Proc. Natl. Acad. Sci. USA
Vol. 77, No. 7, pp. 3870–3874, July 1980
Biochemistry

Transport of vesicular stomatitis virus glycoprotein in a cell-free extract

(endoplasmic reticulum/Golgi complex/oligosaccharide processing/membrane assembly)

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Communicated by I. Robert Lehman, April 7, 1980

ABSTRACT **We describe a cell-free system in which the membrane glycoprotein of vesicular stomatitis virus is rapidly and efficiently transported to membranes of the Golgi complex by a process resembling intracellular protein transport. Transport *in vitro* is energy-dependent and is accompanied by terminal glycosylation of the membrane glycoprotein (dependent upon UDP-GlcNAc and resulting in resistance to endo-β-*N*-acetylglucosaminidase H).**

The elucidation of the mechanisms of assembly of cellular membranes and of the organelles that these membranes define poses a major challenge to cell biologists. In particular, it will be important to understand how distinct sets of proteins are delivered to the different subcellular organelles so as to confer upon them their distinct functions. How is this highly specific intracellular transport and sorting of proteins accomplished?

An essential step towards obtaining a molecular description of these complex cellular events will be to achieve conditions under which the same transport of nascent proteins can proceed in cell-free extracts, for only then can the tools of biochemistry be fruitfully applied. We describe here experiments demonstrating that it is possible to obtain transfer of protein between specific organelles in a cell-free extract by a reaction resembling that of intracellular protein transport. For these studies we have utilized Chinese hamster ovary (CHO) cells infected with vesicular stomatitis virus (VSV) as a well-defined system in which to investigate the transport of a membrane protein destined for the plasma membrane (1).

The viral glycoprotein (G protein) that will reside in the membrane of the mature virion is the only glycoprotein synthesized in VSV-infected cells. Like cellular surface glycoproteins, G is synthesized in the endoplasmic reticulum (ER), is transported to the Golgi complex,* and is then transported to the plasma membrane (1). Clathrin-coated vesicles appear to mediate both of these transport steps (6). The small genome of VSV (encoding only five proteins, all found in virions) provides assurance that the maturation of G follows host-specific pathways.

During its synthesis in the ER, G acquires two mannose-rich oligosaccharides that are subsequently processed as G passes through the Golgi complex (2, 3, 7). This change in oligosaccharide structure provides an indirect means for assaying the arrival of G at the Golgi complex. Endo-H has proved useful in this regard, because this enzyme will cleave the mannose-rich precursor oligosaccharides of G, causing a marked reduction of apparent molecular weight, but will not attack the Golgi-processed oligosaccharides (7).

The general approach we have taken to obtain transport of G *in vitro* is to prepare extracts (postnuclear supernatants) of VSV-infected CHO cells that had been briefly incubated with [^{35}S]methionine *in vivo* so as to label G in the ER. This results in an extract in which all of the labeled G protein still bears the precursor oligosaccharides that can be cleaved by Endo-H. The production of an Endo-H-resistant form of G after incubation of the cell-free extract would be taken as initial evidence of transport to the Golgi complex *in vitro*.

To ensure that only events resulting in the transfer of G between organelles would be detected by this indirect assay, we have utilized extracts prepared from a VSV-infected mutant CHO cell line (clone 15B) and an *in vitro* complementation scheme. Clone 15B cells lack UDP-GlcNAc glycosyltransferase I, an enzyme found in the Golgi complex (3) that is needed to initiate processing of the oligosaccharides from "high Man" to Endo-H-resistant "complex" structures (2, 8, 9). G is transported normally within infected 15B cells (10) but always remains sensitive to Endo-H (11). When extracts of [^{35}S]methionine-labeled, VSV-infected, clone 15B cells are incubated with extracts of uninfected wild-type CHO cells, the appearance of Endo-H-resistant forms of G provides strong evidence for the transfer of G from 15B cell membranes to the Golgi complex derived from the wild-type cell, the site of oligosaccharide processing.

MATERIALS AND METHODS

Cells and Viruses. CHO cells (referred to as "wild type," although not the immediate parent of clone 15B) were maintained in suspension (12). The CHO cell mutant (clone 15B, obtained from Stuart Kornfeld, Washington University) was grown as a monolayer (8). Stock of VSV (Indiana strain) was prepared from CHO cells (12).

