Transport of Newly Synthesized Vesicular Stomatitis Viral Glycoprotein to Purified Golgi Membranes

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ABSTRACT In a previous communication we reported that the newly synthesized membrane glycoprotein of vesicular stomatitis virus could be transported in crude extracts of CHO cells from endoplasmic reticulum-derived membranes to membranes of the Golgi complex. This conclusion was an indirect one, based on the terminal glycosylation of this glycoprotein, a reaction that was dependent upon a Golgi-specific enzyme, UDP-GlcNAc transferase I. We show here that the Golgi fraction of rat liver will substitute for members of CHO cells as a source of transferase I in this reaction. The use of highly purified fractions of liver Golgi membranes, coupled with the ability to recover these membranes from incubations, has now permitted a direct demonstration of net transport of G protein to these heterologous Golgi membranes. This transport reaction is specific, in that the smooth endoplasmic reticulum fraction will not substitute for the Golgi fraction, is quantitatively significant, involving at least 30% of the viral glycoprotein, and is sustained only in the presence of both ATP and a soluble, cytosol fraction of liver cells.

An essential step towards elucidating the mechanism by which distinct sets of proteins are delivered to the different subcellular organelles will be the reconstitution of these complex cellular events in cell-free extracts. The intracellular transport of the membrane glycoprotein (G protein) of vesicular stomatitis virus (VSV) provides an experimental system (1, 2) well suited for investigations of the mechanism of targeting of proteins to the plasma membrane. G protein, virtually the only glycoprotein made by VSV-infected cells, is inserted into the endoplasmic reticulum (ER) membrane during its synthesis. Then, like cellular surface glycoproteins, G is transported to the plasma membrane via the Golgi complex, and is finally incorporated into the envelope of progeny virions as nucleocapsids bud out of the cell. The limited genetic capacity of VSV (1, 2), in contrast with the striking genetic complexity of the process of intracellular transport (3), provides assurance that the maturation of G protein follows pathways provided by the host and not the virus.

In a recent communication (4), we reported experiments suggesting that newly synthesized G protein could be rapidly and efficiently transported to membranes of the Golgi complex in a cell-free system. The reaction studied was energy-dependent and resulted in the terminal glycosylation of G by UDP-GlcNAc transferase I (5), a Golgi-associated enzyme (6). The assay used (4) was based on an in vitro complementation scheme, employing a mutant line of Chinese hamster ovary cells (CHO clone 15B) that lacks transferase I (7, 8). Extracts (postnuclear supernates) of VSV-infected 15B cells, bearing [^{35}S]methionine-labeled G protein in ER-derived membranes, were mixed with extracts of uninfected wild-type CHO cells, and the action of transferase I (from wild-type membranes exclusively) upon G protein (from 15B cell membranes) was detected indirectly by the conversion of the oligosaccharide of G to a form resistant to attack by the high mannose-specific glycosidase (9), endoglycosidase H (Endo H).

Because of the localization of transferase I to Golgi membranes in tissues such as liver (6), the production of Endo H-resistant forms of G in vitro was presumed to reflect transport of G from 15B cell membranes to the Golgi complex derived from wild-type cells. G protein seemed to be specifically transferred to Golgi membranes, for over one-half of the G protein was modified by transferase I, even though Golgi membranes comprise but a small fraction of the membranes present in crude extracts. To test these inferences decisively would require the extensive purification of the Golgi complex and of other organelles present in CHO extracts. Unfortunately, such a separation has yet to be satisfactorily achieved for any cultured cell line.

To circumvent this difficulty, we have turned to rat liver, a tissue that has been successfully fractionated to yield all of the major organelles in both high yield and purity (10–12). We show here that the Golgi fraction and cruder subcellular fractions of rat liver will substitute for membranes of CHO cells as a source of transferase I and also act as acceptors of G protein donated by extracts of VSV-infected 15B cells. Furthermore, the use of highly purified fractions of liver Golgi membranes,

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together with the ability to reisolate these same membranes from incubations, has now made it possible to demonstrate directly an appreciable net transport of G protein to heterologous Golgi membranes in vitro.

MATERIALS AND METHODS

Preparation of Extracts of CHO Cells

Extracts of VSV-infected clone 15B CHO cells were prepared as described (4) from cells that had been pulse-labeled for 5 min with [^{35}S]methionine and then further incubated with carbonyl cyanide *m*-chlorophenylhydrazone as described. Such extracts are referred to as [^{35}S]VSV/15B. Extract of uninfected and unlabeled wild-type CHO cells was also prepared as before (4), except that 10^8 cells were homogenized in 1.5 ml of buffer. The total membrane fraction of CHO cells was obtained by centrifugation of 0.6 ml of the crude wild-type CHO cell extract at 40,000 rpm for 60 min at 4°C in the Beckman SW50.1 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). The membrane pellet was resuspended in 0.3 ml of extract buffer (75 mM KCl, 5 mM magnesium acetate, 1 mM dithiothreitol (DTT), 45 mM HEPES-KOH, pH 7.5) with the aid of a Teflon-glass homogenizer. The supernate obtained (after removing the fatty zone at the top of the centrifuge tube) was taken as the cytosol fraction. Both fractions were frozen in liquid N_2 and stored at −80°C.

