Transient Activity of Golgi-like Membranes as Donors of Vesicular Stomatitis Viral Glycoprotein In Vitro

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ABSTRACT Previous reports demonstrated that the vesicular stomatitis viral glycoprotein (G protein), initially present in membranes of a Chinese hamster ovary mutant cell line (clone 15B) that is incapable of terminal glycosylation, can be transferred in vitro to exogenous Golgi membranes and there glycosylated (E. Fries and J. E. Rothman, 1980, *Proc. Natl. Acad. Sci. U. S. A.* 77:3870–3874; and J. E. Rothman and E. Fries, 1981, *J. Cell Biol.* 89:162–168). Here we present evidence that Golgi-like membranes serve as donors of G protein in this process. Pulse-chase experiments revealed that the donor activity of membranes is greatest at ~10 min of chase, a time when G protein has been shown to have arrived in Golgi stacks (J. E. Bergmann, K. T. Tokuyasu, and S. J. Singer, 1981, *Proc. Natl. Acad. Sci. U. S. A.* 78:1746–1750). Additional evidence that the G protein that is transferred to exogenous Golgi membranes in vitro had already entered the Golgi membranes in vivo was provided by observations that its oligosaccharides had already been trimmed, and that its distribution in a sucrose density gradient was coincident with that of enzymatic markers of Golgi membranes. The capacity of this Golgi-like membrane to serve as donor is transient, declining within 5 min after "trimming" in vivo as the G protein enters a "nontransferable" pool. The rapidity of the process suggests that both the "transferable" and "nontransferable" pools of G protein reside in Golgi-like membranes.

The reconstitution in cell-free systems of segments of the pathway of intracellular protein transport will be a key element in the elucidation of the molecular mechanisms of protein sorting. Animal cells infected with vesicular stomatitis virus (VSV) provide a suitably simple system (19) for this purpose. The viral-encoded glycoprotein (G protein) is synthesized and initially glycosylated in the rough endoplasmic reticulum (ER) membrane and is then transported to the cell surface where it becomes incorporated into budding virions. Earlier reports from this laboratory (7, 24) have established that postnuclear supernates prepared from VSV-infected clone 15B Chinese hamster ovary (CHO) cells (8) can serve as donors of G protein in a process that results in the maturation of G protein's asparagine-linked oligosaccharides in vitro to forms that are resistant to attack by the endoglycosidase, Endo H (23, 30). Terminal glycosylation in this cell-free system required incubation with membranes from wild-type CHO cells (7) that serve as a source of the Golgi-associated glycosyltransferase (UDP-GlcNAc glycoprotein glycosyltransferase I) specifically missing from the clone 15B mutant (9, 27, 29). Incubation of donor with a highly purified Golgi fraction from rat liver resulted in both oligosaccharide processing and the appearance of G protein in the added Golgi membranes (24). Together, these findings suggested that Golgi membranes can act as acceptors of G protein and process its oligosaccharides in vitro. Left unresolved was the important issue of which membrane(s) from the infected 15B cell can serve as the donors.

Here we present experiments that point to a Golgi-like membrane as the principal donor of G protein under the conditions employed. But the activity of these membranes as donor is remarkably transient. Within 5 min in vivo, G protein passes from an intracellular site or state that can serve as a donor to one that can not. This finding may have important implications for the organization of the Golgi stack.

MATERIALS AND METHODS

Materials

[^{35}S]Methionine ([^{35}S]Met; 780–900 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.) and [1-^{3}H]galactose (10 Ci/mmole) was from

Amersham Corp., Arlington Heights, Ill. Pure endo-β-*N*-acetylglucosaminidase H was kindly provided by Dr. P. Robbins, Massachusetts Institute of Technology (MIT; Cambridge, Mass.). Creatine phosphokinase and UDP-GlcNAc were from Sigma Chemical Co. (Sigma; St. Louis, Mo.).

Cells and Virus

A wild-type line of CHO cells (originally from Dr. H. Lodish, MIT) was maintained in suspension (15). The CHO cell mutant clone 15B (obtained from Dr. S. Kornfeld, Washington University, St. Louis, Mo.) was grown in monolayers (18). Stock of VSV (Indiana strain) was prepared as described (15) and was typically about 1×10^{10} plaque-forming units (pfu)/ml.

Labeling of Cells

A variation of our previous procedure (7) was employed. Four plates (10 cm diam) with cells at near confluence ($\sim 10^7$ cells per plate) were infected with 10–20 pfu of VSV per cell. At 4–4.5 h after infection, the cells were removed from the plates by trypsinization (7) and transferred to 10 ml Joklik's minimal essential medium containing nonessential amino acids (Grand Island Biological Co., Grand Island, N. Y.) and 20 mM HEPES-NaOH, pH 7.3. For experiments with [^{35}S]Met the medium lacked methionine. (When [^{3}H]galactose was used, the medium contained Met but had 10% the normal level of glucose.) After 10-min equilibration at 37°C, [^{35}S]Met (0.5–1.0 mCi) was added. Cell suspensions were continuously and gently stirred during all incubations. Chase conditions were obtained by the addition of unlabeled methionine to give a final concentration of 2.5 mM. Chase was terminated by the mixing of the cell suspension with 30 ml of ice-cold phosphate-buffered saline (with Mg^{++} and Ca^{++}; PBS; see reference 7).

