Effect of Trypsin on the Cell Surface Proteins of Hepatoma Tissue Culture Cells

CHARACTERIZATION OF A CARBOHYDRATE-RICH GLYCOPEPETIDE RELEASED FROM A CALCIUM-BINDING MEMBRANE GLYCOPROTEIN*

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Heinz Baumann‡ and Darrell Doyle

From the Department of Molecular Biology, Roswell Park Memorial Institute, Buffalo, New York 14263

Concentrations of trypsin that bring about aggregation of hepatoma tissue culture (HTC) cells also release from the cell surface an \( M_r = 55,000 \) glycopeptide fragment. This glycopeptide fragment also accumulates in the medium, including serum-free medium, as a normal consequence of membrane protein turnover. The trypsin-released glycopeptide is labeled when cells are grown in the presence of fucose or leucine before treatment of the cells with the protease. Similarly, the glycopeptide fragment can be labeled by reacting cells \textit{in situ} by lactoperoxidase-catalyzed radioiodination or by tritiated borohydride reduction of cells treated first with neuraminidase and galactose oxidase. The trypsin glycopeptide fragment was purified by concanavalin A-Sepharose chromatography, and hydroxyapatite chromatography in the presence of dodecyl sulfate. The amino acid and carbohydrate composition was determined, as was the sensitivity of the purified glycopeptide to a variety of endo- and exoglycosidases. The purified glycopeptide contains an average of 17 sialic acid residues and hence, shows charge heterogeneity after electrophoresis in isoelectric focusing gels. The charge heterogeneity can be eliminated completely by treatment with neuraminidase. The glycopeptide after this treatment is homogeneous. The trypsin-sensitive membrane glycoprotein which is the source of the \( M_r = 55,000 \) glycopeptide was identified by two-dimensional gel electrophoretic analysis of labeled cells, treated or not treated with trypsin. This glycoprotein, which has an apparent molecular weight of 85,000 and forms a homodimer in the presence of calcium ions, was purified and its identity as the parent of the \( M_r = 55,000 \) glycopeptide was confirmed by showing that the same \( M_r = 55,000 \) fragment was released by trypsin from the purified glycopeptide as was released from the intact cells.

Plasma membrane proteins offer, by way of their externally exposed portions, targets for degradation by extracellular proteases and glycosidases. That such a mode of membrane protein degradation does, in fact, contribute to the general degradation of surface proteins was shown by us previously for rat hepatoma cells in culture (1). Experimentally, proteolytic digestion. In this manuscript we show that treatment of HTC cells with trypsin at a concentration which results in cell-cell aggregation, releases an \( M_r = 55,000 \) glycopeptide fragment from an \( M_r = 85,000 \) membrane glycoprotein. The \( M_r = 55,000 \) glycopeptide is much more resistant to further degradation by proteases; it also accumulates in the medium during normal cell growth. This membrane glycopeptide, the isolation and characterization of which are described here, could help provide biochemical information about the parent membrane glycoprotein and is a useful probe to examine the mechanism of biogenesis and turnover of the protease-sensitive glycoprotein in the plasma membrane.

EXPERIMENTAL PROCEDURES

Enzymes—Endo-\( \beta \)-N-acetylglucosaminidase D and H and a mixture of exoglycosidases of \textit{Turbo corvus}, containing the following enzymes in units/g of protein: \( \alpha \)-mannosidase, 166; \( \beta \)-mannosidase, 106; \( \alpha \)-glucosidase, 3.2; \( \beta \)-glucosidase, 20; \( \alpha \)-galactosidase, 24; \( \beta \)-galactosidase, 125; \( \alpha \)-l-fucosidase, 58; \( \beta \)-xylosidase, 12; \( \alpha \)-N-acetylglucosaminidase, 2.5; \( \beta \)-N-acetylglucosaminidase, 383; \( \alpha \)-N-acetylgalactosaminidase, 46; and \( \beta \)-N-acetylgalactosaminidase, 30, were purchased from Seikagaku Kogyo, Tokyo; neuraminidases of \textit{Vibrio cholerae} were from Calbiochem, that of \textit{Clostridium perfringens} (grade VI) from Sigma; and trypsin (tsoyphenylalaninechloromethyl ketone-treated, 276 units/mg) from Worthington. Galactose oxidase (3000

3935
units/mg) was a generous gift of Dr. M. Ettinger, State University of New York at Buffalo.

The glycoprotein-containing fractions were dialyzed at a molar ratio of 10% formaldehyde and then lyophilized. The extent of aggregation was determined by separation of the hexitols was done on a gas-liquid chromatograph (Hewlett Packard 402 B) equipped with a column of 3% OV-225 using a temperature gradient of 160-230°C. Inositol was used as internal reference and either a mixture of hydrolyzed and reduced free hexoses or fetuin were used as standards for quantification. The sialic acid content was determined by the method of Hammond and Papermaster (28).