Preparation of Fractions of Rat Liver

Method 1 is that of Fleischer and Kervina (10) as modified by Carey and Hirschberg (11). Briefly, a rat liver (10–15 g) was homogenized in 5 vol of 0.25 M sucrose (buffered at pH 7 by 1 mM Tris-HCl) in a motor-driven Potter-Elvehjen homogenizer at 750 rpm, and employing pestles of 0.026- and 0.012-inch clearances, as specified (10). The postnuclear (2,000 *g*, 10 min) supernate and the postmitochondrial (11,000 *g*, 10 min) supernatant fractions were then prepared (10). The cytosol (high-speed supernate) and microsome fraction were obtained by centrifuging the postmitochondrial supernate at 39,000 rpm for 50 min in the Beckman Ti50 rotor. The microsomal pellet was resuspended by homogenization in a volume of 0.25 M sucrose equal to one-half the volume of the postmitochondrial supernate used. For many experiments, the cytosol fraction was concentrated about fivefold by ultrafiltration using Amicon filter PM10 (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). The Golgi fraction was isolated from the microsome fraction by flotation essentially as described (11). For these purposes, a microsomal suspension was adjusted to 43% (wt/wt) sucrose in a final volume of ~0.7 ml/g liver; ~5 ml of this suspension was overlaid in a Beckman SW27.1 centrifuge tube successively with 4 ml of 36% (wt/wt) sucrose, 4 ml of 33% (wt/wt) sucrose, and 4 ml of 8.2% (wt/wt) sucrose. After centrifugation for 90 min at 26,000 rpm, the Golgi fraction (the 33/8.2% sucrose interface) was harvested by centrifugation at 100,000 *g* for 1 h. The Golgi pellet was homogenized in ~0.3 ml of buffer A (5 mM $MgCl_2$, 50 mM sodium maleate, pH 6.5) containing 0.5 M sucrose. The plasma membrane fraction was isolated by flotation from the nuclear pellet as described (11), and was resuspended in buffer A containing 0.5 M sucrose before use. All fractions could be frozen in liquid N_2 and stored at −80°C.

Method 2 is based on Leelavathi et al. (12) as modified by Tabas and Kornfeld (6). The postnuclear supernate and crude smooth membrane fractions were prepared exactly as described (6). The Golgi fraction was isolated by flotation as described, except that the SW27.1 rotor was employed, and the volumes of the sucrose layers (all sucrose solutions were in buffer A) used in the density gradient were changed accordingly as follows: 4 ml of 1.25 M sucrose; ~6 ml of the crude smooth membrane suspension in 1.10 M sucrose (obtained from ~10 g rat liver); 3.5 ml of 1.00 M sucrose, and 3 ml of 0.5 M sucrose. The Golgi fraction was the 1.0/0.5 M sucrose interface; the material at the 1.1/1.0 M sucrose interface gave the smooth ER fraction. These and cruder fractions were frozen in liquid N_2 and stored at −80° until use. For most experiments, thawed aliquots of membrane fractions were diluted with buffer A, pelleted in the Beckman Airfuge at 22 psi for 15 min and then homogenized in a small volume of buffer A containing 0.5 M sucrose before use.

Incubation to Achieve Transfer of G Protein

Exact conditions are given in each figure legend. An incubation in 100 μl final volume contained 60 μl of incubation cocktail (2.9 mM sodium ATP, 14 mM sodium creatine phosphate, 11 U/ml of creatine phosphokinase, 1.4 mM sodium UDP-Gal, 1.4 mM sodium UDP-GlcNAc, 1 mM dithiothreitol, 4.5 mM magnesium acetate, 75 mM KCl, and 67 mM 4-morpholineethanesulfonic acid-KOH at pH 6.3), and, additionally, 15 μl of [^{35}S]VSV/15B extract, 10 μl of concentrated liver cytosol fraction, and up to 10 μl of the subcellular fraction to be tested as an