Preparations of Cytoplasmic Fractions

The cells were washed, swollen, and homogenized as described previously (7). For crude fractions (sometimes referred to as extracts [7]) of untreated wild-type CHO cells, 7×10^7 cells were used and the postnuclear supernate was prepared, frozen in liquid N_2, and stored at −80°C as described (7). Membranes of CHO cells were isolated by centrifugation of 600 μl of postnuclear supernate for 60 min at 40,000 rpm in a Beckman SW50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The pellet was resuspended by homogenization in 300 μl of extract buffer (75 mM KCl, 67 mM MES-KOH pH 7.4, 4.5 mM magnesium acetate, and 1 mM dithiothreitol [7]). Cytosol was prepared from rat liver (method *1* of reference 7). For some experiments the liver cytosol was concentrated five times by ultrafiltration (using a PM10 filter; Amicon Corp., Lexington, Mass.) and then passed over a Sephadex G-25 column equilibrated with extract buffer. The void volume was used as a source of this concentrated, gel-filtered cytosol fraction.

In Vitro Processing Assay

For standard assays (7), 10 μl of [^{35}S]Met-labeled postnuclear supernate of VSV-infected 15B CHO cells were mixed with 20 μl of postnuclear supernate from uninfected, unlabeled wild-type CHO cells and 45 μl of a reaction cocktail. The cocktail contained 2.9 mM ATP (Na form, pH 7), 14 mM creatine phosphate (Na form), 7 IU/ml of rabbit muscle creatine kinase (Sigma), 1.4 mM UDP-GlcNAc (Na form), 1.0 mM dithiothreitol, 4.5 mM magnesium acetate, 75 mM KCl, and 67 mM 4-morpholinoethanesulfonic acid-KOH (MES-KOH), pH 6.5. The final pH of the incubation mixture was ~6.8. The incubation was carried out at 37°C. Aliquots were taken and processed for SDS PAGE with or without prior treatment with Endo H, as described (7).

Subcellular Fractionation

Subcellular fractionation was based on the procedure of Knipe et al. (14). The postnuclear supernate to be analyzed was mixed with 4.5 ml of ice-cold 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, and centrifuged in an SW50.1 rotor at 40,000 rpm for 60 min. The pellet was resuspended in 4.5 ml of 1 mM EDTA, 1 mM Tris-KOH, pH 8.0, and homogenized with eight strokes of a tight-fitting Dounce homogenizer. The solution was centrifuged again. This pellet was resuspended in 1.5 ml of 45% (wt/wt) sucrose in the Tris-EDTA buffer and homogenized as described above. The suspension was mixed with 3 ml of 60% (wt/wt) sucrose in the Tris-EDTA buffer and overlaid in an SW27.1 centrifuge tube with 2 ml each of 40, 35, 30, 25, and 20% sucrose (all wt/wt) in the Tris-EDTA buffer. The gradient was spun at 25,000 rpm for 15 h. Fractions were collected with a pump from the bottom of the tube through a capillary tube. The density of each fraction was determined from its refractive index. For further analysis, each fraction was diluted by adding 3 ml of the Tris-EDTA solution, and then centrifuged for 60 min at 40,000 rpm in an SW50.1 rotor. To each pellet, either 50 μl of electrophoresis (17) sample buffer (when Endo H was not used) or 30 μl of 0.1 M Tris-HCl pH 6.8, 20 g/liter SDS, 30 mM dithiothreitol (for Endo H digestions) were added. The tubes were then heated for 3 min at 100°C. In the former case the samples were run directly in a 10% SDS polyacrylamide gel (17). In the latter case, 20 μl of a solution of Endo H (75 ng/ml) in 0.3 M sodium citrate, pH 5.5, containing 0.1% SDS was added, and the mixture was incubated for 12–18 h at 37°C. After precipitation with 0.3 ml of 10% TCA and centrifugation for 2 min in the Eppendorf microfuge (Brinkman Instruments, Inc., Westburg, N.Y.), these samples were dissolved in 50 μl of electrophoresis buffer, with an addition of 5 μl of 1 M Tris base, boiled, and analyzed by electrophoresis as described (7).

RESULTS

The Extent of Processing In Vitro Depends upon the Location of G Protein In Vivo

To help ascertain which membranes from the infected clone 15 B cell serve as donors of G protein in vitro, we prepared crude fractions (postnuclear supernates) from VSV-infected 15B cells that had been "chased" for increasing times after a 5-min pulse-label with [^{35}S]Met. These fractions were then tested as donors during prolonged incubations in vitro under standard conditions to determine the maximum amount of G protein that could be processed to Endo H resistance by wild-type membranes (Fig. 1). This capacity to serve as donor can then be correlated with the time the pulse-labeled wave of G protein had been allowed to progress along the ER/Golgi/plasma membrane transport pathway (2) in vivo before homogenization. It is important to note that the carbonyl cyanide m-chlorophenylhydrazone (CCCP) treatment used in earlier work (7, 24) was not employed in these experiments. This distinction will be discussed in detail later.

^{35}S-labeled G protein present in the crude membrane fraction of 15B cells prepared immediately after the pulse (0-min chase) was not processed to a significant degree by wild-type membranes (Figs. 1 and 2). Therefore, the bulk of rough ER membranes are not significant donors under these conditions. The capacity to serve as donor in vitro increased dramatically with subsequent chase in vivo, and was maximal at ~10 min (Fig. 2), when ~40% of G could be processed. After this peak, the capacity to donate G protein in vitro fell precipitously, by about a factor of two for every 5 min of additional chase in vivo. Because G was being delivered to the cell surface during this same period (25), the plasma membrane cannot be a major donor.

