Gruner, S. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3665–3669.
Hutton, W. C., Yeagle, P. L., & Martin, R. B. (1977) *Chem. Phys. Lipids 19*, 255–265.
Israelachvilli, J. N., Mitchell, D. J., & Ninham, B. W. (1976) *J. Chem. Soc., Faraday Trans. 2 72*, 1525.
Israelachvilli, J. N., Marcelja, S., & Horn, R. G. (1980) *Q. Rev. Biophys. 13*, 121.
Litman, B. J. (1973) *Biochemistry 12*, 2545.
Litman, B. J. (1974) *Biochemistry 13*, 2844.
Rance, M., & Byrd, R. A. (1983) *J. Magn. Reson. 52*, 221.
Schmidt, C, F., Barenholz, Y., Huang, C., & Thompson, T. E. (1977) *Biochemistry 16*, 3948–3953.
Sen, A., Brain, A. P. R., Quinn, P. J., & Williams, W. P. (1982) *Biochim. Biophys. Acta 686*, 215–224.
Sen, A., Hui, S. W., & Yeagle, P. L. (1986a) *Biophys. J. 49*, 434a.
Sen, A., Hui, S. W., Yeagle, P. L., & Mantsch, H. H. (1986b) *Biophys. J. 49*, 322a.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121.
Tanford, C. (1980) *The Hydrophobic Effect*, Wiley, New York.
Thayer, A. M., & Kohler, S. J. (1981) *Biochemistry 20*, 6831–6836.
Yeagle, P. L., & Martin, R. B. (1976) *Biochem. Biophys. Res. Commun. 69*, 775.
Yeagle, P. L., & Young, J. (1986) *J. Biol. Chem. 261*, 8175–8181.
Yeagle, P. L., Benson, J., Boni, L., & Hui, S. W. (1982) *Biochim. Biophys. Acta 727*, 39.

Membrane Biogenesis in Embryonal Carcinomas: Glycoproteins Destined for the Cell Surface Are Delayed in a Pre-Golgi Compartment†

Raymond J. Ivatt

Department of Tumor Biology, The University of Texas, M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77030

Received June 3, 1986; Revised Manuscript Received July 23, 1986

ABSTRACT: Embryonal carcinoma and early embryonic cells assemble a family of unusually large and complex carbohydrates. These glycans are highly branched, repeating copolymers of the sugars galactose and *N*-acetylglucosamine, referred to as polylactosamines, and are frequently decorated with fucose, sulfate, and sialic acid. We have previously shown that in teratocarcinoma cells these glycans are part of a large spectrum of glycans assembled on mannose cores derived from a common precursor glycan. Metabolic studies revealed a large excess of high-mannose glycans at a time when complex-type glycans cease to accumulate. The present studies demonstrate that these high-Man glycans are not degraded internally or secreted directly but are on glycoproteins destined for the cell surface. These unprocessed glycoproteins replace material lost during the extensive membrane turnover that occurs in these cells. Their export to the cell surface is delayed in a pre-Golgi compartment.

Murine teratocarcinoma stem cell lines resemble the cells of the inner cell mass of the preimplantation mouse embryo in their biological (Kleinsmith & Pierce, 1964; Pierce et al., 1964; Brinster, 1973; Mintz & Illmensee, 1975; Papaioannou et al., 1975), serological (Artzt et al., 1973), and biochemical (Bernstine et al., 1973) properties. A class of unusually large and complex carbohydrates has been identified on teratocarcinoma stem cells and on the early embryo (Szulman, 1964; Muramatsu et al., 1978, 1980). These carbohydrates are progressively lost during development (Szulman, 1964; Muramatsu et al., 1978, 1980). These glycans have been examined (Fukuda et al., 1985; Muramatsu et al., 1983) and appear to have structural characteristics in common with erythroglycan—a polylactosamine-containing glycan on the surface of red blood cells (Finne et al., 1978; Jarnefelt et al., 1978; Fukuda et al., 1979).

Several observations have implicated these developmentally programmed carbohydrates as an adhesive system for teratocarcinoma stem cells. In particular, Grabel et al. (1979, 1981, 1983) have demonstrated a cell surface carbohydrate-binding component from teratocarcinoma stem cells that has apparent specificity for fucose-rich glycans, Oppenheimer and Humphreys (1971, 1975) have identified an adhesive factor that requires terminal galactose, and Shur (1982, 1983) has demonstrated a cell surface galactosyltransferase that will bind polylactosaminoglycans that are either on other cells or immobilized on a substrate. Our own studies demonstrated that embryonal carcinomas can recognize a subset of their own carbohydrates—specifically branched, sulfated polylactosamines—and have implicated these glycans in cellular adhesiveness.

We investigated the assembly of glycoproteins in embryonal carcinomas, paying particular attention to the biosynthesis of the polyactosamine-containing glycoconjugates described above. Complex-type glycans assembled on mannose cores are the products of a long and complex biosynthetic pathway (Elbein, 1979; Parodi & Leloir, 1979; Spiro & Spiro, 1979; Kornfeld & Kornfeld, 1980; Struck & Lennarz, 1980; Hubbard & Ivatt, 1981). These glycan products are the result of competition between rival glycosylation pathways (Paulson et al., 1978; Beyer et al., 1981; Ivatt, 1981) for a common acceptor glycan derived by extensive trimming from the lipid-linked precursor glycan. We identified a lipid-linked precursor glycan, identical in properties to the one described for fi-

† This investigation was supported by Grant 1-972 from the March of Dimes Birth Defects Foundation and Grant CA42650 from the National Cancer Institute.

