Biogenesis of Plasma Membrane Glycoproteins in Hepatoma Tissue Culture Cells

(Received for publication, April 1, 1977)

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Total membrane glycoproteins were labeled by growing hepatoma tissue culture (HTC) cells in the presence of [14C]fucose, and externally disposed plasma membrane glycoproteins and proteins on the same cells were labeled by reduction of oxidized galactose residues with tritiated sodium borohydride or by lactoperoxidase-catalyzed iodination. The latter labeling methods show that the plasma membrane contains a set of fucose-labeled glycoproteins that is externally disposed. However, the fucose incorporated into membrane glycoproteins does not fractionate solely with the plasma membrane. Rather, a significant amount of the fucose-labeled glycoprotein fractionates with an internal membrane system(s) of the cell. The fucose-containing glycoproteins in the different cell fractions were compared by electrophoresis in dodecyl sulfate-polyacrylamide gels: both the internal membrane system and the plasma membrane contain a similar, and probably identical, set of fucose-labeled glycoproteins as resolved by this method. Furthermore, many of these proteins are present in the same relative proportion to each other in both the plasma membrane and the internal membrane system(s) of HTC cells. The glycoproteins are released from both membrane fractions by the same concentration of deoxycholate, a concentration higher than that needed to release microsomal contents. Autoradiographic analysis of thin sections of HTC cells grown in the presence of [3H]fucose confirm the presence of fucose-containing glycoproteins in an internal membrane compartment. Pulse-chase experiments with fucose as precursor show that part of the membrane-bound glycoprotein in the internal pool(s) is transient and transferred to the plasma membrane within 3 h after fucosylation, while another part remains associated with the internal membrane system for a considerable length of time. Based both on these results and our previous studies on the turnover of HTC cell membrane proteins, it is proposed that, during biogenesis fucose-containing glycoproteins of the cell are assembled as a membrane-limited structure in the intracellular compartment. Part of this presynthesized membrane is then supplied directly to the plasma membrane, but part of it is added to a relatively large internal pool of membrane also having the same glycoprotein composition as the plasma membrane.

While glycoproteins are characteristic constituents of membrane systems in animal cells, their physiological function is, for the most part, not yet understood. The plasma membrane is enriched in this class of proteins, and glycoproteins have a definite topographical organization in this membrane. The carbohydrate residues are confined to that end of the protein which is exposed on the external surface of the lipid bilayer (1–3). Important roles in many surface-mediated cell phenomena have been proposed for these externally disposed glycoproteins, including recognition and binding of small and large molecules (4–7), cell-cell recognition (8, 9), normal growth phenomena (10, 11), and an involvement in the transformation of cells from normal to malignant growth (12–14). Whether such surface glycoproteins are confined solely to the plasma membrane or whether they are present in other cellular organelles has important implications both for their function and for their route of biogenesis. In this paper, we examine the distribution of membrane glycoproteins in HTC cells.1 We show that a set of fucose-containing glycoproteins are externally disposed on the plasma membrane of HTC cells. But, confirming the works of others, particularly Warren, Glick, and their coworkers (15–17), we also show by biochemical, histochemical, and cell fractionation techniques that these carbohydrate-containing proteins are not unique to the plasma membrane. Rather, they are also found associated with an internal membrane system(s) of this cell. Part of this internal membrane system functions as a direct precursor to the plasma membrane and part may form an intracellular reservoir of preformed plasma membrane.

MATERIALS AND METHODS

Cell Culture—A cloned line of HTC cells was maintained and...
grown in suspension culture in Eagle's minimal essential medium containing 10% fetal calf serum as described previously (18, 19). Addition to this medium is indicated in legends to the tables and figures.

Radioactive Labeling - Cells were iodinated with 125I or 131I as described previously in detail (10). The method is essentially that of Phillips and Morrison (20) as modified by Hubbard and Cohn (21). Carrier-free radioactive 125I and 131I were from Amersham/Searle. Viability after iodination was between 90 and 100% as judged by trypsin blue exclusion. Total cell glycoproteins for labeling were growing the cells in the presence of L-[3H]fucose (Amersham/Searle, 18.5 Ci/mmol) or L-1-[14C]fucose (Amersham/Searle, 60 mCi/mmol). Usually cells at a density of between 0.5 and 1 × 10^6 were exposed to the labeled sugar at a concentration of 1 μCi/ml of normal Eagle's culture medium. The time of exposure varied depending on experiment; details are given in the legend to the appropriate figure or table.

