ECTO-GANGLIOSIDE-SIALIDASE ACTIVITY OF HERPES SIMPLEX VIRUS-TRANSFORMED HAMSTER EMBRYO FIBROBLASTS

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ABSTRACT

Cellular location of ganglioside-sialidase activity was determined in confluent hamster embryo fibroblasts transformed with herpes simplex virus type 2. Approximately equal specific activities of ganglioside-sialidase activity were found to be associated with the crude lysosomal and crude plasma membrane fractions isolated from whole cell homogenates. Whole transformed cells hydrolyzed exogenous ganglioside substrate, suggesting a partial location of the cellular sialidase on the outer surface of the plasma membrane of these cells. Intact cells were treated with the diazonium salt of sulfanilic acid, a nonpenetrating reagent inhibitory to ecto-enzymes (DePierre, J. W., and M. L. Karnovsky. 1974. *J. Biol. Chem.* **249:**7111–7120). Cytoplasmic lactate dehydrogenase activity was not inhibited by this treatment, and mitochondrial succinate dehydrogenase activity was inhibited only 10%, indicating that intracellular enzymes were not affected. 5′-Nucleotidase activity was diminished 90%, and sialidase very rapidly lost 40% of its exogenously directed activity. These results show that, in herpes simplex virus-transformed fibroblasts, ganglioside-sialidase is both a lysosomal and a plasma membrane enzyme. The plasma membrane sialidase is capable of acting on endogenous plasma membrane sialolipids and also functions in the cultured transformed cell as an ecto-enzyme which can attack exogenous substrates.

Cell surface sialic acid has been implicated in cell-cell recognition (10), contact inhibition (3), and crypticity of immunogenic loci (4). Changes in lipid-bound (2, 30) and protein-bound sialic acid (28) reportedly occur upon cell transformation. Yogeeswaran and Hakomori have reported that membrane-associated sialidase (neuraminidase, *N*-acetyl neuraminosyl glycohydrolase, EC 3.2.1.18) activity is suppressed at the "touching" stage of cell-cell contact of normal cells, but remains unaltered at any stage of cell contact in transformed cells (29). In previous studies, we have shown that transformed fibroblasts in culture have membrane-bound sialidase activity directed towards exogenous substrate but that control untransformed fibroblasts do not (17). We have found a parallel trend between exogenous ganglioside-sialidase activity and oncogenicity of herpes simplex virus-transformed fibroblasts (15). Visser and Emmelot reported an enriched sialidase activ-

ity in the plasma membrane of hepatoma cells grown in vivo (25). In normal liver cells, sialidase activity has been found to be associated with a number of subcellular fractions, e.g., plasma membrane (16), lysosomes (9, 24), and Golgi apparatus (11). The present work was undertaken to determine the location of the exogenously directed ganglioside-sialidase of herpes simplex virus-transformed cells. We have found that a major proportion of the sialidase of the oncogenic cell functions as an ecto-enzyme, i.e. located on the outer surface of the intact oncogenic cell, functionally facing outwards (5), and potentially capable of removing sialic acid from sialoglycoproteins in the intercellular matrix and on the surface of other cells. This information may lead to a better understanding of the loss of growth control in oncogenic cells. We have previously shown that the enzymatic cleavage of a very small amount of sialic acid from the surface of intact mammalian cells in culture immediately enhances their pyrophosphatase (21) and cholinesterase (20) activities.

MATERIALS AND METHODS

Culture

Herpes simplex virus type 2-transformed hamster embryo fibroblasts, line 333-2-29 (15), were grown in monolayer culture in 8-oz flint glass culture bottles in Medium 199 supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth, and 0.075% $NaHCO_3$. Cultures were harvested at confluence ($1.5\text{-}2 \times 10^7$ cells/bottle) without trypsin and washed four times with a pH 7.4, 24 mM Tris-HCl, buffered isosmotic salt solution in order to remove residual medium. Cultures were routinely assayed for the presence of mycoplasma (19).

