Preferential Degradation of the Terminal Carbohydrate Moiety of Membrane Glycoproteins in Rat Hepatoma Cells and after Transfer to the Membranes of Mouse Fibroblasts

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ABSTRACT Glycoproteins in the plasma membrane of rat hepatoma cells were labeled at their externally exposed tyrosine residues with $^{131}$I and at their galactose and sialic acid residues with $^3$H. The degradation of both isotopes in the total cell protein fraction, in glycoproteins purified by concanavalin A, and in glycoproteins separated on two-dimensional gels was determined. Similarly, the total cellular membrane glycoproteins were metabolically labeled with $[^35]$S methionine and $[^3]$H fucose. The fate of both incorporated labels was followed by lectin chromatography or by precipitation of the proteins with specific antibodies followed by electrophoretic gel separation. In both labeling experiments, the carbohydrate markers were lost from the ligand-recognized fraction with similar kinetics as from the total cell protein fraction. In some glycoprotein species which were separated by two-dimensional gel electrophoresis, the polypeptide portion exhibited up to a twofold slower rate of degradation relative to that of the carbohydrate moiety. This difference is most pronounced in carbohydrate-rich glycoproteins. To corroborate this finding, double-labeled membrane glycoproteins were incorporated into reconstituted phospholipid vesicles which were then transferred via fusion into the plasma membrane of mouse fibroblasts. Both the polypeptide and carbohydrate moieties of the transferred membrane glycoproteins were degraded with the same relative kinetics as in the original hepatoma cells. The rate of degradation is mostly a function of the structural properties of the membrane components as shown by the preservation of metabolically stable fucogangliosides of Reuber H-35 hepatoma cells transferred onto the fibroblasts. The technique of insertion of membrane components into the plasma membrane of another cell should assist in the elucidation of the exact route and mechanism of membrane protein destruction.

In a previous study, we demonstrated that glycoproteins of rat hepatoma cells labeled in situ with galactose oxidase and $[^3]$H NaBH₄ are degraded in a biphasic manner (4). During the first phase, which we define as the time period from 0 to 48 h after introduction of the labeled precursor, two-thirds of the label is lost from the protein fraction, yielding a half-life of about 20 h. Subsequently, in the second phase, after 48 or more hours, the degradation rate decreases, yielding a half-life of about 75 h or longer. Identical turnover was found for the externally oriented plasma membrane proteins of macrophages which were marked by in situ attachment of an antigenic hapten group (20). The kinetics of degradation of membrane glycoproteins in hepatoma cells is markedly different from that of the majority of the cell surface proteins accessible to iodination with lactoperoxidase and $[^13]$I. This class of externally oriented proteins represents mainly non- or minimally glycosylated molecules (5, 14), which turn over with a constant long half-life of about 70-100 h (4, 28).