An essential aspect of this experiment is that the data in Figs. 1 and 2 measure the extent and not the rate of processing in vitro. The total amount of G protein converted to Endo H–resistant forms was the same after 20 and 40 min of incubation for each postnuclear supernate tested as donor in Fig. 1; only data for the 40-min time-point are plotted in Fig. 2. A detailed time-course is shown in Fig. 3. In fact, several Endo H–resistant forms of G protein are produced in vitro. These forms are considered together in Fig. 2 and are collectively denoted G_R in the autoradiograph shown in Fig. 1. Although the total amount of Endo H–resistant material does not increase beyond 20 min of incubation in vitro, slower-migrating forms that are presumably more completely glycosylated become increasingly prominent with time of incubation in vitro (Fig. 3).

Because the extent of reaction was measured, the assay distinguishes two post–rough ER pools of G protein present in 15B cell membranes in vivo, the first able to be donated for processing in vitro, the second not. Because G protein processed

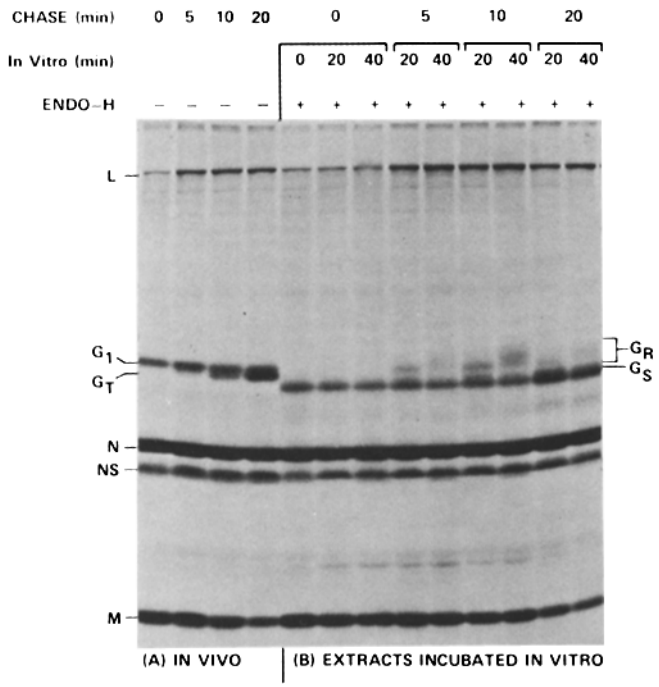

FIGURE 1 VSV-infected 15B cells were pulse-labeled for 5 min with [^{35}S]Met, and chased for 0, 5, 10, or 20 min as indicated, and then postnuclear supernates were prepared as decribed in Materials and Methods. (*A*) An aliquot of each postnuclear supernate was electrophoresed to permit the extent of "trimming" to be determined. (*B*) Each postnuclear supernate was incubated with postnuclear supernate of wild-type cells under standard conditions (see In Vitro Processing Assay under Materials and Methods). Samples taken after 0, 20, or 40 min as indicated (*In Vitro*) were treated with Endo H, and then electrophoresed. G_1 is the freshly synthesized form of the viral glycoprotein. G_T is the most mature form of G protein appearing in 15B cells, and is believed to be the "trimmed" form that contains five mannose units. G_S is the form of G protein whose oligosaccharides have been cleaved by Endo H. G_R includes the several Endo H-resistant forms that are synthesized in vitro. *L*, *N*, *NS*, and *M* are other VSV-encoded proteins.

in vitro can be found in exogenous Golgi membranes (7, 24), the pool that can be processed will be termed the "transferable pool"; the subsequent population of G unable to be processed in vitro will be referred to as a "nontransferable pool."

The data in Fig. 2 suggest that pulse-labeled G protein enters the transferable pool after synthesis in the rough ER, and occupies this pool maximally at 10 min of chase. This wave of G protein then moves from the transferable pool into the nontransferable pool with a half-time of ~5 min. It is remarkable that G protein enters the nontransferable pool so rapidly because the half-time for transit of G protein to the CHO cell surface is ~1 h (25).

Thus, both the site of G protein's synthesis (the rough ER) and its cellular destination (the plasma membrane) can be ruled out as donors on kinetic grounds. Instead, it would appear likely that a major compartment in between, a Golgi-like membrane, is the principal donor that houses the transferable pool of G protein. The experiments that follow point to this conclusion.

Transferability In Vitro Coincides Closely with Oligosaccharide Trimming In Vivo

The degree of maturation of Asn-linked oligosaccharides can delineate how far the attached protein has progressed along the intracellular transport pathway. Two oligosaccharides are added in precursor form (20) to the nascent G protein in the rough ER, and are later processed to the mature forms while in less dense intracellular membranes (11). Oligosaccharide processing of G protein in wild-type CHO cells proceeds in two major stages: first, three glucose units as well as four of the precursor's original nine mannose units are removed ("trimming"; 10, 16, 20, 23, 28, 29); second, trisaccharide sequences of -NAcGlc-Gal-sialic acid are added ("terminal glycosylation"), closely coupled to the removal of two more mannose units (9, 22, 27). In 15B CHO cells, the first stage of oligosaccharide processing takes place, whereas the second stage does not, because this mutant specifically lacks UDP-GlcNAc glycoprotein glycosyltransferase I, the enzyme needed to initiate terminal glycosylation (8, 9, 27, 29). Therefore, the end-product of oligosaccharide processing of G protein in VSV-infected 15B cells is the five-mannose-containing product of the trimming stage (27). This form of G protein, bearing trimmed oligosaccharides, electrophoreses discernibly faster in SDS polyacrylamide gels than the forms of G that bear the larger, precursor oligosaccharides (13).

