Journal of Supramolecular Structure 8:89–109 (1978)
Cell Surface Carbohydrates and Biological Recognition 525–545

Glycoprotein and Protein Precursors to Plasma Membranes in Vesicular Stomatitis Virus Infected HeLa Cells

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Vesicular stomatitis virus is known to mature at HeLa cell plasma membranes. To study the process, cells, infected with vesicular stomatitis virus, were fractionated after short term labeling studies (1 min pulse, 1 min chase) to determine the assembly kinetics of G protein and M protein into plasma membranes. Newly synthesized M protein was found released in the supernatant from which free polysomes were sedimented during sucrose gradient analysis of these polysomes. If this M protein is particle bound, it must have a density of less than 1.08 g/ml. About 40% of this M protein so labeled was not sedimentable at 165,000 X g for 16 h. This newly synthesized M protein had not yet assembled into plasma membrane and thus must represent an internal pool. This and previous studies show that it has a subsequent transit time to the plasma membrane of about 2 min. Once associated with plasma membranes, M protein decayed in an approximately logarithmic fashion indicating that newly synthesized M randomly mixes (and turns over) with preexisting M protein. G protein was particle bound in a 1 min pulse, 1 min chase, and was never found released in a soluble form. At the later time when fucose is added to G protein, the oligosaccharide moiety is near to complete, and on completion is about 2,000 in molecular weight. Evidence is presented showing that fucose is probably attached to the N-acetylglucosamine of the protein carbohydrate linkage. G protein to which fucose had just been added was located internally on a membranous fraction of density 1.14 g/ml in sucrose; its subsequent transit time from this pool (which in uninfected cells is between 1–2% of the total cell fucosyl glycoprotein) was about 15 min. Because their densities were different and their transit times were different, internal newly synthesized M and fucosyl G protein which assemble into plasma membranes were not on the same internal membranous component. Association of M protein with the plasma membranes may thus occur from a nonsedimentable soluble cytoplasmic pool by a process of direct adsorption.

Key words: plasma membrane assembly, HeLa cells, purified plasma membranes, intracellular membrane pools, membrane bound and free polysomes, fucose and glucosamine, glycopeptide synthesis, M protein, G protein, vesicular stomatitis virus

Some viruses maturing at the plasma membrane are known to contain as part of their own membrane a glycoprotein (G protein) and in some cases a nonglycosylated species (M protein). In uninfected HeLa cells, glycoproteins and nonglycosylated proteins assemble into membrane by different pathways (1) and in vesicular stomatitis virus in-

Received March 24, 1977; accepted June 24, 1977.

0091-7419/78/0801-0089$03.80

fected HeLa cells G and M proteins associate with the HeLa plasma membrane at different rates after their synthesis (2). For the nonglycosylated matrix proteins similar observations have been made for fowl plague virus (4), Rauscher leukemia virus (5), and Newcastle disease virus (6). In the same context, Meier-Ewert and Compans (7) have concluded that the different polypeptides of influenza virus were assembled into virions by distinct pathways as in Sendai virus assembly (8). Data from our previous publication (Atkinson et al., Ref. 2) is consistent with the hypothesis that there are different routes of assembly of G and M proteins of vesicular stomatitis virus into HeLa cell plasma membranes. Both these proteins have a discernible internal pool (2, 33) precursor to plasma membrane though little else is known about the properties of the M protein pool.

In this paper the state of glycosylation of G protein in transit to the plasma membrane is more fully defined and internal M protein precursor to plasma membrane protein is shown to be in a soluble form or a form of very low sedimentability. It is shown that G protein and M protein are not on the same physical entity prior to the insertion of the former into the plasma membrane. In addition, data are presented showing the turnover characteristics of M and G protein from the plasma membranes, and also detailing the time and position of fucose addition to the carbohydrate chain.

METHODS

Cells, Virus Infection, and Radioactive Labeling

Stock and experimental HeLa S_3 cells were grown at 37°C in Eagle's minimal essential medium (9) in Earle's suspension powder (Grand Island Biological Company, Grand Island, New York, Catalog No. F-14) in the absence of antibiotics. The final glucose concentration was 2 g/liter instead of half this amount as stated under this catalog number. The growth medium was supplemented with 3.5% calf serum, 3.5% fetal calf serum (Grand Island Biological Company), and 1% glutamine. In these conditions, cells grew logarithmically from 10×10^4 cells/ml to 100×10^4 cells/ml, with a generation time of 23 h and were used for experiments after growth to a density of $50–70 \times 10^4$ cells/ml. Cells were tested monthly for Mycoplasma contamination by the culturing procedure of Levine et al. (10) and by Levine's enzymatic assay (11); by both these criteria they were free of such contamination. For long labeling periods (more than 3 h) cells were used in their normal growth density range ($1.5 \times 10^5 – 8.0 \times 10^5$ cells/ml). For infection and short term labeling periods, unless otherwise stated, cells were collected by centrifugation and resuspended in growth medium minus serum supplemented with glutamine (2 mM) at 5–10 times their previous density. Cells were infected with 10 plaque forming units/cell of vesicular stomatitis virus (VSV: Indiana serotype). At 1 h postinfection, actinomycin D (kindly donated by Merck and Company, Inc., Rahway, New Jersey) was added to a concentration of 1 μg/ml and at 1.5 h postinfection fetal calf serum was added to 5% concentration. At times ranging from 3.5 to 5 h after infection when host protein synthesis is maximally inhibited (12), cells were grown in medium containing various radioactively labeled precursors: L-[1-^{14}C] fucose (48.66 mCi/mmole) was used at 0.1 μCi/ml concentration and L-[6-^{3}H] fucose (13.4 Ci/mmole), was used at 1–20 μCi/ml concentration, ^{14}C-labeled amino acids mixture, 15 amino acids (80–400 mCi/mmole) was used at concentrations of 0.1–1.0 μCi/ml, L-[6-^{3}H] fucose (13.4 Ci/mmole) was used at concentrations of 1–20 μCi/ml. D-[1-^{14}C] glucosamine HCl (45–55 mCi/mmole) was used at a concentration of 3–4 μCi/ml. L-[^{35}S] methionine

(157 Ci/mmole) was used at concentrations of 5–50 μCi/ml. These compounds were purchased from New England Nuclear Corporation, Boston, Massachusetts.

Isolation and Purification of HeLa Cell Plasma Membrane Ghosts

After experimental manipulations, cells were harvested from culture by low-speed centrifugation (800 × g • min) and further processed as previously described (2) utilizing Earle's balanced salt solution for washing at pH 6.8 (adjusted on the day of the experiment). Plasma membranes were prepared by one cycle of zonal centrifugation according to the procedure of Atkinson and Summers (13), except for the omission of iodoacetate and azide. Generally, 4 discontinuous gradients were used to isolate plasma membranes from 1×10^8 cells. The ouabain sensitive Na^+, K^+-ATPase recovery and enrichment, RNA and DNA content, morphological appearance, and the presence of actin- and myosin-like proteins in uninfected plasma membranes prepared identically has recently been described (15). Protein was determined by the Lowry method as modified by Ceccarini and Eagle (16).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

The sodium dodecyl sulfate-polyacrylamide gel (SDS-gel) electrophoresis system of Maizel (17) was used without substantial modification as previously detailed (2): analytical gels were 23–25 cm in length.

Preparation of Polysomes

Membrane bound polysomes and free polysomes were prepared by the method of Grubman, Weinstein, and Shaffritz (18), except sodium deoxycholate (DOC) was substituted for NP40 in the polysome analysis step. Before this, cells were vigorously disrupted in a dounce homogenizer in RSB (19), the nuclei and large plasma membrane fragments removed by brief low speed centrifugation and the remaining material was centrifuged at 16,000 rpm (~ 30,000 × g) in a Sorvall SS-34 rotor for 30 min. The supernatant was made 1% with respect to DOC and the free polysomes were sedimented and separated on 35 ml 7–52% wt/wt sucrose gradient by centrifugation for 16 h at 16,000 rpm in a SW27 rotor at 0°C. The gradients were buffered with RSB and contained 100 μg/ml heparin. In a control experiment, omission of the DOC made no difference to the sedimentation profile of the polysomes and monosomes. The pellet, obtained above after centrifugation of the homogenate at 30,000 × g, was resuspended in RSB, 100 μg/ml heparin and sedimented through a 15–30 (or 35%) wt/wt sucrose gradient for 30 min at 25,000 rpm in the SW27 rotor at 0–4°C. The pellet so obtained was resuspended in RSB, 100 μg/ml heparin, and 1% DOC and analyzed for polysomes as described above. Polysome profiles of absorbance at 260 nm were determined utilizing a Gilford Spectrophotometer with a flow-cell attachment and fractionated into approximately 40 1.0-ml fractions. Regions containing polysomes and supernatant proteins were separately pooled, precipitated with 10% trichloroacetic acid, washed with 100% acetone, and resuspended in SDS-gel electrophoresis running buffer for analysis on SDS polyacrylamide gels.