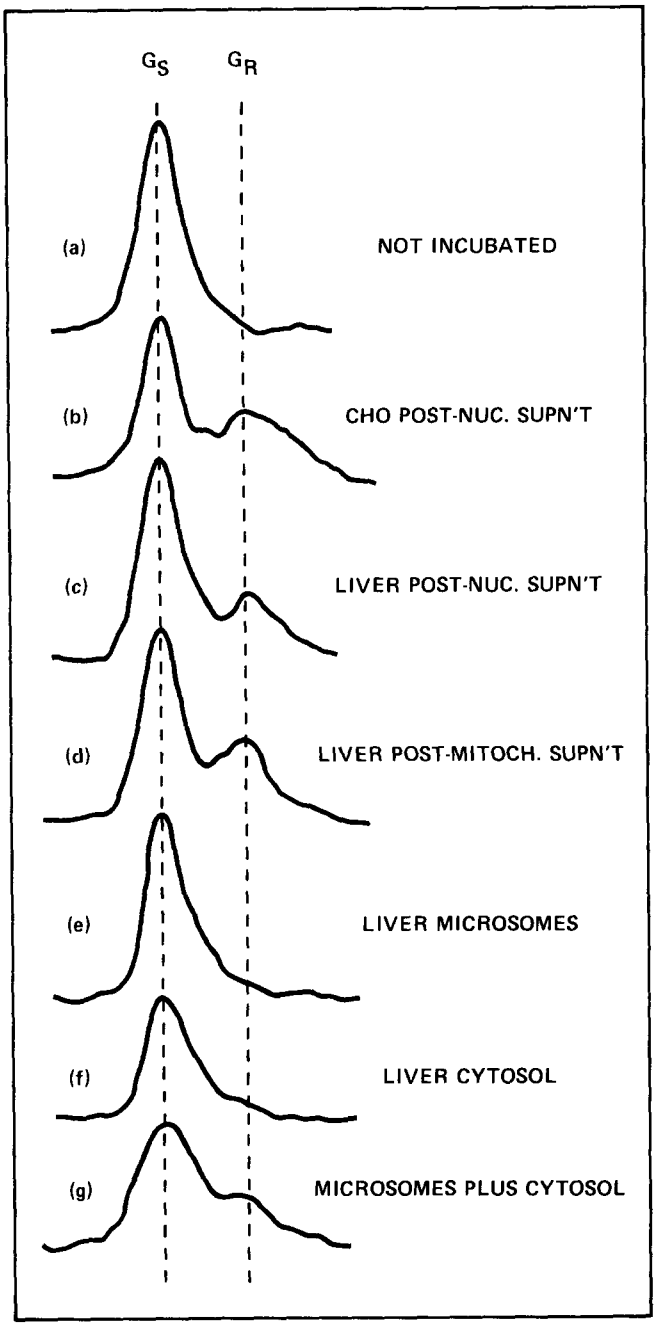

FIGURE 1 Rat liver fractions will substitute for crude extracts of CHO cells. Incubations contained 10 μl of [^{35}S]VSV/15B extract, 30 μl of incubation cocktail, up to 10 μl of the indicated subcellular fraction, and 0.25 M sucrose to achieve a final volume of 50 μl. Samples (15 μl) taken after 40 min of incubation at 37°C were digested with Endo H as described (4) and then electrophoresed (13) and autoradiographed. (*a*) Control, not incubated in vitro. (*b*) Crude extract (postnuclear supernate) of wild-type CHO cells, 10 μl. (*c*) Postnuclear supernatant (method 1) of rat liver, 10 μl. (*d*) Postmitochondrial supernate (method 1) of rat liver, 10 μl. (*e*) Microsomal fraction of rat liver (method 1), 5 μl containing 15 μg protein. (*f*) Cytosol fraction (method 1, not concentrated) of rat liver, 5 μl. (*g*) Microsomal fraction, 5 μl, and cytosol fraction, 5 μl. Only a limited portion of the densitometer tracing of the autoradiograph containing G protein is shown. G_S denotes the position of the "sensitive" form of G whose oligosaccharides have been cleaved by Endo H. G_R is the principal Endo H-resistant form. The percent of total G protein converted to Endo H resistance was estimated as described earlier (4).

acceptor of G protein (usually in buffer A containing 0.5 M sucrose). Incubation was carried out at 37°C for 30–45 min.

Fractionation of Incubations for Analysis

The incubation mixture (0.1 ml) was chilled on ice and adjusted to a final sucrose concentration of 1.1 M by adding: 0.57 ml of 1.3 M sucrose (all sucrose solutions in buffer A), 0.63 ml of 1.1 M sucrose, and finally 0.1 ml of crude smooth membranes (~20 mg/ml, in 1.1 M sucrose) as carrier. This mixture was layered over 1 ml of 1.25 M sucrose in a tube for the Beckman SW50.1 rotor and then overlaid with 1.4 ml of 1.0 M sucrose and 1 ml of 0.5 M sucrose. After centrifugation at 49,000 rpm for 90 min at 4°C, a distinct and sharp band (representing the carrier) was present at each of the three interfaces. All visible material was collected from interface B (1.1/1.0 M) and interface C (1.0/0.5 M) with a Pasteur pipette in the minimum possible volume (usually ~0.5 ml). For analysis, material from each interface was diluted to exactly 1.0 ml volume with water, and a sample (from 0.2–1.0 ml) was precipitated with 5% trichloroacetic acid. The precipitates were dissolved in 50 μl sample buffer for gel electrophoresis by (*a*) adding 1 M Tris-free base until the color turned from yellow to blue, and then (*b*) incubating at 37°C for 30 min before boiling. Polypeptides were separated in a 10% polyacrylamide gel according to Laemmli (13), and the gels were treated with ENHANCE (New England Nuclear, Boston, Mass.) before drying and autoradiography. Exposures of 1–5 d proved sufficient. Densitometer tracings of the x-ray film were made, and the area under each peak was used to determine in arbitrary units the amount of G protein present at each interface. A sample of the entire incubation mixture was also electrophoresed on the same gel, and was used as a reference from which to calculate the percent of the total ^{35}S-labeled G protein present in the whole incubation that had been recovered from each interface. Protein was determined by the Lowry method (14).

RESULTS

Subcellular Fractions of Rat Liver Will Substitute for Crude Extracts of CHO Cells

Extract prepared from VSV-infected 15B cells that had been pulse-labeled with [^{35}S]methionine was incubated (together with ATP, an ATP-regenerating system, and UDP-GlcNAc) either with an extract of wild-type CHO cells or with various subcellular fractions of rat liver (prepared by method 1). As described previously (4), incubation with CHO extract resulted (Fig. 1) in the conversion of ^{35}S-labeled G protein to Endo H-resistant forms (31% converted, tracing *b*). The postnuclear (2,000 *g*) supernatant fraction of liver substituted for CHO extract in this reaction (tracing *c*, 24% converted). Activity was quantitatively retained in the liver postmitochondrial (11,000 *g*) supernate (tracing *d*, 30% of G resistant). The extent of processing of G was found to be proportional to the quantity of liver fraction added (data not shown). Further centrifugation of the postmitochondrial supernate to yield a cytosol fraction (high-speed supernate) and a microsomal pellet resulted in two largely inactive fractions (tracings *e* and *f*, respectively) that reconstituted activity when recombined (tracing *g*, 31% resistant). Preliminary results suggest that the cytosol factor is provided by discrete soluble species that can be fractionated by gel filtration (E. Fries and J. E. Rothman, unpublished observations). The required factors from the microsomal pellet presumably include Golgi membranes, since these would provide the transferase I (6), whose action is being monitored in these assays and is needed to complement the 15B cell extract.