**RESULTS**

**Effect of Treatment with Trypsin on the Composition of the HTc Cell Surface**—When HTc cells, after removal of the medium serum proteins, are exposed to low concentrations of trypsin for a short period of time, the cells develop an affinity for each other. When a suspension of such trypsin-treated cells is gently shaken, the cells immediately form aggregates (Fig. 1). However, between 20 and 30% of the cells are not aggregated. When the suspension is gently shaken with microprobe attachment, the sonicated mixture or homogenate was centrifuged for 60 min at 150,000 X g. The supernatant solution was centrifuged for 60 min at 200,000 X g.

In order to separate isolated glycoproteins with trypsin in vitro, even trace amounts of sodium dodecyl sulfate had to be removed. This was achieved either by precipitation of glycoprotein with trichloroacetic acid and extraction with ethanol, or by precipitation and extraction with acidic acetone at -20°C. The glycoproteins then dissolved in 0.2 M ammonium bicarbonate at concentrations ranging between 0.1 and 1 mg/ml. The various conditions used for trypsin digestion are presented in the legends to Figs. 8 and 10. After digestion, the incubation mixture was lyophilized.

**Isolation of Glycoproteins**—All buffers used contained 0.1 mM phenylisethanol sulfonyl fluoride and 2-mercaptoethanol, and the isoelectric focusing slab gel (first dimension) contained the following Ampholine composition: 0.06% pH 3.5 to 10, 0.06% pH 4 to 6, 0.06% pH 6 to 8, and 0.3% pH 9 to 11. In all cases, the second dimension dodecyl sulfate gel consisted of a uniform concentration of 7.5% acrylamide. The following molecular weight markers were used: myosin (210 X 10^6), β-galactosidase (130 X 10^6), phosphorylase a (93 X 10^6), albumin (68 X 10^6), ovalbumin (43 X 10^6), and myoglobin (17 X 10^6). Fluorographic detection of tryptic peptides was carried out as outlined by Bonner and Laskey (31). For densitometric analysis of the autoradiogram, a densitometer from Corning (model 750) was used.

The glycoproteins, present either in the trypsin-released fraction of intact cells or deoxycholate extracts of the cell membrane fraction, were bound to columns (1 X 5 cm) of concanavalin A-Sepharose (Pharmacia). The nbound material was eluted with 300 ml of deoxycholate/Tris buffer at a flow rate of 40 ml/h. The bound glycoproteins were eluted after incubation of the column with 0.5 M a-methyl mannoside in deoxycholate/Tris buffer for 1 h. The membrane pellet was sonicated once in the same Tris-HCl buffer and was centrifuged again. The glycoproteins were extracted by sonication of the membrane pellet in 1% sodium deoxycholate, 10 mM Tris-HCl, pH 8.0 (deoxycholate/Tris buffer) in twice the volume used for the homogenization. The extract was centrifuged for 1 h at 200,000 X g.

The glycoproteins solution in dodecyl sulfate was applied at room temperature to a column of hydroxyapatite (Bio-Gel HTP, Bio-Rad) equilibrated in 10 mM sodium phosphate buffer, pH 6.8, containing 0.05% (w/v) sodium dodecyl sulfate. The trypsin digests were eluted from the column with a linear gradient of sodium phosphate (details are described in the legend to Figs. 5 and 7). The glycoprotein-containing fractions were dialyzed against several changes of distilled water and then lyophilized.

Purification of glycoproteins by preparative isoelectric focusing was carried out in tube gels (4-mm diameter) composed of 0.1% (w/v) polyacrylamide, 2% (v/v) N,N,N',N'-tetramethylethylenediamine N,N,N',N'-tetraacetate (1 mM) was present during trypsin treatment.

The effect of this treatment with trypsin on the pattern of...
polypeptides, which were labeled at the surface by in situ iodination, was examined and is illustrated in Fig. 2. Almost all surface proteins which are labeled by lactoperoxidase-catalyzed iodination are sensitive to some degree to proteolysis by trypsin. The proteolysis results in an increase in the amount of membrane-bound material appearing on the gel in the region of lower molecular weights. One noticeable example of a protein which appears resistant to trypsin is that with an $M_r = 140,000$. The electrophoretic pattern of the labeled peptides released by trypsin from the membrane into the soluble fraction includes a broad band with an apparent $M_r = 55,000$ and several bands with $M_r < 20,000$. The majority (87%) of the radioactivity released from the labeled surface proteins, however, is in the form of small molecular weight material soluble in acid. When radiiodinated cells were treated with higher concentrations of trypsin (up to 20 $\mu$g/ml) for 10 min at 37°C, the release of radioactivity was almost complete (90%, also see Ref. 32).