Externally exposed glycoproteins were labeled by sodium borohydride reduction of galactose oxidase-treated cells (22-24). HTC cells, washed twice with phosphate-buffered saline, were reacted for 5 min at room temperature with unlabeled sodium borohydride, 1 mM. The cells were then washed and treated with neuraminidase (Calbiochem, 5 units/ml) to remove terminal sialic acids. The cells next were treated with galactose oxidase (a generous gift of Dr. M. Ettinger, State University of New York at Buffalo, 5 units/ml) for 15 min at 37°C. The cells (5 × 10^6/ml) then were washed twice with phosphate-buffered saline, and the aldehydes were reduced with tritiated NaBH₄ at a concentration of 1 mM/ml of cells for 5 min at room temperature. Tritiated NaBH₄ from New England Nuclear. A flow diagram of the cell fractionation is presented in Fig. 1.

Membrane glycoproteins labeled with 125I or 14Cfucose were isolated from HTC cells by extraction with deoxycholate, 1%, in 50 mM Tris, pH 7.6, followed by lectin affinity chromatography on concanavalin A-Sepharose (28). The lectin column (1.3 × 2.5 mm containing 2 ml bed volume of concanavalin A-Sepharose), was equilibrated with deoxycholate, 1%, in 50 mM Tris, pH 7.6, and nonbound proteins were eluted with 20 ml of this same buffer. The column was then washed with 30 ml of the buffer. Bound glycoproteins then were eluted with 0.2 ml α-methylmannoside in deoxycholate, 1%, and 50 mM Tris, pH 7.6 (10 ml). The flow rate was approximately 1 ml/min.

 Autoradiography of Fucose-labeled HTC Cells - Cells that were exposed to [3H]fucose for 48 h or cells that were exposed to 14Cfucose for 48 h followed by a 72 h period of growth in unlabeled fucose were washed three times with Earle's balanced salt solution. These cells were suspended in saline at a concentration of 1 × 10⁶ cells/ml and were equal volumes of 0.1 M sodium phosphate buffer (pH 7.2), 6.6 mM CaCl₂, was added. Following fixation for 1 h at 4°C, the cells were washed for 2 h in several changes of 0.1 M sodium phosphate buffer, pH 7.9, containing 0.6 mM CaCl₂. The cells then were postfixed for 1 h in 1% OsO₄ at 4°C. Next, the cells were dehydrated in ethanol, followed by two changes of propylene oxide and embedded in Epon. Sections, 1 μm, were cut on a Porter Blum microtome. The sections were coated with Kodak NTB-3 liquid emulsion according to the procedure of Kopriwa and Leblond (28). Exposure time varied from 4 to 40 days. Unstained sections after development were viewed by phase contrast microscopy.

Counting Procedures - Aliquots of cell fractions containing incorporated labeled fucose, uridine, or iodide were placed onto 2.4-cm Whatman No. 3-mm filter papers. The filter papers containing fucose-labeled fractions were washed five times with cold 5% trichloroacetic acid containing 1 mM unlabeled fucose followed by a quick rinse in ethanol/ether (1:1). Filter papers containing radioactive iodide were washed as previously described (18). In early experiments, acid-insoluble material was extracted with chloroform/methanol (2:1) for 1 h at 4°C. However, this "lipid" extraction was later avoided because little or none of the acid-insoluble fucose or iodide was soluble in chloroform/methanol. In HTC cells there is little lipid or glycolipid labeling by these precursors.2 The filter papers were placed in scintillation vials and Protocol (New England Nuclear), 1 ml, was added. After 1 h at 55°C, toluene-based scintillation fluid was added and the samples were counted in a Beckman LS-100

2 H. Baumann and C. Porter, unpublished observations.
spectrometer. Filter papers containing radioactive iodide were counted directly in a Beckman Biogamma spectrometer. Efficiencies were determined using internal standards. In some experiments, cells were labeled with both \(^{13}C\) and \(^{15}I\). In these cases, the \(^{15}I\) was first counted in the \(\gamma\) spectrometer. Then, the filter paper was prepared for counting in the liquid scintillation spectrometer. A window was chosen, so that approximately 25% of the \(^{15}I\) counts were detected in the \(^{13}C\) channel.

Acrylamide Gel Electrophoresis - One-dimensional discontinuous acrylamide slab gel electrophoresis in buffers containing sodium dodecyl sulfate was performed as described previously (18, 19). Essentially, the system is that of Laemmli (30). The acrylamide concentration of the separating gel was 9%, while that of the stacking gel was 3%, except as noted in legends to specific tables and figures.

The gels were stained with Coomassie brilliant blue R-250 in 45% methanol, 10% acetic acid. After staining and destaining, gel slabs containing iodinated polypeptides were dried and prepared for autoradiography as described previously (18, 19). Gel slabs containing \(^{125}I\) fucose-labeled polypeptides were prepared for fluorography by washing the destained gel with water, then impregnating the gel with 2,5-diphenyloxazole (PPO) in dimethylsulfoxide exactly as described by Bonner and Laskey (31). Kodak RP Royal X-OMAT medical x-ray film was used for both fluorography and autoradiography. Staining, destaining, and preparation of the gels for fluorography did not affect recovery of the applied radioactivity, which was in the range of 80 to 100%.