Analysis of Pronase-Digested Glycopeptides

Production of glycopeptides by pronase, their subsequent digestion with glycosidases, sizing in gel filtration, glycopeptide standards, high voltage paper electrophoresis, and paper

chromatography have been described in detail elsewhere (20, 1, 2) and in general we have used Thyroglobulin Unit A glycopeptide (mol wt 1,800), Thyroglobulin Unit B glycopeptide (mol wt 3,000), [^{14}C]acetyl-Asn-(GlcNAc)$_2$(Man)$_5$ (mol wt 1,393) and [^{14}C]acetyl-Asn-(GlcNAc) (mol wt 379).

RESULTS

Fractionation of HeLa cells into purified plasma membranes allowed observation of the transfer of newly synthesized VSV structural components from a pool inside whole cells to the plasma membranes. In this way "transit times" (1, 2) can be determined, and by extending the time span of the experiments, turnover characteristics can also be observed.

M and G Protein

Our previous paper established the transit times of M and fucosyl G proteins to the plasma membranes from the site of synthesis (2). The present study confirms that M protein continues to accumulate in purified plasma membranes (Fig. 1A) for not more than 5 min after labeling of M protein has ceased in the intact cells (Fig. 1B). This continued accumulation, though of short duration indicates an internal pool of M protein. The maximum transit time of M protein from this pool to the cell surface also, must be 5 min or less. G protein labeled with fucose stopped accumulating in the plasma membranes about 20 min (Fig. 1A) after labeling of fucosyl G protein in the whole cells had ceased (Fig. 1B). This continued accumulation (20 min) is the transit time of fucosyl G protein. G protein labeled with amino acids (i.e., all G protein including the incompletely glycosylated G protein precursor to fucosyl G protein, see below) continued to accumulate in the plasma membranes for the duration of the chase (Fig. 1A). Thus, the transit time of incompletely glycosylated G protein is not less than the duration of the chase in this experiment (75 min) and has not been defined in this study. Chase times were not extended long enough in our previous study (2) to observe turnover of M and G protein and in order to determine the decay or turnover characteristics of these proteins, the chase period was continued over an extended time period. M protein was observed to chase out the purified plasma membranes in near logarithmic decay (Fig. 1A). This turnover behavior is consistent with complete mixing of virus M protein with preexisting molecules in the plasma membrane and random loss from the cell – as has been previously discussed by Witte and Weissman (22) in their observations on oncornavirus glycoprotein. This would imply that M protein does not arrive in the plasma membrane as a complete virion complement of proteins and depart from the cell in the same manner, but rather it is inserted at various multiple sites with a subsequent remixing of newly inserted and previously inserted molecules. There was no obvious logarithimic decay of fucosyl G protein though this may only reflect that the chase period was not long enough or there were insufficient data points to observe such a chase for these species. Newly synthesized N protein in the whole cells stops labeling immediately on the addition of the amino acid chase (Fig. 1C): accumulation of newly synthesized N protein in the plasma membrane does not stop for another 15 min thereafter, which is therefore the N protein transit time. It is of interest to note that this is near the transit time of fucosyl G protein.

To lessen the possibility that cross contamination of subcellular fractions may affect interpretation of the data, the rate of accumulation of polypeptides labeled with ^{14}C-

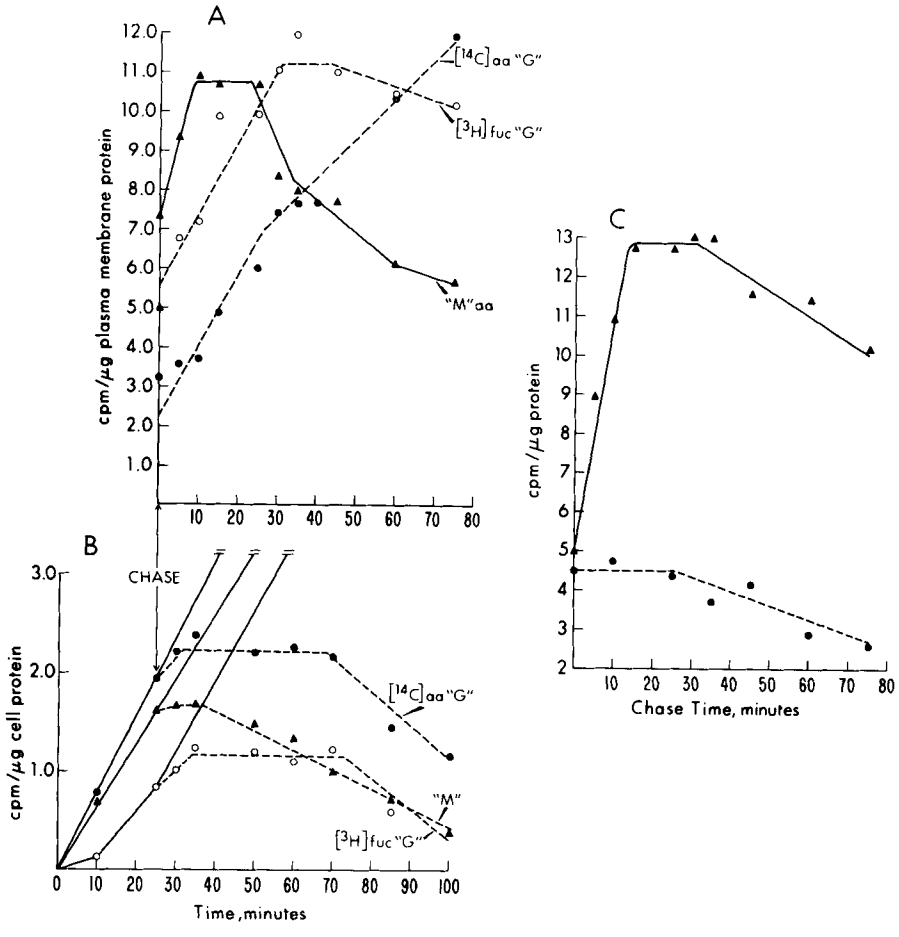

Fig. 1. Chase of 25 min labeled VSV structural proteins into plasma membranes. Cells (1.2×10^9 cells at a density of 3×10^6 cells/ml) were infected 4 h and 25 min with VSV, resuspended in fresh medium at 1.02×10^7 cells/ml then labeled with 34 μCi/ml [^{3}H] fucose and 2.8 μCi/ml ^{14}C-labeled amino acid mixture. Samples were removed from a portion of this culture 10 and 85 min after the radioactive precursors were added to the main culture and further processed for preparation of plasma membranes, measurement of protein, and radioactivity. "Chase" conditions were obtained in the other culture by adjusting it to approximately 10 × amino acids (essential and nonessential), also, 10 × glutamine, 1 × Earle's balanced salt solution, 0.01 mg/ml phenol red for pH adjustment with 10 N NaOH, and 1.25 g (final concentration 40 mM) fucose. 16.5-ml samples (~ 1×10^8 cells) were removed and processed as above for plasma maembrane preparation 0, 5, 10, 15, 25, 30, 35, 45, 60, and 75 min after initiation of the chase. VSV structural proteins in homogenates and in the purified plasma membranes were separated and the radioactivity in them quantitated by SDS-polyacrylamide gel electrophoresis. A) The chase of M protein (▲—▲), fucosyl-G protein (o- - -o), and total G protein (●- - -●) into and from the plasma membranes. B) The chase of M protein (▲- - -▲), fucosyl G protein (o- - -o), and total G protein (●- - -●) from the unfractionated cells. The solid lines in this figure show how the respective proteins labeled, without application of the chase. C) The chase of N protein into and from the plasma membranes (▲—▲) and from the cells (●- - -●).