0006-2960/86/0425-7522$01.50/0

broblastic cells, and studied its metabolism (Ivatt, 1985). We also examined the fate of the glycan after transfer to protein. These studies revealed a large excess of high-Man glycans at a stage when the level of complex-type glycans is stabilized. The rapidly and slowly processed high-Man glycans are on the same spectrum of carrier glycoproteins (Ivatt, 1985). We now show that these high-Man glycans are not degraded internally or secreted unprocessed but rather are processed slowly and accumulate in the extracellular medium. However, they are not secreted directly. These glycans are on glycoproteins that have a residence period at the cell surface and are shed into the medium during membrane turnover. In contrast to glycoproteins that are secreted directly (Kelly, 1985), the export of these cell surface glycoproteins is delayed in a pre-Golgi compartment.

Materials and Methods

Materials. Endo-β-galactosidase was a gift from Drs. Michiko Fukuda and Minoru Fukuda of La Jolla, CA. Endo-β-acetylglucosaminidase H (endo-H) was prepared from *Streptomyces plicatus* according to the method of Tarentino et al. (1978). Bio-Gel P4 and P10 were from Bio-Rad Laboratories (Richmond, CA). D-[2-^{3}H]Man (10–20 Ci/mmol), Gal, Fuc, and [^{131}I]NaI were from New England Nuclear (Boston, MA). Lactoperoxidase and Pronase CB were purchased from Calbiochem (San Diego, CA), and glucose oxidase was purchased from Sigma Chemical Co. (St. Louis, MO).

Culture Conditions. F9 murine teratocarcinoma cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum at 37 °C in a humidified 5% CO_2 and air atmosphere.

Cell Labeling. For the pulse and pulse/chase experiments, F9 cells were washed with glucose-free medium 3 times and then metabolically labeled with Man (1 mCi/mL), Gal, or Fuc (0.1 mCi/mL) in glucose-free medium for 20 min. Chases were performed, as described in Results, in complete medium containing 10% fetal calf serum and, after three washes, with complete medium. After different chase periods the chase medium was collected and the cells were digested with trypsin. The chase medium was lyophilized and exhaustively digested with Pronase. The cells, prior to trypsinization, were washed with calcium- and magnesium-free phosphate-buffered saline (PBS); then they were digested with 0.1% trypsin in calcium- and magnesium-free PBS containing 2 mM EDTA for 5 min at 25 °C. The cells were centrifuged and the material that remained in the supernatant, after trypsinization, was dried down and then exhaustively digested with Pronase. The cell pellet was extracted with chloroform/methanol/water (10:10:3) and then exhaustively digested with Pronase as described previously (Ivatt, 1985). In experiments in which the recovery from trypsin treatment was examined, cells were resuspended in 2 mL of PBS and incubated in 10-cm tissue culture dishes for 15 min at room temperature. In the absence of competing protein, the cells were readily adsorbed and then 10 mL of warm (37 °C), complete medium was added, and the cells were incubated at 37 °C.

For the cell surface iodination experiments, the F9 cells were radiolabeled as monolayer culture by using the lactoperoxidase–glucose oxidase method described by Teng and Chen (1976). Iodinated cells were washed twice with PBS and 3 times with complete medium containing 10% fetal calf serum and then incubated for varying periods of time. After different chase periods, the chase medium was collected and the cells were digested with trypsin. Prior to trypsinization, the cells were washed twice with calcium- and magnesium-free PBS; then they were digested for 5 min with trypsin at 0.1% as above. Cells were centrifuged and the materials that were pelleted with the cells and that remained in the supernatant were collected.

For the cell surface biotinylation experiments, the F9 cells were metabolically labeled with Man for 20 min and then chased either for 5 or 60 min. At this time the cells were washed twice with PBS and then incubated in PBS at 4 °C for 30 min in the presence or absence of *N*-hydroxysuccinimidobiotin. The cells were then washed twice with complete medium and incubated in complete medium for varying periods of time. The medium (5 mL) was collected from the cells after these varying periods of time. The proportion of the radioactivity in the medium that was adsorbed by immobilized avidin was determined by incubating the samples with avidin–Sepharose (100 μL) for 30 min and then washing the beads with PBS (2 × 5 mL).

Preparation and Analysis of the Glycopeptides. Cells were metabolically labeled, serially extracted, and exhaustively Pronase-digested as described previously (Ivatt, 1985). Sensitivity to endo-H was tested in 0.1 M citrate (pH 5.5) with 90 milliunits of enzyme at 37 °C for 4 h under a toluene atmosphere. Exhaustive α-mannosidase digestion was performed as described by Liu et al. (1979). Sensitivity to endo-β-galactosidase was tested in 0.1 M citrate (pH 5.8) with 50 milliunits of enzyme, at 37 °C for 24 h with 0.05% NaN_3 (Fukuda & Matsumura, 1976). Gel filtration was performed as described previously (Ivatt, 1985).