RESULTS

Plasma Membrane Glycoproteins of HTC Cells - Plasma membrane glycoproteins having externally oriented galactose residues can be labeled \emph{in situ} by tritiated borohydride reduction of galactose oxidase-treated cells (22-24). The tritium incorporated into HTC cells via this procedure is localized primarily at the cell periphery as assessed by electron microscopic autoradiography. As shown in Fig. 2, HTC cells do contain a set of externally disposed glycoproteins which have galactose residues accessible for reduction after the cells have been treated with neuraminidase and galactose oxidase. As also shown in Fig. 2, glycoproteins with similar mobilities to the externally labeled glycoproteins are labeled when HTC cells are grown in the presence of \(^{14}C\)fucose for 48 h. That is, many, but not all, of the fucose-labeled glycoproteins in whole cell extracts appear to co-migrate under the conditions of dodecyl sulfate-acrylamide gel electrophoresis of Fig. 2 with plasma membrane glycoproteins externally labeled via the tritiated borohydride reduction method. There is fucose-labeled material that may be inaccessible to external labeling via tritiated borohydride reduction of oxidized galactose particularly in the region of the gel between the \(M_r = 68,000\) and the \(M_r = 135,000\) markers. Possibly some of the fucose-labeled glycoprotein in this region may lack galactose residues. We next attempted to determine whether these fucose-labeled membrane glycoproteins were also accessible to iodination.

But, because of the complexity of the pattern of iodinated polypeptides, it was not possible to equate specific fucose-labeled polypeptides with specific iodinated polypeptides in a

**Fig. 2.** Dodecyl sulfate-polyacrylamide gel electrophoresis of fucose-labeled glycoproteins and externally oriented glycoproteins of HTC cells. One aliquot (250 ml, \(1 \times 10^6\) cells/ml) of a culture of HTC cells was grown in the presence of \(^{14}C\)fucose, 1.0 \(\mu\)Ci/ml, for 48 h. Externally oriented galactose residues of a second identical aliquot of the same culture of cells were labeled \emph{in situ} by tritiated borohydride reduction of oxidized galactose oxidase-treated cells (22-24). The tritium incorporated into HTC cells via this procedure is localized primarily at the cell periphery as assessed by electron microscopic autoradiography. As shown in Fig. 2, HTC cells do contain a set of externally disposed glycoproteins which have galactose residues accessible for reduction after the cells have been treated with neuraminidase and galactose oxidase. As also shown in Fig. 2, glycoproteins with similar mobilities to the externally labeled glycoproteins are labeled when HTC cells are grown in the presence of \(^{14}C\)fucose for 48 h. That is, many, but not all, of the fucose-labeled glycoproteins in whole cell extracts appear to co-migrate under the conditions of dodecyl sulfate-acrylamide gel electrophoresis of Fig. 2 with plasma membrane glycoproteins externally labeled via the tritiated borohydride reduction method. There is fucose-labeled material that may be inaccessible to external labeling via tritiated borohydride reduction of oxidized galactose particularly in the region of the gel between the \(M_r = 68,000\) and the \(M_r = 135,000\) markers. Possibly some of the fucose-labeled glycoprotein in this region may lack galactose residues. We next attempted to determine whether these fucose-labeled membrane glycoproteins were also accessible to iodination.