Chemicals

Gangliosides were extracted from bovine brain with chloroform-methanol (2/1, vol/vol) followed by partition dialysis (7). Individual gangliosides were separated on a silica gel H column with a discontinuous gradient of chloroform-methanol-water of increasing methanol-water content as described previously (17). Fractions obtained were identified by thin-layer chromatography on silica gel. The chromatograms were developed in chloroform-methanol-2.5% aqueous ammonia (65-35-8, vol/vol/vol) (26) and visualized with resorcinol spray (22). Thin-layer chromatography plates, silica gel (hard Q-gel) inert binder (0.25-mm thickness), were obtained from Quantum Industries, Fairfield, N. J. The diazonium salt of sulfanilic acid was prepared as described by DePierre and Karnovsky (5). All other chemicals and solvents were obtained commercially and were of reagent grade quality. They were used without further purification.

Subcellular Fractionation and Analysis

$1.8\text{-}2.4 \times 10^9$ cells were harvested and subcellular fractions prepared as described by Touster et al. (23). Subcellular fractions were diluted with an equal volume of water and centrifuged at $100{,}000\,g \times 1$ h to pellet the membrane fractions. The pellets were washed once with water to remove residual sucrose, centrifuged as before, and the pellets were homogenized in water. Protein concentration was determined by the procedure of Lowry et al. (13), using bovine serum albumin as standard.

Plasma membrane 5′-nucleotidase was assayed according to the procedure of Touster et al. (23), with adenosine 5′-monophosphate as the substrate. Released phosphate was determined colorimetrically by the procedure of Fiske and SubbaRow (6). Mitochondrial succinate dehydrogenase was determined as described previously (16). Lysosomal acid phosphatase was assayed according to the method described by Gianetto and de Duve (8), with β-glycerophosphate as substrate.

Sialidase activity towards endogenous and exogenous substrate was measured, as previously described (15). To allow for hydrolysis of available endogenous substrate, samples were incubated for 90 min. A 60-min reaction time was used for assaying exogenous activity. After purification on Dowex 1-X10 columns (Dow Chemical Co., Midland, Mich.) (9), released sialic acid was measured spectrophotometrically using Warren's thiobarbituric acid procedure (27). For measurement of sialidase activity of intact 333-2-29 fibroblasts, the cells were incubated at 37°C for 2 h in 0.01 M sodium acetate buffer, pH 4.2, in isosmolar pentaerythritol (1) with 0.4 mg of added disialo- and trisialoganglioside substrate. The prolonged reaction time of 2 h was used in order to allow for exhaustion of endogenous substrate which is acted upon preferentially by the enzyme. Endogenous release was monitored by incubating cells under the same conditions in the absence of added substrate. Appropriate enzyme and substrate blanks were included in all enzymatic assays.

Treatment of the Cells with the Diazonium Salt of Sulfanilic Acid

Confluent cells were rinsed nine times with Krebs-Ringer phosphate solution to remove residual growth medium (5). The cells were incubated at 37°C with 5 ml of a 1.8 mM solution of the diazonium salt from 0 to 16 min. At the end of the incubation period, the solution was decanted and the cell layer rinsed nine times with Krebs-Ringer phosphate solution to remove residual diazonium salt. To determine the effect of this treatment on the intactness of the plasma membrane, the cells were incubated with 5 ml of 1.8 mM or 7 mM diazonium salt in Krebs-Ringer phosphate buffer from 0 to 32 min. The cells were rinsed and kept subsequently in 5 ml of Krebs-

Ringer phosphate buffer at room temperature. Samples of the buffer were removed at 5, 15, and 30 min, and released lactate dehydrogenase activity was determined (12).

RESULTS

Enzyme Activities in Subcellular Fractions

The specific activities of 5′-nucleotidase, acid phosphatase, succinate dehydrogenase, and endogenous and exogenous sialidase in the subcellular fractions are shown in Table I. In the fractions obtained upon differential centrifugation (designated as P), the specific activity of sialidase is essentially the same in P_2 (lysosomal and mitochondrial pellet) and in P_3 (microsomal fraction). P_2 contained acid phosphatase and succinate dehydrogenase at higher specific activities than did P_3, in which 5′-nucleotidase had a higher specific activity. Therefore, to study the possible distribution of sialidase activity in the plasma membrane, fractions P_3 (microsomes with some plasma membrane) and P_4 (nuclei with some plasma membrane) were taken for further purification of enriched plasma membrane fractions. The enrichment in specific activity of exogenously directed sialidase activity in the fractions obtained from P_3 (M) and P_4 (N) can be seen to parallel that of the plasma membrane constituent, 5′-nucleotidase.