Preparation of Extracts. *Infection of 15B cells.* Six plates (10-cm diameter) at near confluency (about 10^7 cells per plate) were infected with 5–10 plaque-forming units of VSV per cell in serum-free growth medium (2 ml per plate) containing actinomycin D at 5 μg/ml. At 1 hr after infection, 8 ml of complete growth medium was added to each plate. At 4 hr after infection, cells were removed by trypsinization as follows: Each plate was rinsed with 5 ml of phosphate-buffered saline (P_i/NaCl; per liter: 0.20 g of KCl, 0.20 g of KH_2PO_4, 8.0 g of NaCl, and 1.15 g of Na_2HPO_4, pH 7.4) and then rinsed quickly with

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Abbreviations: VSV, vesicular stomatitis virus; G, viral glycoprotein; ER, endoplasmic reticulum; CHO, Chinese hamster ovary; Endo-H, endo-β-*N*-acetylglucosaminidase H; P_i/NaCl, phosphate-buffered saline; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

* We use the term "Golgi complex" to refer to the intracellular sites in CHO cells in which the oligosaccharides of G are processed by trimming of Man residues and addition of terminal sugars (GlcNAc, Gal, sialic acid), making them resistant to cleavage by endo-β-*N*-acetylglucosaminidase H (Endo-H). For other cell types these modifications have been shown by subcellular fractionation and electron-microscopic autoradiography to occur in the Golgi complex (2–5) as it is defined morphologically.

3 ml of P_i/NaCl containing 0.5 g of trypsin and 0.2 g of EDTA per liter. After 5–10 min, cells were suspended, washed in ice-cold P_i/NaCl, and then suspended in 15 ml of methionine-free Joklik's minimal essential medium (GIBCO) containing 10% dialyzed fetal calf serum, 1% nonessential amino acids (GIBCO), and 20 mM Hepes–NaOH (pH 7.3).

Labeling of cells. After a 5- to 10-min equilibration at 37°C, [^{35}S]methionine (1–5 mCi, >1000 Ci/mmol, Amersham; 1 Ci = 3.7×10^{10} becquerels) was added. After a 5-min pulse-labeling, 30 ml of ice-cold P_i/NaCl containing 0.10 g of $CaCl_2$ and 0.059 g of $MgSO_4$ per liter was added. Cells were centrifuged at 4°C, quickly washed once with the same ice-cold buffer, and resuspended in 6 ml of ice-cold P_i/NaCl (with Mg^{2+} and Ca^{2+}) containing 5 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and kept on ice for 5 min before the addition of another 24 ml of warm P_i/NaCl (with Mg^{2+} and Ca^{2+}) containing 5 μM CCCP. The cells were then incubated for 20 min at 37°C.

Lysis of cells. All subsequent manipulations were at 0–4°C. Cells were centrifuged, washed once with P_i/NaCl, and swollen in 10 ml of 15 mM KCl/1.5 mM magnesium acetate/1 mM dithiothreitol/10 mM Hepes–KOH, pH 7.5. After centrifugation, the total volume was reduced to 0.9 ml, and the cells were resuspended and then disrupted with 20 strokes of a 7-ml tight-fitting Dounce homogenizer (Wheaton, Millville, NJ). Then, 90 μl of a concentrated buffer was added to yield the following final concentrations: 45 mM Hepes–KOH at pH 7.5, 75 mM KCl, 5 mM magnesium acetate, and 1 mM dithiothreitol. The homogenate was centrifuged at $600 \times g$ for 5 min. The resulting postnuclear supernatant (referred to hereafter as "extract") was removed and was either used at once or frozen in portions in liquid N_2 and stored at −80°C. Frozen extracts were stable for several months. For extracts of untreated 15B and of wild-type CHO cells, 10^8 cells were swollen, lysed, and processed as described above.