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**Fig. 3.** Separation of \(^{125}I\)-labeled and \(^{14}C\)fucose-labeled HTC cell glycoproteins by concanavalin A-Sepharose chromatography. One aliquot of an HTC cell culture was iodinated \emph{in situ} via lactoperoxidase catalyzed iodination, while a second aliquot of the same culture was grown for 48 h in the presence of \(^{14}C\)fucose, 1.0 \(\mu\)Ci/2.5 \(\times 10^6\) cells. Glycoproteins were extracted with deoxycholate, 1%, 50 mM Tris/chloride, pH 7.6, from a crude total cell membrane preparation of each aliquot of the cells (18). The deoxycholate extract was applied to a column of concanavalin A-Sepharose. Bound glycoproteins were eluted with 0.2 M \(\alpha\)-methylmannoside in the deoxycholate/Tris buffer. The fucose-labeled glycoproteins in the crude membrane fraction (A), and that bound to Concanavalin A (B) were analyzed in 7.5% acrylamide gels containing dodecyl sulfate by fluorography. Similarly, iodinated glycoproteins that bound to the plant lectin (C) were resolved by x-ray analysis of the 7.5% acrylamide gels.
fraction of HTC Cells and Distribution of Fucose-containing Glycoproteins Relative to Iodinated Plasma Membrane Glycoproteins – A protocol for the isolation of a plasma membrane fraction of HTC cells was presented in Fig. 1. Most of the incorporated iodide (85-90%) is present in the pellet resulting from centrifugation of the homogenate at 400 x g for 30 min (12,000 x g-min centrifugation). We showed previously that the incorporated iodide is a valid marker for the plasma membrane of HTC cells (18). When HTC cell membrane glycoproteins are labeled by growing cells in [3H]fucose for 48 h before iodination, the incorporated fucose does not co-fractionate with the incorporated iodide. Rather, the incorporated fucose is divided between the pellet and the supernatant fraction resulting from the 12,000 x g-min centrifugation. It is possible that vesiculization of the plasma membrane contributes some material to the 12,000 x g-min supernatant fraction. But, this seems unlikely unless the iodinated polypeptides are excluded from such vesicles. Most of the incorporated fucose in the 12,000 x g-min low speed supernatant fraction sediments after centrifugation at 240,000 x g for 1 h. The pellet resulting from this latter centrifugation is also enriched in microsomes and Golgi as indicated by the activities of glucose-6-phosphatase and sialyltransferase, respectively (Table I). This crude microsomal fraction was further separated into a "light microsomal fraction" and a "heavy microsomal fraction" by a step sucrose gradient centrifugation. The light microsomal fraction is enriched relative to the homogenate for incorporated fucose (6-fold), 5'-nucleotidase (5-fold), glucose-6-phosphatase (5- to 6-fold), and sialyltransferase (3-fold) (Table I). This fraction and the heavy microsomal fraction, which bands at 1.4/1.6 sucrose interface contain little of the incorporated iodide. Much of the iodide radioactivity is present in a "plasma membrane" fraction, which was derived from the original 12,000 x g-min pellet by a step sucrose gradient centrifugation. The plasma membrane fraction bands at the 1.6/1.8 mg sucrose interface and is enriched for both acid-insoluble [125I]radioactivity, 15-fold, and [14C]fucose radioactivity, about 3-fold (Table I). This membrane fraction, as well as the heavy and light microsomal fractions when centrifuged again through the step sucrose gradients band again at their same respective regions of the gradient with little relative change in marker enzyme profile (data not shown).

It is important to point out that the highest specific activity for incorporated iodide is in the plasma membrane fraction, while the highest specific activity for incorporated fucose is in the light microsomal fraction (Table I). That is, the plasma membrane fraction is enriched over the homogenate only 3-fold for acid-insoluble fucose radioactivity, while the microsomal fraction is enriched 6-fold. This result suggests that much of the incorporated fucose is not present in the plasma membrane as defined by the incorporated iodide. Furthermore, the acid-insoluble fucose in the plasma membrane and in the "microsomal" fraction cannot be due to cross-contamination; the microsomal fraction has little of the incorporated [125I], a highly specific marker for the plasma membrane of these cells (18), while the plasma membrane has little of the glucose-6-phosphatase activity characteristic of microsomes.

Treatment of intact HTC cells with trypsin will release as much as 80% of iodide incorporated into externally oriented plasma membrane proteins (18), but only 25 to 30% of the incorporated fucose. Almost all of the incorporated fucose is protease-sensitive in deoxycholate or Triton extracts of cells, again suggesting that much of the incorporated fucose is inaccessible to the protease at the HTC cell surface. This distribution of incorporated fucose is not peculiar to HTC cells. Similar studies by Buck et al. (15) also have shown that only about 20% of the fucose-labeled glycopeptides of intact baby hamster kidney cells are sensitive to proteases.

Fucose-containing Glycoproteins of Heavy and Light Microsomal Fractions, and Plasma Membrane – In Fig. 4 is shown...
a fluorogram comparing the fucose-labeled glycoproteins in the plasma membrane fraction to those of the other HTC cell fractions. Each fraction appears to contain the same or a very similar set of fucose-containing glycoproteins, again as resolved by dodecyl sulfate-polyacrylamide gel electrophoresis. Furthermore, as more clearly shown in a densitometric trace of the fluorograms (Fig. 5), the plasma membrane and the internal cell membrane fraction contain these glycoproteins in about the same relative proportion to each other, suggesting that there is a membrane-limited structure inside the cell very similar or identical in glycoprotein composition to the plasma membrane.

**Autoradiographic Localization of Incorporated Fucose in HTC Cells**—Thus far, we have relied mainly on cell fractionation methods and the differential partitioning in the fractionation of $^{125}$I and labeled fucose to show that there are two membrane compartments in HTC cells containing the same or a similar set of glycoproteins. One of these compartments, that defined by the iodide incorporated via lactoperoxidase-catalyzed iodination, is presumably the plasma membrane, catalyzed iodination, is presumably the plasma membrane, that defined by the iodide incorporated via lactoperoxidase-catalyzed iodination or tritium incorporated via borohydride reduction of galactose oxidase-treated cells. In the latter cases, the grains are confined almost exclusively to the periphery of the cell$^2$ (18).

