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

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

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Concentrations of trypsin that bring about aggregation of hepatoma tissue culture (HTC) cells also release from the cell surface an M, = 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 in situ by lactoperoxidase-catalyzed radiiodination or by initiated 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, = 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, = 55,000 glycopeptide was confirmed by showing that the same M, = 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, proteolysis of externally exposed proteins is routinely used for dissociating tissues and tissue culture cell monolayers to harvest individual cells for subculturing. The treatment of cells in situ with proteases can bring about diverse biological effects such as alterations in cell shape (2-4), increased cell growth rate (5-8), cell-cell aggregation (9-11), etc. Some of these latter responses appear to be a general response of the cell to perturbation and not a result of the specific action of a protease. In some cases, however, the loss of distinct surface proteins can be correlated with a change in growth behavior (10, 12, 13). Related to these effects of proteases on growth control is the finding that transformed cells appear to secrete more cellular proteases than do "normal" cells (14-16) and the consistent finding that the surface of transformed cells is different in glycopeptide composition than "normal" cells (17-21). Hence, it is possible that cells have the capability to modify their own surface and in this way carry out a type of growth control.

Many recent studies have shown differences between "normal" and transformed cells in the type of acid-soluble fragments released from the surface of intact cells after extensive digestion with proteases (20, 22). However, in most of these studies, the total spectrum of protease-sensitive surface peptides, usually glycopeptides, was analyzed. Actually, little attention has been given to the analyses of specific membrane proteins or glycoproteins which are normal substrates for 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, = 55,000 glycopeptide fragment from an M, = 85,000 membrane glycoprotein. The M, = 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-β-N-acetylglucosaminidases D and H and a mixture of exoglycosidases of Turbo cornutus, containing the following enzymes in units/g of protein: α-mannosidase, 166; β-mannosidase, 186; α-glucosidase, 3.2; β-glucosidase, 20; α-galactosidase, 24; β-galactosidase, 125; α-L-fucosidase, 58; β-xylosidase, 12; α-N-acetylgalactosaminidase, 2.5; β-N-acetylglucosaminidase, 383; α-N-acetylgalactosaminidase, 46; and β-N-acetylgalactosaminidase, 30, were purchased from Seikagaku Kogyo, Tokyo; neuraminidases of Vibrio cholerae were from Calbiochem, that of Clostridium perfringens (grade V) from Sigma; and trypsin (tosylphenylalanincyloromethyl ketone-treated, 276 units/mg) from Worthington. Galactose oxidase (9000

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

Figs. 5 and 7). The glycoprotein-containing fractions were dialyzed at gradient of sodium phosphate (details are described in the legends to pH 6.8, containing 0.05% sodium dodecyl sulfate. The glycoprotein precipitated with 10% trichloroacetic acid. The precipitate was washed twice with ethanol and dissolved in 10 mM sodium phosphate.

The amount of isotopes incorporated into proteins was determined after precipitation with 10% trichloroacetic acid and extraction with ethanol, either by counting directly in a Biogamma counter (Beckman), or, in cases of tritium, after solubilization in NCS solubilizer (Amersham) by liquid scintillation counting in a Beckman spectrometer.

In order to degrade isolated glycoproteins with trypsin in vitro, even trace amounts of sodium dodecyl sulfate had to be removed. This was achieved either by precipitation of trypsin with trichloroacetic acid and extraction with ethanol at -20°C or by precipitation and extraction with acidified acetone at -20°C. The glycoproteins then were 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 phenylmethylsulfonyl fluoride and 2 mM EDTA. The pH of 6.8 was adjusted by addition of sodium phosphate buffer, pH 6.8, containing 0.05% sodium dodecyl sulfate. The eluates were dialyzed for 24 h against 50 mM Tris-HCl, pH 7.2, containing 0.5% sodium dodecyl sulfate. For determination of the glycoprotein profile, aliquots of the eluates were counted in a liquid scintillation spectrometer. Fractions of interest were pooled, dialyzed for 24 h at 4°C against distilled water, and then lyophilized. Preparative separations of glycoproteins based on size were done in 1.5-mm-thick slabs of 7.5% sodium dodecyl sulfate-polyacrylamide gels. Protein bands were cut out according to their relative migrations and were eluted by shaking for 48 h in 10 ml of 50 mM Tris-HCl, pH 7.8, containing 0.5% sodium dodecyl sulfate. The eluates were dialyzed for 24 h at 4°C against distilled water and then lyophilized.

RESULTS

Effect of Treatment with Trypsin on the Composition of the HTCC Cell Surface—When HTCC 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 included in the aggregates and remain as individual cells in suspension. This ratio of aggregated to nonaggregated cells is not altered even when the cell density ranges between 5 x 10^4 and 5 x 10^5/ml of treated suspension. The nonaggregated cells, however, are not an aggregation-resistant subpopulation in the HTCC cell culture, because when the nonaggregated cells are cultured for an additional 24 h, aggregation again occurs in response to trypsin. Again, 20 to 30% of the cells are excluded from the aggregates. The extent of aggregation was also not affected when either Ca^{2+} (2 mM), ethylenediaminetetraacetate, or ethylene glycol bis(β-aminoethyl ether) 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 of 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 μg/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/NaB[3H]H₄ procedure was achieved by incubating cells with trypsin at a concentration of 10 μg/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 tryptic 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).