Incubation of Extracts. For most experiments, 5 μl of [^{35}S]methionine-labeled extract from CCCP-treated, VSV-infected 15B CHO cells was mixed with 25 μl of extract from untreated wild-type CHO cells and 45 μl of an incubation cocktail. The cocktail contained 2.9 mM ATP (Na form pH 7), 14.3 mM creatine phosphate (Na form), rabbit muscle creatine kinase (Calbiochem) at 11 international units/ml, 1.4 mM UDP-Gal (Na form), 1.4 mM UDP-GlcNAc (Na form), 1.0 mM dithiothreitol, 4.5 mM magnesium acetate, 75 mM KCl, and 67 mM 4-morpholineethanesulfonic acid (Mes)–KOH at pH 5.9. The final pH of the incubation mixture was 6.3. Incubation was at 37°C. Samples (15 μl) of incubations were mixed with 15 μl of 0.1 M Tris·HCl, pH 6.8/20 g of $NaDodSO_4$ per liter/30 mM dithiothreitol and then boiled for 3 min. Then, 20 μl of 0.3 M sodium citrate buffer, pH 5.5, containing $NaDodSO_4$ at 1 g/liter and pure Endo-H (kindly provided by P. Robbins, Massachusetts Institute of Technology) at 75 ng/ml was added. After 12–18 hr at 37°C, proteins were precipitated with 0.3 ml of 10% trichloroacetic acid, dissolved in $NaDodSO_4$, and electrophoresed in a 10% polyacrylamide gel according to Laemmli (13). Dried gels were autoradiographed with fluorographic enhancement and exposed for 1–7 days.

RESULTS

Preparation of Extracts Capable of Oligosaccharide Processing. A postnuclear supernatant ("extract") was prepared from VSV-infected 15B cells after a 5-min pulse label with [^{35}S]methionine. Incubation of a mixture of this extract with a comparable extract from unlabeled, uninfected, wild-type CHO cells did not result in the production of any Endo-H-resistant ^{35}S-labeled G protein (Fig. 1*A*, group 1). From this result we concluded that G could not be transferred all of the way from its site of synthesis in the rough ER to the Golgi complex *in vitro*.

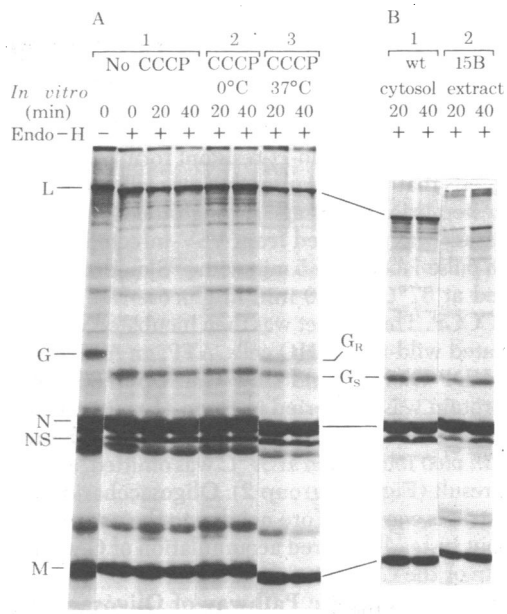

FIG. 1. (*A*) Appearance of Endo-H-resistant forms of G protein upon incubation of cell-free extracts. Extracts were prepared from VSV-infected clone 15B cells that had been treated in different fashions after a 5-min pulse label of [^{35}S]methionine. All three protocols are variations of that described in *Materials and Methods*. (*1*) No CCCP. Cells kept on ice for 20 min after a [^{35}S]methionine pulse, then lysed. (*2*) CCCP, 0°C. Cells kept on ice for 20 min in the presence of CCCP (5 μM). (*3*) CCCP, 37°C. Standard protocol. Cells were incubated at 37°C for 20 min in presence of CCCP (5 μM). Incubations were as described in *Materials and Methods*, and samples were taken at the indicated times and electrophoresed with (+) or without (−) prior digestion with Endo-H. Autoradiograph of dried gel is shown. (*B*) Incubations in which wild-type extract was replaced by (*1*) cytosol fraction ($48{,}000 \times g_{average}$ 2-hr supernatant) obtained from wild-type (wt) CHO cell extract or (*2*) extract from unlabeled, uninfected, 15B cells. G_S denotes the "sensitive" form of G whose oligosaccharides have been cleaved by Endo H. G_R is the principal "resistant" form. N, NS, L, and M denote the other VSV-encoded proteins.

