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DISPOSITION OF GLYCOPROTEINS IN PLASMA MEMBRANE OF CULTURED RAT EMBRYONIC FIBROBLASTS

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Summary

Plasma membrane fractions were isolated from untreated and trypsin- or neuraminidase-treated rat embryo fibroblasts and their sialic acids contents per mg membrane protein were determined. The difference represented enzyme releasable sialic acid exposed on the medium side of the cell membrane. It was 14 to 23% of the total membrane bound sialic acid. Isolated plasma membrane fraction from untreated and enzyme treated cells were then subjected to trypsin or neuraminidase treatment to obtain enzyme-releasable sialic acid from both faces and from the cytoplasmic face of the membrane respectively. Between 30 and 50% of the total membrane bound sialic acid was released from both the faces and 14 to 30% from the cytoplasmic face. An average of 59% was insusceptible to these enzymes. As an alternative to a cytoplasmic location of sialic acid containing membrane constituents, inaccessibility of enzymes to some of these constituents present on the surface of intact cells is considered.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of plasma membrane fractions isolated from untreated and trypsin treated cells and of trypsinized plasma membrane fraction was carried out to know the number and gel migration of proteins and glycoproteins which are exposed on each of the two faces of the plasma membrane and are sensitive or insensitive to trypsin. The results obtained were confirmed by SDS-polyacrylamide gel electrophoresis of untreated and trypsin-treated cells and of isolated plasma membrane fraction after subjecting them to enzymatic radioiodination.

Introduction

Cultured fibroblasts grown in the presence of labeled carbohydrates liberate 10—25% of the label on trypsin treatment [1]. The loss of glycoprotein material does not affect the viability of cells and the lost material is rapidly regener-

ated under appropriate growth conditions [2]. By radioiodination studies, a number of investigators have reported the presence of glycoproteins on the surface of normal cells that are trypsin sensitive and are present in reduced amounts on the surface of transformed cells [3—5]. Investigations by Warren and coworkers [1,6,7] have shown that some of the cell surface components degraded by trypsin give rise to soluble fucosialoglycopeptides. In other investigations involving protease treatment, it has been reported [8] that mild protease treatment of phenotypically normal cells results in a lowering in the concentration of lectin required for their agglutination. Nicolson [9] suggested that mild protease treatment may cause a change in the conformation of membrane proteins. So far no evidence has appeared to support this view.

In contrast to trypsin, in a recent study [10], neuraminidase (*N*-acetylneuraminic acid hydrolase, EC 3.2.1.18) treatment of normal cells only slightly altered their agglutination behaviour although it is known to alter electrophoretic mobility of the cells [11] and to augment their immunogenicity [12]. Neuraminidase treatment of intact cells is often employed for the determination of cells surface associated sialic acid.

Although most of the studies on cell surface carbohydrates concern neuraminidase or trypsin action on the glycoproteins which are exposed on the cell surface (medium side) of the plasma membrane, the cytoplasmic side of the plasma membrane has not been investigated to the same extent. Steck et al. [13] observed that inverted membrane vesicles (inside-out vesicles) derived from erythrocyte membranes are less sensitive to protease digestion than normally oriented vesicles. Zachowski and Paraf [14] have shown that right-side-out membrane vesicles prepared from plasma membranes of a murine plasmocytoma contain more neuraminidase sensitive sialic acid than inside-out membrane vesicles. Nicolson and Singer [15] showed that concanavalin A does not bind to the cytoplasmic side of the plasma membrane. No efforts were made to see if steric factors played a role in the inability of lectin to bind to the carbohydrates present on the cytoplasmic side of the plasma membrane. We have earlier shown that neuraminidase sensitive sialic acid blocks the binding of a bacterial glycolipid mR595 to normal rat embryo fibroblasts [16].

Our interest in the study of glycoprotein disposition in plasma membranes arose because plasma membrane fractions isolated from trypsin treated cells still retained nearly 80% of the membrane bound sialic acid, although certain studies [17,18] and all membrane models indicate their exposure exclusively on the medium face of the plasma membrane. This led us to entertain the possibility that (a) detachable glycoproteins of diverse origin may reside on the surfaces of cells and (b) sialic acid containing constituents may be present on the cytoplasmic side of the plasma membrane or so disposed on the cell surface that they are not available for enzyme attack.

This study presents data on the release of sialic acid by trypsin and neuraminidase from the medium face and from both faces of the plasma membrane. In addition, sodium dodecyl sulfate-polyacrylamide gel profiles of plasma membrane fraction isolated from untreated and trypsin treated as well as trypsinized membranes are provided. Exposed polypeptides present on the surface of untreated cells and trypsin treated cells and on plasma membranes were identified by their mobility on gel after enzymatic iodination.