Effect of Diazonium Salt on Sialidase Activity

The results obtained upon treatment of the intact cells with the diazonium salt of sulfanilic acid showed that at least 40% of the sialidase is located in the plasma membrane on its outer surface where it may act as an ecto-enzyme. Fig. 1 shows the cellular release of the soluble cytoplasmic enzyme, lactate dehydrogenase, after treatment of the cells with the diazonium salt of sulfanilic acid at two concentrations. Conditions of treatment (incubation of the cells in 5 ml of 1.8 mM salt at 37°C for up to 16 min) were chosen to minimize damage to the plasma membrane. As shown in Fig. 2, cellular lactate dehydrogenase activity was not inhibited over this time span. The effects of the diazonium salt of sulfanilic acid on the ecto-enzyme, 5′-nucleotidase, and the mitochondrial

TABLE I

Distribution of sialidase, 5′-nucleotidase, acid phosphatase, and succinate dehydrogenase in the subcellular fractions

Fraction*	Sialidase‡		5′-Nucleotidase	Acid phosphatase	Succinate dehydrogenase
	endogenous	exogenous			
Total sedimentable fraction	1.17	3.11	0.27	0.84	6.6
P_2	2.70	18.8	0.50	1.2	6.5
P_3	2.58	18.5	1.4	0.74	0.0
P_4	2.17	7.04	0.40	0.45	1.2
M_2	34.0	55.5	4.1	0.89	–
M_3	49.8	23.7	2.2	1.3	–
M_4	4.16	14.6	1.0	1.0	–
N_2	47.5	29.9	2.5	0.79	–
N_3	7.27	14.7	0.9	0.24	–
N_4	5.28	0	0.07	0.05	–

* The cell fractions were isolated by the procedure of Touster et al. (23). The total sedimentable fraction refers to the particulate portion of the cell homogenate used for the isolation of the subcellular fractions obtained by centrifugation of the cell homogenate at 100,000 g 1 × h. P_2 refers to the lysosomal-mitochondrial membrane fraction; P_3 to the microsomal membrane fraction; and P_4 to the nuclear fraction. The M subfractions are the membrane fractions obtained from centrifugation of P_3 in a discontinuous sucrose gradient. The N subfractions are the membrane fractions obtained from centrifugation of P_4 in a discontinuous sucrose gradient.

‡ The endogenous and exogenous sialidase activities are given as nanomoles sialic acid released/90 min/mg protein and nanomoles sialic acid released/60 min/mg protein, respectively. Endogenous activity represents sialidase catalyzed hydrolysis of more than 90% of the total endogenous sialic acid containing substrate present in these fractions and is therefore a measure of the amount of available endogenous substrate. The units for 5′-nucleotidase activity are micromoles of phosphate released/30 min/mg protein while for acid phosphatase activity the units are micromoles of phosphate released/60 min/mg protein. Succinate dehydrogenase activity is expressed as nanomoles of succinate oxidized/min/mg protein. For experimental details, see Materials and Methods.

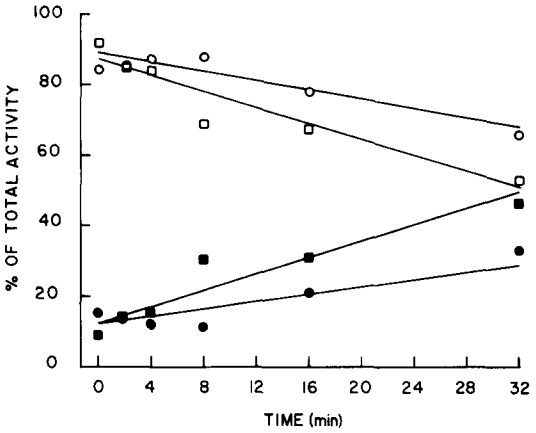

FIGURE 1 Release of lactate dehydrogenase from cells after treatment of the cells with the diazonium salt of sulfanilic acid (5). The cells were incubated with 5 ml of 1.8 mM or 7 mM diazonium salt in Krebs-Ringer phosphate buffer for the time indicated. At the end of the reaction time, the cells were rinsed nine times with Krebs-Ringer phosphate buffer and then kept in 5 ml of the same buffer at room temperature for 30 min. The percent of the total lactate dehydrogenase activity recovered in the buffer or associated with the cells treated with 1.8 mM diazonium salt is shown by (●—●) and (○—○), respectively. (■—■) and (□—□) refer to comparable values obtained with 7 mM diazonium salt.