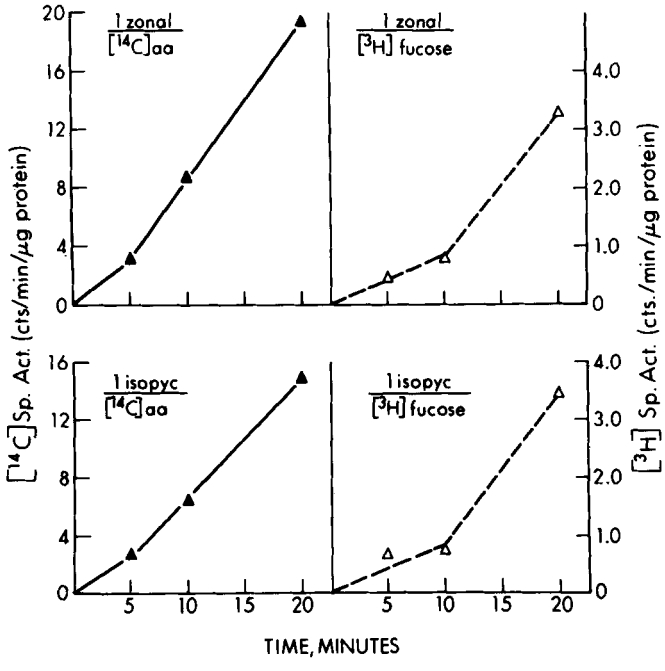

Fig. 2. Effect of more highly purifying plasma membranes on the rapid accumulation of M protein. Cells, 50 ml at 5 X 10^6 cells/ml, were infected with VSV for 4 h 45 min, at which time 2 μCi/ml ^{14}C-labeled amino acids and 16 μCi/ml [^{3}H] fucose were added. Samples were withdrawn and processed for the plasma membrane preparation at 5, 10, and 20 min of labeling. Protein concentration and radioactivity were determined. Upper Panels. Incorporation of ^{14}C-labeled amino acid-labeled proteins (▲—▲) and [^{3}H] fucosyl glycoproteins (△- - -△) into plasma membranes purified by 1 cycle of zonal centrifugation. Lower Panels. Incorporation of ^{14}C-labeled amino acid-labeled proteins (▲—▲) and [^{3}H] fucosyl glycoproteins (△ - - - △) into plasma membranes purified as above but further purified by banding isopycnically half of the material from the zonal gradient on a 50-ml 0–45% wt/wt sucrose gradient by centrifugation for 16 h, at 45,000 rpm in a SW50 rotor. The band was removed, diluted, and the ghosts collected by centrifugation prior to resuspension in a small volume of 10 mM Tris for analysis of protein and radioactivity.

labeled amino acids was determined in plasma membrane preparations which were more highly purified by centrifugation to their isopycnic density of 1.16–1.17 g/ml in sucrose. Our previous results (2) have shown that the bulk of newly synthesized protein associating with plasma membranes to be M protein when the labeling time with radioactive amino acids is very short (minutes). There was little difference in the lag time (2–3 min) in the rate of accumulation of molecules labeled with radioactive amino acids in plasma membranes purified by rate zonal sedimentation (Fig. 2A) and those purified, in the same experiment, to a higher level by isopycnic banding of the membranes at their density of 1.16–1.17 g/ml (Fig. 2B). The ^{14}C-labeled amino acid specific activity in the membranes purified by isopycnic banding did drop slightly consistent with the removal of soluble molecules such as labeled N and NS proteins. Our previous results (3) had shown that once M protein is bound to plasma membrane ghosts it could not be removed by further sucrose

gradient purification, washes, or even with mild detergents known to be capable of removing cytoplasmic tabs from nuclei (19). Hence, loss of labeled molecules is almost certainly the loss of soluble species and not bound M protein. Thus, a procedure known to purify HeLa plasma membranes tenfold (15) with respect to homogenate protein does not significantly alter our transit time estimate for M protein as being 2–3 min. The latter estimate is based on use of the rapid rate zonal purification of plasma membranes (Methods) and the plasma membrane preparations described in the rest of this study were purified only by this method.

Nature of Material 10 Minute Pulse With [^{3}H] Fucose

Our previous results (2) and this study show that the transit time of fucosyl G protein from the site of synthesis to the plasma membranes is 15–20 min. Hence, G protein labeled 12 min with [^{3}H] fucose should all be internal. The nature of this material was explored more thoroughly in this section. VSV-infected HeLa cells, 12-min pulse labeled with [^{3}H] fucose, contain membrane bound material which SDS gel analysis (not shown) demonstrated to be labeled G protein. Pronase digestion of this material followed by high voltage paper electrophoresis at pH 1.9 and pH 6.5 demonstrated that the large proportion of the fucose was attached to glycopeptides. Cellular homogenates from which intact nuclei had been removed, subfractionated on a rate zonal gradient as previously described for the preparation of plasma membranes (14) gave rise to 5 fractions (Table 1): a clear

TABLE I. Distribution of Pulse-Labeled and Long-Term-Labeled Fucosyl G Protein in Cell Fractionation

Cell fraction	Long term labeled [^{14}C] fucose specific activity, cpm/μg protein (relative specific activity)	Recovery %	12 min pulse labeled [^{3}H] fucose specific activity, cpm/μg protein (relative specific activity)	Recovery %
Homogenate	4.5 (1)	100	1.5	100
500 × g · min pellet (nuclei)	4.8 (1.1)	27.7	0.7 (0.4)	11.1
500 × g · min supernatant	4.0 (0.9)	59.7	1.6 (1.1)	72.3
Zonal Fraction I from 500 × g · min supernatant	1.4 (0.3)	9.5	1.0 (0.6)	19.9
Zonal Fraction II	4.3 (1.0)	17.4	3.3 (2.2)	39.0
Zonal Fraction III	3.9 (0.9)	8.0	3.5 (2.3)	21.8
Zonal Fraction IV[a]	22.6 (5.0)	25.9	1.0 (0.7)	3.5
Zonal Fraction V	4.8 (1.1)	1.5	0.6 (0.4)	0.6
Purified plasma membranes	37.9 (9.7)	18.2	0.7 (0.5)	0.8

[a]Contains the partially purified plasma membrane ghosts.

layer on top of the 30% wt/wt sucrose layer containing soluble protein (Fraction I), a white turbid layer containing, among other things, membrane vesicles (Fraction II) which is on top or just penetrating the 30% sucrose layer, a faintly turbid 30% layer containing assorted particles, e.g., vesicles and mitochondria (Fraction III), a turbid band at the 30–45% interface containing plasma membrane ghosts and fragments (Fraction IV) and a clear 45% layer (Fraction V). It can be seen (Table I) that the highest specific activity and the greatest quantity of 12 min ^{3}H pulse-labeled glycoprotein is in Fraction II and III with most of this in Fraction II, the vesicle layer. The plasma membrane layer contains the highest specific activity of the ^{14}C long-term-labeled material which is included as a marker for cell surface fucosyl glycoprotein (35). Glycoprotein pulse labeled 12 min, when removed from Fraction II and centrifuged on a 20–50% wt/wt sucrose gradient banded almost homogeneously at a peak density of 1.14 g/ml (Fig. 3A). Similar observa-