Materials and Methods

Materials

Crystalline trypsin (180 units/mg) was obtained from Worthington Chemical Corp., Freehold, N.J., U.S.A. *N*-Acetyl neuraminic acid and *Clostridium perfringens* neuraminidase type IV (2 units/mg protein, with neuraminlactose as substrate) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. All other chemicals were of reagent grade purity obtained from various commercial sources. Protein iodination grade ^{125}I was secured from New England Nuclear Corp., Boston, Mass., U.S.A.

Pregnant female Wistar rats, calf serum and minimum essential medium were supplied by Flow Laboratories, Rockville, Md., U.S.A. Tryptose phosphate broth and crude trypsin were products of Difco Laboratories, Detroit, Mich., U.S.A.

Cell culture and cell cultivation

This was carried out as described previously [16]. Cell cultures growing in a number of bottles were divided into two equal groups. Growth medium was removed from both groups by decantation and the attached cells were washed three times with phosphate buffered saline. Cells from one group of bottles were removed by gently scraping with a rubber policeman. The scraped cells were kept over ice. The other half of the bottles were treated with 0.1% solution of crystalline trypsin in phosphate buffered saline. The bottles were left in a rocker platform for 15 or 30 min at 37° C. This resulted in the detachment of a large number of cells. After gently pipetting the cell suspension over the attached cells, nearly all cells could be detached from the substratum. The cell suspension was then removed and kept over ice. Both cell suspensions were centrifuged at 1500 rev./min in a refrigerated International centrifuge (Head no. 811). The supernatant was removed and kept over ice, and the sedimented cells were washed once with a small volume of phosphate buffered saline, centrifuged and the second supernatant combined with the first. Approximately 0.3 ml of packed cells was recovered by scraping as well as by trypsinization from 20 bottles. When cells were trypsinized for 30 min instead of 15 min, no significant difference in the content of sialic acid contained in the isolated membranes/mg plasma membrane protein was observed.

Neuraminidase treatment of cells

Cells were scraped from 40—50 bottles and were sedimented by centrifugation. They were washed with phosphate buffered saline that contained 0.005 M $CaCl_2$ and the pH of which was adjusted to 6.4 by the addition of a concentrated solution of KH_2PO_4. The cells were suspended in 5 ml of phosphate buffered saline (pH 6.4). 2 ml of the cell suspension were transferred to 2 tubes and the tubes were placed in a shaking water bath at 37° C. Neuraminidase solution (0.05 ml of 1 unit/ml in phosphate buffered saline, pH 6.4) was added to one tube and 0.5 ml of phosphate buffered saline, pH 6.4, to the control tube. The cells were incubated with agitation for 90 min. They were sedimented by centrifugation and the supernatants and cells were recovered. The cells were

washed with 1 ml of phosphate buffered saline, pH 6.4 and the washing was combined with the previous supernatant. The supernatant solution was used for the analysis of sialic acid and the sedimented cells were employed for the isolation of plasma membranes. The time course of the release of sialic acid from rat embryo fibroblasts by neuraminidase was also checked. It was observed that after a fast initial release during the first 30—45 min, a very slow increase in sialic acid continued up to 90 min. An incubation period of 90 min was routinely employed.

Isolation of plasma membrane fraction

Plasma membrane fractions were initially prepared according to Brunette and Till [19]. Since these preparations were slightly contaminated with nuclei, the procedure was modified to reduce nuclei content. The modified procedure is described below.

Packed cells (0.3 ml, scraped, trypsin-treated or neuraminidase-treated) were suspended in 25—30 ml of distilled water. After 1—2 min, enough 0.1 M $ZnCl_2$ solution was added to give a $ZnCl_2$ concentration of 1 mM. The cell suspension was left at room temperature for 10 min and was shaken intermittently. The cell suspension was then kept over ice for 5 min before transferring to a cold Dounce homogenizer with a tight-fitting piston. The cells were homogenized in the cold by 20—50 up and down strokes of the piston. The breakage of the cells was monitored by a phase contrast microscope. The homogenate was centrifuged at 1400 rev./min in an International Refrigerated centrifuge PR2 (Head no. 811) for 10 min. The sediment was resuspended in cold 1 mM $ZnCl_2$ solution (10—15 ml) and rehomogenized in the Dounce homogenizer by giving 5—10 strokes of the piston. This resulted in the breakage of unbroken cells. Centrifugation as described above was repeated and the sediment was washed with 1 mM $ZnCl_2$ solution. The resulting pellet contained plasma membrane sheets and fragments, nuclei and some unbroken and non-swollen cells. The pellet was suspended in 3 ml of distilled water and the suspension centrifuged at 300 rev./min for 10 min. The supernatant was recovered and recentrifuged at 500—600 rev./min for 10 min. The supernatant was found to contain mainly the plasma membrane fragments and the sediment contained nuclei and some plasma membrane sheets. By repeating slow speed centrifugation and examining the supernatant by phase contrast microscope, fractions containing only plasma membrane fragments and sheets and occasionally a nuclei could be obtained. These fractions were combined and centrifuged at 1400 rev./min to sediment the plasma membranes. When the plasma membranes preparation obtained by this method was subjected to the two phase system described by Brunette and Till [19], the membrane accumulated at the interface. The specific activity of (Na^+, K^+)-ATPase in the membrane fraction ranged from 1.5—4.5 when expressed as μmol phosphorus released/mg protein per h. No succinic dehydrogenase activity was obtained in plasma membrane fractions. The observed NADH diaphorase activity was 0—7% of that contained in the homogenate and is comparable to that reported by Brunette and Till [19]. Although the purity of the membrane fraction is not improved from that described by Brunette and Till, as judged by determination of enzyme activities, it was felt that this procedure was simpler and reliable for sugar estimation in plasma