Fig. 1 *A* demonstrates this decrease in the apparent molecular weight of G protein during pulse-chase experiments carried out with infected 15B cells. That this change in mobility results from reductions in size of the oligosaccharide moieties of G protein (13) is further substantiated by the fact that selective removal of the oligosaccharides with Endo H yields one band with the same mobility at all times of chase (Fig. 1 *B*). The extent of conversion of the larger percursor form (denoted G_1) to the smaller form (denoted G_T) can thus be used as an indirect

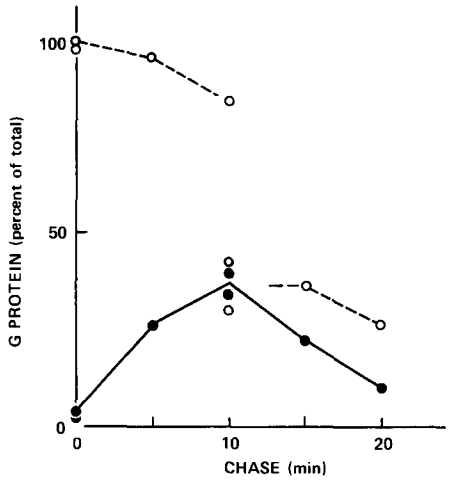

FIGURE 2 Dependence of the extent of processing of G in vitro (●) and of trimming *in vivo* (○) upon time of chase in VSV-infected 15B cells. These curves derive from the experiment in Fig. 1 (0-, 5-, 10-, 20-min chase) and two other independent experiments (0-, 10-, 15-min chase for one; 10-min chase only for the other), and were obtained by integrating densitometer tracings of the autoradiographs. To determine the maximal extent of conversion of G to Endo H resistance in vitro (●), the 40-min time-point was used and all resistant forms (G_R) were considered together. Results with the 20-min time-point were the same (data not shown). The determination of the percent of untrimmed G protein remaining (○), (100 $G_1/(G_1 + G_T)$, from densitometric scans is qualitatively accurate but should be regarded as only an estimate because G_1 and G_T were not completely resolved in this experiment. In this analysis, it was assumed that each form had a Gaussian distribution in the gel. The manner in which the dashed line is drawn at the 10-min points is arbitrary.

assay to follow the arrival of G protein at the site of trimming in the 15B cells. As shown in Fig. 2, G protein is not yet trimmed after 5 min of chase, but most has been trimmed by 15 min of chase. The extent of trimming observed at 10 min of chase is highly variable between independent experiments, whereas the degree of trimming at the earlier and later time-points is quite reproducible. Because a 5-min pulse label was employed and time-points were taken at 5-min intervals of chase, the variability of the 10-min point shows that most of the pulse must have proceeded synchronously into the site of trimming at ~10 min of chase. Unfortunately, the large number of subsequent manipulations makes it difficult to sample the culture more frequently than at 5-min intervals.

Therefore, the ability of G protein to be processed in vitro coincides closely with the time at which its oligosaccharides are being trimmed in vivo. Given the likelihood that the pulse of G protein proceeds nearly synchronously through the transferable pool, it may be useful to simply add up the amounts of G protein that can be processed in vitro at each of the time-points in Fig. 2. This total would then represent the sum of the instantaneous contents of the transferable pool assayed at intervals equal to the duration of the pulse. The sum of the content of the transferable population present at 0, 5, 10, 15, and 20 min of chase is 98% of the ^{35}S-label incorporated into the G protein during the pulse. This suggests that most if not all of the G protein passes through the transferable pool at one time or another of chase, and that the G protein present in this pool in vivo is very efficiently processed in vitro.

The Transferable Pool of G Protein Is Found in Low-density Membranes that Fractionate like Golgi Membranes

To characterize the membranes of 15B cells that act as donors of G protein in a different way, the postnuclear supernate of infected cells that had been labeled for 5 min with [^{35}S]Met and then chased for 10 min was fractionated on an equilibrium sucrose density gradient. The manner in which ER and Golgi membranes distributed in this gradient was determined by using the most pertinent biochemical markers. The ER membrane, the site of incorporation of nascent G protein (19), was marked by the distribution of G protein labeled in a 3-min pulse with [^{35}S]Met (Fig. 4*B*, open circles). The distribution of Golgi membranes in the gradient, considered to be the site of terminal glycosylation (3, 5, 6, 9, 12, 18, 26, 28, 31), was marked by (*a*) the distribution of UDP-Gal:glycoprotein glycosyltransferase activity (Fig. 4*C*, closed circles) and (*b*) the distribution of G protein pulse-labeled with [^{3}H]galactose in VSV-infected wild-type CHO cells (Fig. 4*B*, closed circles).

As shown in Fig. 4*A* (closed circles) most of the pulse-labeled G protein at 10 min of chase was found in low-density membranes whose distribution was similar to that of the Golgi markers. No more than ~20% of the G protein could have been found in the distribution characteristic of denser rough ER membranes. Therefore, by 10 min of chase, the majority of G protein had left the rough ER and now resided in membranes cofractionating with the Golgi markers.

