Concentrations of trypsin that bring about degradation 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 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 purified glycopeptide contains an average of 17 sialic acid residues and hence, shows charge heterogeneity after electrophoresis in isoelectric focusing gels. 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, 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 $M_r = 55,000$ 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-$b$-$N$-acytetylgalacosaminidases D and H and a mixture of exoglycosidases of *Turbo coruscus*, containing the following enzymes in units/g of protein: $a$-mannosidase, 166; $b$-mannosidase, 196; $a$-glucosidase, 3.2; $b$-glucosidase, 20; $a$-galactosidase, 24; $b$-galactosidase, 125; $a$-$L$-fucosidase, 58; $b$-xylosidase, 12; $a$-$N$-acytetylgalacosaminidase, 2.5; $b$-$N$-acytetylgalacosaminidase, 383; $a$-$N$-acytetylgalactosaminidase, 46; and $b$-$N$-acytetylgalactosaminidase, 30, were purchased from Seikagaku Kogyo, Tokyo; neuraminidases of *Vibrio cholerae* were from Calbiochem, that of *Clostridium perfringens* (grade VI) from Sigma; and trypsin (tosylphenylalanylchloromethyl ketone-treated, 276 units/mg) from Worthington. Galactose oxidase (3000 U/g) was from Sigma; and trypsin (tosylphenylalanylchloromethyl ketone-treated, 276 units/mg) from Worthington. Galactose oxidase (3000 U/g) was from Sigma.
units/mg) was a generous gift of Dr. M. Ettinger, State University of New York at Buffalo.

Cells—A cloned cell line of hepatoma tissue culture cells (HTC cells) was grown in suspension culture in Eagle's minimal essential medium containing 10% fetal calf serum (1).

Radioactive Labeling The cell surface proteins were enzymatically radioiodinated with 125I as described previously (23). For radioiodination of glycoproteins with chloramine-T, the method of Hunter and Greenwood (24) was followed. The externally disposed glycoproteins on the cell surface were labeled by sodium borohydride reduction of neuraminidase and galactose oxidase-treated cells according to the method of Gahlenberg and Hakomori (25) with sodium thioglycollate (1).

The glycoprotein-containing fractions were dialyzed at 4°C against several changes of distilled water and then lyophilized.

Preparation of glycopeptides was carried out in tube gels (4-mm diameter) composed of 0.5% (w/v) NP-40, 0.6% (w/v) NP-40, and 0.6% (w/v) each of the following Ampholines (LKB), pH 2.5 to 4, pH 4 to 6, and pH 3.5 to 10. The samples were dissolved immediately before application onto the gels in 9 M urea containing 2% NP-40, 2% mercaptoethanol, and 2% Ampholine, pH 3.5 to 10. The isoelectric focusing occurred for 16 h at 500 V and one additional hour at 100 V. The gels were cut in 5-mm slices and each slice was eluted for 24 h under shaking in 9 ml of 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 glycopeptides based on size were done in 1.5-mm-thick slabs of 1.6% sodium dodecyl sulfate-polyacrylamide gels. Protein bands were cut out according to their relative migrations and were eluted by shaking for 48 h in 1 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.