Liver Golgi Membranes Can Be Recovered from Incubations

The ability of subcellular fractions of rat liver to substitute for crude CHO cell extracts provides an opportunity to add purified liver organelle fractions to incubations and to then recover them and thus test directly for transfer of G protein.

The Golgi fraction was prepared from rat liver by method 2, the procedure of Leelavathi et al. (12) as modified by Tabas and Kornfeld (6). This fraction was 160-fold enriched in the Golgi marker enzyme galactosyl transferase (Table I) and has also been reported to be highly enriched in transferase I and related activities (6). Table I also presents the specific activities of marker enzymes (galactosyl transferase, 5′-nucleotidase, glucose-6-phosphatase) for the subcellular fractions used in this work.

The last sucrose gradient employed in the purification of this Golgi fraction consisted of layers of 1.25, 1.10, and 1.00, and 0.50 M sucrose was also used routinely to reisolate the Golgi membranes after incubations. The incubation mixture to be fractionated was initially contained in the 1.10-M sucrose layer. In the preparation, smooth ER fractions were harvested from the 1.10/1.00 M sucrose interface (denoted interface B), while the Golgi fraction consisted of interface C (1.00/0.50 M interface). Material at interface A (1.25/1.1 M) was generally discarded.

To determine whether liver Golgi membranes could indeed be recovered after incubation, we prepared a ^{3}H-labeled Golgi fraction from a rat injected with 5 mCi of [^{3}H]leucine 16 h before sacrifice. Fig. 2*A* (closed circles) shows that this ^{3}H-

TABLE I

Specific Activities of Marker Enzymes in Subcellular Fractions of Rat Liver

		Glucose-6-Phosphatase*		5′-Nucleotidase‡		Galactosyltransferase§	
Method	Fraction	sp act	Purity	sp act	Purity	sp act	Purity
		μmol/ min/ mg	*-fold*	*μmol/ min/ mg*	*-fold*	*nmol/ h/ mg*	*-fold*
1	2,000 *g* Supernate	0.040	[1]	0.013	[1]	1.4	[1]
	Golgi	0.078	1.9	ND‖	ND	72.0	51.0
	Plasma membrane	0.083	2.1	0.13	10.0	2.0	1.4
2	Postnuclear supernate	0.086	[1]	0.043	[1]	0.10	[1]
	Crude smooth membranes	0.26	3.0	0.18	4.2	0.57	5.7
	Smooth ER	0.20	2.3	0.089	2.1	0.26	2.6
	Golgi	0.033	0.38	0.024	0.56	16.0	160.0

* Glucose-6-phosphatase was assayed according to Aronson and Touster (15).

‡ 5′-Nucleotidase was assayed according to Johnson et al. (16) with 5′-AMP as substrate and 3′-AMP as the blank.

§ Galactosyltransferase was measured with ovalbumin as substrate essentially as described by Brew et al. (17) in a reaction mixture containing in 50 μl volume: 2 μCi/ml UDP-[^{3}H-Gal] (New England Nuclear, 12 Ci/mmol), 100 μM UDP-Gal (unlabeled), 7 mg/ml ovalbumin, 20 mM $MnCl_2$, 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.5. It is unclear why the specific activities of this enzyme are so different for fractions prepared by the two different methods, but this has been observed consistently.

‖ ND, not determined.