One-dimensional electrophoretic analysis at selected times after labeling at the cell surface revealed that the radioactivity was lost uniformly from most of the membrane proteins and glycoproteins (4, 20). These findings and similar results in other systems strongly suggest that the plasma membrane of mAhalian cells is turned over as a unit, probably via interiorization and fusion of the interiorized unit with a lysosome (12, 19). However, an important question remains to be answered: Is the entire molecule of any membrane glycoprotein destroyed at one time or is the molecule subject to step-wise degradation.
were treated for 30 rain at 37°C with neuraminidase glycoproteins (2) or to release surface adsorbed and labeled medium proteins. Then, after an additional wash, HTC cells were suspended at a density of 0.5 x 10^6 cells/ml in phosphate-buffered viability of the cells, when periodate concentrations higher than those indicated (4), and then suspended at a density of 5 x 10^6 cells/ml in phosphate-buffered saline (PBS) containing 5 mM sodium metaperiodate. After a 15-min incubation at 0°C in the dark, the cells were washed three times with PBS. The oxidized products of the sialic acid residues were subsequently reduced by reaction of the cells for 15 min at 0°C with [3H]NaBH₄ (6-9 Ci/mmole), using 1 μCi/ml of cell suspension (5 x 10^6 cells/ml). Although this labeling procedure did not affect the viability of the cells, the subsequent periodate concentrations higher than those indicated were used the long-term viability of the cells was drastically reduced. The specific tritium incorporation into the cell proteins was relatively low, ranging from 1 to 3 x 10^3 cpm/mg of total cell protein. When, immediately after labeling, the cells were treated for 30 min at 37°C with neuramidase (Fixobilia chelites, 0.02 IU per 5 x 10^6 cells in 1 ml PBS), 60-80% of the tritium was specifically removed from the cell protein fraction. In double-labeling experiments, the lactoperoxidase treatment preceded the galactosidase enzyme treatment; but in the case of periodate oxidations, the labeling of the carbohydrate moieties was performed before the iodination. This was done so that the more difficult treatment of the two was first, thus allowing assessment of the cell viability during the course of labeling. Metabolic labeling of HTC cells was carried out by incubation of cells (7 x 10^5/ml) for 24-72 h in medium containing either L-[6-3H]fucose (16.2 Ci/mmole) or the combination of L-[6-3H]fucose and or-p-toluenesulfonyl phenylmethylsulfonyl fluoride (PMSF) at a constant cell density of 5 x 10^6/ml. The lysates were cleared by centrifugation for 10 min at 2,000 g, followed by 60 min at 200,000 g. The supernatant fractions were passed in tandem through a Sepharose CL-4B column (0.5 ml) and an immunoglobulin-containing column. The proportions of cell extract to immunoadsorbant were maintained in the linear range of binding. Then, the immunocolumns were washed with 50-100 vol of 10 mM sodium phosphate pH 7.6 containing 1 M NaCl, 1% NP-40, and 0.1 mM EDTA, distilled water, 0.1 M NaCl, and 1 M NaCl-guanidine- HCl buffer. After a brief rinse of the columns were eluted with 6 M guanidine-HCl in 100 mM glycine-HCl, pH 2.5, 1% NP-40 and 0.1 mM PMSF. After adjusting the pH to 7.4, the eluted fractions were dialyzed against 0.1 M NH HCO₃ and lyophilized. Comparative chromatography of cell lysates showed that columns containing immobilized preimmune globulins bound <2% of the radioactivity retained by columns with immune globulins. Purification of Glycoproteins and Glycolipids: The total membrane glycoproteins of HTC cells used for electrophoresis and in the transfer studies were prepared by chromatography on concanavalin A-Sepharose exactly as recently described (5, 8). The plasma membrane glycoprotein with M, = 85,000 and pl = 4 was purified from the concanavalin A bound fraction of metabolically labeled HTC cells (3 x 10^6) in its calcium-mediated dimer form (M, = 175,000) by preparative polyacrylamide gel electrophoresis as previously described (6). This protein was also purified from cell-surface labeled cells (H and 125I) by hydroxyapatite chromatography (6). The fucogangliosides of H-35 cells were purified as outlined by Baumann et al. (9). Transfer of Glycoproteins and Glycolipids: Purified glycoproteins of HTC cells and fucogangliosides of H-35 cells were incorporated into reconstituted phospholipid vesicles by the detergent dialysis method as reported earlier (8). The protein-lipid ratio (wt/wt), however, was 1:7 instead of 1:4. Fluorescently labeled vesicles albumin was entrapped by vesicle reconstitution in the presence of 5 mg of the modified albumin/m. The material was transferred the vesicles to L-929 cells by fusion with the aid of polyethylene glycol 1000 as previously described (8). The sole modification consisted of using half the amount of phospholipid vesicles (50 μg phospholipid and protein) for fusion into 5 x 10^5 L-929 cells. Gel Electrophoresis: The separation of the purified glycoproteins was carried out by two-dimensional gel electrophoresis as described by O'Farrell (24). The following proteins served as molecular weight markers: myosin (210 x 10^3), phosphorylase A (94 x 10^3), albumin (68 x 10^3), and ovalbumin (43 x 10^3). The radiolabeled pattern was visualized by fluorography (11). To determine the radioactivity present in the separated proteins, selected spots were cut from the gel according to the fluorographic image. For background measurements, portions were cut from the gels in areas which did not contain fluorographically detectable protein spots. Except in experiments with dual isotopes 125I/3H, the gel pieces were rehydrated with 100 μl of water and then incubated with 1 ml of NCS-tissue solubilizer (Amersham) and 10 ml of tolutene-based scintillation fluid at 37°C for 16 h under constant shaking. After acidification of the fluid and storage of the vials for 24 h at 4°C, the radioactivity was measured in a liquid scintillation counter with automatic quench correction (Beckman LS-6000; Beckman Instruments, Inc., Fullerton, CA). Counting was routinely done for a minimum of 10 min, or in samples with low radioactivity, until a counting error of 1% was achieved. In experiments using the dual isotopes 125I/3H, the spill-over in the selected channels and quenching was verified by internal standards. The contribution of 125I to the 3H counts was calculated on the basis of the loss of counts during 10-15 min. The contribution of 125I to 3H isotope, the gel pieces were counted in a Beckman Biogamma counter (Beckman Instruments, Inc.). RESULTS Degradation of Cell-Surface Labeled Glycoproteins in HTC Cells After in situ labeling of the surface of HTC cells with 125I on accessible tyrosine residues, and 3H on accessible galactosyl and galactosaminyl residues, or sialic acid residues, it was seen during subsequent culture that the tritium label was lost more quickly than the iodide marker (Fig. 1). This finding corroborates previous results obtained from cells labeled with tritium or iodide alone (1) and indicates that one chemical modification does not produce a gross alteration in the degradation kinetics of the compounds labeled by the other treatment. From Fig. 1, it appears that, in the plasma membrane, externally exposed polypeptide structures are more stable than the carbohydrate structures. Iodination of the surface of HTC cells, however, leads to the radioactive modification of mostly non-
4% of 131 from the cell protein fraction. The cells in A were separate cultures. The change in cell density during the chase period in the absence or presence of 0.025 IU neuraminidase (NeuAmidase-galactose oxidase-[3H]NaBH4. (B) The same amount of their surfaces first with lactoperoxidase-Na131 and then with neuraminidase (Vibrio cholerae). This treatment resulted in a specific removal of 69% of 3H and 4% of 131 from the cell protein fraction. The cells in A were maintained in one suspension culture. Those in B were kept in two separate cultures. The change in cell density during the chase period shown was similar in all other turnover experiments performed on HTC cells and reported here. The results in both panels are expressed as mean values of duplicate determinations of each culture and are related to 106 mi of original cell suspension.