Isolation of Glycoproteins—The cell-surface proteins were enzymatically released by trypsin treatment to ensure the complete removal of serum proteins. Then, the cells were dispersed in phosphate-buffered saline at a concentration of 5 x 10^6 cells/ml and were incubated in the presence of trypsin under conditions described in the legends to Figs. 5 and 7. The glycoprotein-containing fractions were dialyzed first against several changes of 10 mM sodium phosphate buffer, pH 6.8, containing 0.05% (w/v) sodium deoxycholate, 10 mM Tris-HCl, pH 8.0 (deoxycholate/Tris buffer) in twice the volume used for initial homogenization. The glycoproteins, present either in the trypsin-released fraction or in deoxycholate/Tris buffer, 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 initial homogenization. The extract was centrifuged for 45 min at 200,000 x g. The glycoproteins, present either in the trypsin-released fraction of intact cells or in deoxycholate extracts of the cell membrane fraction, were bound to columns (1 x 5 cm) of concanavalin A-Sepharose (Pharmacia). The nonbound 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 mM a-methyl mannoside in deoxycholate/Tris buffer for 16 h. When further fractionation was carried out, the glycoprotein-containing fractions were dialyzed first against several changes of 10 mM Tris-HCl, pH 8.0, for 48 h and then against 10 mM sodium phosphate buffer, pH 6.8, containing 0.05% (w/v) sodium deoxycholate. The tryptic modification of cell surface proteins was carried out in tube gels (4-mm diameter) composed of 0.5% (w/v) NP-40, 0.6% (w/v) sodium deoxycholate, and 10 mM Tris-HCl, pH 8.0 (deoxycholate/Tris buffer) in twice the volume used for initial homogenization. The precipitate was washed twice with ethanol and dissolved in 10 mM sodium phosphate, pH 6.8, containing 0.05% sodium deoxycholate. The precipitate in 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, pH 6.8, containing 0.05% (w/v) sodium deoxycholate. The adsorbed glycoproteins were eluted with a linear gradient of sodium phosphate (details are described in the legends to Figs. 5 and 7). The glycoprotein-containing fractions were dialyzed at 4°C against several changes of distilled water and then lyophilized.

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 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 10^6/ml of treated suspension. The nonaggregated cells, however, are not an aggregation-resistant subpopulation in the HTC 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 mM), ethylenediamine-tetraacetate, or ethylene glycol bis(β-aminoethyl ether) 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 pg/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]4 procedure was achieved by incubating cells with trypsin at a concentration of 10 pg/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 $p$1 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 $p$1 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 \(3 \times 10^8\) were labeled at the surface with \(\text{NaB}^{[3H]}\text{H}_4\) 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, \(5 \mu\text{g} / \text{ml}\), for 10 min at 37°C. The cells were collected by centrifugation for 5 min at 400 \(\times \text{g}\) and the supernatant fraction was centrifuged for 60 min at 200,000 \(\times \text{g}\). Aliquots of both the untreated and treated cells, each containing 100,000 acid-insoluble cpm, were dissolved in 10 \(\mu\text{l}\) 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 (tryptic 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 \(\text{NaB}^{[3H]}\text{H}_4\) 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 \(\alpha\)-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 \([\text{H}]\text{fucose, before (1)}\) 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 \(\alpha\)-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 \([\text{H}]\text{leucine (1)}\) were treated with trypsin. The released peptides (2) were chromatographed over concanavalin A-Sepharose. After elution of the bound fraction by \(\alpha\)-methylmannoside (3), the glycopeptide was chromatographed over hydroxypatite (4) (see Fig. 5) and compared with \([\text{H}]\text{fucose-labeled glycopeptide (5). All lanes contained 50,000 acid-insoluble cpm. BPB, bromphenol blue.}
labeled either enzymatically at the surface by NaB\[^{3H}\]H\(_4\) reduction after treatment of the cells with neuraminidase and galactose oxidase \[^{3H}\]fucose or \[^{3H}\]leucine (5 \(\mu\)Ci) 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 \(\alpha\)-methylmannoside was treated with 10% trichloroacetic acid. The precipitates were washed twice with ethanol, dissolved in 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>
<thead>
<tr>
<th>Fraction</th>
<th>Neuraminidase, [^{3H}]fucose</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 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 \[^{3H}\]leucine or \[^{3H}\]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 6.5 \(\mu\)l \[^{3H}\]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 x 10\(^6\)) 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 x 16 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 diazylized 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.

sulfate (Fig. 5). This step removed most, if not all, of the other materials contaminating the \(M_r = 55,000\) glycopeptide (Fig. 4C). The purity of the isolated peptide after hydroxylapatite chromatography was judged not only by radioactivity but also by protein staining after separation by dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 6). Chromatography on concanavalin A-Sepharose results in a considerable contamination of the glycopeptide fraction with lectin, which leaks off the Sepharose support. Attempts to separate concanavalin A from the glycopeptide by chromatography over diethylaminoethylcellulose failed, although several other contaminating proteins could be removed. However, hydroxylapatite chromatography did separate concanavalin A from the cell glycopeptide (see Fig. 6).