enzyme, succinate dehydrogenase, are also shown in Fig. 2. The nonpenetrating reagent had little inhibitory effect on the succinate dehydrogenase activity (about 10%) while it inhibited about 90% of the 5′-nucleotidase activity. The effect on both endogenous and exogenous sialidase activity is shown in Fig. 3. About 40% of both activities was inhibited. During the first 4 min, exogenous activity was destroyed even more rapidly than 5′-nucleotidase.

Sialidase Activity of Whole Cells

The intact cells maintained at pH 4.2 in pentaerythritol displayed sialidase activity towards exogenous ganglioside substrate with a sp act of 1.8 nmol of sialic acid released/h/mg protein. Endogenous sialidase activity of these cells towards native substrate was comparable to that for whole cell homogenates. At the end of the experiment, the cells were examined by phase-contrast light microscopy. They appeared intact. However, they slowly took up Trypan blue, indicating plasma membrane leakiness.

DISCUSSION

Study of the subcellular distribution of ganglioside-sialidase activity showed sialidase activity to be present in both the lysosomal and plasma membrane fractions at comparable specific activities in each fraction. 5′-Nucleotidase was present at a higher specific activity in P_3 than in P_2. The lysosomal marker, acid phosphatase, displayed highest specific activity in P_2. This bimodal distribution of sialidase activity is in agreement with previous results (25) obtained in studies of the distribution of sialidase in rat liver. The distribution and enrichment of exogenous sialidase activity in the fractions obtained from P_3 and P_4 parallel those of 5′-nucleotidase activity. This finding supports identification of some sialidase as a plasma membrane constituent.

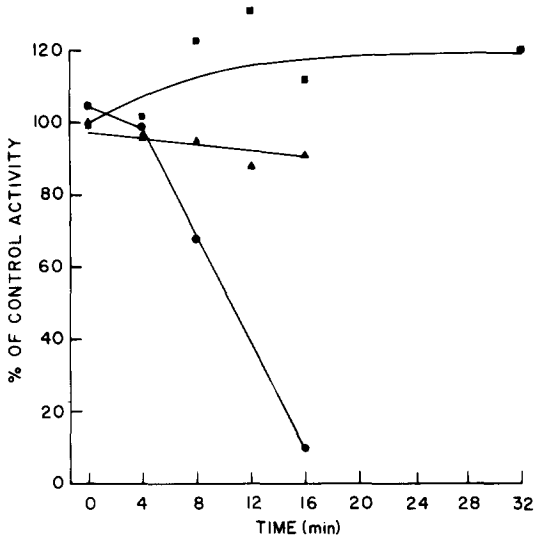

FIGURE 2 The effect of the diazonium salt of sulfanilic acid on the activity of lactate dehydrogenase (■—■), succinic dehydrogenase (▲—▲), and 5′-nucleotidase (●—●) of 333-2-29 fibroblasts. The cells were treated with 5 ml of 1.8 mM diazonium salt of sulfanilic acid in Krebs-Ringer phosphate buffer for the lengths of time indicated. After rinsing the cells nine times with Krebs-Ringer phosphate to remove residual salt, the enzymatic activities were determined. For experimental details, see Materials and Methods. The control enzymatic activities were determined for cells not exposed to the diazonium salt. The zero time sample refers to cells to which the diazonium salt solution was added, immediately removed, resulting in less than a 5-s exposure time, and the cells were rinsed nine times with Krebs-Ringer phosphate buffer.