Flow Cytometry. F9 cells were dispersed by trypsin treatment and filtered through 20-μm Nytex filters. The single-cell suspensions in PBS were plated on tissue culture dishes at 2 × 10^6/10 cm dish. In the absence of competing protein the cells were readily adsorbed and after 15 min 10 mL of complete medium was added. The cells were incubated at 37 °C for various intervals of time. For flow cytometry the cells were displaced from the dishes in calcium–magnesium-free PBS and filtered through 20-μm Nytex filters. The cells at 2 × 10^5 cells/mL were incubated with various fluorescein isothiocyanate (FITC) treated lectins in the concentration range 0.1–30 μg/mL. Binding of the lectins to the cells was measured on an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL) by using the 488-nm line for excitations at 500 mW (Coherent Innova 90 laser). The instrument was calibrated with plastic microspheres of 10-μm diameter, labeled with fluorescein. With 3–14° narrow-angle, forward light scatter gating, the integral FITC fluorescence per cell was calculated and, for each sample, 10 000 cells were measured for fluorescence levels.

Results

Excess of High-Man Glycans after Complex-Type Glycans Cease To Accumulate. Embryonal carcinoma cells assemble a broad spectrum of protein-linked carbohydrates on Man cores derived from a lipid-linked precursor glycan (Ivatt, 1985). The glycans initially transferred to protein are endo-H sensitive and similar in size to the lipid-linked precusor. With time, complex-type glycans accumulate. The time course for this conversion can be seen in Figure 1A. During the chase period, there is a net decrease in the high-Man glycans and an accumulation of complex-type glycans. However, these two processes are not obviously coupled. For example, at a time when complex-type glycans cease to accumulate—about 60 min of chase—there is a large excess of high-Man glycans; these glycans are subsequently lost with no net accumulation of complex-type glycans. During this delayed loss of high-Man glycans, there is a change in the size distribution of the glycan

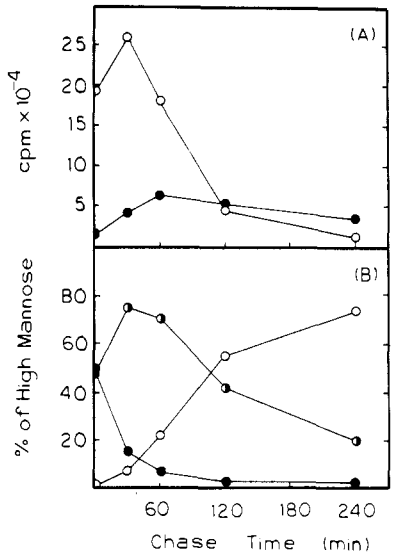

FIGURE 1: Processing of mannose-labeled glycans by F9 cells: (A) amount of radiolabel present in endo-H-sensitive (open circles) and endo-H-resistant (solid circles), protein-linked glycans as a function of increasing chase period following a 20-min pulse label with [^{3}H]Man. (B) Size distribution of the endo-H-sensitive, protein-linked glycans during processing. The size distribution of the endo-H-sensitive, protein-linked glycans was measured at increasing chase times following a 20-min pulse label with [^{3}H]Man. The proportion of protein-linked radiolabel in a particular species is plotted against time: solid circles, more than nine hexoses attached to GlcNAc; halved circles, nine hexoses attached to GlcNAc; open circles, less than nine hexoses attached to GlcNAc. The number (in parentheses) of hexose units attached to *N*-acetylglucosamine (GlcNAc) identifies the size of the glycan.

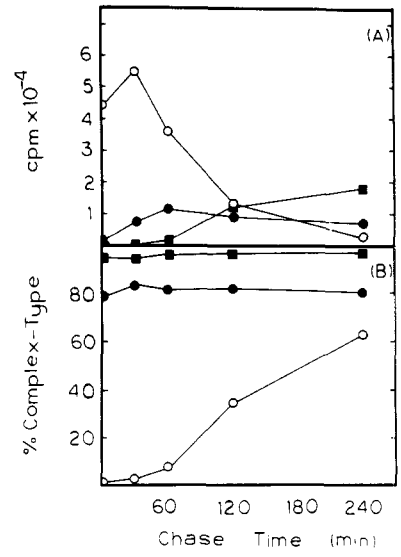

FIGURE 2: Cellular location and composition of protein-linked glycans during processing. Cellular location (A) and composition (B) of protein-linked radiolabeled glycans as a function of increasing chase length following a 20-min pulse label with [^{3}H]Man. Cellular location was determined by sensitivity to trypsinization. The cells were trypsinized at different times following the pulse label and then centrifuged. The material remaining in the supernatant after trypsinization (closed circles) and the material sedimenting with the cells (open circles) were separately pooled. The material spontaneously released into the cultue medium from cells prior to trypsinization was also collected (squares). The composition of these materials was determined by exhaustively digesting them with Pronase and endo-H and then analyzing them by gel filtration on Bio-Gel P4 columns.

species that are sensitive to endo-H (Figure 1B). For example, after a 60-min chase, when the complex-type glycans cease to accumulate, the major endo-H-sensitive glycan species contain at least nine Man residues, while after a second 60-min chase the bulk of glycans have been substantially trimmed. The high-Man glycans processed during the first 60 min of chase and those processed subsequently appear to be in different metabolic pools.

Fate of the Excess High-Man Glycans. We explored the fate of the high-Man glycans present when complex-type glycans cease to accumulate by examining the nature of the glycans spontaneously released into the medium, those associated with the cell surface, and those retained within the cell. Specifically, we wanted to identify the proportion of these high-Man glycans that are shed intact into the medium, the proportion that are degraded intracellularly with no resultant formation of complex-type glycans, and the proportion that are processed in a normal but delayed fashion to form complex-type glycans.