Origin of the Tryptic Glycopeptide—Before proceeding to a chemical analysis of the tryptic glycopeptide, we attempted to identify the glycoprotein in the membrane which is the parent of the tryptic glycopeptide. Results presented in Figs. 3 and 4A and the relatively large amount of radioactivity (Table I) recovered in the \(M_r = 55,000\) glycopeptide suggest that this glycopeptide derives from the major trypsin-sensitive galactose-labeled glycoprotein of the cell with \(M_r = 85,000\) and pl of 4.2 to 4.9 (G-1 in Fig. 3). To prove this more rigorously, glycoprotein G-1 was first purified from HTC cells and then was reacted in vitro with trypsin. For this purpose, the glycoproteins from cells labeled at the surface by the neuraminidase, galactose oxidase, and NaB\[^{3H}\]H\(_4\) procedure were separated from bulk cell proteins by concanavalin A-Sepharose chromatography. The glycoprotein fraction was then subjected to chromatography over hydroxyapatite (Fig.
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-1 was subjected to partial digestion with trypsin. The electrophoretic separation of the trypic digest reveals a band at \( M_r = 55,000 \) as well as bands at \( M_r = 35,000 \) and 25,000 (Fig. 8). Fragments of \( M_r = 35,000 \) 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 \( M_r = 18,000 \) and 19,000 are observed also in the trypsin digest of the \( M_r = 55,000 \) 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\(^{3H}\)H\(_4\). 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 \textit{in vitro} with neuraminidase under similar conditions as used for \textit{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 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.
The disappearance of spot F-l upon neuraminidase treatment can be explained by either degradation by a protease contaminating the neuraminidase or by a breakdown of this \( M_r = 175,000 \) protein into subunits appearing after neuraminidase treatment.
**Tryptic Modification of Cell Surface Proteins**

The glycopeptide released by trypsin from two batches of $4 \times 10^9$ HTC cells, was purified by concanavalin A-Sepharose and hydroxypatite chromatography. Each of the two preparations showed the same degree of purity after electrophoresis on polyacrylamide gels as illustrated in Fig. 6. Lyophilized aliquots of each preparation were used for amino acid and carbohydrate analysis. The values represent the averages of single determinations of the two preparations.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Residues per $M_r = 55,000$ peptide</th>
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<tbody>
<tr>
<td>Amino acids</td>
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<tr>
<td>Aspartic acid</td>
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<tr>
<td>Threonine</td>
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<td>Serine</td>
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<td>Cysteine</td>
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<table>
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<tr>
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<td>4.7</td>
</tr>
<tr>
<td>Galactose</td>
<td>27.5</td>
</tr>
<tr>
<td>Mannose</td>
<td>25.1</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>21.9</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>9.7</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>17.1</td>
</tr>
</tbody>
</table>

**Fig. 9.** Purification of $[^3]H$fucose-labeled glycoproteins. Peak I from hydroxypatite chromatography of $[^3]H$fucose-labeled glycoproteins (see Fig. 5) was analyzed by two-dimensional gel electrophoresis (A): two aliquots each containing 100,000 cpm were dissolved in 20 μl of 50 mM Tris-HCl buffer, pH 6.9, and incubated either in the absence or presence of neuraminidase (Vibrio cholerae, 0.5 unit) for 15 min at 37°C. Glycoprotein F-1 was isolated by preparative isoelectric focusing followed by preparative electrophoresis on 7.5% sodium dodecyl sulfate-polyacrylamide gels in the presence of 5 mM CaCl₂. Two aliquots of the purified glycoprotein F-1 (75,000 cpm) were treated as described above prior to the two-dimensional separation (B). Another aliquot of purified glycoprotein F-1 (100,000 cpm) was dissolved in 40 μl of 10 mM Tris-HCl, pH 7.6, containing 5 mM ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetate. Then, after addition of 20 μl of 100 mM Tris-HCl, pH 6.7, the sample was divided into two parts and incubated for 15 min at 37°C, one part in the presence of 0.5 unit of neuraminidase (C).