To better assess the turnover of the glycoprotein structures, we decided to measure the degradation kinetics of individual glycoprotein species. To do that, we labeled HTC cells at the tyrosine and galactose residues. The glycoproteins were purified from the cells 1 and 48 h after labeling by chromatography on concanavalin A-Sepharose. The lectin-bound fractions were then separated on two-dimensional polyacrylamide gels. As shown in Fig. 2, the pattern of the double-labeled lectin receptors remained qualitatively constant during the 48 h of culture. The glycoproteins migrated generally to more basic positions on the gels than did metabolically labeled glycoproteins (see below; and Fig. 4). As previously shown, this is a result of neuraminidase treatment, which is performed to increase the efficiency of tritium incorporation in penultimate galactose residues (4). Next, the radioactivity present in each of the ten major glycoprotein species identified was assessed. The values of two separate experiments were then used to estimate the apparent half-lives of the separated polypeptides as well as the relative turnover of the iodide to the tritium marker. Table I indicates that most of these proteins have similar half-lives ranging between 17 and 24 h. However, 2 glycoprotein species show apparent half-lives which are longer (glycoprotein 4) or shorter (glycoprotein 6) than the average.

In addition, Table I illustrates that terminal galactose residues were lost from some, but not all, glycoproteins faster than the polypeptide label (most notable are glycoproteins 1, 5, and 8). The accuracy of the half-life determination in Table I is dependent upon reproducible recovery from affinity chromatography and from two-dimensional PAGE. To corroborate that the half-lives calculated in Table I represent valid estimates, we carried out an additional turnover experiment using HTC cells labeled with 125I and 131I (Table II). The advantage of these two isotopes for measuring half-lives of polypeptides in plasma membranes is that the values are not influenced by variation in sample preparation and recovery from purification steps (28). The half-life values obtained by this method (Table II) agree with those in Table I. In both absolute and relative numbers.

The glycoconjugates on the cell surface are amenable to radioactive modification not only on their galactose residues but also on their terminal sialic acid residues (Fig. 1B). Both protein-bound carbohydrates are lost during the first 48 h after labeling at similar rates, but during the subsequent period glycate residues appear to be more stable than the modified glycosylated proteins. (28). It is, therefore, conceivable that the polypeptide backbones of membrane glycoproteins, which contain only about 10% of the iodide marker, are turned over at a rate similar to that of the carbohydrate portion; but in Fig. 1, this fact is masked by the total iodinated material.

FIGURE 2 Degradation of plasma membrane glycoproteins of cell surface-labeled HTC cells. HTC cells, 6 x 107, were labeled at their surfaces with 131I and 3H as described in Fig. 1A. After labeling, the washed cells were incubated in 100 ml of culture medium for 1 h to remove surface-adsorbed and labeled proteins. After change of the culture medium, one-half of the cell suspension was used immediately and the other half after 48 h in culture for purification of the glycoproteins by concanavalin A-chromatography. The total lectin-bound fractions (850,000 131-cpm at 1 h; 230,000 131-cpm at 48 h) were separated on two-dimensional gels. The fluorograph of the 1-h pattern was obtained after 14 h of exposure, that of the 48-h pattern after 2 d. The numbers indicate the spots chosen for radioactivity measurements (see Tables I and II).
sialic acid residues (Fig. 1). Unfortunately, the low specific activities obtained by the sialic acid labeling does not allow an analysis of individual glycoprotein species as was done for the galactose-labeled cells. It is, however, possible to demonstrate a preferential loss of sialic acid residues from membrane glycoproteins. Variation in the number of sialic acid residues we have to resort to metabolic labeling. The incorporation of radioactive precursors such as \[^{35}\text{S}\]methionine, \[^{1}\text{H}\]fucose or \[^{1}\text{H}\]mannose into membrane glycoproteins has the advantage that the metabolic stability of the molecules is not changed by this type of labeling, which is not necessarily the case in cell surface-labeled molecules. Metabolic labeling, however, has the intrinsic problem of reutilization of the precursor (1). To minimize this, we added large excess of unlabeled precursor (1). To minimize this, we added large excess of unlabeled precursor (1). To minimize this, we added large excess of unlabeled precursor (1). To minimize this, we added large excess of unlabeled precursor (1). To minimize this, we added large excess of unlabeled precursor (1).

We chose \[^{1}\text{H}\]fucose as a radioactive precursor for specific labeling of glycoproteins, because this monosaccharide is incorporated with high efficiency into intrinsic membrane proteins (5, 20) and does not show any significant metabolic conversion (9, 28). Studies on subcellular localization have indicated that the labeled glycoproteins were present not only in the plasma membrane but also in intracellular membranes (5, 13). However, the qualitative and quantitative composition and the turnover kinetics of the glycoproteins of both the intracellular membrane and the plasma membrane are very similar (5, 12, 13, 14, 28). Therefore, we used the total cellular glycoproteins (denoted as "membrane glycoproteins") for turnover studies.

