycerides are slightly increased in the TRK-73 cells exposed to phospholipase C in the absence of calcium but are increased approximately 4 × the control values in cells treated with phospholipase C in the presence of calcium. The amounts of released phosphoryl choline and generated diglycerides were approximately the same from EDTA and EGTA cells.

Under the experimental conditions used, the generation of diglycerides could be explained only through the hydrolysis of glycerophospholipid molecules. The fact that these partial glycerides were found predominantly in cells treated with phospholipase C in the presence of calcium indicates that calcium is required for hydrolysis and that limited phospholipid hydrolysis occurred without calcium in the cell system which we have examined.

Table 1
Release of phosphoryl choline and production of diglycerides subsequent to exposure to phospholipase C in the presence or in the absence of C^{2+}*

				Cell associated		
		% Cells viable after treatment	[^{3}H] Phosphoryl choline released (cpm/10^6 cells) x 10^3	[^{14}C] Diglycerides (cpm/10^6 cells) x 10^3		[^{3}H]Phosphoryl choline (cpm/10^6 cells) X 10^3**
Control	EGTA cells	90	13.5	0.78		13.3
	EDTA cells	77	15.6	0.73		33.3
Phospholipase C in PBS	EGTA cells	9	103.4	0.86	(+10)***	2.3
	EDTA cells	5	111.0	0.86	(+18)	3.3
Phospholipase C with Ca^{2+}	EGTA cells	56	100.4	3.2	(+310)	21.0
	EDTA cells	49	102.4	3.2	(+344)	35.7

* Phosphoryl choline is reported as ^{3}H cpm after subtraction of labeled choline (see Materials and methods); diglycerides are reported as ^{14}C cpm found in the corresponding areas of the chromatogram after thin layer chromatography; viability was determined by the Trypan Blue dye exclusion method.

** Determined in the upper phase of a Folch extract of TRK-73- treated cells after purification by Sephadex [9] and reineckate precipitation [8] to remove ^{3}H choline.

***Percent increase relative to control value.

These results also indicate that the release of phosphoryl choline is not an accurate measure of phospholipase C hydrolysis of cellular lecithin. More accurate values for phosphoryl choline release specifically due to phospholipase C hydrolysis can be calculated from the amount of diglycerides produced as follows, given the assumption that most of the increase in diglycerides is due to phospholipase C hydrolysis of lecithin, although a small amount of diglycerides may be generated from hydrolysis of glycerophospholipids other than lecithin, for example, phosphatidylethanolamine. Thus, we isolated lecithins by bi-dimensional thin layer chromatography [14] from TRK -73 cells labeled with [^{3}H] choline and [^{14}C] glycerol, and found that they had a ^{3}H/^{14}C ratio of 31. Using this ratio and the known amount of [^{14}C] glycerol released as diglycerides, an equivalent amount of [^{3}H]phosphoryl choline was calculated as being released by phospholipase C hydrolysis. From these calculations, we found 3–4% and 80% of the total labeled phosphoryl choline found in the media of cells treated with phospholipase C in the absence and in the presence of calcium, respectively, could be accounted for by phospholipase C hydrolysis. Therefore, release of phosphoryl choline from the cells treated with phospholipase C in the absence of calcium was presumably effected through other mechanisms.

An indication of a possible mechanism involved is furnished by an analysis of the upper (hydromethanolic) phase obtained after extraction and partition of the lipids from the treated TRK -73 cells by the method of Folch et al. [13]. By this procedure, hydrosoluble products such as phosphoryl choline appear in the hydromethanolic phase. The ^{3}H label due to phosphoryl choline was found to be lower in the cells treated with phospholipase C in the absence of calcium, in comparison to the amount obtained from either the untreated control cells or the cells treated with phospholipase C in the presence of calcium (table 1). These results indicate that comparatively more of the cellular phosphoryl choline pool is maintained inside the cells during incubation with phosphate buffer or phospholipase C in the presence of calcium. In the absence of calcium, there is presumably leakage