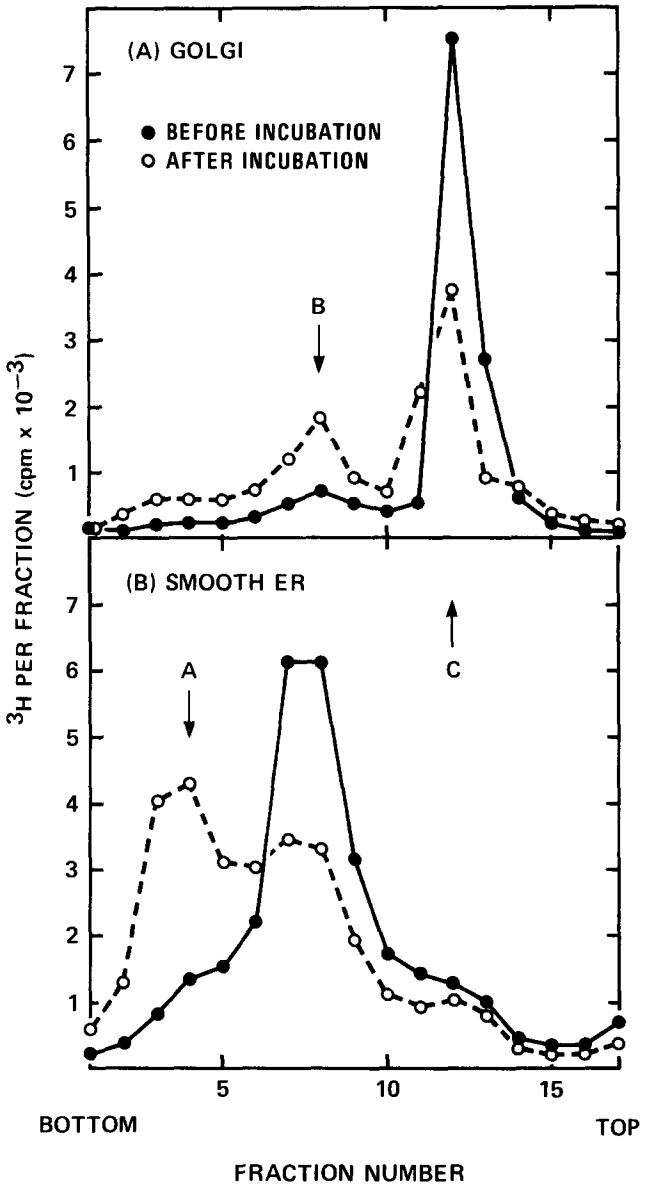

FIGURE 2 Rat liver Golgi and smooth ER membranes can be recovered from incubations. (*A*). The Golgi fraction (method 2) was prepared from a rat ~16 h after intraperitoneal injection with 5 mCi of L-[4,5-^{3}H]leucine (New England Nuclear) and resuspended in 0.4 ml of buffer A containing 0.5 M sucrose, at 4.2 mg/ml of protein. The specific activity was 250 cpm/μg protein. Closed circles: A sample (63 μg) of ^{3}H-Golgi fraction was fractionated on a sucrose gradient as described in Materials and Methods ("Fractionation of Incubations for Analysis"). Open circles: A mock incubation containing 60 μl of incubation cocktail, 15 μl of postnuclear supernate of unlabeled VSV-infected 15 B CHO cells, 15 μl (63 μg) of ^{3}H-Golgi fraction, and 10 μl of concentrated liver cytosol fraction was carried out for 45 min at 37°C, and the incubation mixture was then fractionated as above. The fractions were collected from below and counted. Arrows indicate the locations of interfaces A (1.25/1.1 M sucrose), B (1.1/1.0 M sucrose), and C (1.0/0.5 M). (*B*). Similar to *A*, except ^{3}H-labeled smooth ER fraction was employed. This smooth ER fraction was from the same preparation as the ^{3}H-Golgi fraction above and had a specific activity of 150 cpm/μg protein. Closed circles, ^{3}H-smooth ER fraction (195 μg) was fractionated on the sucrose gradient. Open circles, a mock incubation was carried out as in *A*, except that the ^{3}H-smooth ER fraction (15 μl containing 195 μg protein) was used in place of the ^{3}H-Golgi fraction.

Golgi fraction can be reisolated at the same interface (C) from which it was harvested originally. After incubation with CHO extract under the same conditions employed for in vitro transport in later experiments reported in this paper some of the ^{3}H was redistributed in the density gradient, mainly to interface B (Fig. 2*A*, open circles). Nevertheless, ~75% of the ^{3}H was still concentrated at interface C, showing that most of the original liver Golgi fraction can be reisolated from this region. The origin of ^{3}H in denser fractions is unclear but probably does not results from fusion, because most can be released treatment with EDTA (data not shown).

A similar experiment using the [^{3}H]leucine-labeled sn ER fraction (Fig. 2*B*, closed circles) shows that the smooth fraction can be reisolated mainly at interface B, from wh this fraction was harvested originally. After incubation (op circles), about one-half of the ^{3}H had redistributed, mainly the region of interface A, with the remainder still concentrate at interface B.

G Protein is Transferred to the Liver Golgi Fraction

[^{35}S]VSV/15B extract was incubated with or without the rat liver Golgi fraction in the presence of ATP, an ATP-regenerating system, the cytosol fraction, and UDP-GlcNAc. Incubations were then adjusted to 1.10 M sucrose, subjected to sucrose density gradient centrifugation, and fractions were analyzed by polyacrylamide gel electrophoresis (Fig. 3). When no liver

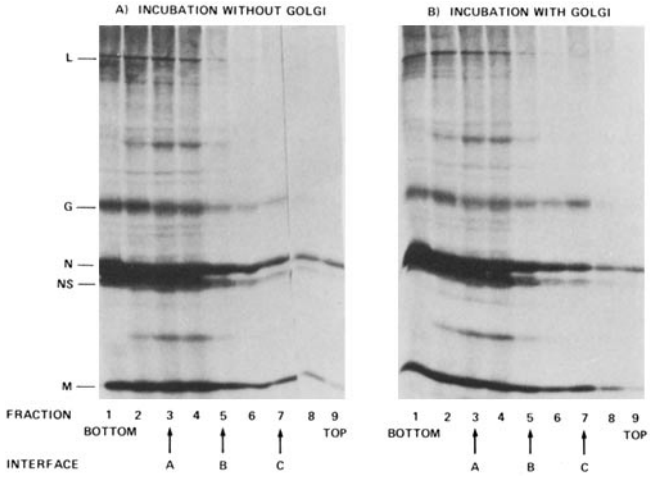

FIGURE 3 Redistribution of G protein after incubation with Golgi membranes. After 30 min at 37°C, incubations were analyzed by sucrose gradient centrifugation, as described in Materials and Methods, and fractions (0.5 ml) were collected from below. Samples (0.2 ml) of each fraction were diluted to 1.0 ml volume with water, precipitated with trichloroacetic acid, and subjected to gel electrophoresis. Only trace radioactivity was found in the small pellet at the bottom of the centrifuge tube. (*A*) Incubation without Golgi fraction, containing 120 μl of incubation cocktail, 20 μl of ^{35}S-labeled VSV/15B extract, 20 μl of concentrated liver cytosol, 700 μg of crude smooth membranes of liver (method 2, in 20 μl of buffer A containing 1.1 M sucrose), and 20 μl of 0.5 M sucrose in buffer A. (*B*) Incubation with Golgi fraction, containing cocktail, ^{35}S-labeled extract, and cytosol as in *A*, and also 80 μg of Golgi fraction of rat liver (method 2, in 20 μl of buffer A containing 0.5 M sucrose) and 20 μl of buffer A containing 1.1 M sucrose. Arrows at *A*, *B*, and *C* denote the approximate locations of the three interfaces analyzed in later experiments. Complete autoradiogram is shown, including all VSV-encoded proteins (L, G, N, NS, and M).