Pulse/chase experiments similar to those described above were performed, and instead of the cellular material being taken as one pool, trypsinization was performed and the trypsin-releasable and the trypsin-resistant materials were collected and analyzed separately. The material spontaneously released by the cells into the medium was also collected and analyzed. The results of this experiment are shown in Figure 2.

Metabolically labeled material was released into the medium slowly during the first 60 min and then accumulated more rapidly over the next 3 h (squares, Figure 2A). The material released into the medium was always greater than 90% complex type in nature (squares, Figure 2B). Metabolically labeled material released from the cells by trypsinization reached a peak after 60 min and then slowly declined during subsequent chase periods (solid circles, Figure 2A). The proportion of complex-type glycans in the material released from the cells by trypsinization remained relatively constant but at about 80% (solid circles, Figure 2B). Metabolically labeled material not released from the cells by trypsinization reached a peak after 30 min and then rapidly decreased during continued chase periods (open circles, Figure 2A). In contrast, the composition of the glycans that remained with the cells after trypsinization changed dramatically during the chase period from being almost exclusively high-Man to being about 60% complex type after 4 h (open circles, Figure 2B).

The distributions of glycan species in the intracellular and cell surface pools, after 30-min and 240-min chases, are shown in Figure 3. The cell surface glycans, as defined by trypsin accessibility (Figure 3A,B) closely resemble each other at the two time points. In contrast, the intracellular glycans (Figure 3C,D) are very different from each other in composition. The glycans present after the earlier chase periods (Figure 3C) are predominantly high-Man in nature with very little complex-type glycan, whereas the glycans present after the later chase period (Figure 3D) are predominantly complex type in nature. Also, at the later time period, the high-Man glycans that are present are extensively trimmed instead of being, after endo-H treatment, predominantly $Man_9GlcNAc$.

The overall recovery of complex-type glycans in the three cellular pools was almost 90% of the amount predicted for the conversion of the lost high-Man glycans to complex type. The conclusions to be drawn from this experiment are that the high-Man glycans that remain after complex-type glycans cease to accumulate are actually processed normally to complex-type glycans, with the difference that this processing occurs much more slowly. The following experiments examined the cellular fate of these slowly processed glycans.

Cellular Fate of the Slowly Processed Glycoproteins. The experiments described above demonstrated that only a small

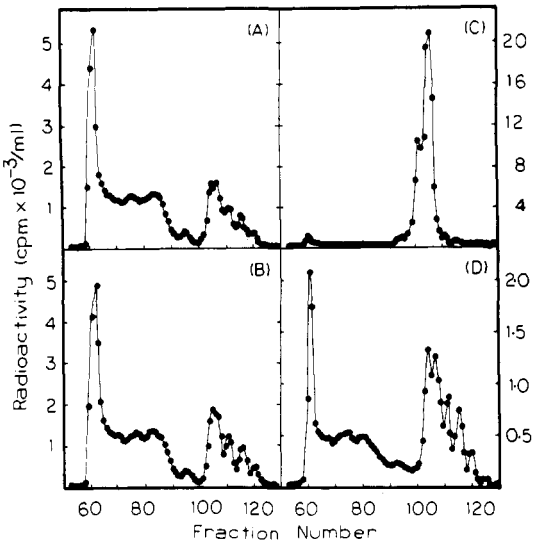

FIGURE 3: Size distribution of the protein-linked glycans in different cellular compartments prepared from cells chased either for 30 (A and C) or 240 min (B and D) after a 20-min pulse label with [^{3}H]Man. Glycans were prepared from the trypsin-resistant (A and B) and trypsin-sensitive (C and D) compartments. After exhaustive digestion with Pronase and endo-H, the glycans were then analyzed by gel filtration on Bio-Gel P4 columns.

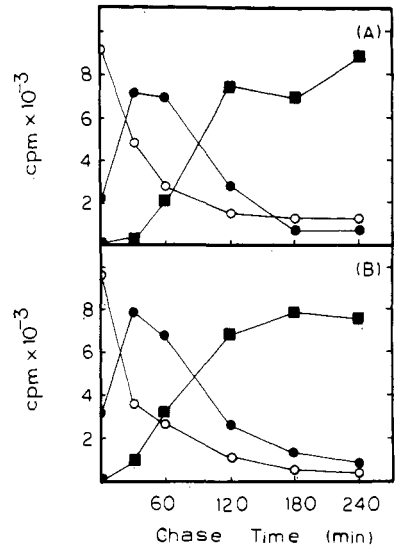

FIGURE 4: Cellular location of protein-linked glycans during processing after metabolic labeling with sugars incorporated in the Golgi apparatus. The cellular location of protein-linked radiolabeled glycans as a function of increasing chase length following a 20-min pulse label either with [^{3}H]Gal (A) or with [^{3}H]Fuc (B). Cellular location was determined by sensitivity to trypsinization as before. The material spontaneously released into the medium (squares) and the cell-derived materials that were either released (closed circles) or not released (open circles) by the trypsin treatment were determined.