membranes since sugar-containing solutions were not used in fractionation, and generally gave plasma membrane fraction free of nuclei.

Trypsinization of plasma membrane fraction

Plasma membrane fractions (1—2 mg protein) were treated with 1 ml of a solution of 0.1% trypsin in phosphate buffered saline at 37°C for 15 min and the mixture was kept in a shaker bath. After incubation, the reaction mixture was cooled over ice and the trypsinized membranes were recovered by centrifugation at 2000 rev./min for 15 min in an International Refrigerated centrifuge. A clear supernatant resulted. Supernatants and the membrane sediments were recovered and were analysed for sialic acid. Membrane sediment was also utilized for the analysis of polypeptides by polyacrylamide gel electrophoresis. When samples could not be subjected to analysis immediately, they were kept at 0°C and analysed the next day. In one experiment, the release of sialic acid after 15 and 30 min of incubation with trypsin was compared. Only a slight increase occured when time of incubation was increased from 15 to 30 min.

Neuraminidase treatment of plasma membrane fraction

This was essentially the same as that described for intact cells, except that smaller volumes were employed.

Sialic acid, protein and enzyme determinations

Bound sialic acid was estimated colorimetrically [20] after hydrolysis in 0.1 N H_2SO_4 at 80°C for 40 min. Free sialic acid released from intact cells and from plasma membrane suspensions by neuraminidase was estimated without prior acid hydrolysis. Synthetic *N*-acetyl neuraminic acid was used as the standard. Protein was determined according to Lowry et al. [21] with crystalline bovine serum albumin as the standard. Activities of (Na^+, K^+)-ATPase and NADH diaphorase were measured according to Wallach and Kamat [22]. Succinic dehydrogenase activity was determined according to Maeno et al. [23].

Polyacrylamide gel electrophoresis

Polyacrylamide gels were prepared in tubes 11—11.5 cm long with 0.8 cm internal diameter. The gel solution contained a mixture of 15 ml of deaerated buffer containing/liter: 7.8 g of $Na_2HPO_4 \cdot 7H_2O$, 38.6 g of $Na_2HPO_4 \cdot 7H_2O$ and 2.0 g of sodium dodecyl sulfate; 13.5 ml of deaerated solution of acrylamide containing/100 ml: 22.2 g of acrylamide, 0.6 g of methylene bisacrylamide and 1.5 ml of ammonium persulfate (7.0 mg/ml); and 0.045 ml of *N,N,N',N'*-tetramethylamine. About 2.5 ml of this mixture was added to each tube capped at the bottom and allowed to polymerize for 1 h at room temperature (25°C). The tubes were then decapped and introduced in larger test tubes containing the gel buffer described above and left at room temperature prior to use.

Known amounts of membrane suspension were sedimented and to the sediment 50—100 µl of a solution containing 1% sodium dodecyl sulfate, 10% glycerol, 0.04 M Tris · HCl buffer, pH 7.4, and 0.4% mercaptoethanol were added, vortexed and the membrane suspension heated at 100°C in a boiling water bath for 5 min and left at 65°C for 30 min. This results in the complete

dissolution of the membranes. Membrane solution (10—50 μg membrane protein) containing added bromophenol blue as a marker was applied on top of the gel and electrophoresis was carried out for about 6 h. The current was 3 mA/tube for 20 min and then 8 mA/tube for about 5.5 h. During this time, the marker reached near the bottom of the tube. Gels were removed and stained in 0.125% Commassie Brilliant Blue in methanol/water/acetic acid (5.4 : 3.2 : 1, by vol.). The gels were left in the staining solution for 1.75 h, removed and destained for 24 h in water/acetic acid/methanol (17.5 : 1.5 : 1, by vol.).

For the detection of glycopeptides, the gels were first fixed in 10% trichloroacetic solution for 30 min, washed overnight in distilled water and then immersed in Schiffs reagent (B.D.H.) for 45 min in the dark. The gels were subsequently washed in running tap water. Faint red bands at the top portion of the gel and diffused ones at the lower portion were observed.