The following reasoning based on observations of secretory proteins (14, 15) served to explain this negative finding as well as to motivate the design of the successful experiments outlined below. Transport vesicles appear to bud off specifically from the smooth "transitional elements" of ER (14, 16). After a brief pulse label followed by homogenization, most of G would not be in vesicles derived from these transitional elements, but rather would be in the rough microsomes. These vesicles would be "dead ends" *in vitro*, unable to package G into the transport vesicles needed to travel to Golgi. It might therefore be essential to prepare extracts from cells in which pulse-labeled G had been trapped in the "transitional elements" of ER or at an even later stage of maturation in order to obtain efficient transport (involving fewer steps) *in vitro*.

Treatment of secretory cells with inhibitors of oxidative phosphorylation immediately after a brief pulse of radioactive amino acids causes the accumulation of the radioactively labeled secretory proteins in the ER and its transitional elements (14, 15). The energy-dependent step blocked by this procedure seems to be the formation of the vesicles that carry secretory proteins from the transitional elements of the ER to the Golgi complex (14, 15). We have found (unpublished data) that the transport of G can be blocked in a similar way; incubation of VSV-infected CHO cells with CCCP (an uncoupler of oxidative

phosphorylation) in glucose-free medium after a 5-min pulse of [^{35}S]methionine prevents the conversion of labeled G to an Endo-H-resistant form. This G protein is not accessible to externally added chymotrypsin; this block is reversible, because removal of CCCP from the medium results in the subsequent conversion of G to an Endo-H-resistant form (data not shown). This suggests that CCCP causes the intracellular accumulation of G at a step prior to passage through the Golgi complex.

An extract was prepared from VSV-infected 15B cells that had been pulse-labeled for 5 min with [^{35}S]methionine, and then incubated at 37°C for 20 min in glucose-free medium containing CCCP. This extract was then incubated with an extract of untreated wild-type CHO cells, ATP, an ATP-regenerating system, UDP-GlcNAc, and UDP-Gal. Up to 60% of the total [^{35}S]G protein was converted to Endo-H-resistant forms (Fig. 1*A*, group 3). When CCCP was added to the cells but the subsequent *in vivo* incubation at 37°C was omitted, active extracts did not result (Fig. 1*A*, group 2). Oligosaccharide processing in the extracts was therefore not due to the presence of CCCP *per se*, but instead required accumulation of G within the cell at the site of the CCCP-induced block.

The Same Enzymatic Pathway of Oligosaccharide Processing is Used *in Vivo* and *in Vitro*. The appearance of Endo-H-resistant forms of G protein absolutely required the presence of membranes from wild-type cells. No reaction occurred when the ^{35}S-labeled extract of VSV-infected 15B cells was incubated with the cytosol fraction of wild-type cells or with an extract of 15B cells (Fig. 1*B*). These experiments show that the *in vitro* reaction requires membrane-bound GlcNAc transferase I, as *in vivo*.

Kinetics of Oligosaccharide Processing *in Vitro*. The amount of Endo-H-sensitive form of G [the form that runs as G_S after cleavage of the oligosaccharides (7)] decreases with time, with the corresponding appearance of the major Endo-H-resistant form, G_R (Fig. 2). A processing intermediate (running between G_S and G_R) is present at intermediate times. This form of G, which cannot be seen *in vivo*, presumably represents G protein with only one of its two oligosaccharides rendered resistant to Endo-H (17). At late times, an Endo-H-resistant form of G with an apparent molecular weight greater than G_R accumulates; this form comigrates with the sialylated form of G denoted earlier as G_2 (12).

Fig. 3 shows the kinetics of the appearance of Endo-H-resistant G quantitatively, as well as the effect of lowering the concentration of wild-type membranes in the incubation mixture. (The Endo-H-resistant bands just described were considered together in determining the percent of Endo-H-resistant G that had been produced.) At the highest concentrations of membranes, processing to Endo-H resistance proceeds to near completion within 20 min, similar to the time course *in vivo* (6, 7). The concentration of wild-type membranes

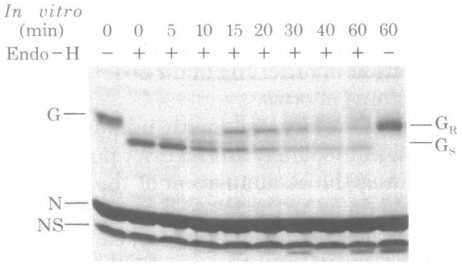

FIG. 2. Time course of oligosaccharide processing *in vitro*. Samples of an incubation mixture were taken at the indicated times and electrophoresed with (+) or without (−) prior treatment with Endo-H. Shown is autoradiograph of the portion of the gel containing G, N, and NS proteins.