In a further experiment, the postnuclear supernate from VSV-infected 15B cells that had been chased for 10 min was incubated with the postnuclear supernate of wild-type CHO cells under conditions that result in oligosaccharide processing. This incubated mixture was then fractionated on the sucrose gradient. The total content of ^{35}S-labeled G protein and also

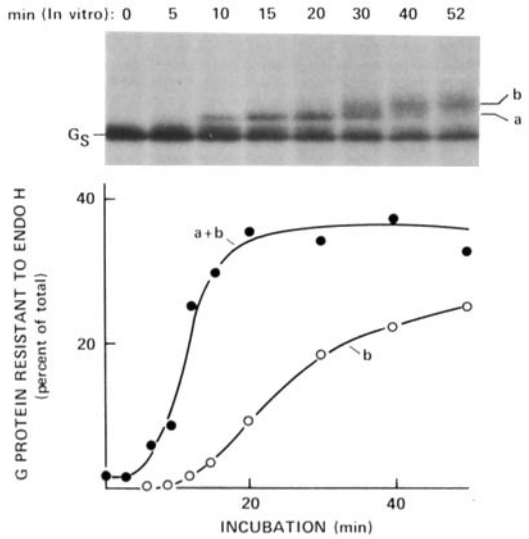

FIGURE 3 Time-course of the conversion of G protein to Endo H-resistant forms. Postnuclear supernate of VSV-infected 15B cells (labeled for 5 min with [^{35}S]Met and chased for 10 min) was incubated with postnuclear supernate of wild-type cells under standard conditions. Aliquots were taken at the times indicated, treated with Endo H, and analyzed by gel electrophoresis. Shown on top is an autoradiograph of the portion of the gel containing G protein. Below, the percent of G protein converted to Endo H-resistant forms was determined by integration of densitomer tracings of the autoradiograph. (●), Total Endo H-resistant forms of G; (○) includes only Endo H-resistant material running slower than band *a*, e.g., the set of band(s) labeled as *b*, measured by subtracting the sharp band *a* from the total amount of resistant material (G_R).

the amount of Endo H-resistant forms of G protein in each fraction were then determined (Fig. 4*A*). The distribution of total G protein (Endo H-sensitive and -resistant forms considered together) was essentially unchanged as a result of the incubation (Fig. 4*A*, open circles); yet, ~40% of the G protein in the incubation had been processed to Endo H-resistant forms. Moreover, the G protein that had been processed in vitro was all found in the light membrane distribution characteristic of the Golgi markers (Fig. 4*A*, triangles). Therefore, most or all of the transferable pool of G protein that had been processed in vitro must have originally resided in 15B membranes found in the less dense, Golgi-like distribution.

To ascertain whether the untrimmed and trimmed forms of G protein (Fig. 1*A*) found in infected 15B cells distributed differently in the sucrose gradient, the postnuclear supernate of cells that had been chased for 10 min was fractionated (Fig. 5). Each fraction was electrophoresed, and the amount of each form estimated as in Fig. 2. Fractionation of the untrimmed (G_1) form was similar to that of the rough ER marker. The trimmed (G_T) form followed the less dense distribution characteristic of the Golgi markers, the membranes that also house the transferable pool of G protein that can be processed in vitro. Therefore, most of the G protein processed in vitro must have already had its oligosaccharides processed in vivo, thereby linking the evidence from cell fractionation in this section to the kinetic evidence in preceding experiments.

Properties of the In Vitro Processing Reaction

The conditions employed here in the preparation of crude fractions for use as donors differ from those previously reported (7, 24) by the omission of a postpulse treatment with CCCP in the absence of glucose. We have shown here that extracts

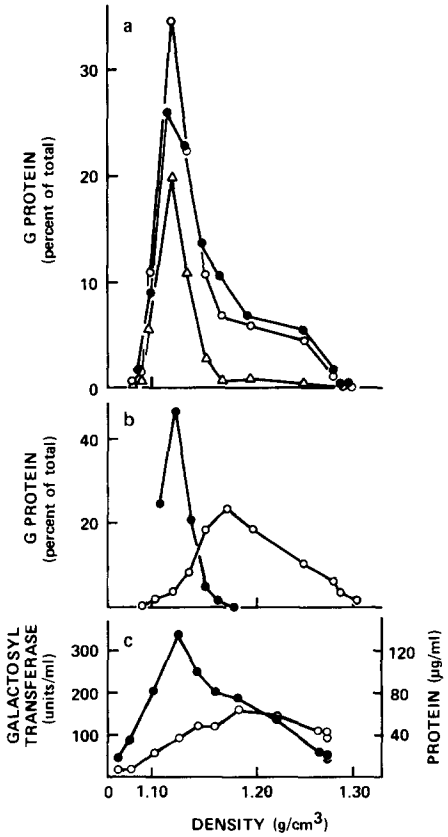

FIGURE 4 (*a*) Subcellular fractionation of postnuclear supernates before and after incubation in vitro. These graphs represent the distribution of ^{35}S-labeled G protein in two gradients. In one case, 100 μl of a postnuclear supernate of ^{35}S-labeled VSV-infected 15B cells (10-min chase) was mixed with 100 μl of postnuclear supernate of wild-type CHO cells and fractionation was begun immediately (see Materials and Methods for details). All of this G protein was found to be sensitive to Endo H (not shown), and its distribution (●) was determined by integrating densitometer traces of autoradiographs of the electrophoresed fractions. The content of G protein in each fraction is expressed as a percent of the total labeled G protein recovered from the gradient in this and other cases. Rather than plotting fraction numbers, we plotted the values against the density of sucrose contained in each fraction. For the other gradient, the same amounts of labeled postnuclear supernates of VSV-infected 15B cells and of wild-type cells were incubated with 300 μl of the standard reaction cocktail for 30 min at 37°C before cell fractionation. The distributions of total G protein (○; i.e., Endo H-sensitive and -resistant forms considered together) and of the Endo H-resistant forms considered separately (△) are shown. (*b*). Markers for ER and Golgi membranes. Shown is the distribution in gradients of ^{35}S-labeled G protein from VSV-infected 15B cells that had been labeled for only 3 min and not chased (○), and the distribution of ^{3}H-galactose incorporated into VSV-infected wild-type CHO cells in a 5-min pulse (●). In each case, a mixture of 100 μl of labeled postnuclear supernate and 100 μl of postnuclear supernate from unlabeled, wild-type CHO cells were run on the gradient. In the latter case, supernate was prepared from 1×10^8 CHO cells that had been labeled with 200 μCi/ml of ^{3}H-galactose in a low glucose medium (see Materials and Methods). The TCA-precipitable radioactivity in each fraction of the gradient was determined, and expressed as a percent of the total radioactivity recovered. No detectable ^{3}H was found in fractions of density >1.17. (*c*) The distributions of galactosyltransferase (●) and of protein (○). Galactosyltransferase activity of the resuspended pellets of gradient fractions was determined according to Brew et al. (4) with the modifications described (24), using ovalbumin as acceptor. 1 U represents 1 nmol of UDP-Gal transferred/h at 37°C. Protein was measured by the Lowry method (21).