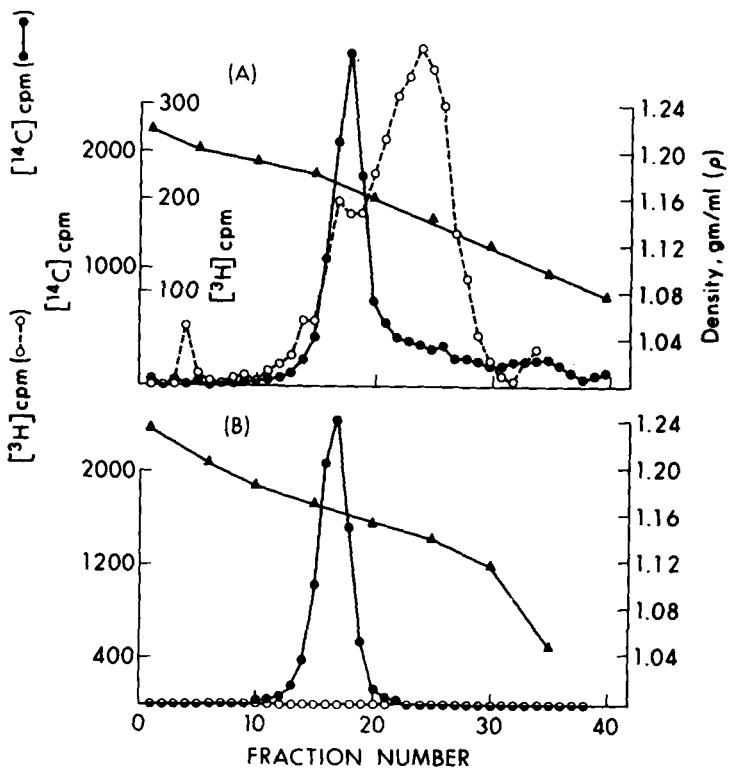

Fig. 3. Comparison of the density of internal fucosyl G protein bearing membrane and plasma membranes. Cells were labeled approximately 16 h with 0.03 μCi/ml [^{14}C] fucose, then infected with VSV (2.25 × 10^8 cells in 50 ml). Approximately 4 h after infection the cells were resuspended at 2 × 10^6 cells/ml and pulse labeled 12 min with 50 μCi/ml [^{3}H] fucose. Plasma membranes are prepared by 1 cycle of zonal centrifugation. In addition, the membranous material located in Zonal Fraction II (Atkinson, 1973) was diluted 25% with 10 mM Tris pH 8, and further analyzed. A) Fraction II material (see also Table I) was mixed with purified plasma membranes and banded isopycnically on a 35-ml 20–50% wt/wt sucrose gradient by centrifugation at 25,000 rpm for 16 h in a SW27 rotor at 4°C. [^{14}C] Fucose-labeled plasma membranes (●—●); [^{3}H] fucose-labeled Fraction II (internal) membranes (○ - - - ○). B) As above except plasma membranes (●—●) were not mixed with Fraction II material. Note the absence of 12 min pulse-labeled fucosyl G protein (○ - - - ○) in the plasma membranes.

tions were made when the unfractionated homogenate was similarly analyzed on a sucrose gradient. The plasma membranes purified from these same cells had little, if any, 12 min pulse-labeled glycoproteins (Fig. 3B). It follows that a 12 min pulse of VSV infected HeLa cells resulted in significant labeling of fucosyl G protein with much of it intracellular as had been suggested from the known transit times. Plasma membranes prepared from infected cells in the same experiment labeled with [^{14}C] fucose were seen to band at a density of 1.17 g/ml (Fig. 3B) at which there is, at most, only a shoulder of 12 min labeled material. This shoulder was almost absent in Fraction II material which had been centrifuged on a similar gradient without the addition of the "marker" plasma membrane material. Thus, the shoulder is probably artifactually adsorbed under these conditions of centrifugation. Hence, material pulse labeled with [^{3}H] fucose for 12 min, was mostly intracellular and was observed at its isopycnic density in a turbid layer at a density typical for vesicles (1.14 g/ml, 13).

State of Completion of the Oligosaccharide Moiety on the Addition of Fucose

In order to more fully assess the meaning of a transit time of fucosyl G protein, the state of completion of the oligosaccharide was analyzed at various labeling times with radioactive fucose. Purified VSV [^{3}H] fucosyl glycopeptides sized on Sephadex G-25 with standard glycopeptides (Fig. 4A) were estimated to be of mol wt 1,950–2,290; when sized on G-50 (Fig. 4B), less ambiguity in the estimate was possible and the glycopeptides appeared to have a mol wt of ~ 2,000. Newly synthesized glycopeptides in infected cell cytoplasm, labeled 5, 10, or 90 min with [^{14}C] fucose appeared (Figs. 5A–C) roughly the same size as the fucosyl glycopeptide in released virus (cf. Fig. 4A) leading to the conclusion that fucose is added near to last in the growing oligosaccharide of fucosyl G protein. [^{3}H] glucosamine in the G glycopeptides formed 3 sizes of substances containing oligosaccharides. The largest size, eluting with blue dextran (Fig. 5C) was either neutral or basic in high voltage paper electrophoresis (HVPE) at pH 1.9 and 6.5 and is probably phosphorylated oligosaccharide arising from a dolichol intermediate (see Discussion). The next size present in the highest molar ratio after 90 min labeling (Fig. 5C) was near to the size of, though distinctly smaller than, the fucosyl glycopeptides. This material was all glycopeptide by HVPE analysis. Glucosamine-labeled glycopeptides in released virus were identical in size to the fucosyl glycopeptides and the apparent lack of this size in infected cells (Figs. 5A–C) may only be a reflection of the fact that the pool of completed G protein within the cell is very small compared with the partially glycosylated pool. The smaller size, mol wt ~ 350–400, appeared in greatest molar ratio in 5-min labeled (Fig. 5A, fractions 52–60) material. This material when [^{14}C] acetylated, was observed to cochromatograph and coelectrophorese (in HVPE) with [^{14}C] acetyl-asparaginyl-N-acetylglucosamine. However, it was also detected in released virus, albeit in very low molar ratio, so it is not known if it is a biosynthetic intermediate. Purified G protein, labeled with [^{14}C] fucose and [^{3}H] glucosamine (Fig. 6A) upon glycosidase digestion gave rise to 2 fucosyl glycopeptides and 3 glucosaminyl products (Fig. 6B). The larger of the 2 fucosyl peaks, also labeled with glucosamine and mol wt ~ 1,600–1,800, was glycopeptide not digested with endo-β-N-acetylglucosaminidase D. Its presence is of interest because it reflects similar endoglycosidase resistance displayed by the uninfected host cells (1) and also quite different to the complete susceptibility of G protein of the same serotype grown in BHK21 cells (23). The smaller of the 2 [^{14}C] fucosyl glycopeptides, colabeled with [^{3}H] glucosamine was pooled and cochromatographed on Sephadex G-25 with standard glycopeptide markers (Fig. 6C). The peak

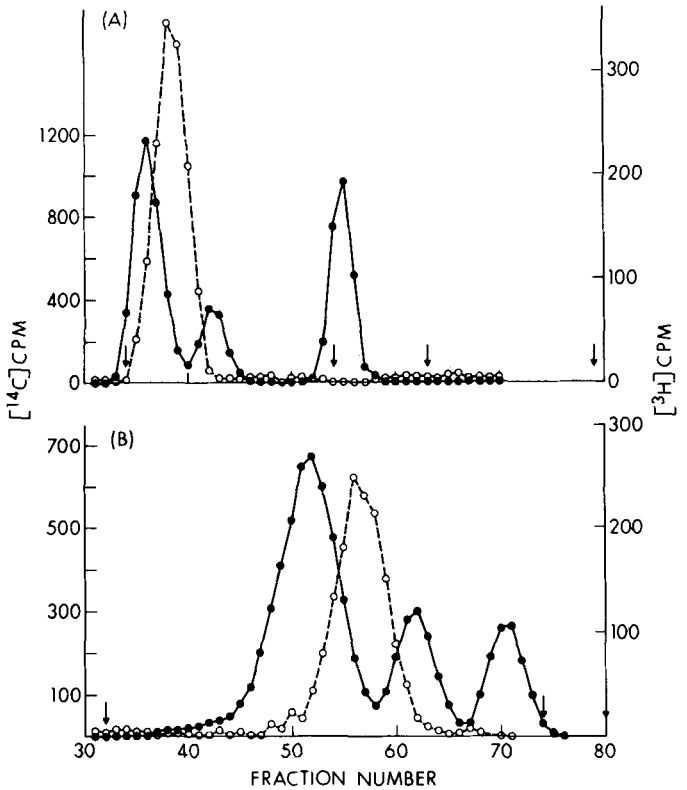

Fig. 4. Molecular weight of VSV fucosyl glycopeptides grown in HeLa cells. VSV labeled with [^{3}H]-fucose was purified, digested with pronase, and chromatographed on Sephadex G-50. The single included peak was pooled, lyophilized, and chromatographed on Sephadex G-25, and the included peak eluting just after the excluded volume mixed with [^{14}C]acetylated Thyroglobulin Unit B glycopeptide (mol wt 3,000), [^{14}C]acetylated Asn-(GlcNAc)$_2$(Man)$_5$(mol wt 1,393), and [^{14}C]acetylated Asn-GlcNAc (mol wt 379). This mixture was chromatographed on Sephadex G-25. A) VSV glycopeptides (o - - - o); [^{14}C]acetylated markers (• - - - •). The order of appearance of glycopeptide peaks is from left to right: [^{14}C]acetyl Thyroglobulin Unit A, [^{3}H]fucosyl G protein, [^{14}C]acetyl-Asn-(GlcNAc)$_2$(Man)$_5$, [^{14}C]acetyl-Asn-GlcNAc. The arrows show the positions of the standard substances from left to right: blue dextran (excluded), stachyose (mol wt 666) fucose (mol wt 164), and sodium azide. B) The same mixture chromatographed on Sephadex G-50. The peaks are from left to right as listed above. The arrows show the elution positions of blue dextran and fucose. NaN_3 elution position is not shown but was at fraction 80.