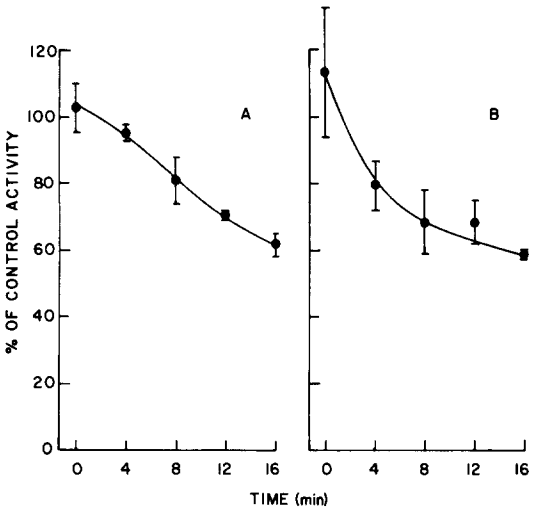

FIGURE 3 The effect of the diazonium salt of sulfanilic acid on the endogenous and exogenous sialidase activities of 333-2-29 fibroblasts. The cells were treated with 5 ml of 1.8 mM diazonium salt of sulfanilic acid in Krebs-Ringer phosphate buffer for the lengths of time indicated. After rinsing the cells nine times to remove residual salt, the endogenous and exogenous sialidase activities were determined. For experimental details, see Materials and Methods. The control sialidase activity was determined for cells not exposed to the diazonium salt. The zero time sample represents cells to which the diazonium salt was added, immediately removed, resulting in less than a 5-s exposure time, and the cells were rinsed nine times with Krebs-Ringer phosphate buffer. The values shown were obtained for two experiments, each done in duplicate on cells grown under similar conditions at passage nos. 62 and 67. The upper end of the error bar refers to sialidase values obtained for cells at passage no. 62 and the lower at passage no. 67. The curves shown represent the average values for these two experiments. Curve A represents endogenous sialidase activity and B, exogenous sialidase activity.

The results obtained upon treatment of the intact cells with the diazonium salt of sulfanilic acid suggest that sialidase in the plasma membrane is an ecto-enzyme. The negligible degree of inhibition of lactate dehydrogenase and succinate dehydrogenase activities is evidence that, under the conditions used, the ecto-inhibitory reagent was unable to enter the cells, acting only on those enzymes exposed on the outer surface of the plasma membrane. Inhibition of 90% of the known ecto-enzyme, 5'-nucleooidase (5), and also of 40% of the sialidase activity was observed. These results allow the conclusion that at least 40% of the cells' total ganglioside-sialidase activity is associated with the plasma membrane, where it is located on the outer surface as an ecto-enzyme.

The demonstration of exogenously directed ganglioside-sialidase activity, comparable to that of whole cell homogenates, upon incubation of the whole cells with added ganglioside substrate suggests that the ecto-sialidase is readily active towards extracellular substrate. The acidic pH of the incubation medium and the fact that, although the cells appeared morphologically normal by phase-contrast light microscopy, they slowly took up Trypan blue suggest that some of the observed activity of whole cells might be a result of availability of substrate to the lysosomal sialidase. For all of the observed ganglioside-sialidase activity to have been due to lysosomal sialidase, however, the exogenous substrate would have to be as readily available to the lysosomal enzyme in the unbroken whole cell as in the cell homogenate. This is not probable since the cells are intact at the initiation of the reaction, and, even though it is leaky at the end of the reaction period, the plasma membrane must still pose a diffusion barrier to the availability of the ganglioside substrate to the intracellular lysosomal sialidase. Therefore, it may be inferred that plasma membrane sialidase is active in degrading the exogenous substrate. We have previously shown that for plasma membrane sialidase to be active towards exogenous substrate, it must first deplete native plasma membrane sialoglycolipid constituents to some extent. The value for plasma membrane sialidase in our procedure for measurement of ecto-ganglioside-sialidase activity of whole cells is bound to understate this activity since predepletion of whole cell endogenous substrate was not carried out.

These studies of ecto-sialidase activity employed gangliosides as substrate. We have shown that gangliosides can associate with membranes under the conditions used to assay ganglioside-sialidase activity (18), and that in liver plasma membranes the endogenous sialidase preferentially acts upon the native ganglioside of these cells, hematoside (16). These results suggest that for exogenous sialyl residues to be available to the enzyme, they may need to be localized near the integral membrane components (14) of the outer cell surface. However, no firm conclusions can yet be made regarding the mode of action of the ecto-sialidase.

From the evidence presented in this study, the hypothesis may be advanced that ecto-sialidase might play a role in regulating sialic acid content of cell surfaces. Changes in sialidase activity might result in an alteration of surface sialic acid, giving rise to modification of cell behavior during cell-cell interactions.

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