**Release of Glycoproteins from Plasma Membrane and Microsomal Fractions with Deoxycholate**—If the same fucose-containing glycoproteins are present in the plasma membrane and in an internal membrane system(s) of the cell, then the question arises whether these glycoproteins are actually secretory in nature and only transiently associated with both the internal membrane system and the plasma membrane. We attempted to answer this question, using low concentrations of deoxycholate to selectively release microsomal contents, thereby differentiating between intrinsic membrane glycoproteins and glycoproteins only transiently associated with the microsomal compartment of the cell. As originally shown by Kreibich and co-workers (36, 37), low concentrations (less than 0.05%) of deoxycholate will release rapidly labeled, presumably secretory, proteins from liver microsomes, while higher concentrations are required to "solubilize" intrinsic membrane proteins. The fucose-labeled glycoproteins of the microsomal fraction and the plasma membrane fraction of HTC cells are released from the bilayer at a concentration of deoxycholate higher than that required to release secretory proteins from liver microsomes (Fig. 7). Deoxycholate, at a concentration of 0.1% will release 70% or more of the fucose-labeled glycoproteins from the membranes. Most of the iodinated proteins are resistant to release by deoxycholate even at this concentration, suggesting that they are more tightly bound to the membrane than the glycoproteins. The concentration-dependent release of the fucose-labeled glycoproteins from the microsomal fraction by deoxycholate is identical to that from the plasma membrane, suggesting that these glycoproteins are organized in a similar way.

**FIG. 4.** Dodecyl sulfate-polyacrylamide gel electrophoresis of the fucose-labeled glycoproteins in HTC cell fraction. Cells were grown in the presence of $[^{3}H]$fucose, $1 \mu$Ci/ml, for 48 h and the following fractions were prepared from a homogenate (H) of these cells as described under "Materials and Methods": crude microsomes, M; light microsomes, LM; heavy microsomes, HM; and plasma membrane, PM. Aliquots of each fraction were dissociated by heating at 100°C for 1 min in 1% dodecyl sulfate, 1% 2 mercaptoethanol. Polypeptides were separated on 9% polyacrylamide slab gels. Gels were prepared for fluorography to reveal the fucose-labeled polypeptides as described in the text.

**FIG. 5** (left). Densitometer trace of the fucose-labeled glycoproteins in the light microsomal and plasma membrane fractions of fig. 4.

**FIG. 6** (right). Localization of incorporated fucose by autoradiography of HTC cell sections. Cells were grown in the presence of $[^{3}H]$fucose, $1 \mu$Ci/ml, for 48 h. Thin sections, 1 $\mu$m, were prepared for autoradiography as described in the text. The autoradiograph was exposed for 4 days. Unstained sections were viewed by phase contrast microscopy and the autoradiographic grains appear white in the photographs. Magnification about $\times 1,000$. 

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**Membrane Proteins of HTC Cells**

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While the other(s) appears to be an internal membrane system. Autoradiography of thin sections from HTC cells that were grown for 48 h in the presence of $[^{3}H]$fucose also shows that this is the case. The long labeling period was chosen because the incorporated label will better reflect the steady state concentration of the fucose-containing glycoproteins in the cell. As shown in Fig. 6, autoradiographic grains representing incorporated fucose are not concentrated at the cell periphery but are present over most of the cell section, with the exception of the nucleus. Similar results were obtained from autoradiographs prepared from cells that were labeled with fucose for 1 and 2 h. That is, even after shorter intervals of labeling acid-insoluble fucose was localized over most of the cell section except for the nucleus. The localization of the incorporated fucose should be contrasted with the localization of iodide incorporated via lactoperoxidase-catalyzed iodination or tritium incorporated via borohydride reduction of galactose oxidase-treated cells. In the latter cases, the grains are confined almost exclusively to the periphery of the cell$^2$ (18).
Fig. 7. Release of labeled proteins from the light microsomal and plasma membrane fractions of HTC cells by deoxycholate (DOC). One aliquot of cells was grown in the presence of [1H]fucose, 1 μCi/ml, for 72 h. The same set of glycoproteins are labeled as at 1, 2, 24, 48, or 72 h. These same cells were exposed to [14C]leucine, 1 μCi/ml, for the last 24 h of the experiment. A second aliquot of HTC cells was incubated with [3H]leucine. Light microsomal and plasma membrane fractions were prepared from both groups of cells. The supernatant fractions were suspended in 0.01 M Tris buffer (pH 7.5), 0.025 M KCl, 0.005 M MgCl₂ and aliquots (0.2 ml, 20 to 200 μg of protein) were treated with the concentration of detergent indicated in the figure. The final volume was 0.5 ml. After 30 min incubation at 4°C, supernatant and pellet fractions were obtained for the determination of acid-insoluble radioactivity by centrifugation at 50,000 rpm for 60 min in the Beckman Ti-50 rotor. The arrow indicates the lowest deoxycholate concentration at which radioactivity is released from a liver microsomal fraction prepared from mice that had been injected with [1H]leucine 1 h prior to killing. The details for this latter experiment were exactly as described in Refs. 36 and 37. Mice were used as a source of rapidly labeled secretory proteins rather than HTC cells because the latter cells secrete very little protein into the medium.