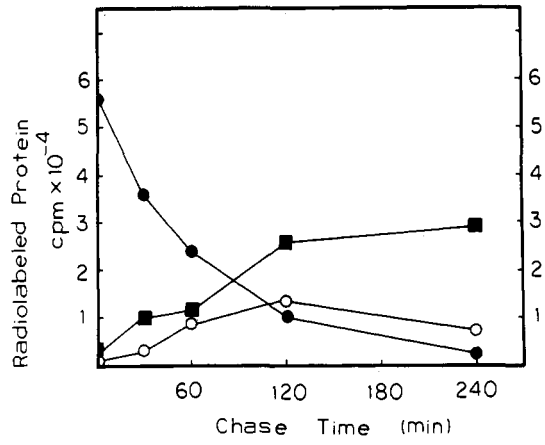

FIGURE 5: Turnover of cell surface material labeled in situ. Cells were labeled by the lactoperoxidase method and trypsinized at different periods following radioiodination. The material remaining in the supernatant (open circles) and the material sedimenting with the cells (solid circles) after trypsin treatment were separately pooled. The culture medium from the cells prior to trypsinization (squares) was collected and the amount of radioiodinated material precipitated by trichloroacetic acid was measured.

amount of the cellular high-Man glycans was lost as a result of degradation and also that very few high-Man glycans were shed unprocessed directly into the medium. The major loss was as a consequence of conversion to complex-type glycans and subsequent accumulation of complex-type glycans in the medium. However, this accumulation of complex-type glycans in the medium could arise by two distinct mechanisms—surface shedding and direct secretion. The following experiments demonstrate that the bulk of the glycans to appear extracellularly have a residency period at the cell surface and that very few are secreted directly.

Pulse/chase metabolic studies, as described for Figure 2, were performed, except that in these experiments the metabolic label was galactose and fucose instead of mannose. Galactose and fucose are incorporated into glycoproteins in the Golgi apparatus after extensive trimming of the Man cores. The time courses shown in Figure 4 demonstrate that very few of the nascent complex-type glycans were on glycoproteins that were secreted directly into the medium. Nearly all of the Gal- and Fuc-labeled glycoproteins were rapidly chased into a pool that was retained by the cell yet accessible to trypsin. As observed in the pulse/chase experiments performed with Man as the metabolic label, there was a slow accumulation in the extracellular space. However, this extracellular accumulation clearly occurs at the expense of the trypsin-accessible cellular pool. The conservation of Man-labeled material at the cell surface during the delayed processing of part of the high-Man pool (as seen in Figure 2) therefore indicates that the material lost from the cell surface is replaced. A combination of approaches demonstrates that the bulk of the high-Man glycans processed in a delayed manner actually replenishes cell surface materials and that the delayed accumulation of complex-type glycans in the extracellular medium is the result of cell surface shedding and not the result of direct export.

Turnover of Cell Surface Material. The first approach measured the rate of turnover of the cell surface material labeled in situ. Cell surface proteins were radioiodinated and the fate of cell surface-derived material was determined. The results of these experiments are shown in Figure 5. After radioiodination, the cells were extensively washed with complete medium and then cultured for various intervals of time. The medium was removed and the trichloracetic acid precipitable iodine was measured. The cells were trypsinized and then centrifuged. The radioiodinated materials that were released from the cells by trypsinization and that remained with the cell pellet were then measured. These results show that there is exponential decay of material from the cell surface. The half-time for radioiodinated material on the cell surface is about 60 min. This is similar to the rate of shedding of Gal- and Fuc-labeled materials from the cell surface (Figure 4) and is similar to the delayed conversion of high-Man to complex-type glycans that occurs during the second hour of processing (Figure 1).

Regeneration of Cell Surface Material. The second approach directly measured the ability of cells to replenish cell surface materials from the intracellular pool. In experiments

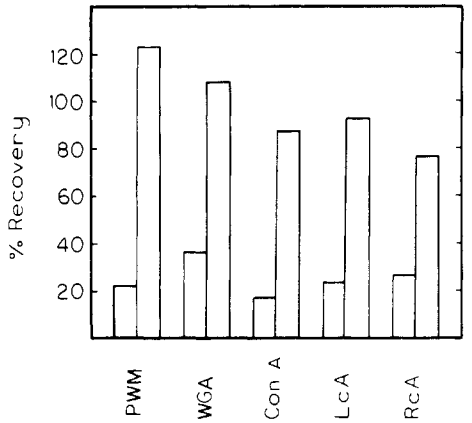

FIGURE 6: Recovery of lectin receptor expression following trypsin treatment. Cells were treated with trypsin, washed, and adsorbed onto tissue culture dishes. The cells were then incubated for either 1 or 24 h. The right-hand panel of each pair compares the levels of expression after 1 and 24 h of recovery. The left-hand panel of each pair compares the levels of expression immediately following trypsin and after 24 h of recovery. The lectins used were PWM pokeweed mitogen and WGA, Con A, LCA, and RCA, the agglutinins from wheat germ, *Canavalia ensiformis*, *Lens culinaris*, and *Ricinus communis*, respectively.