Total cellular glycoproteins of \[^{1}\text{H}\]fucose-labeled HTC cells follow degradation kinetics similar to those of plasma membrane glycoproteins labeled at their sialic acid residues (compare Fig. 3 with Fig. 1 B). The loss of the protein-bound radioactivity can be attributed mostly to degradation of the glycoproteins to small molecular weight material, detected as

| Glycoprotein | cpm at 1 h | Ratio | cpm at 48 h | Ratio | \(t_{1/2}\) h |
|-------------|-----------|-------|-------------|-------|-------------|
| Experiment I | \(^{3}H\) | \(^{1}H\) | \(^{3}H/^{1}H\) | \(^{3}H\) | \(^{1}H\) | \(^{3}H/^{1}H\) | \(^{3}H\) | \(^{1}H\) | \(^{3}H/^{1}H\) |
| 1 | 73,000 | 240,700 | 0.34 | 16,560 | 55,120 | 0.30 | 22 |
| Spot series 1 | 448 | 954 | 0.47 | 52 | 179 | 0.29 | 19 |
| 2 | 4,645 | 18,394 | 0.25 | 809 | 2,935 | 0.27 | 18 |
| 3 | 4,333 | 15,565 | 0.28 | 630 | 2,443 | 0.26 | 18 |
| 4 | 5,903 | 14,115 | 0.42 | 1,851 | 5,396 | 0.35 | 25 |
| 5 | 2,574 | 6,602 | 0.39 | 302 | 1,258 | 0.24 | 20 |
| 6 | 4,509 | 21,068 | 0.21 | 264 | 1,115 | 0.23 | 11 |
| 7 | 391 | 1,739 | 0.23 | 53 | 189 | 0.28 | 15 |
| 8 | 1,512 | 9,779 | 0.40 | 354 | 889 | 0.28 | 23 |
| 9 | 9,400 | 17,733 | 0.55 | 1,288 | 2,639 | 0.48 | 18 |
| 10 | 379 | 924 | 0.41 | 43 | 146 | 0.29 | 18 |
| Experiment II | 115,680 | 174,880 | 0.66 | 23,426 | 43,382 | 0.54 | 24 |
| Spot series 1 | 1,270 | 693 | 1.83 | 173 | 174 | 0.99 | 23 |
| 2 | 4,040 | 7,792 | 0.52 | 354 | 1,957 | 0.44 | 24 |
| 3 | 4,608 | 8,604 | 0.54 | 987 | 2,216 | 0.35 | 24 |
| 4 | 6,473 | 7,788 | 0.83 | 2,636 | 3,708 | 0.71 | 44 |
| 5 | 3,008 | 5,112 | 0.74 | 712 | 1,276 | 0.56 | 23 |
| 6 | 4,344 | 11,273 | 0.39 | 731 | 1,672 | 0.44 | 17 |
| 7 | 833 | 1,584 | 0.53 | 197 | 415 | 0.48 | 24 |
| 8 | 2,065 | 2,367 | 0.87 | 643 | 948 | 0.68 | 36 |
| 9 | 24,714 | 12,057 | 2.05 | 4,791 | 2,559 | 1.87 | 21 |
| 10 | 701 | 522 | 1.34 | 144 | 126 | 1.14 | 23 |

In two separate experiments (I and II) using different preparations of enzymes and label, HTC cells were double-labeled at the cell surface with \(^{3}H\) (tyrosine residues) and \(^{1}H\) (galactose and \(^{1}H\)galactosamine residues) as described in Figs. 1 A and 2. The cellular glycoproteins were isolated by concanavalin A chromatography 1 and 48 h after labeling. The recoveries of the protein-bound radioactivities from the lectin columns were 25-33% of \(^3H\) and 5-8% of \(^{1}H\) for the 1 h samples, and 27-30% of \(^3H\) and 5-8% of \(^{1}H\) for the 48-h samples. The concanavalin A-bound fractions were separated by two-dimensional electrophoresis (see Fig. 2). Identical areas of the gels containing the glycoproteins indicated in Fig. 2 were measured for \(^3H\) and \(^{1}H\) radioactivities. All values for \(^{1}H\) labeled glycoproteins were calculated from the loss of the total radioactivity determined in the selected gel pieces assuming decay with first-order kinetics during 48 h (see Fig. 1 A). The rate constants of degradation, \(k_d\), were calculated from the equation:

\[
\text{cpm at 48 h} = \text{cpm at 1 h} \times e^{-k_d t}
\]

and used to determine the half-lives from the equation: \(t_{1/2} = \ln 2/k_d\).
Table II

Half-Lives of Cell Surface Glycoproteins of HTC Cells

| Glycoprotein | Mₜ₀ | 0 h | 48 h | 48 h | 1/2 |
|--------------|-----|-----|------|------|-----|
| Acid-soluble | 1.87 | 1.93 | 0.52 | 25   |
| Co-culture   | 1.87 | 1.93 | 0.52 | 25   |