Fig. 8. Transfer of the fucose-labeled glycoproteins from the internal membrane system to the plasma membrane. A culture of HTC cells, 1 × 10⁶ cells/ml, was suspended in medium containing [1H]fucose, 1 μCi/ml. At the end of 2 h, the cells were collected by centrifugation, washed once, and resuspended in medium containing 2 μCi unlabeled fucose. At the times indicated in the figure, aliquots of the culture were taken for the determination of acid-insoluble radioactivity in total cell protein, the 12,000 × g-min supernatant fraction, the 12,000 × g-min pellet fraction and a plasma membrane fraction isolated from the 12,000 × g-min pellet. The procedures for the preparation of the different cell fractions are given in the text. The cells were incubated at a concentration of 1.5 × 10⁵/ml. At this density, cell growth is arrested (18, 19), and no cell growth occurred during the course of the experiment.

with respect to the lipid bilayer in the two different membrane systems. That is, they are not sequestered within the lumen of a vesicle in the microsomal fraction.

Transfer of Fucose-labeled Glycoproteins from Internal Membrane System to Plasma Membrane—As shown in Fig. 1, most of the plasma membrane, 85 to 90%, and as identified by incorporated [14C]leucine is separated from the other membrane systems containing fucose-labeled glycoproteins in the first step of the HTC cell fractionation. That is, the 12,000 g-min supernatant fraction contains little of the plasma membrane. Even if the 12,000 g-min pellet is somewhat contaminated with the other membrane systems of the cell, this separation of the different cell membranes is sufficiently good to examine the kinetics of protein glycosylation and transfer of the synthesized glycoproteins from the internal membrane compartment to the plasma membrane. As shown in Fig. 8, the total amount of fucose incorporated into both total cell protein and the proteins in the 12,000 g-min supernatant fraction increases progressively during a 2-h administration of labeled fucose to a culture of HTC cells. When the labeled fucose in the culture medium is removed and replaced with unlabeled sugar, incorporation of label into total cell protein stops, indicating an effective "chase," and there is a loss of acid-insoluble label from the 12,000 × g-min supernatant fraction. The label lost from the 12,000 × g-min supernatant fraction, which contains the internal membrane system, corresponds almost quantitatively to the increase in radioactivity in the 12,000 × g-min pellet fraction. The increase in specific radioactivity of protein in a plasma membrane fraction isolated from the 12,000 × g-min pellet is in parallel to the increase in total radioactivity of this pellet. About 3 additional hours of chase with unlabeled fucose are required for the fucose label in the plasma membrane to reach maximum. These results show that part (representing about 40% of the fucose incorporated in the 2 h of administration) of the membrane-bound fucosyl glycoproteins of the internal membrane system is a direct precursor to the plasma membrane with a 3-h transit time required after glycosylation to move these proteins into the surface membrane.

While some of the fucose-labeled glycoprotein of the plasma membrane originates directly from the internal membrane source, much of the glycoprotein in the internal membrane compartment is not transferred rapidly to the plasma membrane. Thus, as also shown in Fig. 8, there is still a significant amount of the incorporated fucose present in the internal membrane compartment after a relatively long period of growth in medium containing unlabeled fucose. The material that is not rapidly transferred to the plasma membrane, representing 50 to 60% of the fucose incorporated into the cell, is lost from the internal membrane pool at about the same rate, or perhaps at a somewhat faster rate (19) than these same glycoproteins from the plasma membrane. But, the degradation is relatively slow from both compartments with a half-life of from 50 to 100 h (Fig. 9; Ref. 19). That there is indeed a reservoir of internal membrane glycoproteins is illustrated more directly in autoradiographs of thin sections from HTC cells that were grown in labeled fucose for 48 h, followed by a 72-h period of growth in unlabeled fucose (Fig. 10). The 48-h interval of fucose incorporation, again gives a better indication of the steady state concentration of the glycoprotein in the different membrane fractions than does
FIG. 9. Long-term loss of fucose-labeled glycoproteins from HTC cell membrane fractions. Experimental details are similar to those of Fig. 8, except the cells were exposed to [3H]fucose for 8 h followed by the chase with unlabeled fucose.