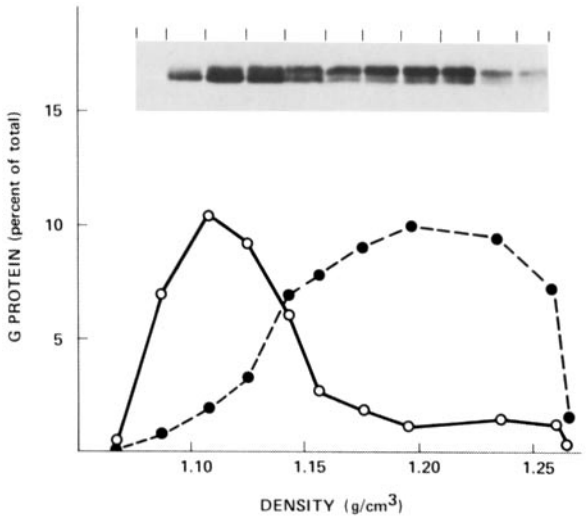

FIGURE 5 Sucrose gradient analysis of 200 μl of the postnuclear supernate of VSV-infected 15B cells that had been pulse-labeled for 5 min with [^{35}S]Met and chased for 10 min before homogenization. The pelleted membranes from each fraction were electrophoresed as in Fig. 1*A*. The portion of the autoradiograph containing G protein is the *inset* above; each lane corresponds to a fraction plotted below. The amounts of the upper (●; untrimmed, G_1) and lower (○; trimmed, G_T) bands in each fraction were estimated from densitometer tracings, and expressed as a percent of the total ^{35}S-labeled G protein recovered from the gradient.

prepared without an energy-poisoning step can also be active as donors of G protein, provided that a period of chase has been allowed before homogenization. It is entirely possible that the time required to wash the cells in between the pulse and the CCCP treatment in the earlier protocol (7) was equivalent to a period of chase in the present procedure. Therefore, the donors in both instances may be Golgi-like membranes. Our previous suggestion that ER membranes are donors after the treatment with CCCP would then be incorrect.

In any case, because the energy-poisoning step is no longer used, it was important to confirm certain key properties of the system. These experiments employed crude membranes from infected 15B cells prepared after 10 min of chase as donor for the in vitro processing reaction. Fig. 6 shows that UDP-GlcNAc glycoprotein glycosyltransferase I activity, provided by wild-type membranes, is needed for processing in vitro. The postnuclear supernatant fraction of 15B cells cannot replace that of wild-type cells. Processing was efficient only when a cytosol fraction (high-speed supernate) was present (Fig. 7) and exhibited a broad pH optimum centered at about 6.8 (Fig. 8).

We reported that the processing reaction observed with crude fractions from CCCP-treated VSV-infected 15B cells as donor was dependent on added ATP (7, 24). This is also true of the activity of postnuclear supernates prepared from infected 15B cells chased for 10 min but not treated with CCCP (Table I), providing ATP has been rigorously removed. Simply dialyzing or washing the membranes was not sufficient to demonstrate the ATP dependence. However, enzymatic depletion of endogenous ATP by preincubation with hexokinase and excess glucose reduced the reaction in the absence of added ATP to <10% of that seen in the presence of ATP and an ATP-regenerating system (Table I, line 5). Presumably, the energy poisoning used in our initial work (7, 24) made it easier to demonstrate an ATP dependence because ATP had been depleted in vivo.

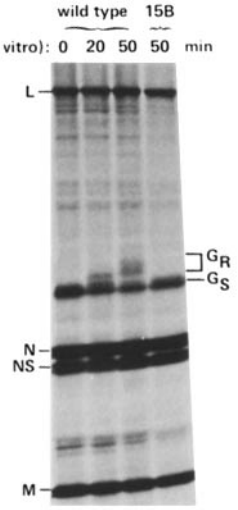

FIGURE 6 Appearance of Endo H-resistant forms of G protein upon incubation of cell-free extracts. Postnuclear supernate prepared from VSV-infected CHO cells of clone 15B, (after pulse-labeling with [^{35}S]Met for 5 min and chase for 10 min) were incubated with either postnuclear supernate of untreated, unlabeled wild-type cells or a comparable fraction of 15B cells, for various times, as indicated. Aliquots (15 μl) were treated with Endo H and analyzed by electrophoresis in a 10% polyacrylamide gel. G_S is the form of the viral glycoprotein whose oligosaccharides have been cleaved by Endo H. G_R denotes the forms of G protein resistant to Endo H. *L*, *N*, *NS*, and *M* are other VSV-encoded proteins.