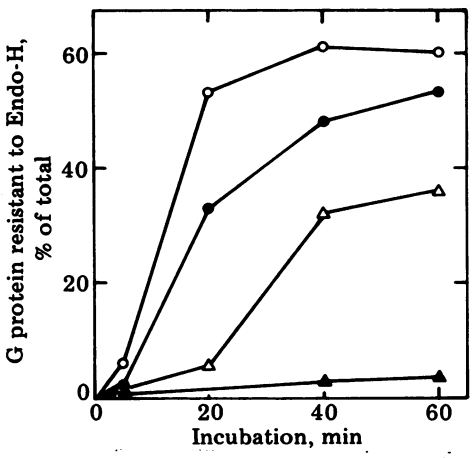

FIG. 3. Kinetics of processing as a function of concentration of wild-type CHO cell membranes. The percent of G protein resistant to Endo-H was determined by integration of densitometer tracings of autoradiographs; all three Endo-H-resistant forms (see text) were considered together. Each incubation mixture contained 45 μl of incubation cocktail, 5 μl of extract from VSV-infected 15B cells labeled with [^{35}S]methionine, and: 25 μl of extract of wild-type CHO cells (○); 4.2 μl of wild-type extract, 21 μl of cytosol fraction of wild-type cells (●); 0.70 μl of wild-type extract, 24 μl of cytosol (△); 25 μl of cytosol (▲).

was reduced by 6-fold or 36-fold by adding wild-type extract diluted with a cytosol fraction in place of undiluted extract. A delay in the appearance of Endo-H resistance that was noticeable at the high membrane concentrations became prominent at the 36-fold dilution. This delay in the processing probably reflects a concentration-dependent transit time required for G to go from its origin in 15B membranes to the site of oligosaccharide processing in the wild-type Golgi membranes.

Transfer of G Occurs Among Sealed Vesicles. Before incubation (Fig. 4, 0 min), 83% of the total G protein in extracts was protected from extensive tryptic hydrolysis and preserved

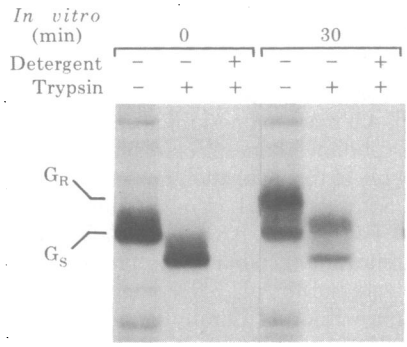

FIG. 4. Accessibility of G protein to trypsin before and after oligosaccharide processing *in vitro*. Measurement was based on the procedure of Katz and Lodish (18). Twenty microliters of extract of [^{35}S]methionine-labeled VSV-infected 15B cells was mixed with 60 μl of extract of wild-type CHO cells and 120 μl of cocktail. One half of the mixture ("incubated") was incubated at 37°C for 30 min and the other half ("unincubated") was kept on ice with 10 μl of 0.1 M EDTA added. To 15-μl samples of the incubated and the unincubated samples were then added 30 μl of 50 mM KCl/50 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$ or 50 mM KCl/50 mM Tris·HCl, pH 7.6, respectively. To these samples were then added (when indicated with +) trypsin (5 μl of 1 mg/ml) with or without deoxycholate (5 μl of 40 g/liter). After 15-min incubation at 37°C, soybean trypsin inhibitor (5 μl of 1 mg/ml) was added to each sample. After another 5 min, samples were precipitated with trichloroacetic acid and then treated with Endo-H before electrophoresis. Shown is the portion of the autoradiograph containing G protein.