of phosphoryl choline from the cellular pool. It is of interest that the % viability is the lowest in cells exposed to phospholipase C in the absence of Ca^{2+} (table 1). This indicates that more cell membrane damage occurred in labeled cells in the absence than in the presence of Ca^{2+}. The mechanism(s) whereby Ca^{2+} may protect the plasma membrane of TRK -73 cells exposed to the phospholipase C preparation utilized is not known: however, an increase in permeability was demonstrated after treatment of cells with chelating agents and treatment with Ca^{2+} has been shown to have a compacting effect on membrane molecular structure [15].

Since maximum hydrolysis by phospholipase C required Ca^{2+}, we wished to determine the degree of fluorescence staining of the SV40 -associated antigen utilizing optimal conditions for phospholipase C hydrolysis. TRK -73 cells were exposed to the crude enzyme with and without Ca^{2+} and the cells were stained and examined by fluorescence microscopy as described [1,2]. Table 2 demonstrates that an equivalent degree of staining was obtained in the absence of Ca^{2+} or at Ca^{2+} concentration of 5 and 10 mM. These results suggest that the exposure of the antigenic determinant cannot be correlated with the extent of phospholipid hydrolysis.

In summary, evidence has been presented that hydrolysis by phospholipase C was probably not responsible for the exposure of SV40 -induced surface antigens on the cell membrane of certain SV40-transformed rabbit and hamster cells. Nevertheless, it is clear that pretreatment of the TRK -73 cells with the crude phospholipase C preparation is required for the subsequent detection of the SV40-induced surface antigen(s) by membrane immunofluorescence [2]. This pretreatment affected the surface membrane of the transformed cells in that the viability decreased and the permeability for small molecules, such as phosphoryl choline, was increased. Furthermore, such pretreatment resulted in the release from cells of 5–6 times more protein than from control cells (data not shown). Only small amounts of phospholipid were released in these experiments, indicating that phospholipase C in the absence of calcium does not cause the detachment of intact portions of the cell membrane. It is most likely that the protein found in the incubation medium was derived from the plasma membrane; however, we cannot rule out the possibility that the protein leaked from within the cell due to membrane damage.

The active principle in the crude phospholipase C preparation that caused the membrane alterations and subsequent exposure of the SV40-induced surface antigen is not known at present, although phospholipase C itself may well have played a partial role because of the limited phospholipid hydrolysis which

Table 2
Detection of SV40-induced surface antigen on TRK-73 cells subsequent to exposure to phospholipase C in the presence or in the absence of Ca^{2+}

Enzyme treatment	Ca^{2+} concentration (mM)	% Positive cells	Fluorescence index
–	–	0.41	–
+	–	23.8	0.23
+	5	21.8	0.21
+	10	27.1	0.27

Enzyme treatment and membrane immunofluorescence technique are described in detail in the text and in [1,2]. Antochthonous immune serum No. 233 [2] used at a dilution of 1:10.

$$\text{Fluorescence Index (FI)} = \frac{\%\ \text{Negative control cells} - \%\ \text{negative test cells}}{\%\ \text{Negative control cells}}$$

FI calculated on the basis of the non-enzyme treated control cells; staining of the test cells in considered significant when FI $\geq$ 0.20 [1].

occurred in the absence of Ca^{2+} (see also [6,7]). Treatment of the transformed cells with neuraminidase, hyaluronidase, or trypsin did not result in uncovering of the antigen [2], so these enzymes are not responsible. Since treatment of cells with purified protease preparations is known to result in uncovering of the certain antigens [16,17], it is possible that some protease in the preparation utilized may have been responsible. However, a preliminary attempt to directly demonstrate protease activity in the phospholipase C preparation utilizing purified myoglobin as substrate did not result in the release of polypeptide-like material as detected by high voltage electrophoresis.

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