molecular weight of the [^{14}C]fucosyl glycopeptide was estimated to be 600–660, possibly corresponding to X-Asn-GlcNAc-Fuc, whereas the peak molecular weight of the glucosamine containing material was 500–550, possibly corresponding to X-Asn-GlcNAc. However, there must also have been some glucosamine-labeled oligosaccharide present (an expected product of the glycosidase mixture which contained endo-β-N-acetylglucosaminidase D) probably of the composition GlcNAc-(Man)$_n$ (23) because the bulk of the glucosamine-labeled material was neutral in HVPE at pH 1.9 (Fig. 6D). The bulk of the fucose-labeled material was basic, probably due to the presence of an amino acid such as Asn. Much of this fucose could be removed by mild acid hydrolysis (0.1 N HCl, 85°C,

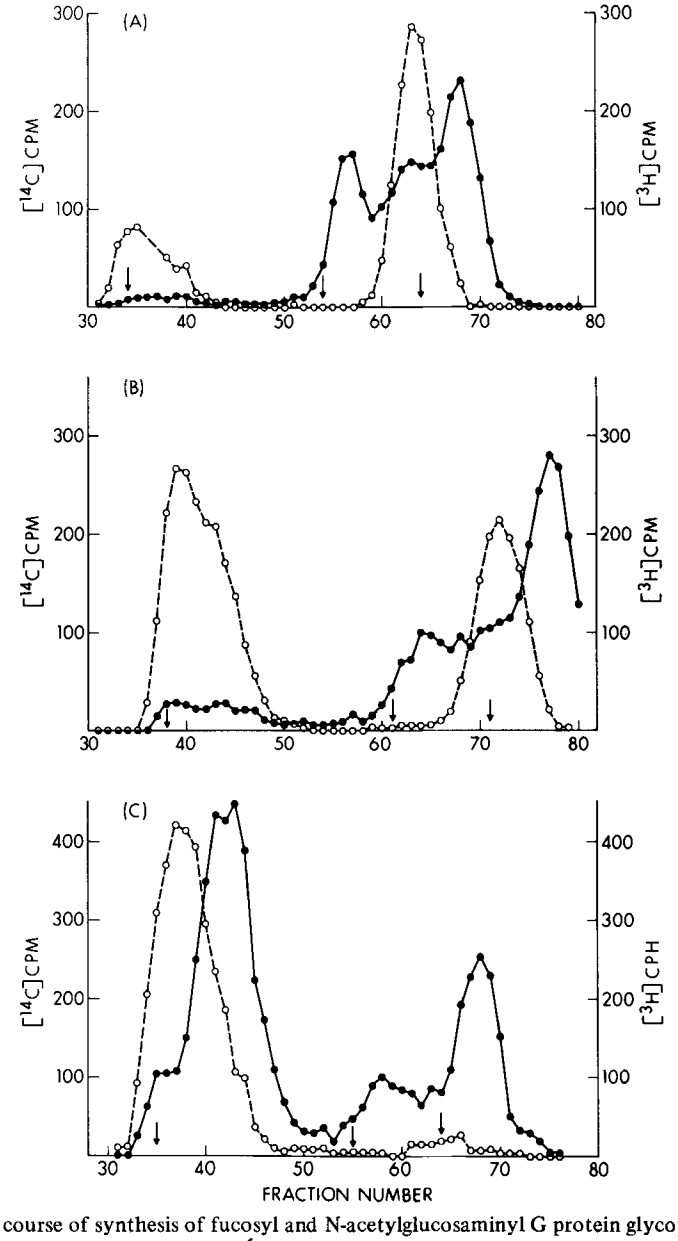

Fig. 5. Time course of synthesis of fucosyl and N-acetylglucosaminyl G protein glycopeptides. Cells, 9.1 X 10^8 cells at a density of 7.6 X 10^6 cells/ml, were infected approximately 5 h with VSV and then pulse labeled with 33 μCi/ml [^{3}H] fucose and 3.3 μCi/ml [^{14}C] glucosamine. At 5, 10, and 90 min of labeling, 20-ml samples were withdrawn and processed as if for plasma membrane preparation. However, after the homogenates had been centrifuged at 500 X g • min the pellet was discarded and the supernatants digested with pronase (10 mg/ml) for 3 days. The digests were chromatographed on Sephadex G-25. A) Five min [^{3}H] fucose-labeled glycopeptides (○ - - - ○); 5 min [^{14}C] glucosamine-labeled glycopeptides (●—●). The arrows from left to right show the elution positions of the standard substances: blue dextran, stachyose, and fucose. The 2 large peaks to the right of the diagram were found to contain labeled GDP-fucose, glucosamine, and fucose, and possibly other soluble intermediates as well (see Ref. 34). B) Ten min [^{3}H] fucose-labeled glycopeptides (○ - - - ○) and [^{14}C]-glucosamine-labeled glycopeptides (●—●). The arrows are the same as above. C) Ninety min [^{3}H] fucose-labeled glycopeptides (○ - - - ○) and [^{14}C] glucosamine-labeled glycopeptides (●—●).

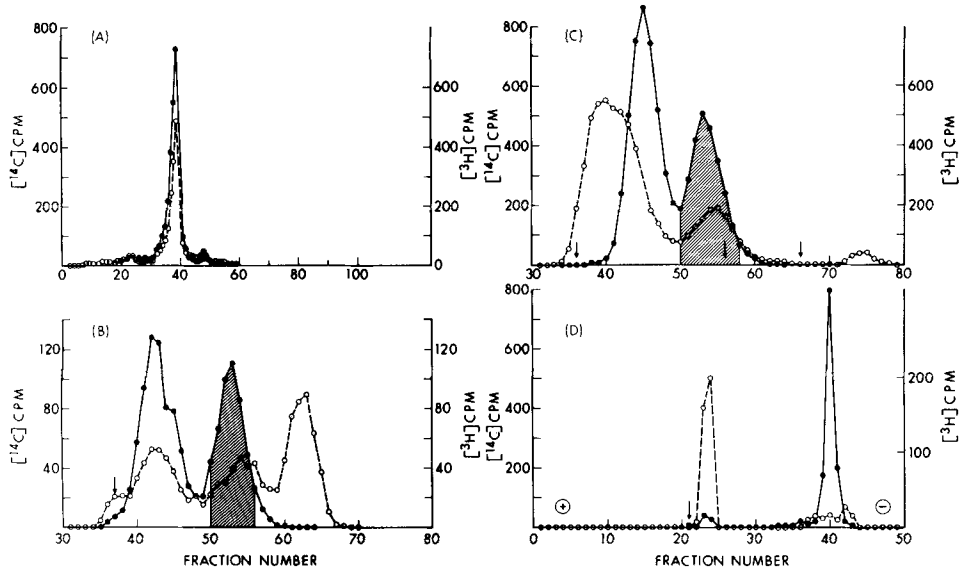

Fig. 6. Fucose in the linkage region of VSV glycopeptides. Cells, 3×10^8 cells at a density of 6×10^6 cells/ml, were infected 1.5 h with VSV then resuspended at 6×10^5 cells/ml. Five hours after infection the cells were labeled with 10 μCi/ml [^{3}H] glucosamine and 0.4 μCi/ml [^{14}C] fucose for 17 h. Virus was then purified from the supernatant medium by the standard methods involving precipitation with polyethylene glycol and banding isopycnically on a 7–52% wt/wt sucrose gradient. The purified virus was then dissociated with 2% SDS-1% mercaptoethanol and 0.01 M phosphate buffer, pH 7.0. A) The dissociated virus was electrophoresed on SDS 7.5% polyacrylamide gels, aliquots of the fractionated gel counted, and the radioactive band pooled for further analysis: [^{14}C] fucose-labeled G protein (•—•); [^{3}H] glucosamine-labeled G protein (○ - - - ○). B) The purified G protein was digested with pronase and then with endo-β-N-acetylglucosaminidase D, β-N-acetylglucosaminidase, β-galactosidase, and neuraminidase. The resultant digest was chromatographed on Sephadex G-25. [^{14}C] Fucose-labeled glycopeptides (•—•); [^{3}H] glucosamine-labeled glycopeptides and oligosaccharides (○ - - - ○). Arrow is elution position of blue dextran; note the double-labeled peak eluting near this which contains endo-β-N-acetylglucosaminidase D-resistant glycopeptides; the shaded area, containing digested material, was pooled for further treatment. C) Pooled material from the previous step was rechromatographed on Sephadex G-25 with standard glycopeptide markers: [^{3}H] acetyl-Thyroglobulin Unit B (mol wt 3,000) eluting in fraction 40; [^{3}H] acetyl-Thyroglobulin Unit A (mol wt 1,800) eluting in fraction 42 (both Thyroglobulin glycopeptides were generous gifts of Dr. A. M. Adamany, Department of Biochemistry) and [^{14}C] acetyl-Asn $(GlcNAc)_2$ $(Man)_5$ (mol wt 1,393) eluting in fraction 46. The peak elution positions of these markers have been determined on the same column when run singly. [^{14}C] Fucose-labeled glycopeptides (•—•, peak eluting at fraction 53); [^{3}H] glucosamine-labeled glycopeptides and oligosaccharides (○ - - - ○, peak eluting at fraction 55). The arrows show the elution position of the standard substances from left to right blue dextran, stachyose, and fucose. The shaded area is the material pooled ("linkage region") from the previous step. D) Pooled material from the column described in C) was subjected to high voltage paper electrophoresis at pH 1.9. The arrow marks the origin. The standard substance galactose runs approximately 3 fractions to the cathode under our conditions: [^{14}C] fucose-labeled glycopeptides (•—•); [^{3}H] glucosamine-labeled material (○ - - - ○).