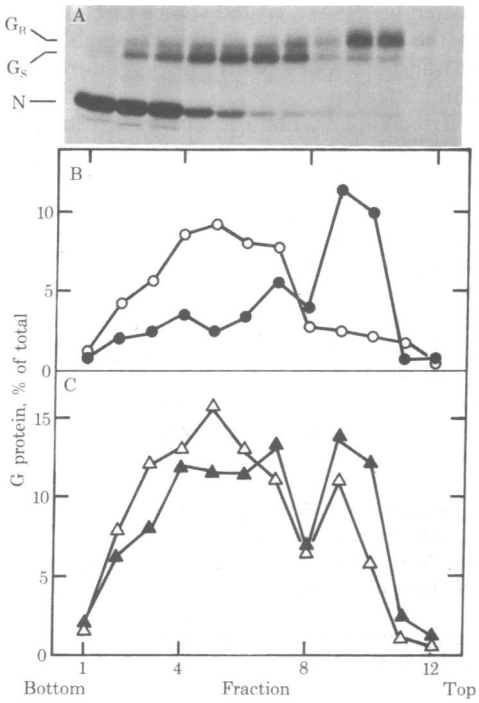

FIG. 5. (*A*) Separation of Endo-H-sensitive and resistant forms of G protein after *in vitro* processing by density gradient centrifugation. The experiment was based on the procedure of Knipe *et al.* (19). One hundred microliters of ^{35}S-labeled extract of VSV-infected 15B cells was mixed with 100 μl of wild-type extract and 300 μl of cocktail. After 30-min incubation at 37°C, 4.5 ml of ice-cold 10 mM Tris·HCl, pH 7.4/10 mM NaCl/1.5 mM $MgCl_2$ was added and the mixture was centrifuged at 30,000 rpm in the Beckman SW 50.1 rotor for 60 min. The membranes were resuspended in 4.5 ml of 1 mM Tris·HCl/1 mM EDTA, pH 8.0, with a Dounce homogenizer and centrifuged again. The pellet was then homogenized in 1.5 ml of 45% (wt/wt) sucrose in the Tris/EDTA buffer and mixed with 3 ml of 60% sucrose in the same buffer. This suspension was overlaid with 2 ml each of 45%, 40%, 35%, 30%, 25%, and 20% (wt/wt) sucrose solutions in the Tris/EDTA buffer and centrifuged for 16 hr at 25,000 rpm in the Beckman SW 27.1 rotor. Fractions were collected from the bottom and were then diluted with 2 vol of Tris/EDTA buffer and centrifuged at 40,000 rpm in the SW 50.1 rotor for 1 hr. The resulting pellets were digested with Endo-H before being analyzed by gel electrophoresis. Autoradiograph of the part of the gel containing G is shown. (*B*) The autoradiograph was scanned with a densitometer and the relative amounts of Endo-H-resistant (●) and -sensitive (○) forms of G protein were determined as for Fig. 3. A mixture identical to the one described in *A*, but which had not been incubated, was analyzed by the same procedure. All G protein from this gradient was Endo-H sensitive (data not shown). (*C*) Distribution of G protein in this later gradient ("unincubated," △) together with the distribution of total G protein obtained from the autoradiogram shown in *A* ("incubated," ▲).

as a large tryptic fragment; the slight decrease in the apparent molecular weight has previously been demonstrated (18) to be due to the loss of approximately 30 amino acids located at the COOH terminus of G on the cytoplasmic side of the microsomes. Protection was abolished by the presence of deoxycholate during the tryptic digestion (Fig. 4). After 30-min incubation, 63% of the Endo-H-resistant form (G_R) and 50% of the Endo-H-sensitive form (G_S) were protected from trypsin in a similar fashion. These results indicate that G occurs in the extract as a transmembrane protein with most of its mass and its oligosaccharides inside sealed vesicles, as in rough microsomes (18). They also suggest that the oligosaccharides had been processed by enzymes also present inside sealed vesicles.

Table 1. Requirements for processing of G protein *in vitro*

Addition or omission*	Endo-H-resistant G protein produced†
1. None (complete)	[1]
2. − ATP	1.14
3. − ATP, − creatine phosphate, − creatine kinase	<0.05
4. − UDP-GlcNAc	0.13
5. − UDP-Gal	1.43
6. + EGTA (10 mM)	1.09
7. + A23187 (20 μM)	0.25
8. + A23187 (20 μM), + EGTA (10 mM)	0.25
9. + CCCP (20 μM)	1.22

Lines 1–5. Of an extract from uninfected CHO cells, 600 μl was centrifuged for 1 hr at 30,000 rpm in an SW 50.1 rotor. The supernatant was dialyzed against 0.25 M sucrose. The pellet was resuspended in 600 μl of 0.25 M sucrose. Each incubation mixture contained (per 50 μl), 5 μl of resuspended pellet, 5 μl of dialyzed supernatant, and 5 μl of ^{35}S-labeled 15B cell extract, 5 μl of 0.25 M sucrose, and 30 μl of cocktail from which select components were omitted when indicated. *Lines 6–9.* Divalent cation ionophore A23187 and CCCP were added from 1 mM stock solutions in dimethyl sulfoxide. Control incubation included this solvent (2%, vol/vol). EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

* Complete reaction mixtures contained ATP, creatine phosphate, creatine kinase, UDP-Gal, and UDP-GlcNAc.