HTC cells, 1 × 10⁸, were suspended in 30 ml of PBS and treated with neuraminidase (Vibrio Choleræ, 0.02 U/ml) for 15 min at 37°C. This treatment was performed to render the surface glycoproteins comparable to those labeled and analyzed in Fig. 2. Then, one-half of the cell preparation was labeled with 10 mCi of ³¹¹I (identical to that of Experiment II in Table I) and acid-soluble radioactivity in the culture medium. To follow the fate of individual species, especially with regard to the relative third was combined with equal amounts of ³¹¹I-labeled cells and frozen immediately as well (0-h time point), and the last third together with one-half of ³¹¹I-labeled cells was suspended in 50 ml of medium and cultured for 48 h (48-h co-culture; control for equal degradation of both labeled proteins). The remaining third of the ³¹¹I-labeled cells was cultured in 25 ml of medium for 48 h and then combined with the aliquot of ³¹¹I-labeled cells frozen at 0 h (48-h time point). The glycoproteins were simultaneously isolated by concanavalin A chromatography and separated by two-dimensional electrophoresis, yielding essentially the same pattern as in Fig. 2. The radioactivities of the two isotopes in the glycoprotein species 1-10 were determined and expressed as the ratio of ³¹¹I/³²⁵I (for the relative distribution of the ³¹¹I radioactivity see Table I, Experiment II). The half-lives were determined by using the ratio values from the 0- and 48-h time points (28).

In the above experiments, we used affinity chromatography on concanavalin A-Sepharose as a means of purifying glycoproteins. Therefore, we have analyzed a subset of the total glycoproteins which are selected on the basis of their specific structures of terminal carbohydrates. If, during its life time, a membrane glycoprotein undergoes modification of terminal carbohydrate moieties resulting in the loss of lectin affinity, we would miss these turnover products. To qualitatively assess whether such structural modification of membrane glycoproteins of HTC cells occurs, we chose immunoaffinity chromatography using polyspecific antibodies prepared against glycoproteins purified by concanavalin A from the total membrane fraction of HTC cells (3). We labeled HTC cells with ³¹¹I-fucose and isolated the antigens at various times during the chase period. The two-dimensional polyacrylamide gel patterns depicted in Fig. 6 show two new findings regarding the turnover of membrane glycoproteins: (a) During the chase period, some glycoprotein species demonstrate a progressive shift toward the basic side. This is most noticeable in the two protein spot series marked with 1 and 2 in Fig. 6. This change in charge is selected a single glycoprotein which is cleanly separated by electrophoresis and migrates with a molecular weight of 175,000 in its calcium-dependent dimeric form. This glycoprotein is indicated as gp17S in Fig. 4 and is identical to the monomeric form labeled as spot 9 in Fig. 2. Quantitation of the ³⁵S and ³H radioactivities in the gp175 spot showed that the polypeptide marker was lost about half as fast as the carbohydrate marker (Fig. 5B). The apparent half-life of the ³⁵Smethionine in gp175 is 26 h while that of ³Hfucose is only 17 h. Moreover, the degradation of the gp175 polypeptide appears to be slightly faster than that of the total lectin-bound fraction (Fig. 5A), but slower than that of the same protein labeled with ³¹¹I on the cell surface (Tables I and II). When the turnover of the carbohydrate markers in gp175/85 is compared by following the change in the relative isotope ratios during the chase period (Fig. 5B and Table I), we noted that the fucose residues are relatively less stable than the galactose residues.

Acid-soluble radioactivity in the culture medium. To follow the fate of individual species, especially with regard to the relative turnover of the polypeptide and carbohydrate moieties, we have used cells labeled with both ³⁵Smethionine and ³Hfucose. Because the use of ³⁵Smethionine leads to a labeling of terminal carbohydrates. The use of ³⁵Smethionine pattern reveals, however, some additional minor spots not present in the ³Hfucose pattern. That these proteins also represent glycoproteins purified by concanavalin A from the total membrane fraction of HTC cells (3). We labeled HTC cells with ³¹¹Ifucose and isolated the antigens at various times during the chase period. The two-dimensional polyacrylamide gel patterns depicted in Fig. 6 show two new findings regarding the turnover of membrane glycoproteins: (a) During the chase period, some glycoprotein species demonstrate a progressive shift toward the basic side. This is most noticeable in the two protein spot series marked with 1 and 2 in Fig. 6. This change in charge is

FIGURE 3 Degradation of the total fucoglycoproteins and fucoglycolipids in HTC cells and H-35 cells. HTC cells and H-35 cells were metabolically labeled with ³¹¹Ifucose 15 µCi/ml for 72 h and 24 h, respectively. At the times indicated, two 10-ml aliquots of the suspension HTC cells and two monolayers of H-35 cells were used for determination of the radioactivity present in the cell proteins and cell lipids. The data for HTC cells represent mean values of duplicate determinations. Those for H-35 cells represent the measured values for each monolayer.
FIGURE 4 Two-dimensional electrophoresis pattern of metabolically labeled membrane glycoproteins of HTC cells. HTC cells were labeled with [35S]methionine (15 μCi/ml) and [3H]fucose (15 μCi/ml) for 72 h. The glycoproteins were purified by concanavalin A-sepharose chromatography from the total cell membrane fraction. Aliquots of the lectin-bound fraction containing 400,000 acid-insoluble cpm were separated by two-dimensional PAGE as in Fig. 2, and the radioactive patterns were visualized by fluorography.

probably caused by the loss of sialic acid residues, since neuraminidase treatment of glycoproteins purified from HTC cells immediately after labeling produces an identical shift in the charge of these proteins (data not shown). (b) During the chase period, the appearance and accumulation of new antigenic glycoproteins take place. Two examples we have labeled as 3 and 4 in Fig. 6. The spot series 3 represents a glycoprotein form that does not bind to concanavalin A and, therefore, does not appear on the gel pattern of steady-state labeled glycoproteins (Fig. 4). Future experiments should show whether these new antigens are metabolically stable proteolytic degradation products of membrane glycoproteins and, if so, from which pulse-labeled glycoprotein they derive.