Since glycoproteins have been proposed as the class of membrane constituents most involved in cell aggregation phenomena, the effect of trypsin on externally oriented HTC cell glycoproteins was next examined. In situ proteolytic digestion of cells labeled at the surface by reductive tritiation, after treatment of the intact cells with neuraminidase and galactose oxidase, indicates that surface glycoproteins with accessible galactose residues are more resistant to the action of trypsin than are the bulk of the surface proteins accessible for in situ iodination (Ref. 1). A maximum release of protein-bound radioactivity introduced by the galactose oxidase/NaBH$_4$ procedure was achieved by incubating cells with trypsin at a concentration of 10 $\mu$g/ml for 10 min at 37°C. The portion of the cell-surface glycoprotein sensitive to trypsin under these conditions comprises about 30 to 35% of the total surface tritiated glycoprotein. A two-dimensional separation of these labeled glycoproteins before and after treatment of the cell in situ with trypsin shows that not all glycoproteins were equally affected by the protease. Rather, there are only a few glycoproteins which are susceptible to trypsin (Fig. 3). Radioactivity is lost completely from three different series of glycoproteins with $M_r = 85,000$, 55,000, and 125,000 and $pI$ 4.2 to 4.9, 5.5 to 6.2, and 5.2 to 6.0, respectively (designated G-1, G-7, and G-16 in Fig. 3). The trypsin-treated cells show, in addition to the specific loss of surface glycoproteins, the appearance of two new glycoproteins with $M_r = 105,000$ and 35,000 and $pI$ 5.0 to 5.5 and 5.0 to 7.0, respectively (tg-1, tg-2). These latter proteins probably represent trypptic fragments still associated with the plasma membrane. Most of the glycoproteins present in HTC cells treated or not treated with trypsin can be recovered by affinity chromatography over concanavalin A-Sepharose. One obvious exception is the glycoprotein fragment G-2 created in the membrane by trypsin (Fig. 3).

A described previously (1), the acid-insoluble material released from the cell by trypsin consists of only one major glycopeptide with an apparent molecular weight of 55,000 and isoelectric point of 3.9 to 4.2 (Fig. 3). This same glycopeptide accumulates in serum-free or serum-containing culture medium of HTC cells, indicating that proteolysis and release of this fragment is a part of the normal turnover process for surface proteins (1). This $M_r = 55,000$ tryptic glycopeptide fragment also binds to concanavalin A-Sepharose, allowing purification and characterization.

**Purification of the Major Glycopeptide Released by Trypsin from the Surface of HTC Cells**—The major glycopeptide present in the soluble fraction after treatment of HTC cells with trypsin can be purified to homogeneity or near homogeneity in only a few chromatographic steps. The trypsin-sensitive fraction derived either from cells labeled at the surface by the neuraminidase, galactose oxidase, and NaB$^4$H$_4$ procedure or from cells labeled metabolically with $[^3]$H]fucose or $[^3]$H]leucine was first passed through a concanavalin A-Sepharose column, resulting in the removal of the majority of nonglycosylated proteins (Fig. 4C) and some other minor glycopeptides (Fig. 4A and B and Table I). Metabolic labeling with fucose or leucine, or both, was used to demonstrate both the degree of purification and to show that the $M_r = 55,000$ tryptic peptide in Fig. 3 is of cellular origin and not a fragment of a serum protein which was firmly bound to the plasma membrane and hence labeled by surface labeling methods. The concanavalin A-bound fraction next was chromatographed on hydroxyapatite in the presence of sodium dodecyl
FIG. 3. Removal of glycoproteins by trypsin from surface-labeled HTC cells. HTC cells (3 x 10⁶) were labeled at the surface with NaB³⁴H₄ after treatment of the cells with neuraminidase and galactose oxidase. One-half of the labeled cells were suspended in 30 ml of phosphate-buffered saline and digested with trypsin, 5 μg/ml, for 10 min at 37°C. The cells were collected by centrifugation for 5 min at 400 x g and the supernatant fraction was centrifuged for 60 min at 200,000 x g. Aliquots of both the untreated and treated cells, each containing 100,000 acid-insoluble cpm, were dissolved in 10 μl of sample buffer containing sodium dodecyl sulfate. The remaining cells were solubilized by sonication in a deoxycholate/Tris buffer. The glycoproteins of the cells as well as those in the trypsin-released material were isolated by concanavalin A-Sepharose chromatography and then precipitated by 10% trichloroacetic acid. The precipitates were washed three times with ethanol. Aliquots containing 100,000 cpm (cell glycoproteins) or 30,000 cpm (trypsinic glycopeptides) were subjected to a two-dimensional electrophoretic separation. The fluorograms after a 1-month exposure are shown. G-1, G-7, and G-16 represent the surface glycoproteins, which are completely sensitive to trypsin (for explanation of the numbering system see Fig. 7); tG-1 and tG-2 are tryptic, membrane-bound fragments, which still contain carbohydrates. BPB indicates the position of the tracking dye, bromphenol blue.