Golgi fraction was added, the content in each fraction of G as well as the other viral proteins (L, N, NS, and M) decreased progressively from the bottom towards the top of the gradient (Fig. 3*A*).

Note especially that very little of the total ^{35}S-labeled G is present in the regions of interfaces B and C. It is precisely this very low background of labeled G protein in the top portion of the gradient (containing interfaces B and C) that makes the experiments in this paper technically possible.

Incubation with liver Golgi fraction causes G protein specifically to assume a bimodal distribution, with a new peak present at interface C (Fig. 3*B*) where most of the Golgi membranes could be reisolated (Fig. 2*A*). This finding suggests that G is redistributed to the Golgi fraction of liver in a manner dependent upon the presence of this fraction.

Transfer to Liver Golgi Membranes is Specific and Energy-dependent

Fig. 4 shows the results of fractionations in which only the membranes concentrated at interfaces B and C were analyzed, for convenience. These interfaces are particularly useful, because most of the reisolated liver smooth ER and Golgi fractions concentrate there (Fig. 2). In contrast, the CHO membranes that contain [^{35}S]G protein do not concentrate at any of the interfaces (Fig. 3) and, in particular, contribute little radioactivity in the vicinity of interfaces B and C.

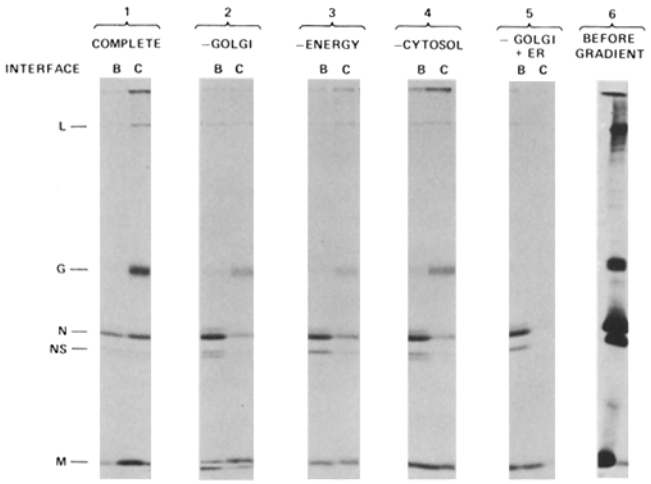

FIGURE 4 Transfer of G protein to liver Golgi membranes. Only the interfaces B (1.10/1.00 M), and C (1.00/0.50 M) of sucrose gradients of incubations were analyzed. For this purpose, samples of interfaces B (20% of total) and C (50% of total) were precipitated with trichloroacetic acid and subject to electrophoresis and autoradiography. The smaller sample of interface B was necessary because of the large amount of protein in this region of the gradient. Incubations (100 μl final volume) were carried out as described in Materials and Methods for 30 min at 37°C. (*1*) Complete incubation, with 60 μl of cocktail, 15 μl of ^{35}S-labeled VSV/15B extract, 10 μl of concentrated liver cytosol, and 15 μg of liver Golgi fraction (method 2, in 15 μl buffer A containing 0.5 M sucrose). (*2*) Incubation in which only the Golgi fraction was omitted and replaced by 15 μl of buffer A containing 0.5 M sucrose. (*3*) Identical to complete incubation, except that, in preparing the cocktail, ATP was replaced by 2.9 mM sodium ADP, and both creatine phosphate and creatine phosphokinase were omitted and replaced by water containing sufficient NaCl to maintain sodium ion at its previous concentration. (*4*) Cytosol alone was omitted and replaced by 10 μl of 0.25 M sucrose. (*5*) The Golgi fraction alone was omitted and replaced by 225 μg of liver smooth ER fraction (method 2, 15 μl in buffer A containing 0.5 M sucrose). (*6*) A sample of the unfractionated ^{35}S-labeled VSV/15B extract was electrophoresed.

In analyzing these gradients, considerable care was taken to remove all of the interfacial material in a minimum volume (usually ~0.5 ml). This was facilitated by the inclusion of a crude microsomal membrane fraction as carrier, because the carrier gives rise to visible, sharp bands at interfaces B and C representing, respectively, smooth ER and Golgi membranes derived from the carrier. All visible material was therefore harvested from the interfaces, thus ensuring a complete and reproducible collection of the cosedimenting radioactive membranes that had been reisolated from incubations. The very low background of labeled CHO membranes near interfaces B and C makes it possible to obtain highly reproducible results without paying careful attention to the exact volume in which all of the interfacial material is collected. A somewhat smaller amount of G is found at interface *B* in Fig. 4 than in the region containing this interface in Fig. 3. This is because of the small number of fractions collected in Fig. 3 without any attention to the exact location of the interfaces.