Degradation of Membrane Glycoproteins of HTC Cells after Transfer into the Plasma Membrane of Fibroblasts

The above approach to the analyses of glycoprotein degradation, like that described by others (16, 20), relies on the preservation of most, if not all, of the antigenic determinants or lectin binding properties of the molecules of interest throughout the course of their catabolism. In an attempt to circumvent this problem, we applied another technique to analyze the degradation of membrane glycoproteins. The glycoproteins were purified from the membranes of suitably labeled cells, they were then incorporated into reconstituted lipid vesicles, and the vesicles were transferred to the plasma mem-

spot of gp175 (indicated in Fig. 4), were cut out and the two isotopes were measured. The results in both panels represent the values of each duplicate determination.
cells released a large portion of the transferred glycoproteins in coproteins incorporated into vesicles and (b) by using fewer efficiency. Previously, we have demonstrated the proper insertion of membrane glycoproteins of HTC cells into reconstituted phospholipid vesicles and subsequent transfer onto the membrane of L-929 cells (8). The conditions used in these previous experiments, however, were not optimal, because the recipient cells carrying vesicles appear to be fused to the recipient cells and degradation of the transferred molecules really the same as in the original cells or is it affected by the new cellular environment? To test this, we have chosen for transfer two membrane components which differ in their metabolic stability. Glycolipids, such as the fucogangliosides of Reuber H-35 cells (9), are found to be much more stable \( t_{1/2} = 30 \) h than membrane glycoproteins \( t_{1/2} = 200 \) h than membrane glycoproteins (Fig. 3). We purified the fucogangliosides from \[^3H\]fucose-labeled H-35 cells and transferred these molecules, via reconstituted vesicles containing nonlabeled membrane glycoproteins of HTC cells, to L-929 cells. As shown in Fig. 9B, the degradation of the fucoglycolipids after transfer is quite distinct from that of the fucoglycoproteins of HTC cells (Fig. 8). The half-life of the transferred glycolipids is the same in L-cells as in the source H-35 cells (Fig. 3). In addition, the rate of degradation is not different from that of the endogenous radioactive compounds present in the lipid fraction of L-929 cells labeled with \[^3H\]fucose (Fig. 9A).

A preferential removal of the carbohydrate marker was found to take place in a complex mixture of transferred glycoproteins (Fig. 8). The question is: Do any individual glycoprotein-species behave similarly? In the experiment shown in Fig. 5, we noticed that the fucose-rich glycoproteins with \( M_r = 85,000 \) (gp85/175) are subject to preferential loss of the fucose residues. Furthermore, gp85/175 offers the advantage that it can be purified in amounts sufficient for reconstitution and transfer studies. To carry out a transfer of this glycoprotein to L-929 cells and to follow its degradation, we isolated the dimeric form of this gp85/175 from HTC cells labeled both with \[^35S\]methionine and \[^3H\]fucose. The radioactive purity of the final preparation is demonstrated in Fig. 10. The glycoprotein was incorporated into reconstituted phospholipid vesicles and then transferred into the plasma membrane of L-929 cells. There, the loss of both isotopes from the cell protein fraction was determined (Fig. 11A). In two independently conducted experiments, a preferential loss of \[^3H\]fucose was found, which is comparable to that in HTC cells (see Fig. 5B). Differing from the degradation kinetics in HTC cells, however, are the relatively long half-lives of both the polypeptide and carbo-
hydrate markers (see summary in Table III). To verify the validity of our measurements by using molecules labeled at other residues, we prepared the same gp85/175 from HTC cells cultured in the presence of [35S]methionine and [3H]mannose. This glycoprotein preparation was transferred onto L-929 cells in the same manner as the fucose-labeled one. As shown in Fig. 11 B, the methionine/mannose-labeled protein has a half-life similar to that of the methionine/fucose-labeled protein (Fig. 12 A). This time, however, no preferential loss of the carbohydrate marker was detectable. This property conforms to the turnover of the similarly labeled protein in HTC cells (data not shown, see Table III).

Above (Tables I and II, and Fig. 5 B), we have described that gp85/175 labeled on the cell surface has a slightly shorter half-life than the same protein labeled metabolically. Whether this phenomenon is a consequence of the chemical modification of the molecule or of the exclusive subcellular localization of the labeled protein is not known. By bringing the glycoprotein from surface-modified cells into the same cellular environment as the metabolically labeled protein, we might at least assess the metabolic stability of that glycoprotein. Therefore, we isolated gp85/175 from HTC cells labeled with lactoperoxidase-131I and neuraminidase-galactose oxidase-[3H]NaBH4, and transferred the glycoprotein, via reconstituted vesicles, onto L-929 cells (Fig. 12). The protein now follows closely the degradation kinetics of the methionine-labeled protein (Fig. 11). The measurements of the isotope ratios indicate that the labeled galactose residues are lost from the protein only slightly faster than the 131I label.