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 were then 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 $M_r = 85,000$ and pI 4.2 to 4.7 (Fig. 9B and C). This experiment (Fig. 9B) shows in addition that similar glycoprotein spots appear in the position where G-2 migrates in Fig. 7B, suggesting that G-2 represents by-products of the desialylation of glycoprotein F-1. The glycoproteins F-1 and F-2 after the final isolation step were also subjected to partial hydrolysis with trypsin and the proteolytic fragments produced were compared by electrophoresis in dodecyl sulfate-acylamide gels with a glycopeptide derived from in vivo-labeled cells (Fig. 10A). Both glycoproteins 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 glycoprotein F-1, however, a glycoprotein band at $M_r = 120,000$ appears. This form could be due to a removal of one $M_r = 55,000$ portion from a dimer of $M_r = 175,000$ while the remaining parts of the dimer stay associated. In fact, this partially degraded form of glycoprotein F-1 ($M_r = 120,000$) 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 $M_r = 55,000$ 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 glycopeptide 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-apolysaccharide cell extracts or to concanavalin A-bound glycoproteins causes a strong enhancement of the band at $M_r = 175,000$ (F-1) with a concomitant decrease in intensity of the $M_r = 85,000$ protein. However, subsequent treatment with the calcium chelating agent ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetate, the $M_r = 175,000$ glycoprotein decreases in intensity while the $M_r = 85,000$ 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 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 were then separated from each other by preparative gel electrophoresis. The purified glycoprotein F-1 now could also be converted by treatment with chelating agent into...
bound, as well as that nonbound, to the lectin contain glycopeptide 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, 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, 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.

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, 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.

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 neuraminidase prior to treatment with trypsin migrates at the same position (Fig. 4). The amino acid and carbohydrate composition of the nonmodified glycopeptide is shown in Table II.

The compositional analyses in Table II indicate that carbohydrates 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 useful in determining 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-acetylgalactosaminidase D and only slightly after treatment with endo-β-N-acetylgalactosaminidase H. Digestion of the glycopeptide with a combination of mixed exoglycosidases, neuraminidase, and both endo-β-N-acetylgalactosaminidases yielded a maximal change in molecular weight from 55,000 to 43,000. A similar treatment of the glycopeptide, slightly after treatment with endo-β-N-acetylgalactosaminidase D and only 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 deglycosylation 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 glycopeptide of similar, if not identical, molecular weight (data not shown).

Membrane glycoproteins of HTC cells consist of families of...
untreated peptide preparation, the number of bands observed
to further removal of additional sialic acid residues. Taking
into consideration the charge heterogeneity present in the
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 quantita-
some partial desialylated forms are less susceptible than oth-
however, does not follow a normal distribution, indicating that
on the sialic acid analysis in Table II. Gradual, limited diges-
to the loss of 1 residue of sialic acid, the
focusing gels is due to the loss of 0.5 ml of distilled and boiled water. After 4 h, the pH
was determined. Lane 10 indicates the positions of all bands observed
fluorogram. B, in vitro radioiodinated glycopeptide from the
experiments described in Fig. 11 was analyzed. Aliquots containing
200,000 cpm or 0.2 µg of glycopeptide were dissolved and treated as
the [3H]fucose-labeled glycopeptide above. The samples were incu-
ated with: 1, no neuraminidase; 2, 0.1 unit; 3, 0.5 unit; and 4, 2.5
units for either 30 min (2 and 3) or 24 h (1 and 4) at 37°C. The
electrophoretic separation occurred on a 12% sodium dodecyl sulfate
polyacrylamide gel. BPB, bromphenol blue.

**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
electrophoresis. This intercellular glycoprotein is not under
formulae 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.
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 glycopeptide is remarkably similar to that of carcinoembryonic antigen but other properties of the HTC cell glycoprotein 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 criterion 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 $M_r = 55,000$ tryptic 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 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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Tryptic Modification of Cell Surface Proteins

**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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