Iodination of cells and plasma membrane fractions and SDS-polyacrylamide gel electrophoresis separation of iodinated polypeptides

A known volume of packed cells (untreated and trypsin-treated) and known amounts of plasma membrane fractions obtained from untreated and trypsin treated cells were suspended in 4 ml of phosphate buffered saline containing 2.5 mM glucose. 20 μl of carrier-free ^{125}I (2 mCi/ml) solution, 10 μl of 0.5 mM sodium iodide solution, 20 μl of lactoperoxidase solution (1.84 mg/ml) and 10 μl of glucose oxidase solution (0.05 unit : 1 unit equals oxidation of 1 μmol glucose/ml at 25°C) were added. Control reaction mixture lacked lactoperoxidase. The reaction mixtures were incubated for 15 min at room temperature. Iodinated cells and membrane fractions were recovered by centrifugation and washed several times with phosphate buffered saline. The washed preparations were dissolved in glycerol-SDS-mercaptoethanol mixture as described earlier. The solution were subjected to electrophoresis in 7.5% polyacrylamide equilibrated with 0.1% SDS according to Laemmli [24]. The gels were cut into slices of 2-mm width after dessication and the slices were counted for ^{125}I in a Gamma counter.

Results

Sialic acid content of plasma membranes from untreated and trypsin-treated cells and of trypsinized membranes and trypsinates

Table I provides data on the sialic acid content of plasma membrane fractions isolated from untreated (A) and trypsin-treated (B) cells, and the sialic acid contained in trypsinates of cells (G) and in trypsinates of plasma membrane fractions obtained from untreated (E) and trypsin treated (F) cells. The sialic acid content of the two plasma membrane fractions after their treatment with trypsin (C and D) are also given. From these data, the amounts of trypsin realeasable sialic acid exposed on the medium face (A—B or E—F), on the cytoplasmic face or cryptically disposed on the medium face (B—C or B—D), and on both faces of the plasma membrane (A—C or A—D) have been calculated (Table I). Table I also gives a percentile distribution when sialic acid contained in the plasma membrane fraction obtained from untreated cells and containing 1 mg protein is taken as 100. In spite of the fact that the sialic acid contents of

TABLE I

SIALIC ACID CONTENT OF PLASMA MEMBRANE FRACTIONS AND TRYPSINATES

The values are means ± S.D. of three experiments. Calculations for A, C and E are based on the protein content of plasma membrane fraction of rat embryo cells and those for B, D and F are based on the protein content of plasma membrane fraction of trypsinized rat-embryo cells. According to Warren et al. [1] approx. 7% of the leucine label from leucine-labelled cells is removed by trypsinization of cells. The values given in G are the average values obtained in two experiments. For comparative purpose this value can be transformed to 11.65 nmol sialic acid/mg plasma membrane protein, if it is assumed that plasma membrane protein constitute a maximum of 10% of the total cell protein.

	Sialic acid content	
	(nmol/mg membrane protein)	(%)
A. Plasma membranes of rat embryo cells	43.40 ± 7.30	100
B. Plasma membranes of trypsinized embryonic rat cells	35.60 ± 5.80	82
C. Trypsinized plasma membranes of rat embryo cells	26.50 ± 1.90	61
D Trypsinized plasma membranes of trypsinized rat-embryo cells	24.56 ± 0.92	56.58
E Trypsinate of rat embryo cell plasma membranes (derived from C)	23.73 ± 11.81	54.67
F Trypsinate of trypsinized rat-embryo cell plasma membrane (derived from D)	14.13 ± 7.65	32.55
G Trypsinate of rat embryo cells (μmol/mg total cell protein)	11.65	
Trypsin-released glycopeptides from the medium face (average of A—B and E—F)	8.73 ± 3.28	20.10
Trypsin-released glycopeptides from the cytoplasmic face or cryptically disposed medium face (average of B—C and B—D)	10.10 ± 5.88	23.67
Trypsin-released glycopeptides from both faces or exposed and cryptically disposed medium face (average of A—C and A—D)	17.90 ± 7.63	41.24

the plasma membrane fractions were slightly different in the three experiments, there was agreement between the sialic acid content of trypsin sensitive glycoproteins exposed on the medium and cryptically disposed on the medium side or exposed on the cytoplasmic side of the plasma membrane in each experiment. Based on the average of the three experiments, 19.5% of the total plasma membrane bound sialic acid was exposed on the medium side and 22.1% was cryptically disposed on the medium side or exposed on the cytoplasmic side. The remainder of the sialoglycoprotein and gangliosides (59.4%) was not degraded from the plasma membrane by trypsin under our conditions of experimentation.

It should also be noted that a significant amount of bound sialic acid was present in the trypsinates of the cells (G). This could have originated from glycoproteins excreted by the cells, serum glycoproteins absorbed on the cells as well as from glycopeptides that were released by trypsin action on the cell surface glycoproteins.