1 h) indicating its terminal position. These data indicate that fucose is probably attached to the glucosamine of the protein CHO linkage, an assertion which is supported by the known specificity of endo-β-N-acetylglucosaminidase D (41). The existence of a small glycopeptide containing fucose, such as seen in Fig. 6C, argues also in favor of fucose being added much later in the synthesis of the entire oligosaccharide chains, since no rapidly labeled intermediate containing fucose of this size appeared (see Fig. 5A).

Internal Newly Synthesized M Protein

The supernatant fractions obtained at the top of sucrose gradients used to analyze the free and membrane-bound polysomes were prepared from VSV-infected HeLa cells and the distribution of newly synthesized, released G protein and M protein ascertained after a 1 min pulse, 1 min chase of [^{35}S] methionine. This regime of pulse-chasing was adopted for 2 reasons. First, released M and G protein could more readily be identified by their co-migration with VSV structural species; pulse labeling without a chase results in a high heterogenous background obscuring the main components. More importantly, the total time (2 min) is within the known transit time of M protein from its site of synthesis to the plasma membrane and hence M protein labeled in this way should substantially be in an internal pool (2). In addition to the soluble protein component of the sucrose gradient used to prepare the polysomes, the polysome region itself was analyzed on SDS-polyacrylamide gels. Released G protein did not significantly accumulate in the supernatant from which free polysomes were sedimented (Fig. 7A) whereas released M protein did (Fig. 7a) with a specific activity of 27.4 cpm/μg total protein. This experiment was repeated such that the fraction containing the free polysomes was sedimented on a 7–52% sucrose gradient in the absence of DOC to test whether M protein seen in the supernatant actually came from a membranous form contaminating the free polysomes and solubilized by DOC. Identical results were obtained and therefore the appearance of M protein in the supernatant from which the free polysomes were sedimented indicated that it is in a relatively non-sedimentable form and that its density, if particle bound, is less than 1.08 g/ml, i.e., less than that of the sucrose at the top of the gradient. M protein was still found in the supernatant to the free polysomes after a 30 min chase, but with a specific activity of only 13.7 cpm/μg protein. It was observed that no M protein could be detected in the supernatant of the gradient on which the membrane-bound polysomes were analyzed after a 1 min pulse, 1 min chase (Fig. 7C). In several other experiments there were minor amounts of M protein in this supernatant probably a contaminant insufficiently removed in the wash step prior to the polysome gradient. After 30 min chase M protein appeared with a relatively low specific radioactivity of 3.9 cpm/μg total protein (Fig. 7D). After a 1 min pulse, 30 min chase plasma membranes contain substantial amounts of labeled M protein which would mix and dilute with preformed unlabeled M protein. This cannot be so after a 1 min pulse, 1 min chase because little newly synthesized M protein is assembled into plasma membrane within 2 min of its synthesis (Ref. 2 and see above). From this belated appearance of M protein and its relatively low specific activity, it is likely that plasma membrane vesicles are present in both the cell supernatant from which the free polysomes were sedimented and also in the particulate fraction from which the membrane-bound polysomes were sedimented. Vesicles and fragments from other sources containing M protein could also be present. The appearance of released G protein in the supernatant of the gradient containing the membrane-bound polysomes after a 1 min pulse, 1 min chase (Fig. 7C) or in a 1 min pulse, 30 min chase (Fig. 7D) might similarly reflect the presence of intracellular membrane and plasma membrane containing G protein solubilized by DOC.

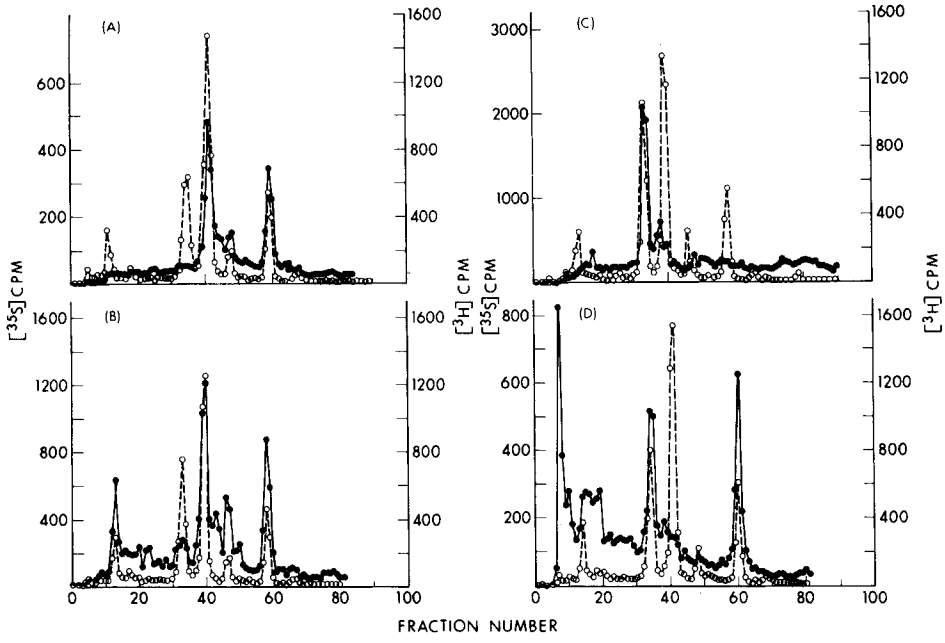

Fig. 7. Analysis of the soluble supernatants of the cell fraction from which free and membrane-bound polysomes were sedimented. Cells, 7.53 X 10^8 cells at a density of 5.6 X 10^6 cells/ml were infected 4 h with vesicular stomatitis virus, resuspended in fresh medium lacking methionine at a density of 2.6 X 10^7 cells/ml, incubated 15 min, and then pulse labeled 1 min with 50 μCi/ml [^{35}S]-methionine (results were essentially the same when these experiments were performed in medium containing complete methionine or at 0.05 normal methionine concentration). The label was then chased by adjusting the culture to 10 X L-methionine. Metabolism was stopped by placing the culture on frozen Earle's solution, after which the culture was washed by centrifugation in Earle's solution. The pellet was resuspended in 8.0 ml RSB and homogenized vigorously in a 0.002-inch clearance dounce homogenizer. Nuclei and the bulk of plasma membrane ghosts and fragments were sedimented at 1,600 rpm for 3 min. The postnuclear supernatant was centrifuged at 31,000 X g for 30 min and this supernatant was saved for analysis of free polysomes. The pellet was resuspended in 6 ml RSB with 100 μg/ml heparin, and sedimented through a 15–30% wt/wt sucrose gradient at 25,000 rpm for 30 min in a SW27 rotor at 0°C. The pellets were resuspended in RSB, 100 μg/ml heparin, and 1% DOC, and sedimented on 7–52% wt/wt sucrose gradients, in RSB, 100 μg/ml heparin, for 16 h at 16,000 rpm at 1°C in a SW27 rotor. A typical free polysome profile was obtained by sedimenting the cell supernatants on 7–52% wt/wt sucrose gradient for 16 h, 16,000 rpm in a SW27 rotor. This profile was substantially similar whether or not 1% DOC was included in the supernatants and whether or not unlabeled methionine was included in the labeling medium of the above composition. The polysome region and the soluble region of the gradient were pooled for further analysis on SDS-polyacrylamide gels. The "membrane bound" polysome region and the soluble region of the gradient were similarly pooled for further analysis. •—•) Pulse and chase of [^{35}S]methionine; ○ - - - ○) purified VSV marker labeled with [^{3}H]amino acids. The structural species of the marker virus from left to right are respectively: L, G, N, NS, and M proteins. A) Supernatant to the free polysomes 1 min pulse, 1 min chase. B) Supernatant to the free polysomes 1 min pulse, 30 min chase. C) Supernatant to the membrane-bound polysomes 1 min pulse, 1 min chase. D) Supernatant to the membrane-bound polysomes 1 min pulse, 30 min chase.