A complete incubation (containing ATP and its regenerating system, the Golgi fraction, and cytosol) results in the selective appearance of G protein at interface C, the Golgi interface (Fig. 4, group *1*), with little G appearing at interface B. G comprised 48% of ^{35}S-labeled viral protein at this interface, whereas G accounted for only 6% of ^{35}S-labeled protein in the unfractionated incubation (group *6*). Omitting only the Golgi fraction (group *2*), replacing ATP and its regenerating system with ADP (group *3*), or omitting only cytosol (group *4*), caused a substantial reduction in the appearance of G at interface C. The effect of omitting liver cytosol was the least dramatic, perhaps because of the contribution of CHO cytosol from the [^{35}S]VSV/15B extract. Moreover, the degree of requirement for cytosol varied among experiments, being either more or less stringent than in the experiment shown here. The explanation for this is unclear at present.

The distribution of G protein at the interfaces of these and other gradients is presented quantitatively in Table II. In the experiment of Fig. 3 (exp I in Table II), 15% of the total ^{35}S-labeled G originally present in 15B cell membranes was transferred specifically to interface C (the liver Golgi interface) in a reaction that depends upon the presence of this Golgi fraction, an energy source, and the cytosol fraction. In other experiments, the extent of transfer was even higher, approaching 30% (Table II, exp III), and when the Golgi fraction prepared by method 1 was used, 36% of G was transferred (exp II, line 4). The crude extract of CHO cells, while capable of promoting extensive oligosaccharide processing (reference 4 and Fig. 1), does not promote the appearance of G at interface C (Table II, exp III), providing further evidence that G found at this interface is present in liver Golgi membranes, and suggesting that CHO Golgi membranes do not band at interface C.

Table II also shows that the smooth ER fraction (exp I, line 5 and exp III, line 3) will not substitute for the Golgi fraction in the transport reaction, even when added in 15 times the amount of the Golgi fraction. Furthermore, smooth ER does not greatly inhibit transfer of G protein to Golgi membranes when both are present (exp II, line 3). It is particularly significant that when liver smooth ER was employed in place of Golgi membranes (Table II, exp I, line 5; Fig. 4, group *5*), no

TABLE II

Requirements for Transport of G Protein to Golgi Membranes In Vitro

exp	Incubation conditions*	[^{35}S]G protein recovered from interfaces (percent of total)	
		B	*C*
I	1. Complete (15 μg Golgi, method 2)	1.0	15.0
	2. −Golgi	1.8	1.9
	3. −ATP, −creatine phosphate, −creatine kinase, +ADP	0.9	2.3
	4. −Cytosol	3.1	4.5
	5. −Golgi, +smooth ER (225 μg, method 2)	2.0	0.6
II	1. Complete (80 μg Golgi, method 2)	6.5	18.0
	2. 0-min incubation	3.4	5.9
	3. +smooth ER (55 μg, method 2)	3.2	13.0
	4. Complete (15 μg Golgi, method 1)	4.5	36.0
III	1. Complete (80 μg Golgi, method 2)	—	27.0
	2. −Golgi, +CHO membranes (180 μg)	—	6.8
	3. −Golgi, +smooth ER (55 μg, method 2)	—	5.4

exp I: The G protein found at each interface shown in Fig. 4 was quantitated and expressed as a percent of the total labeled G protein present in the unfractionated incubation mixture, as described in Materials and Methods. Incubations 1–5 are detailed in the legend to Fig. 4.

exp II: (1) Complete incubation (37°C for 30 min) was identical to that in Fig. 4, except that 80 μg of the Golgi fraction (method 2) was used. (2) A complete incubation mixture was fractionated immediately after it was made. (3) Incubation contained smooth ER fraction (55 μg, method 2) in addition to Golgi fraction (80 μg). (4) Complete incubation in which the Golgi fraction prepared by method 1 (15 μg in 15 μl of buffer A containing 0.5 M sucrose) replaced the Golgi fraction prepared by method 2.

exp III: (1) Complete incubation (37°C, 45 min) using CHO cell cytosol instead of liver cytosol. Contained 10 μl of [^{35}S]VSV/15B extract, 60 μl cocktail, 20 μl of cytosol of wild-type CHO cells, and liver Golgi fraction (80 μg, method 2) in a final volume of 100 μl. (2) Golgi fraction was omitted and replaced by 180 μg of the total membrane fraction of wild-type CHO cells (in 10 μl extract buffer). (3) Golgi fraction was replaced by 55 μg of smooth ER (method 2). Only material from interface C was analyzed.

* Complete incubations contain [^{35}S]VSV/15B extract, liver Golgi fraction, liver cytosol fraction, ATP, an ATP regenerating system (creatine phosphate and creatine phosphokinase), UDP-GlcNAC, and UDP-Gal.

increment in the amount of G recovered at interface B was detected. Yet, about one-half of the smooth ER reisolates after incubations at interface B (Fig. 2 *B*). These experiments provide evidence for specificity in the transport reaction, with Golgi membrane as the preferred acceptor. Preliminary experiments also suggest that the rat liver plasma membrane will not act as an acceptor.

About 46% of the G protein transferred to interface G was Endo H-resistant (Fig. 5). It is not clear why all of the G protein transferred to liver Golgi membranes was not converted to Endo H-resistance. In contrast, all of the G transferred to CHO Golgi fractions was made resistant (4). Moreover, despite the extensive processing of G protein observed with cruder liver fractions (Fig. 1, typically 30–40% resistant), when purified Golgi fractions were employed, processing of only ~15% of G was observed (Fig. 5), even with saturating amounts of Golgi fraction. It seems possible that during purification, the liver Golgi membranes may have been disrupted to yield remnants, some of which possess acceptor activity but lack glycosyltransferases. In fact, it appears that purified liver Golgi membranes separate into two principal subfractions, one rich in glycosyltransferases and derived from *trans* cisternae, the other depleted in these enzymes and derived from *cis* elements (18, 19).