During the 72-h period of growth in unlabeled medium, the specific activity of the incorporated fucose decreased about 10-fold, due mainly to two cell divisions and one half-life of glycoprotein degradation. Yet, the distribution of grains is very similar to that in cells not grown in unlabeled medium (compare Figs. 6 and 10). Note that the 10-fold difference in incorporated radioactivity between Figs. 6 and 10, and the 10-fold difference in exposure time results in a similar number of grains over the two sets of cells. Similar results were obtained when cells were pulsed with [3H]fucose for 1 or 2 h and chased with unlabeled fucose for 24 or 48 h.

There is little apparent difference between the set of fucose-labeled membrane glycoproteins that is transferred rapidly to the plasma membrane and the set that is held in the intracellular membrane compartment. This is shown by the densitometric scans of fucose-labeled glycoproteins as separated by electrophoresis in dodecyl sulfate-polyacrylamide gels. The analysis in Fig. 11 is of the different fractions from the fucose pulse-chase experiment of Fig. 8. The same glycoproteins are present in the 12,000 × g-min supernatant fraction immediately after 2 h of labeling with fucose, as are present after 2 h of labeling followed by a 2-h chase with unlabeled fucose. Also, the same set of glycoproteins are present in the plasma membrane 3 h after the addition of unlabeled fucose to the cell. These are also the same glycoproteins present in the plasma membrane and in the internal cell membranes when cells are long term labeled with fucose (Figs. 4 and 5).

FIG. 10. Autoradiographic analysis of the loss of fucose-labeled glycoproteins from HTC cells. Experimental details are similar to those of Fig. 6, except that the autoradiographs were made from cells labeled with fucose for 48 h followed by a 72-h period of growth in the presence of unlabeled fucose. Exposure time was 40 days.

Indirectly via interiorization and internal storage of plasma membrane vesicles. We attempted to answer this question by following the subcellular distribution of plasma membrane proteins and glycoproteins labeled with [125I] as a function of time that the cells were in culture after external labeling. In an experiment identical in design to that of Fig. 1 and Table I, the externally disposed polypeptides accessible to iodination co-fractionated (that is, showed similar enrichment and distribution of incorporated iodide in the different cell fractions) with the plasma membrane, irrespective of whether the fractions were prepared from cells immediately after iodination or from iodinated cells that were in culture for 24 h. This result supports the study of Fig. 8, indicating that most of the large internal pool of membrane glycoproteins arises from synthesis and not interiorization of plasma membrane vesicles containing these glycoproteins. The results of the experiment, however, do not rule out the possibility that some (albeit
we have purified to apparent homogeneity a subset of these different membrane fractions of HTC cells are homologous, demonstrate unequivocally that the glycoproteins in the two homologous. We attempted to further resolve these glycoproteins using a two-dimensional electrophoretic system modified for the production of plasma membrane proteins is probably very similar to that for secretory proteins (see Ref. 39 for review). The secretory and membrane proteins may only differ in their relative affinity for the lipid bilayer. Actually, a similar model to that of Fig. 12 has also been proposed recently by Scanlin and Glick for the turnover of the plasma membrane (42).

DISCUSSION

The major points we wish to emphasize from the results of the present study are: (a) in HTC cells, a set of fucose-containing glycoproteins is externally oriented on the plasma membrane and is also present in an internal membrane system of the cell; (b) the fucose-containing glycoproteins comprising this set are present in the same relative proportion to each other in each of the two membrane systems, (c) part (40 to 50%) of the labeled fucose incorporated into glycoproteins of the internal membrane system in a 2-h pulse behaves as a direct precursor to the plasma membrane, requiring about 3 h after the addition of fucose for this set of labeled glycoproteins to reach the surface, (d) the other part (50 to 60%) of the fucose incorporated into the set of glycoproteins remains associated with the internal membrane system during the chase with unlabeled fucose; and, (e) the set of fucose-labeled glycoproteins which chases to the surface appears to be similar to the set that remains associated with the internal membrane system.