Sialic acid content of plasma membranes of untreated and neuraminidase-treated cells, of neuraminidase-treated plasma membranes and of released sialic acid

The experiments are identical to those described in Table I except that trypsin was replaced by neuraminidase.

The averages of values obtained in two experiments are presented in Table II. It was found that the sialic acid content of plasma membranes was higher in experiment 1 than in experiment 2. The cultured cells were in their second passage in experiment 1, whereas they were in their fifth passage in experiment 2. The results, when expressed on the basis of percentage, are complementary to those obtained with trypsin (Table I). Thus a significant amount of sialic acid was released from neuraminidase treatment of intact cells (G), although this enzyme removed a mere 15% of sialic acid of the plasma membrane fraction. As observed in case of trypsin, the percentage of sialic acid released from plasma membrane isolated from untreated cells was about twice as high as that released from plasma membranes obtained from neuraminidase-treated cells. On an average, more neuraminidase-releasable sialic acid was cryptically disposed on the medium side or was present on the cytoplasmic side of the membrane (22.67%) than on the medium side of the plasma membrane (15.3%). These differences may be significant. The percentage of plasma membrane-bound sialic acid that is insensitive to the action of neuraminidase is 58.2 and this figure is comparable to that obtained with trypsin (59.9%).

Gel electrophoresis of plasma membranes of untreated and trypsin-treated cells and of trypsinized membranes

Plasma membranes of embryonic rat cells contain at least 18 polypeptides

TABLE II

NEURAMINIDASE-RELEASABLE AND NON-RELEASABLE SIALIC ACID OF PLASMA MEMBRANE FRACTIONS

The values given over the average values obtained in two experiments.

	Sialic acid content	
	(nmol/mg membrane protein)	(%)
A Plasma membranes of embryonic rat cells	36.6	100
B Plasma membranes of neuraminidase-treated cells	29.7	80.87
C Neuraminidase-treated plasma membranes of embryonic rat cells	22.6	61.74
D Neuraminidase-treated plasma membranes of neuraminidase-treated cells	20.0	54.60
E Sialic acid released from plasma membranes of embryonic rat cells by neuraminidase	16.0	43.71
F Sialic acid released from plasma membranes of neuraminidase-treated cells by neuraminidase	11.6	31.70
G Sialic acid released from embryonic rat cells neuraminidase (nmol/mg cell protein)	11.3	
Sialic acid released by neuraminidase from the medium face of the plasma membrane (mean of A—B and E—F)	5.6	15.3
Sialic acid released by neuraminidase from the cytoplasmic face or cryptically disposed medium face of the plasma membrane (mean of B—C and B—D)	8.3	22.67
Sialic acid released by neuraminidase from both faces of exposed and cryptically disposed medium face of the plasma membrane (mean of A—C and A—D)	15.3	47.9

bands (Fig. 1A), the most prominent being bands 1, 2, 3, 5, 7, 14, 17 and 18. Leaving aside band 14, the other (1—3, 5, 7, 17, 18) stain with periodic acid Schiff's reagent to varying intensities (not shown) and hence they are glycoproteins. The same statement cannot be made for other bads of lesser intensity since periodic acid-Schiff's stain is relatively less sensitive, and will miss glycopeptides the concentration of which is low in the membrane. When the polypeptide pattern of plasma membranes of untreated cells (Fig. 1A) is compared with the polypeptide pattern of membranes from trypsinized cells (Fig. 1B), a variety of changes are observed. The major band 3 was not affected, but bands 5 and 18 were completely removed and bands 1, 2, 7, 14 and 17 were partially removed. The bands which were removed gave rise to degraded peptides which banded at positions marked TC1-TC12 (Fig. 1B). Thus, degraded products still remain associated with the membrane. Periodic acid-Schiff staining of such a gel gives no clear bands except a diffused area below band 16 extending lower than band 18 (not shown).

Fig. 1C is the gel electrophoretogram of trypsinized membranes. It was apparent from this figure that there was considerable loss of proteins on trypsinization of membranes. Many of the polypeptides that were observed after trypsinization of one side of the membrane (TC1, TC2, TC5, TC9, TC10, TC12) were also removed. Polypeptides 1 and 2 were degraded and appear as TM1 and TM2 and polypeptides TC3 and TC6 were removed. Polypeptides 6, 10, 13 and 14 could still be seen and degraded polypeptides TC7 and TC11 were reduced. New sharp bands TM3, TM4 and TM5 and broad bands TM6, TM7 and TM8 made their appearance. Periodic acid-Schiff staining of this gel