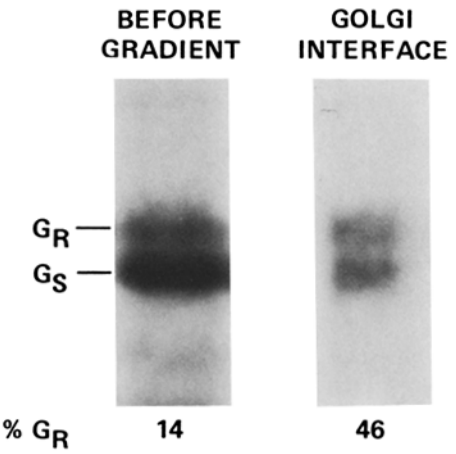

FIGURE 5 Endo H-resistant forms of G are selectively found in Golgi fractions. A complete incubation identical to that described in Fig. 4 (group 1) was carried out, followed by a sucrose gradient. The track on the left represents the electrophoretic pattern obtained when a sample (15 μl) of the incubation was taken before the sucrose gradient and digested with Endo H as described (4). 14% of G was Endo H-resistant. The track on the right represents the pattern obtained when the material at interface C was digested with Endo H. 46% of G was found to be Endo H-resistant. For this purpose, a sample of interface C was diluted with 0.25 M sucrose and centrifuged at 49,000 rpm in the Beckman SW50.1 rotor for 2 h, and the pellet was dissolved by boiling in 60 μl of 50 mM Tris-HCl, pH 6.8, containing 1% $NaDodSO_4$ and 15 mM dithiothreitol, and digested with 40 μl of pure Endo H (75 ng/ml, kindly provided by Dr. P. Robbins of M.I.T., in 0.3 M sodium citrate, pH 5.5, containing 0.1% $NaDodSO_4$) as described (4). Control experiments in which a similar amount of Endo H-sensitive [^{35}S]G protein was digested together with unlabeled interface C showed complete conversion of G to the G_S form, so the limited digestion observed in the experiment is not the result of limiting amounts of Endo H or of excess substrate. Only the portion of the autoradiograph containing G proteins is shown here.

DISCUSSION

Our previous work (4) strongly suggested that G protein could be transported in cell-free extracts from ER-derived membranes of mutant 15B CHO cells to Golgi membranes from wild-type CHO cells. This inference was based on the finding that terminal GlcNAc residues were added in a reaction requiring a Golgi-localized enzyme (5, 6), transferase I, found only in membranes from wild type (8). Although plausible, this conclusion was necessarily indirect. Decisive evidence that G was transported to the Golgi apparatus would require a satisfactory purification of this and other subcellular organelles from CHO cells, and this has not yet been possible for any cell line. Nevertheless, it seemed unlikely that these events resulted from extensive and nonspecific fusion of mutant with wild-type membranes during incubations because (*a*) the inferred transport reactions were ATP-dependent (4), and (*b*) Endo-H-resistant and Endo-H-sensitive forms of G protein were well resolved by sucrose gradient centrifugation (4), with the Endo-H-resistant forms concentrated in less dense, Golgi-rich fractions. But, in the absence of a more satisfactory subcellular fractionation, trivial explanations of our results could not be completely dismissed.

The ambiguities resulting from fractionation of tissue culture cells have now been circumvented by employing extracts and subcellular fractions of rat liver as a source of the Golgi membranes putatively required to obtain the oligosaccharide processing and terminal glycosylations reported earlier.

Using this approach, it has been possible to confirm that the Golgi fraction of liver contains the activity needed to complement in vitro the defect of 15B cells (Fig. 5). Furthermore, a transfer of G to these Golgi membranes has now been unambiguously demonstrated (Fig. 4 and Table II). The smooth ER fraction will not substitute for the Golgi fraction as an acceptor of G protein. The appearance of G in the reisolated Golgi fraction is clearly the result of transfer to liver Golgi membranes and not some redistribution of G protein among endogenous CHO cell membranes because: (*a*) G does not appear at interface C when liver Golgi fraction is omitted (Table II, exp I, line 2), and (*b*) G also does not appear at interface C when liver Golgi fraction is replaced by a crude extract of wild-type CHO cells (exp III, lines 1 and 2) even though extensive oligosaccharide processing of G takes place (Fig. 1). Evidently the added rat liver Golgi fraction is competing efficiently with endogenous CHO Golgi fraction for the pool of G protein being transported. Incubations contain a great excess of liver Golgi fraction over Golgi fraction present in the crude [^{35}S]-VSV/15B cell extract, as about equal weights of 160-fold purified liver Golgi fraction and of crude CHO cell extract are typically added.

In addition to delineating the specific role of Golgi membranes in the in vitro transport reaction (4), the experiments reported here also establish that this reaction is in fact energy-dependent and is greatly stimulated by the presence of the cytosol fraction. It thus appears likely that the transport observed in cell-free extracts follows pathways resembling those of intracellular protein transport, as our results would be quite difficult to attribute to nonspecific fusion events. It is hoped that further fractionation of the Golgi membranes and of the cytosol fraction will yield insights into the mechanisms of transport of proteins to the Golgi apparatus and the sorting of proteins within this complex organelle.

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