In Table III, we have summarized the half-life values for radioactive residues in the glycoproteins native to HTC cells and after their transfer to L-929 cells. These measurements, in

**FIGURE 7** Fusion of L-929 cells with reconstituted vesicles containing entrapped fluoresceinated albumin. L-929 cells (B) 5 x 10⁶ were fused with reconstituted vesicles containing 50 μg phospholipids plus nonlabeled membrane glycoproteins of HTC cells (7:1 weight ratio). Fluoresceinated albumin was entrapped within the vesicles during reconstitution. Control cells (A) were first exposed to the fusogen, then to the vesicles as described previously (8). Immediately after fusion and washing, the cells were analyzed under a Leitz fluorescence microscope. Left, light microscopic picture. Right, fluorescent image of the same cells.
canavalin A-Sepharose chromatography from the total cell membrane and [3H]fucose for 72 h as described in the experiment shown in Fig. 5. HTC cells were labeled with [35S]methionine and [3H]fucose for 72 h as described in the experiment shown in Fig. 5. The double-labeled glycoproteins were purified by con
canavalin A-Sepharose chromatography from the total cell mem-
brane fraction. From the final double isotope-labeled preparations, 50 μg of glycoproteins, with specific activities of 4.1 × 10⁴ cpm/μg protein and 11.8 × 10⁴ cpm/μg protein for the 3H and ³⁵S, respectively, were combined with 400 μg of phospholipids for vesicle reconstitution. One-half of the resulting vesicles were fused with 2.5 × 10⁷ L-929 cells, the other half was used for treatment of the
control cells. The cells of each batch were plated into eight 3.5-cm dishes. After 16 h, the media and nonadhered cells were removed and 2 ml of fresh culture media were added to the cultures. The cell monolayers were now used for determination of the radioactivity present in the acid-soluble and insoluble fractions of the medium and in the cell protein fraction. The values per culture dish of the experimental cells are shown. The presentation of radioactivity associated with the control cells is omitted due to the low values (at 16 h after fusion; the control cells contained only 7% of the acid insoluble radioactivity of the experimental cells). At any time the radioactivity present in the acid-soluble fraction of the cells did not exceed 1% of the total cell-associated radioactivity.

FIGURE 8 Degradation of double-labeled membrane glycoproteins of HTC cells in L-929 cells. HTC cells were labeled with [35S]methi-

DISCUSSION

In this manuscript, we tried to evaluate the usefulness of the technique of insertion of membrane components into the plasma membrane of a different cell as a means to analyze the degradation pathway of specifically labeled glycoconjugates. Previously, the major techniques for studying plasma membrane turnover have involved chemical or enzymatic labeling of cell surface components or metabolic pulse labeling of cells with amino acid or sugar precursors. The loss of the label from the cells (4, 28), from isolated membrane fractions (13, 19), and from specific membrane components recovered by specific immunoprecipitation (16, 20) or immobilized lectins (4, 21) was used to assess turnover properties. Each of the methods has certain disadvantages: Chemical modification of surface components might alter the metabolism of the modified constituents and affect their stability. Or, when metabolic labeling is used, reutilization of labeled precursor(s) is a distinct possibility. If cell fractionation is a prerequisite step in measuring the turnover of membrane constituents, the lack of purity of membrane fractions and variable recovery can be a problem. Furthermore, the degradation of the plasma membrane, or of any cellular membrane, probably occurs preferentially in the lysosomal compartment (12), the consequence of which is a constant redistribution of membranes in other parts of the cell. Turnover measurements on membrane fractions purified by procedures whose goal is high purity discount, therefore, all those (plasma) membrane proteins and their metabolic intermediates present in other cellular compartments at the time of cell fractionation. When degradation of membrane proteins is

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conjunction with the qualitative assessment of changes in the glycoprotein structures during the turnover, suggest that the various membrane glycoprotein species are generally broken down uniformly, regardless of molecular weight or charge. The complete degradation of the individual molecules to acid-soluble material, however, does not always occur at once but can occur in a certain temporal sequence. Two elements were recognized: proteolytic fragmentation, with accumulation of stable products; and preferential loss of terminal carbohydrate units such as fucose or sialic acid residues.

FIGURE 9 Degradation of endogenous fucoglycoproteins and fu-
colipids in L-929 cells. (A) L-929 cells were trypsinized from mono
layer and then exposed to polyethylene glycol under conditions similar to those for cells used for fusion studies. The cells were placed in 3.5-cm dishes and labeled for 24 h with [3H]fucose (5 μCi/ml). After washing of the cells, the amount of radioactivity present in the culture medium and in cell protein and the cell lipid fraction was determined. After 72 h, the culture medium in the remaining dishes was replaced. (B) [3H]fucoglycosides isolated from H-35 cells (3 × 10⁶ cpm) were combined with 50 μg of nonlabeled membrane glycoproteins purified from HTC cells and 400 μg phospholipids for vesicle reconstitution. The resulting vesicles were fused into L-929 cells and the cells were subsequently cultured as those in Fig. 8. Only the cell lipid-associated radioactivities were determined. Sixteen hours after fusion, control cells contained 1,200 cpm per monolayer, representing 6% of the values of the experimental cells. In both panels, the data shown are the values per culture dish.
components, not more than 1% of the cell-associated radioactive activity was found to be present in the precursor pool of the cell lectin (Figs. 3, 6, and 7), the accuracy of the results relies on the preservation of the antigenic determinants or a loss or alteration of these sites would, of course, escape detection.