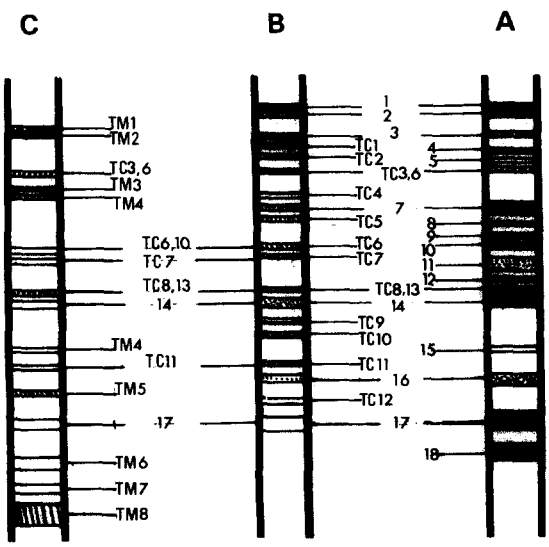

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of the plasma membrane fraction of (A) untreated and (B) trypsin-treated rat embryo fibroblasts. Gel C is derived from the plasma membrane fraction of trypsin-treated cells subjected to further trypsin treatment. Approx. 50 μg of membrane protein was put on gels A and B, whereas gel C contained that amount of protein which remains associated with membrane after plasma membrane fraction of trypsin-treated cells containing 50 μg protein are further subjected to trypsinization. Details of trypsinization, gel preparation and staining are described in the Materials and Methods section. The gels were stained with Coomassie Blue.

gave no clear bands except for a high intensity broad band constituting the area around bands TM6—TM8. Gel electrophoretograms of the three types of membranes were repeated 3—4 times with essentially similar observations.

Iodination of untreated and trypsin-treated cells and of their membrane fractions

Enzymatic radioiodination has been used to label proteins. The label is incorporated mainly into the accessible tyrosine residues of proteins. Application of this procedure to intact cells and to cell membranes is supposed to label proteins that are exposed on the medium face of the cell and on the two faces of the cell membranes if they are in the form of sheets.

Iodination of untreated and trypsin-treated cells was carried out as described in the Materials and Methods section and the iodinated cells were subjected to