Since we have relied upon electrophoresis in dodecyl sulfate-polyacrylamide gels as a resolving system for these membrane glycoproteins, the question arises whether the fucose-labeled glycoproteins in each of the two membrane systems are really homologous. We attempted to further resolve these glycoproteins using a two-dimensional electrophoretic system modified after that of Ames and Nikaido (38). The fucose-labeled glycoproteins do show charge heterogeneity in this system. Moreover, the glycoproteins of both the plasma membrane fraction and the internal membrane system again appear very similar now with respect to charge as well as size. Realizing that a rigorous biochemical analysis is required to demonstrate unequivocally that the glycoproteins in the two different membrane fractions of HTC cells are homologous, we have purified to apparent homogeneity a subset of these proteins from both the plasma membrane and the internal cell membrane fractions. We will present a detailed characterization of these glycoproteins elsewhere. Suffice it to say here, that we have as yet been unable to detect any differences between the fucose-labeled glycoproteins purified from these different HTC cell fractions. Hence, the results of these studies taken together argue strongly that the two membrane systems contain the same set of fucose-containing glycoproteins.

Based on results presented here, and on our previous studies (18, 19, 39), we propose the scheme presented in Fig. 12 for the biogenesis of the plasma membrane of HTC cells. The essential features of this model are not new. Similar schemes have been proposed or suggested for the route of biogenesis of secretory proteins (40, 41) and the biogenetic pathway for the production of plasma membrane proteins is probably very similar to that for secretory proteins (see Ref. 39 for review). The secretory and membrane proteins may only differ in their relative affinity for the lipid bilayer. Actually, a similar model to that of Fig. 12 has also been proposed recently by Scanlin and Glick for the turnover of the plasma membrane (42).

The mode of membrane biogenesis includes turnover via interiorization of packets or units of plasma membrane. Each packet is relatively complex in HTC cells containing as many as 100 different proteins all present in different molar amounts. The membrane proteins so interiorized are degraded by fusion of the interiorized packet with a primary lysosome to form a secondary lysosome (19, 39, 43, 44). The interiorized packet of membrane polypeptides is then most probably degraded to its constituent amino acids. The interiorized packet contains both lipid and protein, because we2 can show that glycolipids labeled via reduction of oxidized galactose with sodium borohydride turn over at about the same rate as the glycoproteins of the membrane. The pool of interiorized plasma membrane must be relatively small at any one time in HTC cells because there is very little incorporated iodide present in the interior membrane system even when cells are fractionated 24 h after iodination. The small size of this interiorized pool is due probably to the slow rate of degradation of the HTC cell membrane (half-life of at least 100 h) and possibly to rapid proteolysis of the membrane polypeptides to smaller acid-soluble molecular species once the rate-limiting step in degradation of the membrane has occurred. Degradation may not be a necessary consequence of interiorization. Actually, most of the interiorized membrane packets in HTC cells may return to the surface via a process of recycling (44). That is, the rate of interiorization of plasma membrane in this cell appears to be much higher than the rate of membrane protein degradation. One way to explain this result is to invoke some type of recycling mechanism, in which the internal pool of membrane arising from recycling must be relatively small at any one time. In this respect, it is conceivable that during recycling, carbohydrate residues (19) but not the amino acid backbone, are removed from, or added to, the membrane glycoproteins, leading to heterogeneity in the turnover of the sugar moieties.

In the model depicted in Fig. 12, the cell replaces a unit of interiorized membrane that has been degraded with a preassembled unit of like composition. These newly synthesized, preassembled units can be added directly to the plasma membrane, requiring a transit time of approximately 3 h.
after fucosylation to reach the surface. However, in HTC cells, there is also a relatively large reservoir of presynthesized, preassembled membrane units, which appear identical in fucoprotein composition to the plasma membrane. As much as 60% of the fucose-labeled polypeptides of the HTC cell are present in this interior cell compartment. The function of this membrane compartment is not known. But, HTC cells may not be unusual in having such an internal reservoir of plasma membrane proteins. For example, recent experiments show that the muscle cell acetylcholine receptor is also present in an internal membrane, some of which is not a direct precursor to the plasma membrane (45-47). This interior membrane compartment, we believe, could have important implications for the control of membrane biogenesis and assembly in animal cells. For example, the question arises of how the cell places only one membrane protein in the plasma membrane. Such specific replacement of a membrane polypeptide might happen when one protein is removed from the surface via some sort of capping mechanism (11), or when the concentration of one protein is modulated by a hormone, such as the modulation of the insulin receptor by insulin (48). Three ways can be envisioned by which the cell might replace a single protein or replace a limited subset of proteins in the plasma membrane. One way would be to assemble a completely new packet or unit, enriched in those proteins which must be replaced in the plasma membrane. A second mechanism might be to use the presynthesized reservoir of plasma membrane proteins and to add new specific proteins to the preassembled packet. A third way might be to reorganize the proteins in the interior membrane units and to concentrate perhaps by a capping-type mechanism those proteins which must be replaced specifically in the plasma membrane. Experiments are currently in progress to find out which, if any, of these mechanisms is used by HTC cells to replace specific proteins in the membrane, and also to determine what is the function of the large, preassembled pool of plasma membrane precursor in the pathway of membrane biogenesis.

REFERENCES

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