The combined results of the turnover measurements (Table I–III), nevertheless, suggest that the polypeptide backbone of some membrane glycoproteins is at least twice as stable as the carbohydrate moiety, depending on the carbohydrate marker used. The technique of transfer of membrane components in reconstituted vesicles is a method which allows the measurement of the degradation rate of glycoproteins without the need of cell fractionation of specific precipitation, without the loss of intermediate forms, and without significant problems of label reutilization. The latter is concluded from the observation that, at any given time after transfer of labeled membrane components, more than 1% of the cell-associated radioactivity was found to be present in the precursor pool of the cell (Fig. 8). This technique is, however, not without problems. As we indicated previously (6) and in Fig. 12, the major drawback is that only a 70–90% rightside-out orientation of the membrane glycoproteins in the reconstituted vesicles can be achieved. Some of the transferred glycoproteins, therefore, will assume a reversed orientation within the recipient plasma membrane.

We limited our analyses in this report mainly to the markers, methionine, galactose, mannose, and fucose. The question arises as to whether other monosaccharides, such as the distal sialic acids or the proximal N-acetyl-glucosamines of glycoproteins, follow the same degradation pattern as fucose or a pattern closer to mannose, galactose, or that of the polypeptide marker. Kreisel et al. (21) reported that a glycoprotein with $M_r$ = 110,000, present in the liver plasma membrane and labeled in

![Figure 11 Degradation of metabolically labeled gp85/175 of HTC cells in L-929 cells](image)

**Figure 11** Degradation of metabolically labeled gp85/175 of HTC cells in L-929 cells. (A) The glycoprotein gp85/175 was purified from two different batches of HTC cells labeled for 72 h with $[^{35}S]$methionine and $[^{3}H]$fucose. Both preparations I and II, showed the same radioactive purity as illustrated in Fig. 10. Gp85/175 of preparation I, containing 710,000 $[^{3}H]$-cpm and 40,000 $[^{35}S]$-cpm, and preparation II, containing 250,000 $[^{3}H]$-cpm and 34,000 $[^{35}S]$-cpm, were each combined with 200 µg of phospholipids for vesicle reconstitution. The resulting vesicles (see also Fig. 10 A, lane 2), containing in incorporated form 68% and 80% of the initially added glycoprotein, respectively, were fused with L-929 cells. The cells were then plated into four 10-cm dishes. After 16 h, the media (15 ml) were changed. At the times indicated, one dish was used for determination of the radioactivities associated with the cell proteins. (B) Gp85/175 was purified as that in Fig. 10 but using the membrane fraction from 3 X 10^9 HTC cells labeled for 72 h with $[^{35}S]$methionine (15 µCi/ml) and $[^{3}H]$mannose (15 µCi/ml). The glycoprotein with 180,000 $[^{3}H]$-cpm and 20,000 $[^{35}S]$-cpm was transferred onto L-929 cells and its degradation there was determined as described in A.
vivo, appears to have lost terminal carbohydrates, fucose, and N-acetyl-neuraminic acid, much faster than the polypeptide markers, methionine and arginine.

Differential degradation of the glycoprotein structure, if true, has important implications to the mechanisms of general membrane biosynthesis and turnover. When a plasma membrane, such as that of an HTC cell, is involved in endocytosis, it will encounter the lysosomal compartment at some point (7) and will be in contact with lysosomal enzymes. It is conceivable that the incoming membrane glycoproteins are exposed both to proteolytic and to glycolytic degradation reactions. As proposed by different groups, these surface-derived membrane packages, modified or not, can leave the lysosome compartment again and will recycle back to the surface (12, 25-27). Because several reports indicate that endocytotic vesicles (or recycling vesicles?) are found also in association with Golgi or GERL structures (10, 18, 23), an exposure of the surface-derived membrane components to the enzymatic activities of these organelles is also possible. Whether repair of the glycoproteins with terminally damaged carbohydrate structures takes place at this point is not known. As Fig. 2 indicates, however, there is no readdition of sialic acid residues to neuraminidase-treated surface glycoproteins, because no detectable acidic shifts in charge of the proteins are detectable even after 48 h in culture. If such a repair mechanism is nonexistent or minor, the membrane will accumulate underglycosylated or even deglycosylated fragments of glycoproteins. This would explain the relatively large concentration of slowly turning over, nonglycosylated proteins on the cell surface of HTC cells and L-929 cells (4, 8, 15, 28) as well as the enhanced presence of new antigens in Fig. 6. If, indeed, membrane glycoproteins are subjected to faster degradation than nonglycosylated proteins, the cell has to compensate for this preferential loss by a relatively higher rate of synthesis of certain membrane components in order to maintain a steady-state level.

The technique described in this report offers an opportunity to elucidate the exact catabolic fate of membrane glycoconjugates. The transfer of chemically well-defined and appropriately labeled membrane components into a nonlabeled cell environment will allow the determination not only of the subcellular redistribution of the transferred species but also of any ongoing biochemical alterations of the transferred molecules.

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