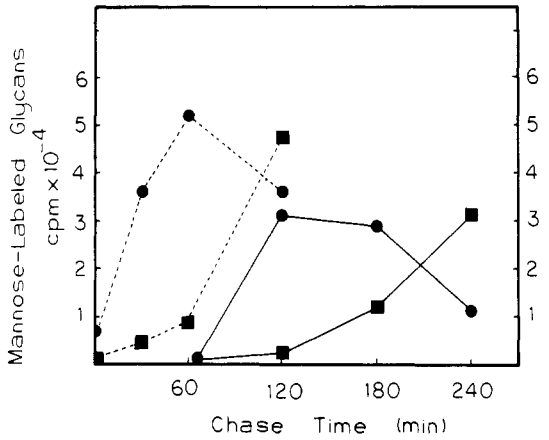

FIGURE 7: Regeneration of cell-surface glycoproteins. Fate of the complex-type glycans produced from high-Man glycans that remain unprocessed after the first hour of chase. Cells were pulse-labeled with [^{3}H]Man for 20 min and then chased for different intervals. At various times the materials shed spontaneously into the medium (squares) and the materials released from the cells by trypsin treatment (circles) were separately collected (dashed lines). Alternatively, cells were pulse-labeled with [^{3}H]Man for 20 min, chased for 60 min, and trypsin treated (solid lines). The cells were allowed to recover for different intervals and the spontaneously released materials (squares) and the materials released at these different times by a second trypsin treatment (circles) were separately collected.

performed for flow cytometric studies we observed that cells rapidly recover their cell surface expression of lectin receptors following trypsin treatment. In Figure 6 the level of expression of various lectin receptors is shown immediately following trypsin treatment (left panel of each pair) and after a 60-min recovery period (right panel of each pair). The values are recorded as a percentage of the level of lectin receptors expressed on cells allowed to recover from trypsin treatment for 24 h (i.e., two generations). The panel of lectins recognizes a broad spectrum of glycans; pokeweed mitogen (PWM) recognizes branched polylactosamines (Irimura & Nicolson, 1983) and wheat germ agglutinin (WGA) recognizes both sialylated glycans (Adair & Kornfeld, 1974) and polylactosamines (Ivatt et al., 1986; Gallagher et al., 1985). With F9 cells very few WGA receptors are lost on neuraminidase digestion; Con A recognizes a variety of mannose-containing glycans including high-Man, hybrid, and biantennary complex type (reviewed: Goldstein & Hayes, 1978), LCA recognizes fucose in the context of biantennary complex-type glycans (Kornfeld et al., 1981), and RCA recognizes terminal Gal and GalNAc residues (reviewed: Goldstein & Hayes, 1978). This spectrum of cell surface lectin receptors is substantially regenerated within a 60-min period. We used this observation to determine if the material that appears on the cell surface during this recovery period is derived from de novo synthesized material or from the preexisting intracellular pool.

Cells were pulse-labeled for 20 min, chased for either 5 or 60 min, and then trypsinized and allowed to recover. The radioactivity released into the medium at different times after trypsinization was collected. After different periods of recovery the cells were subjected to a second digestion with trypsin and the radioactivity released from the cells by this subsequent trypsinization was collected. The results of this experiment are shown in Figure 7.

In this experiment, approximately 50000 cpm was released from the cells digested after 60 min of chase (circles, dashed lines). During the recovery periods this cell surface material was partially replaced (circles, solid lines); for example, approximately 30000 cpm was released upon redigestion 60 min later. This demonstrates that the internal pool of radiolabeled glycoproteins *can* act as a reservoir for the cell surface. This additional release of radioactivity upon redigestion is not the result of excessive cellular damage as less than 2500 cpm was released if retrypsinization was performed after only a 5-min interval. During the recovery period some material is released spontaneously into the medium (squares, solid lines); for example, 4000 cpm was released during a 60-min recovery period. In contrast, cells that had not had their cell surface pools depleted by trypsin treatment spontaneously released 40000 cpm into the medium during the second 60 min of chase (squares, dashed lines). The trypsin treatment by itself did not substantially affect the overall distribution of radiolabeled material. When trypsin treatment was performed only 5 min after the 20-min pulse, very little radiolabel was present on the surface. After a delay, radiolabeled material appeared in the cell surface and extracellular pools with no significant difference between the trypsin-treated and trypsin-untreated cells. The spectrum of glycoproteins present on the trypsin-treated cells after a 60-min recovery period was very similar to the spectrum of glycoproteins present at steady state (Ivatt, 1985). The major species in both situations have apparent molecular weights on sodium dodecyl sulfate–polyacrylamide gel electrophoresis of 250, 170, and 105 kDa and minor species of 130 to 140 and 95 kDa.

The large reduction in the amount of radiolabeled material to appear in the extracellular pool as a consequence of trypsin treatment at 60 min suggests that the source of the extracellular material is the cell surface. As the total amount of radiolabeled material at the cell surface is conserved during this chase period, a comparable amount of material must be mobilized to replace the surface shedding. This material ultimately appears in the extracellular pool after a residence period on the cell surface. This analysis and the results with Gal and Fuc labeling demonstrate that there is very little direct secretion with these cells.

Origin of the Extracellular Material. The third approach measured the proportion of material found in the medium that was derived from the cell surface. Cells were pulse-labeled for 20 min, chased for either 5 or 60 min, and biotinylated. The radioactivity released into the medium at different times after biotinylation was collected. This material was then incubated in immobilized avidin and the proportion of ra-