As 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[3H]H₄ procedure or from cells labeled metabolically with [3H]fucose or [3H]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 10^5) were labeled at the surface with NaB[3H]H_4 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 (trypsic glycopeptides) were subjected, together with the solubilized cells, 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 NaB[3H]H_4 after neuraminidase and galactose oxidase treatment, before (1) and after (2) exposure to trypsin. The corresponding fractions 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 hydroxyapatite (7). Lanes 1 and 2 contained 30,000 acid-insoluble cpm, Lanes 4 to 7 contained 50,000 cpm. B, cells labeled metabolically with [3H]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 hydroxyapatite (4) (see Fig. 5) and compared with [3H]fucose-labeled glycopeptide (5). All lanes contained 50,000 acid-insoluble cpm. BPB, bromphenol blue.
The glycopeptides derived from trypsin-treated cells, which were labeled metabolically either with \([3H]\)fucose or \([3H]\)leucine, 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, 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 I

**Isolation of the glycopeptide released by trypsin from sugar and amino acid-labeled HTC cells**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Neuraminidase, galactosidase oxidase (NaB[3H]H4)</th>
<th>([3H])Fucose</th>
<th>([3H])Leucine</th>
</tr>
</thead>
<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 g 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 \([3H]\)fucose or \([3H]\)leucine, were purified by concanavalin A-Sepharose chromatography. The samples were each applied to a column (0.6 × 15 cm) of hydroxyapatite and the adsorbed material eluted in fractions of 1.76 ml by a linear gradient consisting of 0 to 0.3 M sodium chloride, each of 10 mM sodium phosphate buffer, pH 6.8, plus 0.05% sodium dodecyl sulfate. Aliquots of 0.5 ml \([3H]\)fucose label or 40 \(\mu l\) \([3H]\)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 × 10⁷) were treated with trypsin (5 \(\mu g/ml\)) 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 × 15 cm) of diethylaminoethyl 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 applied to a column (1 × 15 cm) of diethylaminoethyl 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.
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-1 was subjected to partial digestion with trypsin. The electrophoretic separation of the trypsin digest reveals a band at Mr = 55,000 as well as bands at Mr = 35,000 and 25,000 (Fig. 8). Fragments of Mr = 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 Mr = 18,000 and 19,000 are observed also in the trypsin digest of the Mr = 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 artificially 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 <3.9, <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 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 glycopeptide G-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 10⁶), 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 purified glycoprotein $F_1$ now could also be converted by treatment with chelating agent into $F_1$ to increase the amount of glycoprotein $F_1$ in polyacrylamide gel. The eluted proteins were treated with 5 mM CaCl$_2$ to sugar-labeled and sodium dodecyl sulfate-polyacrylamide gels in the presence of 5 mM CaCl$_2$. This protein treatment as glycoprotein $nF_2$ or $nF_3$. However, after subsequent treatment with the calcium chelating agent ethylene glycol bis(&aminoethyl ether)N,N'-tetraacetate, the M, = 175,000 glycoprotein decreases in intensity while the M, = 85,000 glycoprotein 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 purified as follows: the two acid components $F_1$ and $F_2$ in fucose-labeled HTC cells. It should be mentioned, however, that the tryptic glycopeptide with $M_r = 55,000$ containing large amounts of sialic acid residues (see below), does not dimerize even in the presence of 10 mM Ca$^{2+}$. 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

### Table II

Amino acid and carbohydrate composition of the tryptic glycopeptide

<table>
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<tr>
<th>Residue</th>
<th>Residues per $M_r = 55,000$ peptide</th>
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<tr>
<td>Amino acids</td>
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<tr>
<td>Aspartic acid</td>
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<td>Threonine</td>
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<td>Glutamic acid</td>
<td>32.2</td>
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<td>Proline</td>
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<td>Glycine</td>
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<tr>
<td>Cysteine</td>
<td>Trace</td>
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<td>Methionine</td>
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<table>
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<th>Carbohydrates</th>
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<tr>
<td>Glucose</td>
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<tr>
<td>Galactose</td>
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<td>Mannose</td>
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<tr>
<td>N-Acetylgalactosamine</td>
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<tr>
<td>N-Acetylglucosamine</td>
<td>9.7</td>
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<td>Sialic acid</td>
<td>17.1</td>
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from intact HTC cells not treated with neuraminidase appears
after dodecyl sulfate-polyacrylamide gel electrophoresis as a
broad band with an average apparent molecular weight of
55,000 (Fig. 6). Identical values for the apparent molecular
weight were obtained when the peptide was electrophoresed
on gels with higher percentages of acrylamide or on gels
containing 8 M urea (data not shown). The glycopeptide
released from cells which were first treated with neuramini-
dase prior to treatment with trypsin migrates at the same
position (Fig. 4). The amino acid and carbohydrate composi-
tion of the nonmodified glycopeptide is shown in Table II.