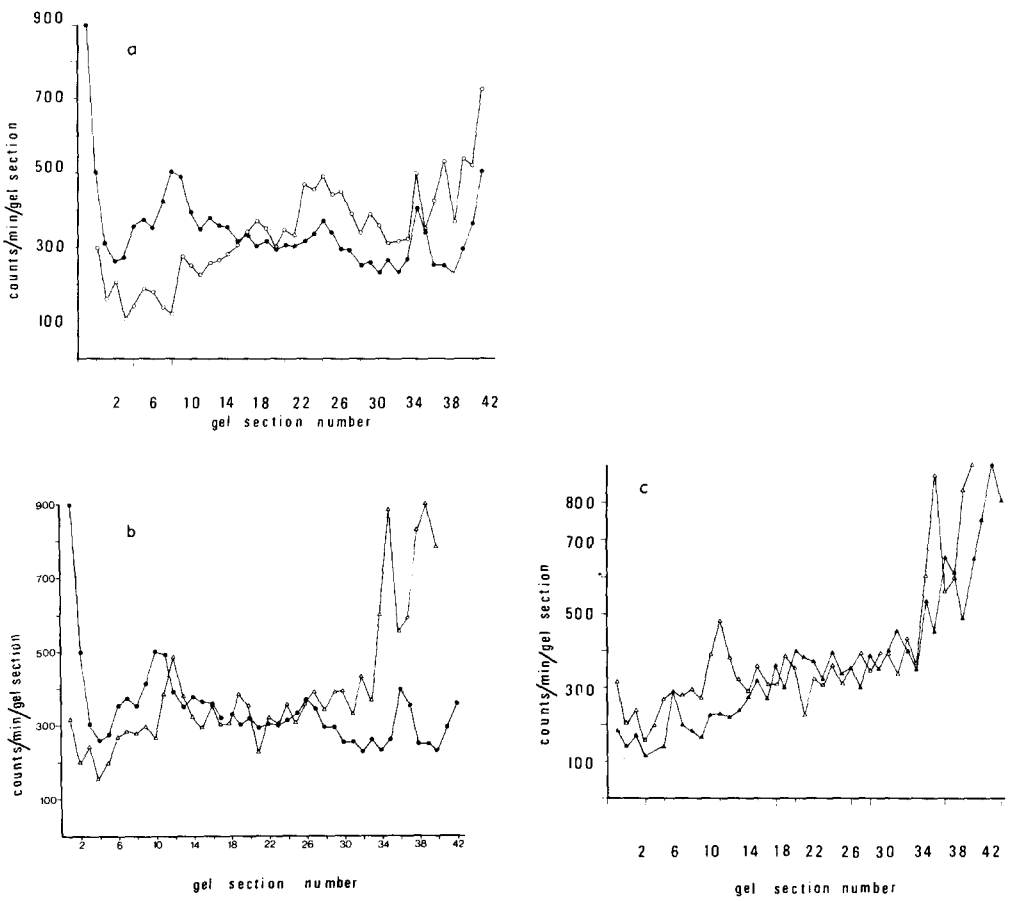

Fig. 2. Distributions of ^{125}I radioactivity in 7.5% polyacrylamide and 0.1% sodium dodecyl sulfate slab gel electrophoretogram of ^{125}I-labelled polypeptides. (a) untreated intact cells (●———●) and trypsinized intact cells (○———○); (b) untreated intact cells (●———●) and plasma membrane fraction of untreated cells (△———△); (c) plasma membrane fraction of untreated cells (△———△) and plasma membrane fraction of trypsin treated cells (▲———▲). The gels were cut into sections of 2 mm thickness and counted for ^{125}I. Experimental details are given in the Materials and Methods section. Nearly equal amounts of radioactivity were put on each gel.

SDS-polyacrylamide gel electrophoresis and the gel plate was sectioned and the sections counted for ^{125}I. Fig. 2A shows the distribution profile of radioactivities in polypeptides of untreated and trypsin treated cells. A number of peaks of radioactivity are observed in both cases. The major difference between the two is the reduction in label in peaks represented by section 7 and 10—11 and increase in label in the region comprising section 24—28, 31, 36 and 39 on trypsinization of cells.

A comparison of the distribution of iodinated polypeptides of intact cells and of iodinated plasma membrane fraction obtained from untreated cells is shown in Fig. 2B. The object of this comparison was to discern those polypeptides that are iodinated in the plasma membrane fraction but not in intact cells. These would be exposed on the cytoplasmic side. Such a comparison failed to give a definitive clue for the existence of highly iodinated polypeptides exposed predominantly on the cytoplasmic side of the membrane. Although Fig. 2B may suggest that polypeptides represented by sections 12, 19, 29—30, 32 and 34 are oriented on the cytoplasmic side, this was negated because of lack of similar peaks in iodinated plasma membranes of trypsinized cells (Fig. 2C). Shifts of one section could be due to the non-reproducible movement of polypeptides in the gel or due to manipulative error. However, increased labelling of the fast moving polypeptides (sections 32—34) in plasma membrane fractions than in intact cells could be indicative of their presence on both faces of the plasma membrane.

Comparison of the profiles of iodinated polypeptides of plasma membrane fractions obtained from untreated and trypsin treated cells (Fig. 2C) showed that polypeptide represented by sections 6 and 7 was reduced to a smaller extent and that represented by section 12 to a greater extent by the action of trypsin on intact cells.

Discussion

The results obtained by us on the treatment of intact cells with trypsin and neuraminidase show that a significant amount of bound and free sialic acid is released from intact rat embryo fibroblasts by these enzymes (Tables I and II, line G). However, the origin of the enzyme-solubilized sialic acid is unknown, and may not be exclusively from the cell surface. It may arise partly from the glycoproteins excreted by the cell during incubation in the presence of the enzymes. A portion may also originate from coating of cells by glycoproteins present in fetal calf serum which is used as a nutrient for cell culture.

Because of the diverse origin of release sialic acid on enzyme incubation of intact cells, we resorted to the use of the difference in membrane bound sialic acid in plasma membrane fractions obtained from untreated and enzyme-treated cells, as a measure of the sialic acid containing constituents sensitive to these enzymes which are exposed on the medium side of the plasma membrane. Using this approach, it was observed that both trypsin and neuraminidase solubilized only a part (15 and 20% respectively) of the total plasma membrane-bound sialic acid of intact cells. Glick et al. [25] have also observed that neuroblastoma cells grown in the presence of labelled glucosamine incorporate 37% of the total cell label into the surface membrane and 7—10% is solubilized by

trypsin. Thus a significant portion of carbohydrates of the plasma membranes remains unsolubilized by trypsin. In contrast to trypsin, great diversity in the amount of sialic acid released by neuraminidase (28—90% of total cell sialic acid) has been reported from various cell types [26—29]. In our experiments, it was observed that about 30% of the total cell sialic acid was releasable by neuraminidase; but it constitutes only 15% of the plasma membrane bound sialic acid (Table II).

It was apparent that the remainder may be unreactive or unavailable to the action of these enzymes because of its location in the plasma membrane. It was felt that if the latter was true because plasma membrane-bound sialic acid was exposed on the cytoplasmic side of the plasma membrane, treatment of isolated plasma membrane sheets with trypsin and neuraminidase would lead to a greater release of plasma membrane-associated sialic acid. We observed that treatment with trypsin or neuraminidase of plasma membrane fraction isolated from untreated cells released 41.6 and 38.7% of the total membrane bound sialic acid (Table I and II). For plasma membrane fractions isolated from cells treated with trypsin or neuraminidase, the values were 22.1 and 23.3% (Tables I and II). Thus, these results supported the concept that nearly equal amounts of enzyme-susceptible sialic acid-containing constituents were exposed on the medium and on the cytoplasmic face of the plasma membrane.