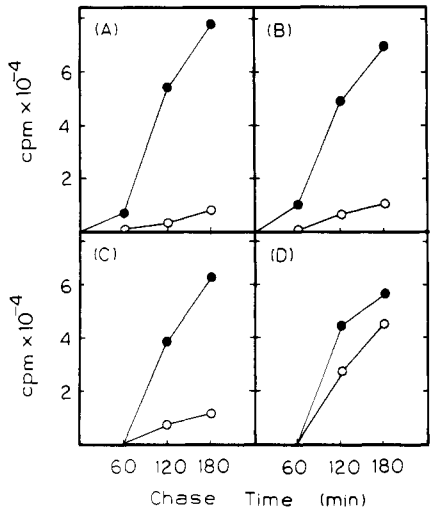

FIGURE 8: Cellular pool that provides the extracellular glycoproteins. Proportion of the glycoproteins found extracellularly that were derived from the cell surface. Cells were pulse-labeled with [^{3}H]Man for 20 min and then chased for either 5 (A and B) or 60 min (C and D). The cells were then incubated with PBS at 4 °C for 30 min either in the presence (A and C) or in the absence (B and D) of NHS Biotin. The material released into the medium by the cells at different time points and after these treatments was measured (solid symbols). The amount of this material adsorbed by immobilized avidin is shown (open symbols).

dioactivity adsorbed by this protein was determined. The results of this experiment are shown in Figure 8. The solid symbols show the amount of material released into the medium and the open symbols show the amount of this material that is adsorbed by immobilized avidin. When biotinylation was performed after a 5-min chase (Figure 8B), apart from a delay caused by the cold incubation, radioactivity appears in the medium at a rate similar to that observed previously (Figure 7). Very little of the material recovered in the medium was adsorbed by immobilized avidin. When biotinylation was performed after a 60-min chase (Figure 8D), there was still extensive release of the material into the medium; however, in this case, the bulk of the radioactivity was adsorbed by immobilized avidin. Experiments, performed in parallel, subjected the cells to the same treatment in terms of washes and cool incubation but in the absence of the biotinylating reagent (Figure 8A). The results showed that biotinylation by itself had very little affect upon the rate that material accumulated extracellularly. These results demonstrate that the cell surface was the major source of the material found extracellularly during the second 60-min chase.

Together these experimental approaches establish that there is extensive shedding of cell surface materials, that the material that is shed from the cell surface is replaced by material from the large pool of unprocessed glycoproteins, and that the material that is found in the extracellular medium is predominantly derived from the cell surface and is not exported directly. The close similarities in the kinetics of these processes suggest that they are coordinated.

Discussion

The present study examined the assembly of Man-containing glycans in embryonal carcinomas. These glycans are a heterogeneous group of compounds that are derived from a common precursor glycan (Ivatt, 1985). The processing of this common precursor in these pluripotent stem cells has been shown to be similar to the pathway established for fibroblastic cells (Elbein, 1979; Parodi & Leloir, 1979; Kornfeld & Kornfeld, 1980; Struck & Lennarz, 1980; Hubbard & Ivatt, 1981). The Man core is assembled on a carrier lipid as part of a much larger glycan and transferred en bloc to protein acceptors. Most of these precursor glycan chains are trimmed rapidly during the maturation of the glycoproteins to expose a core structure. The glycans assembled on this core structure are very heterogeneous in size and include an unusual class of glycans containing repeating lactosamine units (polylactosamine glycans).

During the conversion of the high-Man precursor glycan to complex-type glycans, there are two discernible classes of high-Man glycan. One class is rapidly and extensively trimmed to form the core structure. The other class is hardly trimmed at all beyond the removal of the three hexose (glucose) residues. The very low steady-state level of partially trimmed glycans during the appearance of complex-type glycans suggests that the reactions involved in processing are very efficiently coordinated and that once processing of a glycan chain begins it rapidly goes to completion. The second class of high-Man glycans is lost very slowly. We examined the secreted material from these cells during more extensive time courses and it appears that the second class of high-Man glycans is converted to complex-type glycans normally but with much slower kinetics than the first class. The kinetics of this processing suggest that the proteins that carry the second class of high-Man glycans equilibrate with an internal pool of glycoproteins. As these glycoproteins remain unprocessed, we identify this pool as being pre-Golgi. This identification is consistent with the absence of a slowly transported pool when metabolic experiments are performed with Gal and Fuc (sugars that are incorporated in the Golgi apparatus).

Complex-type glycans that are shed from the cell surface can be replaced from this pool of unprocessed glycans. We used both metabolic and in situ labeling in conjunction with trypsinization to estimate the average rate of turnover of cell surface materials. Our conclusions are that cell surface material on these cells is shed with similar kinetics to the slow conversion of high-Man to complex-type glycans, i.e., having a half-life of about 60 min. The small contribution that direct export makes to the extracellular accumulation of metabolically labeled material is demonstrated by the biotinylation experiments described in Figure 8. Here the bulk of the material released during the second 60-min chase period is derived from material present at the cell surface.

It appears most likely that this reservoir of unprocessed material is slowly converted to complex-type glycans and that the carrier proteins for these glycans are destined to appear on the cell surface. Previous studies (Ivatt, 1985) showed that there were similar spectra of protein carriers expressed at different times during processing and that the different kinetic pools of high-Man glycans were not on different pools of carrier glycoproteins. When the levels of high-Man and complex-type glycans have been compared, there has routinely been an excess of high-Man glycans during pulse/chase experiments at a time when the level of complex-type glycans has stabilized. Our studies suggest that these excess high-Man glycans are eventually processed to replace the carrier glycoproteins lost during membrane turnover.

Acknowledgments

We acknowledge the excellent technical assistance of John W. Reeder and Zahra Mansouri. We thank Nancy Edwards for help in preparing the manuscript.

References

Adair, W. L., & Kornfeld, S. (1974) *J. Biol. Chem. 249*, 4696–4704.