The overall conclusion from these observations is that the M protein in the supernatant from which free polysomes were sedimented after 1 min pulse, 1 min chase is an intracellular form of M protein and quite probably a soluble form (28). G protein by contrast is particle bound and not found in this fraction. When the cell supernatant (30,000 × g supernatant) containing released M protein pulse labeled 1 min with [^{35}S]-methionine and chased 1 min was centrifuged at 165,000 × g for 16 h, a substantial portion of M protein (40%) was again found to be nonsedimentable. The sedimented and the "soluble" M protein had nearly identical specific activities so it is not known if this represents a subfractionation of M protein. It was concluded from this section of the results that the bulk of M protein after a 1 min pulse, 1 min chase accumulated in the free polysome supernatant, possibly in the cytosol and if particle bound had a different density than the internal G protein labeled for the same period of time.

DISCUSSION

Addition of fucose to VSV G protein in HeLa cells is among the later steps in the synthesis of the molecule, and, on the addition of fucose the glycopeptide is near that of the completed size in the purified virus (mol wt ~ 2,000) as found in this paper. This size is a more accurate estimate than the estimate in our previous paper (mol wt ~ 1,800) (26) and is consistent with that of Robertson et al. (27) for HeLa spinner cells. It is of interest to note that the average mol wt of VSV glycopeptides from virus grown in BHK21 monolayer cultures is 3,150 daltons (23). G protein backbones are made on membrane-bound polysomes (24, 25) where the initial events in glycosylation possibly occur (36); the present data do not pertain to this point. However, fucose is not added to growing oligosaccharides in the polysomes because ^{14}C-labeled amino acid-labeled G protein (i.e., not necessarily glycosylated) continued to chase into the plasma membranes at least 45 min after fucosylated G had ceased to do so. This argues that though the transit time of fucosylated G is ~ 15–20 min, there is another G component which is only partially glycosylated (2). This glycoprotein has a transit time of not less than 75 min and hence G protein is being processed on its way to the plasma membrane at least 55 min prior to the addition of fucose. The mechanism and site of synthesis of protein backbone of this precursor to fucosyl G protein has been studied by others (36). Hunt and Summers (29) observed that core sugars, such as mannose and glucosamine, seemed to be added in the rough endoplasmic reticulum (er) whereas, fucose, a terminal sugar, is added in the smooth er. Their suggestion that the initial glycosylation events may be the en bloc addition of a mannose and glucosamine oligomer receives support from our observation (A. M. Adamany and P. H. Atkinson, unpublished) that a dolichol-bound oligosaccharide could be isolated from VSV-infected HeLa cells labeled for 30 min with [^{3}H] glucosamine. This material was extracted with chloroform:methanol:H_2O (10:10:3) and adsorbed on DEAE-cellulose (DE-52) from which it was eluted with an ammonium acetate in methanol gradient (30, 31). An N-acetylglucosamine-mannose-containing oligosaccharide could be released from the lipid saccharide after hydrolysis in 0.01N HCL/10% methanol at 100°C for 10 min and was found to have a molecular weight of approximately 2,000 as judged by gel filtration on Biogel P-6. Addition of fucose is near to the last events in the glycosylation of G protein and presumably could occur after addition of such a possible glucosamine containing oligosaccharide. There was no evidence

of any small glycopeptide intermediates in the biosynthesis of fucosyl glycoprotein. The possibility of an intermediate containing one glucosamine only (see text and Fig. 5A) will have to be further investigated to decide on its validity. G, M, N, NS, and L proteins have been observed to differentially associate with different density membrane fractions. In these studies (34), M protein was found mainly in rough endoplasmic reticulum (density 1.17–1.20 g/ml) and plasma membrane (density 1.15–1.17 g/ml) in a 2–4 min pulse whereas G protein was found mainly in the rough endoplasmic reticulum, but eventually (8 min on) became associated with the light membranes (density 1.12–1.15 g/ml). This latter association is probably similar in time to that posttranslational time when fucose is added to the internal G protein [and uninfected cell glycoprotein, Yurchenco and Atkinson (35)]. The light membrane fraction of Hunt et al. (34) may be similar to the internal membranous component bearing fucosyl glycoprotein of density 1.14 g/ml observed here (Fig. 3).

Our previous paper (2) and that of David (33) showed that there was a small internal pool of M protein precursor to plasma membrane M protein but it was not further characterized. "Soluble" M protein detected in infected cells by us (3, 28) and others could be a variety of forms of M protein including that which was removed from membrane material during cell disruption, and that which never assembles into plasma membranes: we have found in HeLa cells that most of the cellular M protein does not assemble into plasma membranes (unpublished data). It is possible that the transit time of M is time required for some posttranslational modification and that once the modification occurs M enters a pool having 2 compartments that are in rapid equilibrium – a soluble compartment and a membrane-bound compartment. In this interpretation, pulse-labeled M protein would first appear in the soluble pool then transfer to the plasma membrane, mix with unlabeled M protein already in the plasma membrane prior to the onset of labeling, and then transfer back to the soluble pool: The decay of labeled M protein from the plasma membrane seen in Fig. 1 might thus be reequilibration of the plasma membrane M protein back to the soluble pool which is, as a function of chasing the labeled amino acids, getting "cold" M in the chase. There are several points weighing against this interpretation. First, in order for the internal pool to become "cold" in the chase it would have to significantly expand with incoming unlabeled M protein diluting out labeled M protein returning from the plasma membrane. This would imply a steadily expanding internal pool throughout the infectious cycle, including during pulse label accumulation without a chase. In this interpretation, M protein should accumulate either in isolated plasma membranes or in released virus with kinetics consistent with such an expanding precursor pool. Mathematical description of these pools and the expected rates of accumulation of products coming from them was discussed in our previous paper (35): Molecules coming from such an expanding pool would accumulate in a "product" pool with exponential kinetics. There is little evidence for this, and, on the contrary, M protein accumulates in plasma membranes (2) and in released virus (34) with linear kinetics consistent with a small rapidly equilibrated internal pool which does not expand during the term of the experiment. In addition, there is convincing evidence that from about 2 h after infection both the rate of synthesis of M protein and the amount of M protein in the cell steadily decreases in a several hour chase (27a). Secondly, there is evidence that M protein is lost from the cell (Fig. 1b) in amounts more than sufficient to account for the decay process seen from the plasma membranes (Fig. 1a) consistent with accumulation of

M protein in released virus previously observed in other laboratories (27a, 34). Third, the initial rate of loss may seem to resemble the rate at which the M chased into the plasma membrane and therefore seem to be an equilibrium process. However, it can readily be determined that when the last 7 or 8 points on the chase curve are plotted on a logarithmic scale (for specific activity) against time the decay is monophasic, linear, with a half time for turnover of 80–85 min. That is to say, half the newly synthesized M protein inserted into the plasma membrane during the initial 10 min of the chase, is mixed with preexisting M protein then randomly lost from the cell in 80–85 min. Such a rate of turnover would not seem to be inconsistent with virus "bud" formation in the plasma membrane noting at the same time that we have no idea how fast this procedure is. Though in vitro association of M protein with plasma membranes during cell disruption was shown able to occur (3), it does not do so in significant quantities to that already inserted in vivo as determined by mixing-dilution experiments (2). It should be emphasized that the plasma membranes utilized in these studies are ghost structures which can be expected to contain all intermediate forms of budding virus. Plasma membranes purified as vesicles from vigorously disrupted cells may have lost both the intermediate forms as well as bound M protein itself. These studies clearly show an insertion, and random mixing of newly synthesized M protein with preexisting M protein. This mixing may be mixing of individual preexisting M molecules with newly synthesized M or mixing of partially completed aggregates containing newly synthesized and preexisting M protein. The data in this paper do not distinguish these possibilities beyond that mixing of some sort must occur before loss of the M protein from the cell.