Fig. 4. Purification of the glycopeptide released by trypsin from labeled HTC cells. The samples used for electrophoresis on a 7.5% sodium dodecyl sulfate-polyacrylamide gel derived from the experiments of Table I. A, cells, labeled with NaB³⁴H₄ after neuraminidase and galactose oxidase treatment, before (1) and after (2) exposure to trypsin. The corresponding fraction released from the cells is shown in (3). The trypsin-released material (4) was applied on a concanavalin A-Sepharose column, and after elution of the nonbound material (5), the bound glycopeptide was released by α-methylmannoside (6). The glycopeptide was chromatographed on hydroxypatite (7). Lanes 1 and 2 contained 30,000 acid-insoluble cpm, Lanes 4 to 7 contained 50,000 cpm. B, cells labeled metabolically with [³⁴H]fucose, before (1) and after (2) exposure to trypsin. The corresponding fraction released from the cells is shown in (3). The trypsin-released material was chromatographed over concanavalin A-Sepharose. After elution of the nonbound material (4), the bound glycopeptides were released by α-methylmannoside (5) and then purified by passing through hydroxypatite (6) (see Fig. 5). Lanes 1, 2, and 4 to 6 contain 50,000 acid-insoluble cpm. C, cells, labeled metabolically with [³⁴H]leucine (1) were treated with trypsin. The released peptides (2) were chromatographed over concanavalin A-Sepharose. After elution of the bound fraction by α-methylmannoside (3), the glycopeptide was chromatographed over hydroxypatite (4) (see Fig. 5) and compared with [³⁴H]fucose-labeled glycopeptide (5). All lanes contained 50,000 acid-insoluble cpm. BPB, bromphenol blue.
Isolation of the glycopeptide released by trypsin from sugar and amino acid-labeled HTC cells

Three batches of HTC cells, each consisting of \(1 \times 10^6\) cells, were labeled either enzymatically at the surface by \(\text{NaB}[^3\text{H}]\text{H}_4\), reduction after treatment of the cells with neuraminidase and galactose oxidase or metabolically by culturing the cells in the presence of 5 mCi of \([^3\text{H}]\text{fucose}\) or \([^3\text{H}]\text{leucine}\) (50 µCi) for 3 days. The cells were washed three times with phosphate-buffered saline, suspended in 200 ml of the same buffer, and digested with 1 mg of trypsin for 10 min at 37°C. After ultracentrifugation, the supernatant fractions were applied to a column of concanavalin A-Sepharose. The bound material released by 0.5 M α-methylmannoside was treated with 10% trichloroacetic acid. The precipitates were washed twice with ethanol, dissolved in 0.5 M sodium dodecyl sulfate, and chromatographed on hydroxyapatite as described in Fig. 5. The values shown in the table represent the total acid-insoluble radioactivity recovered in each of the fractions.

<table>
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<tr>
<th>Fraction</th>
<th>Neuraminidase, galactose oxidase</th>
<th>([^3\text{H}]\text{fucose})</th>
<th>([^3\text{H}]\text{leucine})</th>
</tr>
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<tbody>
<tr>
<td>Cells before trypsin treatment</td>
<td>45.5</td>
<td>115.0</td>
<td>236.0</td>
</tr>
<tr>
<td>Cells after trypsin treatment</td>
<td>34.7</td>
<td>103.7</td>
<td>228.3</td>
</tr>
<tr>
<td>Trypsin-soluble material (200,000 x supernatant)</td>
<td>7.9</td>
<td>8.3</td>
<td>3.5</td>
</tr>
<tr>
<td>Concanavalin A-bound</td>
<td>3.9</td>
<td>2.9</td>
<td>0.37</td>
</tr>
<tr>
<td>After hydroxyapatite chromatography</td>
<td>2.9</td>
<td>2.1</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Fig. 5. Purification of the tryptic glycopeptide by hydroxyapatite chromatography. The glycopeptides derived from trypsin-treated cells, which were labeled metabolically either with \([^3\text{H}]\text{leucine}\) or \([^3\text{H}]\text{fucose}\), were purified by concanavalin A-Sepharose chromatography (details described in Table I). The peptides were precipitated with 10% trichloroacetic acid, washed twice with ethanol, and dissolved in 3 ml of 10 mM sodium phosphate buffer, pH 6.8, containing 0.05% sodium dodecyl sulfate. The samples were each applied to a column (0.6 x 15 cm) of hydroxyapatite and the adsorbed material eluted in fractions of 1.76 ml by a linear gradient consisting of 50 ml each of 10 mM and 300 mM sodium phosphate buffer, pH 6.8, plus 0.05% sodium dodecyl sulfate. Aliquots of 0.5 ml \([^3\text{H}]\text{fucose}\) label) or 40 ml \([^3\text{H}]\text{leucine}\) label) of each fraction were used for the determination of radioactivity. The solid bar indicates the fractions pooled for further studies.