The enzymatic liberation of 22—23% of the total plasma membrane-bound sialic acid from plasma membrane fractions isolated from enzyme-treated cells cannot be due to their contamination with endoplasmic reticulum because (a) plasma membranes, unlike endoplasmic reticulum membrane fragments, were in the form of the entire cell envelope or large membrane sheets, (b) activity for microsomal enzyme, NADH diaphorase, was very low or absent in our preparations, and (c) the release of such a large amount of sialic acid from endoplasmic reticulum alone would require a gross contamination of our plasma membrane fraction by endoplasmic reticulum, which if present, would have been detected by an enrichment of NADH diaphorase activity.

The above concept would, however, be in conflict with the results of Nicolson and Singer [15] and Benedetti and Emmelot [18] who showed that carbohydrate reacting lectin [15] and colloidal iron [18] localize only on the medium face of the plasma membrane. If it were to be accepted that carbohydrate chains exist only on the medium face of the plasma membrane, our observations would suggest that carbohydrate containing constituents exist in at least three forms in the membrane. First, in a non-cryptic or exposed form, where they are easily accessible to trypsin or neuraminidase; second, an equal portion in a cryptic form which becomes exposed on isolation of the plasma membrane and third, in a non-reactive or inaccessible form.

In order to get an insight into the nature of polypeptides and glycopolypeptides on the surface of plasma membranes which are reactive or non-reactive to trypsin in intact cells and those which are non-reactive to trypsin treatment of isolated membranes, SDS-polyacrylamide gel electrophoresis patterns of plasma membrane fractions isolated from untreated and trypsin-treated cells and of trypsinized plasma membranes were carried out. It was observed that seven polypeptides (5, 8, 9, 11, 12, 15 and 18, see Fig. 1, a and b) were susceptible to trypsin action on intact cells, and hence these could be considered as primarily

exposed on the medium side of the plasma membrane. Four polypeptides (1, 2, 7 and 17, see Fig. 1) were partially degraded by trypsinization of intact cells and fully on trypsinization of plasma membrane fraction (compare Fig. 1b with 1c). These polypeptides could be exposed both on the medium as well as the cytoplasmic face of the plasma membrane or could exist in two forms, cryptic and non-cryptic, on the medium face of the membrane. Since these polypeptides were also stained with periodic acid-Schiff's reagent, they are derived from glycoproteins and are presumably responsible for nearly equal release of sialic acid from the two faces of the plasma membranes described above. Of the remaining seven polypeptides (3, 4, 6, 10, 13, 14 and 16) that were not affected by trypsinization of cells, only one (3) was completely removed by trypsinization of plasma membrane fraction, and hence it would be the only polypeptide exposed primarily on the cytoplasmic face of the membrane.

The results obtained in radioiodination studies tend to support those obtained on SDS-polyacrylamide gel electrophoresis of membranes described above. Thus, polypeptides of high molecular weight (sections 5—11, Fig. 2a) are readily iodinated in intact cells and are reduced quantitatively on trypsin treatment of intact cells. Greater iodination of polypeptides in the middle region in case of trypsinized cells (section 22—26, Fig. 2a) is presumably due to degraded polypeptides that remain associated with plasma membrane. Iodination patterns of polypeptides of intact cells and of isolated plasma membrane fraction were very similar (Fig. 2b) which would indicate that few proteins, if any, that can be iodinated are exposed on the cytoplasmic face of the membrane. Further, since the iodination of the high molecular weight polypeptides with respect to other polypeptides was not enhanced in the membrane fraction, this would seem to indicate that these high molecular weight polypeptides cannot be iodinated from the cytoplasmic face, in case they traverse the entire membrane or if they are exposed on the cytoplasmic face of the plasma membrane. The comparison of the iodination patterns of plasma membrane fractions isolated from untreated and trypsin-treated cells were also very similar and confirmed the existence of high molecular weight polypeptides in membranes of untreated cells and their reduction on trypsin treatment.

The results of gel patterns, therefore, reduce the likelihood of the existence of glycoproteins on the cytoplasmic face of the membrane. They tend to support a cryptic or non-reactive disposition of membrane proteins and glycoproteins. Only conjectures can be made regarding the cryptic disposition. It is possible that membrane glycoproteins exist as enzyme-sensitive single units (a non-cryptic form) and as enzyme-insensitive aggregates (cryptic form). Some of these aggregates may undergo disaggregation during membrane isolation and provide reactive substrate for the enzymes. Conversely, it is also possible that all protein molecules do not penetrate the lipid bilayer to the same extent and hence they exist in a reactive and non-reactive form.

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