† The fraction of G converted to Endo-H resistance is expressed relative to the amount of G converted for a control incubation (such as line 1). Lines 2–5 and 6–9 were separate experiments resulting from incubations of 50 min, in which the control values were 30% and 34% Endo-H resistant, respectively.

Fractionation of Extracts Before and After Incubation. Fractionation of an incubated extract by isopycnic centrifugation in a sucrose density gradient (19) showed that the Endo-H-resistant and sensitive forms of G distributed quite differently (Fig. 5 *A* and *B*). Endo-H-resistant forms of G were concentrated in the lighter fractions, with a distribution similar to that found (data not shown) for galactosyltransferase (4), a marker enzyme of Golgi membranes. The Endo-H-sensitive form was concentrated in the denser fractions, with a distribution similar to that found (data not shown) for the site of incorporation of [^{35}S]methionine into G protein after a 5-min pulse label of VSV-infected CHO cells and for other markers of ER reported earlier (19).

Fig. 5*C* shows the distribution of total G protein before and after incubation *in vitro*. Before incubation, all of G was sensitive to Endo-H (not shown) and distributed similarly to the Endo-H-sensitive form that remained after incubation (Fig. 5*B*). A shift in total G protein from denser fractions (1–7) to lighter fractions (8–12) was observed (Fig. 5*C*), due to the Endo-H-resistant G protein, which specifically appears in the light fractions after incubation. This small net shift is consistent with a transfer of about 30–40% of the total G protein from the distribution pattern of the ER markers to that of the Golgi marker enzyme, with all of the transferred G protein processed to Endo-H resistance. However, the poor resolution of subcellular organelles of tissue culture cells (19) makes this point difficult to establish firmly.

Properties and Requirements of the *in Vitro* System. The *in vitro* reaction required energy in the form of nucleoside triphosphates (Table 1, line 3). This requirement probably reflects a need in the transport process rather than in oligosaccharide processing, because the isolated processing enzymes are not energy dependent (2, 3).

Importantly, UDP-GlcNAc was specifically required for processing to Endo H resistance (Table 1, lines 4 and 5), providing strong evidence that the production of Endo-H-resistant

G protein *in vitro* involves the terminal glycosylation of G with at least one GlcNAc unit, in accordance with the *in vivo* pathway (2). With our assay it was not possible to establish whether Gal was also added to the oligosaccharide when UDP-Gal was present (line 5); however, this appears likely because long incubations resulted in the production of higher molecular weight forms of G protein that migrated in electrophoresis as do the sialylated forms made *in vivo* (Fig. 2).

External Ca^{2+} was not required, because EGTA did not inhibit. Interestingly, processing was inhibited by the divalent cation ionophore A23187 (in the presence of Mg^{2+}), suggesting that divalent cations other than Mg^{2+} are needed on the inside of sealed vesicles. This ionophore blocks the intracellular transport of secretory proteins at a site between ER and Golgi (15) and also blocks transport of G protein *in vivo*, causing an irreversible accumulation in an Endo-H-sensitive form (unpublished experiments).

DISCUSSION

Our experiments suggest that G protein has been transported *in vitro* from membranes derived from the mutant 15B CHO cell to membranes from wild-type cells. It seems likely that passage to the wild-type Golgi complex has taken place, because the enzymes that mediate the oligosaccharide processing we have detected (resistance to Endo-H) have been localized to this organelle (2–5) in several cell types.