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified tryptic glycopeptide. HTC cells (4 x 10⁶) were treated with trypsin (5 µg/ml) for 10 min at 37°C. The released glycopeptide was purified by concanavalin A-Sepharose chromatography and recovered by precipitation with 10% trichloroacetic acid. The precipitate was washed twice with ethanol. One-tenth aliquot of it was dissolved in sample buffer (Slot 2). The remaining glycopeptides were divided in half. One-half was dissolved in 5 ml of 10 mM Tris-HCl buffer, pH 7.8, containing 1% Triton X-100 and applied to a column (1 x 16 cm) of diethylaminomethyl cellulose (DE52, Whatman) equilibrated in the above buffer. The glycopeptide was eluted by a linear gradient (200 ml) of sodium chloride (from 0 to 0.3 M) and appears as one peak at a sodium chloride concentration between 0.11 and 0.15 M. The pooled fractions were dialyzed against distilled water and lyophilized. One-fifth of the material was dissolved in sample buffer (Slot 3). The second half of the lectin-purified peptide fraction was subjected to hydroxyapatite chromatography as described in Fig. 5. The glycopeptide-containing fractions were dialyzed against distilled water and lyophilized. One-fifth of the preparation was dissolved in sample buffer (Slot 4). All samples plus concanavalin A (Pharmacia) (Slot 1) and molecular weight standard proteins (Slot 5) were electrophoresed on a sodium dodecyl sulfate gel of 10% acrylamide and stained for protein with Coomassie brilliant blue.
7A), resulting in separation into different subsets of glycoproteins. The hydroxyapatite glycoprotein fractions could be further resolved by either preparative isoelectric focusing or gel electrophoresis in sodium dodecyl sulfate (Fig. 7B). The glycoprotein of interest (G-1) appears in the first peak off the hydroxyapatite column. A second glycoprotein, G-3 in Peak I, which is present in considerable amount, could be removed by preparative isoelectric focusing in polyacrylamide gels. After elution from the isoelectric focusing gel, glycoprotein G-I was subjected to partial digestion with trypsin. The electrophoretic separation of the trypsin digest reveals a band at M, = 55,000 as well as bands at M, = 35,000 and 25,000 (Fig. 8). Fragments of M, 35,000 and 25,000 also accumulate when the glycopeptide released from the cell surface by trypsin is treated further with trypsin. The two minor bands with M, = 18,000 and 19,000 are observed also in the trypsin digest of the M, = 55,000 glycopeptide when it is radioiodinated in vitro (compare slots 2 and 8 of Figure 8). Possibly then, these two bands represent not tryptic glycopeptides labeled at terminal galactose residues, but rather fragments which were artifically labeled in the polypeptide chain by NaB[3H]H4. This type of labeling can happen, as demonstrated previously (1). Nevertheless, the pattern of the trypsin digest of the isolated and purified glycoprotein G-1 shows unequivocally that the glycopeptide detectable in the trypsin-released fraction of intact cells derives from this glycoprotein (G-1).

The glycoprotein G-1 in Fig. 8 does not, however, represent the "native" form of this glycoprotein in situ, because prior to the extraction of total glycoproteins, the cells underwent a partial desialylation in order to expose subterminal galactose residues to the galactose oxidase/borohydride reduction. Hence, to determine whether the "native" glycoprotein yields the same glycopeptide upon trypsin hydrolysis, the membrane glycoproteins from metabolically, rather than surface-labeled, cells were purified. Chromatography of [3H]fucose-containing glycoproteins on hydroxyapatite yields a similar profile as the galactose-labeled surface glycoproteins (Fig. 7A). The elution of the first three fucose-labeled peaks from the hydroxyapatite, however, occurs at a lower phosphate molarity and the major component in Peak VI is more clearly separated from that in Peak VII. A two-dimensional separation of the glycoproteins present in the pool of Peak I from fucose-labeled cells shows a quite different pattern than that of Peak I from surface galactose-labeled glycoproteins (Fig. 9A). Three major components (F-1, F-2, and F-3) are present in fucose-labeled Peak I and are characterized by apparent molecular weights of 175,000, 85,000, and 90,000 and isoelectric points of <3.5, 3.5 to 3.9, and 4.2 to 5.4, respectively. However, when these glycoproteins were treated in vitro with neuraminidase under similar conditions as used for in situ surface labeling, the two-dimensional pattern shows that the positions of the glycoproteins F-2 and F-3 are shifted more toward the basic end of the acrylamide gel, nF-2, nF-3, whereas a spot related in molecular weight to glycoprotein F-1 is no longer detectable. The migration of the fucose-labeled glycoprotein after neuraminidase treatment, nF-2, is the same as for the [3H]galactose-labeled glycoprotein G-1 (Fig. 7B), indicating identity of the molecules. Recent studies have shown that the glycoprotein F-3 is not accessible to enzymatic surface labeling with either galactose oxidase or lactoperoxidase and is not degradable by treatment of intact cells with neuraminidase or trypsin, whereas this protein is affected by these modifications when internal membrane fractions were used. This suggests an exclusively intracellular location of glycoprotein F-3 and would explain why no corresponding surface-labeled spot in Figs. 3 and 7B was detected.
Tryptic Modification of Cell Surface Proteins