The compositional analyses in Table II indicate that car-
bohydrates represent about 38 weight % of the peptide or that
the molecular weight of the carbohydrate-free polypeptide
should be about 33,000. Because of the finding that digestion
of glycoproteins by endoglycosidases can be used in deter-
mining the molecular weight of the carbohydrate-free protein
moiety (33), we examined the endoglycosidase sensitivity of
in vitro radioiodinated glycopeptide. However, as shown in
Fig. 11, the molecular weight did not change appreciably after
treatment with endo-β-N-acetylglucosaminidase D and only
slightly after treatment with endo-β-N-acetylglucosaminidase
H. Digestion of the glycopeptide with a combination of mixed
exoglycosidases, neuraminidase, and both endo-β-N-acetyl-
glucoaminidases yielded a maximal change in molecular weight
from 55,000 to 43,000. A similar treatment of the glycopeptide,
but in the presence of 0.075% sodium dodecyl sulfate, caused
the complete destruction of the glycopeptide, probably due to
the action of proteolytic enzymes contaminating one or more
of the glycosidase preparations. Even after maximal deglyco-
sylation with various glycosidases, the peptide still retained a
low affinity for concanavalin A. Surprisingly, the fraction
bound, as well as that nonbound, to the lectin contains gly-
copeptide of similar, if not identical, molecular weight (data
now shown).

Membrane glycoproteins of HTC cells consist of families of
comparison, radioiodinated 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, glycoprotein
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 electropho-
resis. BPB, bromphenol blue.

Fig. 10. Tryptic mapping of glycoproteins F-1, F-2, and F-3. The
glycoproteins F-1, F-2, and F-3, isolated from Peak I resulting from
hydroxyapatite chromatography (see Figs. 5 and 9), were treated
similarly to the glycoprotein G-1 used in Fig. 8. Each sample contained
50,000 cpm and was dissolved in 50 μl of 0.2 M ammonium bicarbonate.
The separation was carried out on a sodium dodecyl sulfate gel
consisting of a linear gradient of acrylamide (9 to 16%). A, glycoprotein
F-1 (2 to 5) and glycoprotein 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

Fig. 11. Influence of glycosidases on the electrophoretic mobility
of the tryptic glycopeptide. Tryptic glycopeptide, derived from 5 × 10^6
HTC cells, was purified by concanavalin A-Sepharose chromato-
graphy and iodinated with chloramine-T and 1 mCi of 125I (12). After
chromatography over Sephadex G-25, the labeled glycopeptide was
purified further by hydroxyapatite chromatography as described in
Fig. 5. The glycopeptide was precipitated by acidified acetone and
the precipitate was washed twice with acetone. Aliquots containing 1 × 10^6
cpm (about 1 μg) were dissolved in 30 μl of 50 mM sodium
citrate, pH 5.5. To some of the samples, 0.1% sodium dodecyl sulfate
and 0.1 M 2-mercaptoethanol was added and the mixtures were boiled
for 2 min. After incubation at 37°C for 24 h, 30 μl of the sample was added
to the mixture. The enzymes were dissolved in the same sodium citrate
buffer and 10 μl of each enzyme solution was used. After incubation
for 2 min, the mixture was boiled for 2 min. Aliquots, one-fifth of the sample, were
electrophoresed on a 12% sodium dodecyl sulfate polyacrylamide gel and
the autoradiograph after a 1-day exposure is shown. Lanes represent the
following incubations: 1 and 10, no enzyme; 2, 1 milliunit of endo-β-
N-acetylglucosaminidase D; 3, 1 milliunit of endo-β-N-acetyl-
glucoaminidase H; 4, 1 milliunit of both endoglycosidases D and H, 5, as in
4 but in the presence of sodium dodecyl sulfate; 6, 0.1 mg of mixed
exoglycosidases plus 10 milliunits of neuraminidase; 7, as in 6 but in the presence of sodium dodecyl sulfate; 8, 1
milliunit of both endo-β-N-acetylglucosaminidases D and H plus 0.1
mg of mixed exoglycosidases plus 10 milliunits of neuraminidase; 9, as
in 8, but in the presence of sodium dodecyl sulfate. BPB, bromphenol
blue.
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 Mₐ = 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 Mₐ = 85,000 glycoprotein of the same or a different HTC cell. In the present paper, we have purified an Mₐ = 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 Mₐ = 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 Mₐ = 85,000 membrane glycoprotein remains to be identified. Similarly, the function of the released Mₐ = 55,000 glycopeptide and its parent Mₐ = 85,000 membrane glycoprotein in HTC cells remains to be elucidated. However, we have presented evidence suggesting that the Mₐ = 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 Mₐ = 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 Mₐ = 85,000 polypeptide calcium linked with another homologous polypeptide but from which the Mₐ = 55,000 glycopeptide fragment has been cleaved. The properties of the Mₐ = 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 Mₐ = 55,000 tryptic glycopeptide fragment to its Mₐ = 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 Mₐ = 85,000 glycoprotein is not confined exclusively to the plasma membrane of those 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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