The current experiments were designed to further characterize the internal pool of M protein precursor to plasma membrane and the labeling conditions were carefully chosen such that the labeled newly synthesized M protein would mostly be internal as distinct from plasma membrane associated M protein. Furthermore, newly synthesized M protein first appeared in the supernatant from which the free polysomes were sedimented, consistent with previous findings that M protein mRNA is found in the free polysomes of VSV-infected cells (24, 25), and not in the supernatant to the membrane bound polysomes. David (40) has also presented evidence that M protein passes through a soluble phase before being added to the plasma membranes. The current studies thus show that there is an internal "soluble" M protein pool at least some of which must be on its way to assembly in plasma membrane and some of its characteristics described in this study distinguish it from internal fucosyl G protein bearing membrane also on its way to assembly in the plasma membrane. Different routes of assembly for fucosyl G protein and M protein can now be argued from the following facts: that fucosyl glycoprotein, representing a practically completed molecule (the state of completion can be determined from Figs. 4, 5, and 6) has an approximate 20 min transit time to the plasma membrane from the site of addition of fucose and that M protein has a less than 5 min transit time from its site of synthesis (this conclusion is obtainable from Fig. 1); that fucosyl G protein is on an internal membranous component with a density of 1.14 g/ml (derived from Fig. 3) as compared with its final density in plasma membrane (1.16 g/ml); most M protein within its transit time (less than 3 min; see Fig. 2) to the plasma membrane can be observed is soluble form and, if particle bound at all, has a density of not more than 1.08 g/ml. The internal form of M protein precursor to plasma membranes is, therefore, quite different from the component to which fucosyl glycoprotein is bound. These facts together allow for differentiation

of assembly routes shown in the model (Fig. 8) for pathways of assembly of these components. The model shows a hypothetical vesicle containing G proteins (knobbed line) with the nonglycosylated end associated with the lipid bilayer [see Mudd (37) for evidence of this orientation and a review of the background evidence and see also Ref. 39]. It also shows M protein (shaded area) in some undefined association with the plasma membrane. A general proviso to Fig. 8 must be that there is no evidence that fucosyl G protein is on a vesicle, as depicted, or that the fucosyl G protein has the orientation shown. In mouse colonic epithelial cells, there is evidence of such a vesicle carrying fucosyl glycoprotein to the cell surface (39). For fucosyl G protein, however, we do know that it is initially on an internal membranous component and that it ends up on the outside of the virus, and therefore, presumably on the outside of the cell prior to virus maturation. Furthermore, though a concentrated "patch" of M and G protein is shown in this set of figures there is no evidence for its initial existence. It is inferred that such a patch must at some stage exist during virus morphogenesis because the virus ends up free of the cell with the equivalent of such a "patch" forming its membranous envelope. However, the mechanisms of patch formation and exclusion of host proteins are not known and are not intended to be the subject of this paper The data in this paper, just summarized above, allow us to distinguish between the 3 alternative pathways of assembly shown in Fig. 8. This study and our previous study (2) would rule out the possibility shown in Fig. 8A because it is inconsistent with the observed kinetics: M and fucosyl G protein do not have the same transit time from the site of synthesis to the plasma membrane as the model implies they do. Neither do they have the same density because G is membrane bound and M appears soluble. The possibility shown in Fig. 8B is unlikely on the basis of the present data: intracellular G protein, once fucosylated and completed, is located on an internal membranous component of density 1.14 g/ml in sucrose and internal (1 min pulse, 1 min chase). Most M protein is in the cell supernatant (also the supernatant from which free polysomes were sedimented) having a peak density in sucrose of 1.08 g/ml. Thus, if M protein is added to a membranous component carrying fucosyl G protein to the plasma membrane prior to assembly into the plasma membrane, the subsequent entry of the conglomerate must be instantaneous, otherwise a sedimentable form of newly synthesized M protein similar to fucosyl G protein should be observed. There is little evidence for this and thus direct addition of M protein from its internal pool to the plasma membrane (Fig. 8C) would seem to explain the observations made in this paper most satisfactorily and also those made on the in vitro binding of M protein to isolated plasma membranes (3, 28). The models are not intended to distinguish the order in which G protein and M protein reach the membrane. However, it is noted (27a) that M and G protein are made at about the same rate from the start of the infectious cycle and thus if M is rapidly synthesized and inserted it may, in fact, be the first VSV protein to modify the plasma membrane. However, the present data do not prove this one way or another but do allow for differentiation of the independent routes G and M proteins take into the plasma membrane. Hay (5) has also proposed the direct addition to plasma membranes of influenza virus matrix protein. Different pathways of assembly have also been proposed for NP protein and M protein in Sendai virus; the latter also being assembled into virions from a small cytoplasmic

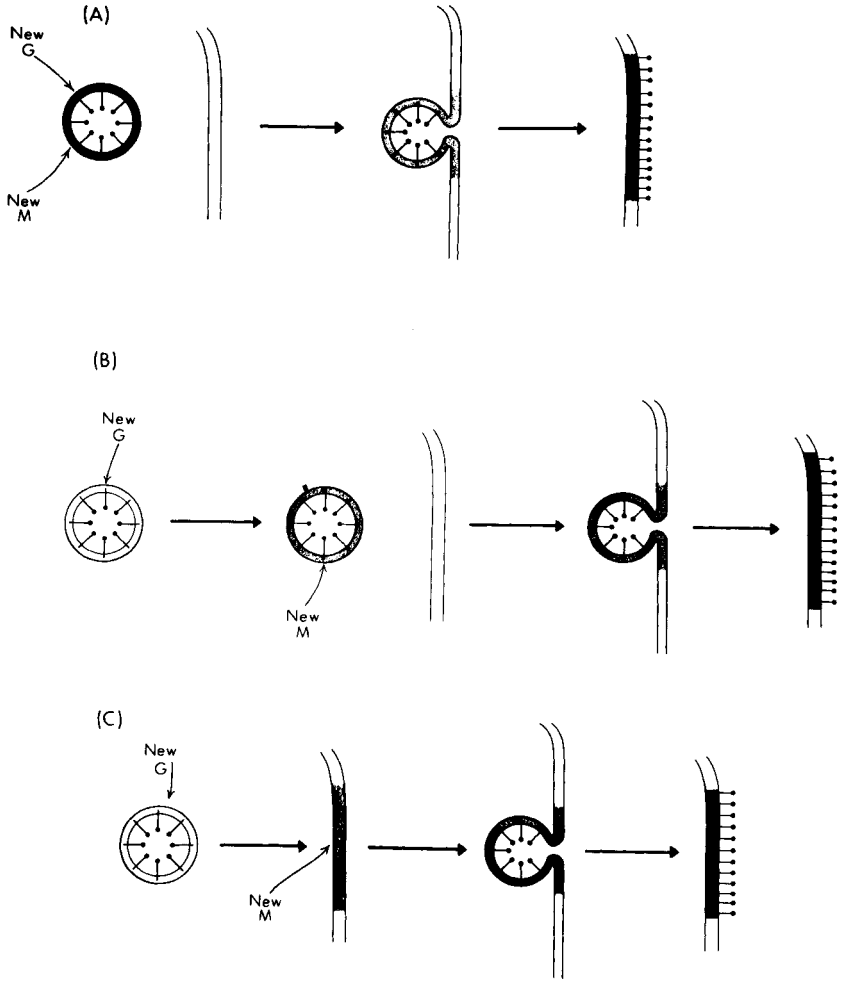

Fig. 8. Three modes of adding newly synthesized fucosyl G protein and newly synthesized M protein to membrane and plasma membrane. In all panels, G protein is represented by a spike and knob, M protein is represented by the shaded area. An internal membranous component is represented by the double membranes circle. However, there is no evidence it is a vesicle; neither is there evidence for the orientation of the G protein. There is also no presumption that the "patch" of M and G protein shown in the plasma membrane (double membranes open structure) assembles in the manner or in the order shown. The M and fucosyl G protein probably present in the plasma membrane prior to the onset of labeling are not shown in these diagrams for sake of clairty. It is possible M and G protein reach the plasma membrane simultaneously or at different times. This model is not intended to distinguish this and the diagram is intended to aid distinction of route only of newly synthesized M protein and newly synthesized fucosyl G protein into the plasma membrane.

pool (32). Hunt et al. have proposed different pathways of assembly for the assembly of VSV components into HeLa cell plasma membranes (34) by different cell fractionation techniques to those used in this study. What sort of interactions M protein would undergo in transition from the relatively nonsedimentable phase to tight binding in the plasma membranes demonstrated (3, 28) is entirely unknown.

ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service Research Grants CA13402 and CA06576. The author is an Established Investigator of the American Heart Association. I thank Ms. Joyce Tsang for superb technical assistance and Dr. Takashi Muramatsu, Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York for glycopeptide substrates of known composition.

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