FIG. 8. Tryptic mapping of glycoprotein G-1. Glycoprotein G-1 present in Peak I after hydroxyapatite chromatography (see Fig. 7) was separated from glycoprotein G-3 by preparative isoelectric focusing. The residual sodium dodecyl sulfate in the glycoprotein preparation was removed by precipitation with 10% trichloroacetic acid followed by washing of the precipitate with ethanol twice. The glycoprotein was dissolved in 0.2 M ammonium bicarbonate. One aliquot containing 30,000 cpm in 50 μl was incubated in the absence (1) and another containing 50,000 cpm in the presence (2) of 0.25 μg of trypsin for 5 min at 37°C. For purposes of comparison, glycopeptides derived by trypsin treatment of NaB[3H]H4-labeled cells (see Fig. 4A) were similarly digested. Aliquots of the tryptic glycopeptide containing 50,000 cpm were incubated in the absence (3), or presence of 0.25 μg of trypsin for 5 min at 37°C (4), 0.5 μg of trypsin for 10 min at 37°C (5), or 1 μg of trypsin for 4 h at 37°C (6). In addition, the glycopeptide derived from HTC cells (1 x 10^6), which were pretreated with neuraminidase, was isolated by concanavalin A-Sepharose chromatography; it was then iodinated in vitro with chloramine-T and 0.25 mCi of 125I. The labeled product was further purified by hydroxyapatite chromatography. Aliquots of the labeled glycopeptide containing 50,000 cpm were treated under conditions identical to the samples mentioned above; one without (7) and one with (8) 0.5 μg of trypsin for 10 min at 37°C. Following proteolytic digestions, samples were lyophilized and the residue was analyzed on a sodium dodecyl sulfate gel consisting of a linear gradient of acrylamide (9 to 16%). BPB, bromphenol blue.

The disappearance of spot F-1 upon neuraminidase treatment can be explained by either degradation by a protease contaminating the neuraminidase or by a breakdown of this M_0 = 175,000 protein into subunits appearing after neuraminidase treatment.
gel electrophoresis. The purified glycoprotein F-1 now could also be converted by treatment with chelating agent into calcium chelating agent ethylene glycol bis(α-aminoethyl ether)N,N'-tetraacetate, in order to increase the amount of glycoprotein F-1. The eluted proteins were treated with 50 μM CaCl₂ to sugar-labeled and sodium dodecyl sulfate-polyacrylamide gel. The tryptic glycopeptide with Mᵋ = 55,000 containing large amounts of sialic acid residues (see below), does not dimerize even in the presence of 10 mM Ca²⁺. In order to illustrate that the tryptic glycopeptide is not a fragment which can be produced by trypsin digestion of membrane glycoproteins other than F-1 and F-2, the glycopeptide pattern of glycoprotein F-3 is shown in Fig. 10B.

Characterization of the Glycopeptide Derived from the HTC Cell Surface—The glycopeptide released by trypsin

treatment as glycoprotein nF-2 or nF-3. The latter possibility is supported by the observation that addition of 2 mM CaCl₂ to sugar-labeled and sodium dodecyl sulfate-solvobilized cell extracts or to concanavalin A-bound glycoproteins causes a strong enhancement of the band at Mᵋ = 175,000 (F-1) with a concomitant decrease in intensity of the Mᵋ = 85,000 protein. However, subsequent treatment with the calcium chelating agent ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetate, the Mᵋ = 175,000 glycopeptide decreases in intensity while the Mᵋ = 85,000 glycopeptide increases.