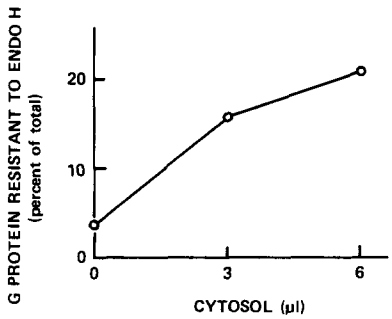

FIGURE 7 Cytosol dependence. Incubations (25 μl, final volume) contained standard cocktail (15 μl), postnuclear supernate of ^{35}S-labeled VSV-infected 15B cells (10-min chase, 2 μl), resuspended membrane pellet of wild-type cells (2 μl) and rat liver cytosol (0–6 μl, as indicated). Cytosol was prepared as described (24), and was not concentrated or gel-filtered.

DISCUSSION

Golgi-like Membranes as Donors of G Protein

The VSV glycoprotein is synthesized and initially glycosylated in the rough ER membrane, and is then transported to the cell surface (11, 13–15, 19, 20). Until recently, the evidence that G protein passes through the Golgi membranes in between the rough ER and the plasma membrane has been indirect and largely based on a detailed knowledge of the biochemical steps in G protein's glycosylation. During the intracellular transport of G protein, its oligosaccharides are extensively modified by the removal or "trimming" of mannose and glucose (10, 16, 20, 23, 28, 29) and by terminal glycosylation (9, 22, 27). Enzymes capable of trimming mannose (28; an α-1,2-mannosidase) and of terminal glycosylation (including *N*-acetylglucosaminyltransferases, galactosyltransferases, sialyltransferases) are concentrated in highly purified Golgi fractions of liver and other

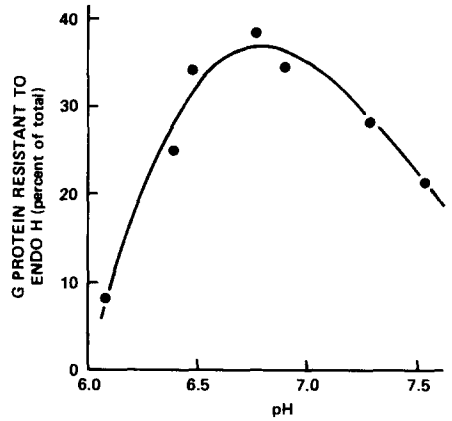

FIGURE 8 pH dependence. Postnuclear supernate of VSV-infected 15B cells (labeled for 5 min with [^{35}S]Met and chased for 10 min) was incubated with postnuclear supernate of wild-type CHO cells using reaction cocktails containing 80 mM MES-KOH buffer at different pHs. Aliquots were taken after a 40-min incubation and analyzed for the total percent of G protein made resistant to Endo H (G_R). The remainder of the incubation mixtures was diluted threefold with water and the pH was measured.

tissues (3, 5, 6, 9, 12, 26, 28, 31), and electron microscope autoradiography has shown that terminal glycosylation occurs in the Golgi complex (18). It has therefore been reasonable to assume that G protein's oligosaccharides are processed as G protein passes through the Golgi complex in infected tissue culture cells. The elegant immunoelectron microscopic experiments of Bergmann et al. (2) have now firmly and directly established the correctness of this assumption by demonstrating the presence of G protein in Golgi stacks. Moreover, G protein was shown to pass successively through three major compartments in VSV-infected CHO cells: from rough ER[1] to Golgi stacks to plasma membrane (2).

The purpose of the experiments reported here was to determine which membrane(s) of the VSV-infected CHO clone 15B cell serve as donor(s) of G protein in the cell-free system. In light of the work of Bergmann et al. (2), as well as earlier biochemical findings (3, 5, 6, 9–11, 13–16, 20, 22, 23, 26, 29), the three prime candidates that could donate a large fraction of the total intracellular pool of G protein are the three major cellular compartments through which G protein passes: rough ER, Golgi, and plasma membrane. Of course, in theory any membrane of the 15B cell harboring G protein could potentially serve as a donor to some extent, because all of the G protein in this mutant cell carries either precursor or trimmed intermediate oligosaccharide forms (27, 29). All of these forms are sensitive to Endo H and are substrates that can be processed in vitro to Endo H–resistant forms by enzymes found in wild-type CHO membranes.

In spite of this diversity of potential donors, we have found that the capacity to serve as donor of G protein for processing in vitro is actually a highly specific property possessed only by certain membranes through which G protein passes rapidly in vivo. Several independent lines of kinetic, physical, and enzymatic evidence, taken together, strongly suggest that the principal but transient donor is a Golgi-like membrane.

[1] CHO cells do not have an extensive smooth ER comparable to that found in liver. Most of the smooth intracellular membranes in CHO cells are readily identified as Golgi stacks or appear to be small vesicles (our unpublished observations).

TABLE I

ATP Dependence of Oligosaccharide Processing In Vitro

Conditions*	Endo H-resistant G protein produced‡
1. Complete	[1]
2. − ATP, − creatine phosphate, − creatine kinase, + ADP	0.54
3. Same as *2*, + hexokinase	0.37
4. Same as *2*, + glucose	0.39
5. Same as *2*, + hexokinase, + glucose	<0.1

* (*1*) Complete incubation contained 10 μl of extract from VSV-infected [^{35}S]Met-labeled 15B cells (10-min chase), 2 μl of concentrated, gel-filtered rat liver cytosol, 5 μl of resuspended membrane pellet of wild-type cells, 30 μl of standard reaction cocktail, and 3 μl of extract buffer. The other incubations (*2-5*) had all of the components of *1* but, instead of the standard cocktail, each received a cocktail lacking ATP, creatine phosphate, and creatine kinase, and containing 2.9 mM sodium ADP plus enough NaCl to maintain sodium ion at its previous concentration. In addition, incubation *3* received 1 μl of a hexokinase solution (1,400 U/ml) prepared by dissolving, in water, centrifuged crystals of an ammonium sulfate precipitate of yeast hexokinase purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; *4* received 1 μl of a 0.25 M solution of glucose; and incubation *5* received 1 μl each of hexokinase and glucose. Samples of incubations taken at 20 min were treated with Endo H and electrophoresed, and the dried gel was autoradiographed.