That the observed processing of G *in vitro* is dependent upon transport events resembling those of intracellular protein transport, and does not result from extensive and nonspecific fusion of membranes, is indicated by the following lines of evidence: (*i*) The same enzymatic pathway processes the oligosaccharides of G with similar kinetics *in vivo* and *in vitro*. (*ii*) ATP is required for processing in the *in vitro* system, as it is for transport *in vivo* (16). (*iii*) Endo-H-sensitive and resistant forms of G can be well resolved by an isopycnic centrifugation, a result inconsistent with nonspecific fusion. (*iv*) Processing was inhibited by A23187, as *in vivo*. The specificity of the transport reaction *in vitro* is underscored by the high degree of processing, suggesting that most of the G protein had been correctly delivered to the Golgi membranes, despite the fact that these make up only a small fraction of the total membrane mass in the extract.

A reasonable working hypothesis based on our results is that G protein is transported in the cell-free extract by coated vesicles that bud from ER-derived vesicles (from 15B cells) and then fuse with vesicles of the Golgi complex (from wild type). This model is plausible because a similar transport block with CCCP is known to prevent exit of proteins from ER in other systems (14, 15). It is certainly possible, however, that G has accumulated not within the ER or its transitional zone after the CCCP-induced block but instead within a transport vesicle, such as a coated vesicle or a coated vesicle that has lost its coat. In this case, the *in vitro* system would presumably involve fusion of the transport vesicle with the Golgi-derived vesicles, without a budding step.

The *in vitro* complementation between extracts of mutant and wild-type cells implicit in the design of our experiments provides assurance that we are studying truly cell-free reactions. It could have been argued that the cells have simply been disrupted in a sufficiently mild manner so as to preserve aspects of three-dimensional cellular topography and structure that are essential to the process of intracellular transport, such as connections between ER and Golgi, or an intact cytoskeleton. But the simple observation of transfer of protein between membranes of two different cell extracts speaks against this and suggests that such organization may not even be necessary in living cells to support such a vital function as the intracellular transport of newly synthesized proteins.

We are grateful to Dr. P. Robbins for a generous gift of Endo-H and to Dr. S. Kornfeld for providing us clone 15B cells. This work was supported by National Institutes of Health Grant GM 25662 and by a European Molecular Biology Organization Fellowship to E.F.

1. Lenard, J. (1978) *Annu. Rev. Biophys. Bioeng.* **7**, 139–166.
2. Tabas, I. & Kornfeld, S. (1978) *J. Biol. Chem.* **253**, 7779–7786.
3. Tabas, I. & Kornfeld, S. (1979) *J. Biol. Chem.* **254**, 11655–11663.
4. Schachter, H., Jabbal, R., Hudgin, R., Pineteric, L., McGuire, E. & Roseman, S. (1970) *J. Biol. Chem.* **245**, 1090–1100.
5. Leblond, C. & Bennett, G. (1977) in *International Cell Biology 1976–1977*, eds. Brinkley, B. & Porter, K. (Rockefeller Univ. Press, New York), pp. 526–567.
6. Rothman, J. E. & Fine, R. E. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 780–784.
7. Robbins, P., Hubbard, S., Turco, S. & Wirth, D. (1977) *Cell* **12**, 893–900.
8. Gottlieb, C., Baenzinger, J. & Kornfeld, S. (1975) *J. Biol. Chem.* **250**, 3303–3309.
9. Narasimhan, S., Stanley, P. & Schachter, H. (1977) *J. Biol. Chem.* **252**, 3926–3933.
10. Schlessinger, S., Gottlieb, C., Feil, P., Gelb, N. & Kornfeld, S. (1976) *J. Virol.* **17**, 239–246.
11. Li, E. & Kornfeld, S. (1978) *J. Biol. Chem.* **253**, 6426–6431.
12. Knipe, D., Lodish, H. & Baltimore, D. (1977) *J. Virol.* **21**, 1121–1127.
13. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
14. Jamieson, J. & Palade, G. (1968) *J. Cell Biol.* **39**, 589–603.
15. Tartakoff, A. & Vassalli, P. (1977) *J. Exp. Med.* **146**, 1332–1345.
16. Palade, G. (1975) *Science* **189**, 347–358.
17. Rothman, J., Katz, F. & Lodish, H. (1978) *Cell* **15**, 1447–1454.
18. Katz, F. & Lodish, H. (1979) *J. Cell Biol.* **80**, 416–426.
19. Knipe, D., Baltimore, D. & Lodish, H. (1977) *J. Virol.* **21**, 1128–1139.