To further clarify the fate of glycoprotein F-1 after neuraminidase treatment and the relationship of F-1 to the glycoproteins F-2 and F-3, glycoprotein F-1 was first purified as follows: the two acid components F-1 and F-2 in fucose-labeled Peak I were isolated by preparative isoelectric focusing in a polyacrylamide gel. The eluted proteins were treated with 5 mM CaCl₂ in order to increase the amount of glycoprotein F-1. They then were separated from each other by preparative gel electrophoresis. The purified glycoprotein F-1 now could also be converted by treatment with chelating agent into glycoprotein F-2 or by neuraminidase into a spot series typical for glycoprotein G-1 with Mᵋ = 85,000 and pI 4.2 to 4.7 (Fig. 9, B and C). This experiment (Fig. 9B) shows in addition that similar glycopeptide spots appear in the position where G-2 migrates in Fig. 7B, suggesting that G-2 represents one of the desialylation of glycoprotein F-1. The glycopeptides F-1 and F-2 after the final isolation step also were subjected to partial hydrolysis with trypsin and the proteolytic fragments produced were compared by electrophoresis in dodecyl sulfate-acrylamide gels with a glycopeptide derived from in vivo-labeled cells (Fig. 10A). Both glycopeptides F-1 and F-2 produce the same spectrum of fragments as those seen for the glycopeptide isolated from the cell surface. Under mild conditions of hydrolysis of glycopeptide F-1, however, a glycopeptide band at Mᵋ = 120,000 appears. This form could be due to a removal of one Mᵋ = 55,000 portion from a dimer of Mᵋ = 175,000 while the remaining parts of the dimer stay associated. In fact, this partially degraded form of glycopeptide F-1 (Mᵋ = 120,000) was also found by two-dimensional gel analysis of metabolically labeled monolayers, but not of suspension cultures of HTC cells. It should be mentioned, however, that the tryptic glycopeptide with Mᵋ = 55,000 containing large amounts of sialic acid residues (see below), does not dimerize even in the presence of 10 mM Ca²⁺. In order to illustrate that the tryptic glycopeptide is not a fragment which can be produced by trypsin digestion of membrane glycoproteins other than F-1 and F-2, the glycopeptide pattern of glycopeptide F-3 is shown in Fig. 10B.

Characterization of the Glycopeptide Derived from the HTC Cell Surface—The glycopeptide released by trypsin

<table>
<thead>
<tr>
<th>Table II: Amino acid and carbohydrate composition of the tryptic glycopeptide</th>
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<tbody>
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<td>Amino acids</td>
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<tr>
<td>Asparagine</td>
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<td>Aspartic acid</td>
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<td>Threonine</td>
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<td>Serine</td>
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<td>N-Acetylgalactosamine</td>
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<td>N-Acetylgalactosamine</td>
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<td>Sialic acid</td>
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The glycopeptides of similar, if not identical, molecular weight (data not shown). The separation was carried out on a sodium dodecyl sulfate gel consisting of a linear gradient of acrylamide (9 to 16%). A, glycopeptide F-1 (2 to 5) and glycopeptide F-2 (6 to 9) were treated as follows: without trypsin (2, 6) or with trypsin, 0.12 μg for 5 min at 25°C (3, 7), 0.25 μg for 5 min at 37°C (4, 8), or 0.5 μg for 10 min at 37°C (5, 9). For comparison, radiiodinated purified glycopeptide (see Fig. 11) (1) and the glycopeptide derived from [3H]fucose-labeled cells (see Fig. 4B) (10) also were electrophoresed. The latter peptide was digested in addition with 0.25 μg of trypsin for 5 min at 37°C (11). B, glycopeptide F-3 was treated under conditions similar to those just described: without trypsin (1) or with 0.25 μg of trypsin for 5 min at 37°C (2) or 0.5 μg of trypsin for 10 min at 37°C (3). For comparison, [3H]fucose-labeled glycopeptide without trypsin (4) or after incubation with 0.25 μg of trypsin for 5 min at 37°C (5) was also subjected to electrophoresis. BPB, bromphenol blue.
untreated peptide preparation, the number of bands observed into consideration the charge heterogeneity present in the digestion of a [3H]fucose-labeled glycopeptide with neuraminidase and subsequent analysis of the products on an isoelectric focusing gel results, in fact, in the appearance of about 23 discrete bands (Fig. 12A). Complete digestion yields one single band with an apparent isoelectric point of 4.6. The quantitative distribution of labeled bands at each digestion step, however, does not follow a normal distribution, indicating that some partial desialylated forms are less susceptible than others to further removal of additional sialic acid residues. Taking into consideration the charge heterogeneity present in the untreated peptide preparation, the number of bands observed in the neuraminidase experiment of Fig. 12 is in reasonable agreement with the number of sialic acid residues determined by compositional analysis (Table II).

The loss of 17 residues of sialic acid would reduce the molecular weight of the glycopeptide by 5,240. Although at the same time 17 negative charges are lost, a reduction of 5,000 in the apparent molecular weight could be observed by sodium dodecyl sulfate-gel electrophoresis (Fig. 12B). At this stage of digestion, the glycopeptide behaves as a homogeneous species in both isoelectric focusing gels and in dodecyl sulfate-polyacrylamide gels.