‡ Determined from densitometer tracings of the autoradiograph, and normalized to the results for the complete incubation.

(*a*) The donor is neither the rough ER nor the plasma membrane (Fig. 2). Little processing in vitro results when G protein is present in vivo mainly in the rough ER (0-min chase) or in the plasma membrane (well after 20-min chase).

(*b*) G protein would be mainly present in the Golgi stack at the time when crude membrane fractions are best able to serve as donors (Fig. 2). Immunoelectron microscopy and immunofluorescence experiments (2) show that G protein reaches Golgi stacks within 11 min after synthesis in CHO cells at 32°C; the capacity to serve as donor rises progressively with chase and is optimal at ~10 min at 37°C.

(*c*) The capacity to donate G protein is maximal at a time of chase when the attached oligosaccharides are just being trimmed, an activity associated with Golgi membranes (Fig. 2).

(*d*) The G protein that is processed in vitro is provided by low-density membranes that distribute in sucrose gradients in the same manner as conventional biochemical markers of the Golgi apparatus (Fig. 4). Of course, these fractions are not pure, so that the presence of the donor in these fractions does not by itself constitute proof that the donor is a Golgi membrane.

(*e*) Almost all of the trimmed form of G protein but little of the untrimmed form was found in the low-density membranes in the sucrose gradient after 10 min of chase (Fig. 5). Therefore, most of the G protein donated by low-density membranes in vitro must already have been trimmed in vivo.

In short, the donor membranes have greatest activity when G protein is independently known to be present in the Golgi stack; the G protein that is donated appears to have already entered Golgi membranes as judged by the trimming of its oligosaccharides and the coincidence of its distribution on a sucrose gradient with that of markers of Golgi membranes. Almost all of the newly synthesized G protein in the cell can be donated in vitro at one time of chase or another and nearly half can be processed at 10 min of chase. This makes it seem likely that the donor is a major reservoir of G protein, such as the Golgi stacks, rather than a transit form, such as transport vesicles. But, because the latter possibility cannot be excluded, the donor will be referred to as a "Golgi-like" membrane. In retrospect, it appears likely that the donor in earlier experiments utilizing CCCP (7, 24) was also a Golgi-like membrane.

Our previous work suggested that the G protein donated by 15B cell membranes can be processed in vitro by enzymes present in exogenous, purified Golgi membranes, and that processing follows the appearance of G protein in these Golgi membranes (7, 24). If the donor in this process were also a Golgi membrane, then G protein originating in one Golgi complex would have to arrive at the site of terminal glycosylation in another Golgi complex in order to explain our findings. There is presently no evidence for such an inter-Golgi transport in vivo, but this process would almost certainly have gone undetected unless distinguishable Golgi complexes were present, as in the in vitro system, where fractions from wild-type and mutant cells are mixed.

Transferable and Nontransferable Pools of Viral Glycoprotein May Both Reside in Golgi Membranes

The capacity of Golgi-like membranes to donate G protein in vitro is remarkably transient, declining with a half-time of ~5 min with additional chase in vivo beyond the 10-min point (Fig. 2). This finding reveals the existence of two populations, or pools, of G protein: a "transferable" pool that can reach terminal glycosyltransferases of another Golgi complex; and another, "nontransferable" pool of G protein that cannot be processed in vitro. G protein rapidly enters the nontransferable pool from the transferable one with a half-time of ~5 min in vivo.

It is important to realize that the distinction between these two in vivo pools of G protein is a valid one that stands irrespective of issues that concern the nature and physiological significance of the in vitro processing reaction itself. The cell-free system has clearly provided an assay that distinguishes these two pools; while the assay most likely measures a reconstituted segment of intracellular transport, the in vivo pools nevertheless exist irrespective of what is actually happening in vitro.

The surprisingly transient nature of the Golgi-like donor raises the possibility that G protein may enter the nontransferable pool more rapidly than it leaves the Golgi complex. Only about half of a pulse of G protein reaches the CHO cell surface in ~1 h (25); yet almost all of the pulse has entered the nontransferable pool by 20 min of chase (Fig. 2). Both pools might therefore be present in Golgi-like membranes.

A simple hypothesis that can explain both the existence of two pools in vivo and the finding of a Golgi-like membrane as donor in vitro is that each of the two pools might reside in a distinct compartment of the Golgi complex. G protein would then pass successively from the first compartment (housing the transferable pool) to the second (housing the nontransferable pool). This would perhaps correlate with the *cis* to *trans* direction of protein transport through the Golgi stack (1, 5, 31). In a cell, G protein would generally be transported from the first to the second compartment within the same Golgi complex. The inter-Golgi transport observed in vitro would represent transfer from the first compartment of one Golgi complex to the second compartment of another. Because transport from the first to the second compartment would be vectorial, G protein residing in the second compartment in vivo might not be transferable to another Golgi in vitro. Given that the appearance of G protein in the transferable pool coincides with

the trimming of its oligosaccharides, and that processing in vitro requires that G protein in the transferable pool arrive at the site of terminal glycosylation in a wild-type Golgi complex, it would then be expected that the α-1,2-mannosidase responsible for trimming would be concentrated in the first Golgi compartment, and that terminal glycosyltransferases (3) would be concentrated in the second Golgi compartment. These activities may prove helpful in future studies as enzymatic markers of the two Golgi compartments that we have postulated to account for our unexpected biochemical findings.

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