Artzt, K., Dubois, P., Bennett, D., Condamine, H., Babinet, C., & Jacob, F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2988–2992.
Bernstine, E. G., Hooper, M. L., Grandchamp, S., & Ephrussi, B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3899–3903.
Beyer, T. A., Sadler, J. E., Rearick, J. I., Paulson, J. C., & Hill, R. L. (1981) *Adv. Enzymol. Relat. Areas Mol. Biol. 52*, 23–176.
Brinster, R. L. (1973) *Biochem. Genet. 9*, 187–191.
Elbein, A. D. (1979) *Annu. Rev. Plant Physiol. 30*, 239–272.
Finne, J., Krusius, T., Rauvala, H., Kekomaki, R., & Myllyla, G. (1978) *FEBS Lett. 89*, 111–115.
Fukuda, M. N., & Matsumura, G. (1976) *J. Biol. Chem. 251*, 6218–6225.
Fukuda, M., Fukuda, M. N., & Hakomori, S.-I. (1979) *J. Biol. Chem. 254*, 3700–3703.
Fukuda, M. N., Dell, A., Oates, J. E., & Fukuda, M. (1985) *J. Biol. Chem. 260*, 6623–6631.
Gallagher, J. T., Morris, A., & Dexter, T. M. (1985) *Biochem. J. 231*, 115–122.
Goldstein, I. J., & Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem. 35*, 127–340.
Grabel, L. B., Rosen, S. D., & Martin, G. R. (1979) *Cell (Cambridge, Mass.) 17*, 477–484.
Grabel, L. B., Glabe, C. G., Singer, M. S., Martin, G. R., & Rosen, S. D. (1981) *Biochem. Biophys. Res. Commun. 102*, 1165–1171.
Grabel, L. B., Singer, M. S., Martin, G. R., & Rosen, S. D. (1983) *J. Cell Biol. 96*, 1532–1537.
Hubbard, S. C., & Ivatt, R. J. (1981) *Annu. Rev. Biochem. 50*, 555–583.
Irimura, T., & Nicolson, G. L. (1983) *Carbohydr. Res. 120*, 187–195.
Ivatt, R. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4021–4025.
Ivatt, R. J. (1985) *Biochemistry 24*, 7314–7320.
Ivatt, R. J., Harnett, P. B., & Reeder, J. (1986) *Biochim. Biophys. Acta 881*, 124–134.
Jarnefelt, J., Rush, J., Y.-T., & Laine, R. A. (1978) *J. Biol. Chem. 253*, 8006–8009.
Kelly, R. B. (1985) *Science (Washington, D.C.) 230*, 25–32.
Kleinsmith, L. J., & Pierce, G. B. (1964) *Cancer Res. 24*, 1544–1552.
Kornfeld, R., & Kornfeld, S. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., Ed.) pp 1–34, Plenum, New York.
Kornfeld, K., Reitman, M. L., & Kornfeld, R. (1981) *J. Biol. Chem. 256*, 6633–6640.
Liu, T., Stetson, B., Turco, S. J., Hubbard, S. C., & Robbins, P. W. (1979) *J. Biol. Chem. 254*, 4554–4559.
Mintz, B., & Illmensee, K. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3585–3589.
Muramatsu, T., Gachelin, G., Nicolas, J. F., Condamine, H., Jakob, H., & Jacob, F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2315–2319.
Muramatsu, T., Condamine, H., Gachelin, G., & Jacob, F. (1980) *J. Embryol. Exp. Morphol. 57*, 25–36.
Muramatsu, H., Ishihara, H., Miyauchi, T., Gachelin, G., Fujisaki, T., Tejima, S., & Muramatsu, T. (1983) *J. Biochem. (Tokyo) 94*, 799–810.
Oppenheimer, S. B. (1975) *Exp. Cell Res. 92*, 122–126.
Oppenheimer, S. B., & Humphreys, T. (1971) *Nature (London) 232*, 125–127.
Papaioannou, V. E., McBurney, M. W., & Gardner, R. L. (1975) *Nature (London) 258*, 70–73.
Parodi, A. J., & Leloir, L. F. (1979) *Biochim. Biophys. Acta 559*, 1–37.
Paulson, J. C., Prieels, J. P., Glasgow, L. R., & Hill, R. L. (1978) *J. Biol. Chem. 253*, 5617–5624.
Pierce, G. G., Beals, T. F., Sri Ram, J., & Midgley, A. R. (1964) *Am. J. Pathol. 45*, 929–962.
Shur, B. D. (1982) *J. Biol. Chem. 257*, 6871–6878.
Shur, B. D. (1983) in *Teratocarcinoma Stem Cells, Coldspring Harbor Conferences on Cell Proliferation* (Silver, L., Martin, G. R., & Strickland, S., Eds.) Vol. 10, pp 185–196, Cold Spring Harbor Press, Cold Spring Harbor, NY.
Spiro, R. G., & Spiro, M. J. (1979) in *Glycoconjugate Research* (Gregory, J., & Jeanloz, R., Eds.) Vol. 2, pp 613–636, Academic Press, New York.
Struck, D. K., & Lennarz, W. J. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., Ed.) pp 25–84, Plenum Press, New York.
Szulman, A. E. (1964) *J. Exp. Med. 199*, 503–516.
Tarentino, A. L., Trimble, R. B., & Maley, F. (1978) *Methods Enzymol. 50*, 574–579.
Teng, N. N. H., & Chen, L. B. (1976) *Nature (London) 259*, 578–580.