**DISCUSSION**

In previous communications (1, 23, 32, 34-36) from this laboratory, we have shown that the composition of the plasma membrane of rat hepatoma cells is complex with as many as 100 different polypeptides accessible in situ to labeling via external methods, including lactoperoxidase-catalyzed iodination and tritiated borohydride reduction after treatment of cells with galactose oxidase and neuraminidase. When the turnover of these externally oriented polypeptides was examined, we found that most of them had similar if not identical rates of degradation (1, 32). These results indicated that the major mechanism used by these cells to remove proteins from the plasma membrane most likely involved interiorization of units of membrane followed by fusion of the interiorized unit...
with a lysosome and degradation of the entire unit, both protein and glycolipid. We proposed (34, 35) and presented evidence for (36) a mode of membrane biogenesis whereby the cell synthesized, assembled, and delivered to the surface units of membrane having the same composition as those removed from the surface by interiorization and degradation.

However, not all surface proteins were degraded by the mechanism of unit interiorization-lysosomal degradation just described. Some externally oriented cell proteins, particularly glycoproteins, showed heterogeneous and much faster rates of degradation relative to the bulk of plasma membrane protein. A most conspicuous member of this class of surface proteins in HTC cells was an \( M_r = 85,000 \) plasma membrane glycoprotein which was externally oriented in that it could be labeled by both lactoperoxidase-catalyzed iodination or tritiated borohydride reduction after galactose oxidase treatment of intact viable cells. This glycoprotein was also very protease-sensitive and indeed a fragment similar in molecular weight to the fragment released from the cell surface by trypsin could be found in the medium of cultured cells. Hence, this membrane protein appeared to be removed from the cell surface by a cell-derived protease which is either secreted into serum-free medium or is still on the surface of the HTC cell and able to act on the \( M_r = 85,000 \) glycoprotein of the same or a different HTC cell. In the present paper, we have purified an \( M_r = 55,000 \) glycopeptide that is released from the surface of HTC cells by trypsin. We also present strong evidence that the source of this glycopeptide, which is the same glycopeptide as that released from the cell in culture as a normal consequence of membrane turnover, is indeed the \( M_r = 85,000 \) externally oriented glycoprotein of the plasma membrane. Most of the other membrane glycoproteins that can be labeled either metabolically with sugar or amino acid precursors or at the cell surface by the labeled borohydride-galactose oxidase procedure are not affected by trypsin. Hence, membrane glycoproteins appear more resistant to trypsin than do the bulk of the cell surface proteins labeled by lactoperoxidase-catalyzed iodination.

The carbohydrate composition of the purified glycoprotein is remarkably similar to that of carcinoembryonic antigen but other properties of the HTC cell glycopeptide and its parent glycoprotein suggest that they are not homologous to carcinoembryonic antigen (37).

The purified glycopeptide contains sialic acids and when the glycopeptide is subjected to isoelectric focusing it shows charge heterogeneity. Most and probably all of this heterogeneity can be eliminated by treatment with neuraminidase. After extensive treatment with neuraminidase, the purified glycopeptide is homogeneous by the criteria of electrophoretic mobility in dodecyl sulfate-polyacrylamide gels and isoelectric focusing gels. The cell-surface or secreted protease that is responsible for the normal turnover of the \( M_r = 85,000 \) membrane glycoprotein remains to be identified. Similarly, the function of the released \( M_r = 55,000 \) glycopeptide and its parent \( M_r = 85,000 \) membrane glycoprotein in HTC cells remains to be elucidated. However, we have presented evidence suggesting that the \( M_r = 85,000 \) glycopeptide can exist as a dimer in the presence of calcium (Figs. 9 and 10). HTC cells can also grow as monolayer cultures and calcium is required for attachment. Actually, an \( M_r = 120,000 \) multimeric form of this protein can be identified on dodecyl sulfate-polyacrylamide gels when and only when the cells are grown as monolayer cultures. This form may represent one intact \( M_r = 85,000 \) polypeptide calcium linked with another homologous polypeptide but from which the \( M_r = 55,000 \) glycopeptide fragment has been cleaved. The properties of the \( M_r = 85,000 \) membrane glycoprotein just described suggest a role for this membrane protein in cell-cell aggregation and experiments are currently in progress to determine if this glycoprotein or its tryptic fragment are involved in normal cell-cell aggregation, or in the growth of these cells in vivo as solid tumors, or in the aggregation of cells brought about by trypsin treatment itself.

Finally, the unequivocal assignment of the \( M_r = 55,000 \) trypsin glycopeptide fragment to its \( M_r = 85,000 \) parent glycoprotein in the membrane as well as the limited sensitivity of the other membrane glycoproteins to trypsin provides us with a system to examine the intracellular route of biogenesis and the kinetics of replacement of a specific protein in the plasma membrane or at the surface of the cell. In this regard, it should be mentioned that the \( M_r = 85,000 \) glycoprotein is not confined exclusively to the plasma membrane of these cells. Like most externally oriented cell glycoproteins, it is also present as part of an intracellular membrane system which is identical or nearly identical in composition to the plasma membrane (36).

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