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Reconstitution of the Transport of Protein between Successive Compartments of the Golgi Measured by the Coupled Incorporation of N-Acetylglucosamine

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Summary

Transport of the VSV-encoded glycoprotein (G protein) between successive compartments of the Golgi has been reconstituted in a cell-free system and is measured, in a rapid and sensitive new assay, by the coupled incorporation of ^3H -N-acetylglucosamine (GlcNAc). This glycosylation occurs when G protein is transported during mixed incubations from the “donor” compartment in Golgi from VSV-infected CHO clone 15B cells (missing a key Golgi GlcNAc transferase) to the next, successive “acceptor” compartment (containing the GlcNAc transferase) in Golgi from wild-type CHO cells. Golgi fractions used in this assay have been extensively purified, and account for all of the donor and acceptor activity in the cells. Together with several other lines of evidence, this indicates that the cell-free system is highly specific, measuring only transport between sequential compartments in the Golgi stack. Transport *in vitro* is almost as efficient as in the cell, and requires ATP and the cytosol fraction in addition to protein components on the cytoplasmic surface of the Golgi membranes.

Introduction

To understand the basis for the specificity of subcellular compartments, we must learn how cells transport proteins to their correct locations and keep them there (Rothman and Lenard, 1984). This extensive traffic in proteins and membranes is mediated by transport vesicles. We must find out how these vesicles bud off from membranes, taking away only a select set of proteins (“protein sorting”), and how each type of transport vesicle succeeds in fusing only with the membrane that envelops its designated target (“vesicle sorting”), ensuring accurate delivery of the desired proteins. Cell-free systems that faithfully reproduce these processes are needed to unravel this biochemistry. In this and two following papers (Braell et al., 1984; Balch et al., 1984), we describe and analyze a cell-free system that seems to reconstitute accurately intercompartmental transport as it occurs in the context of the Golgi stack. It seems likely that both the budding of transport vesicles (from one set of Golgi cisternae) and their select fusion (with the next set of cisternae) take place.

The Golgi is an organelle particularly well suited for a functional reconstitution because its stack can be isolated

essentially intact (Fleischer, 1974) and because it is specialized for protein transport (Farquhar and Palade, 1981; Rothman, 1981; Tartakoff, 1982). Thus membrane components needed for transport should be especially concentrated in Golgi fractions. In addition, the actions of the series of glycosyltransferases present in the sequential cisternae of the stack offer a ready means to measure the transport of a protein between these successive compartments, making cumbersome physical separations unnecessary. Three distinct compartments, each consisting of a small number of cisternae, are distinguished within the stack by several, quite independent lines of evidence (Tartakoff, 1982, 1983; Rothman, 1981; Dunphy et al., 1981; Roth and Berger, 1982; Griffiths et al., 1982; Roth, 1983; Dunphy and Rothman, 1983; Goldberg and Kornfeld, 1983; Deutscher et al., 1983; Rothman et al., 1984a, 1984b). These compartments have been termed *cis*, *medial*, and *trans*, in the order of their encounter by transported glycoproteins (Griffiths et al., 1983). Galactose residues are added to the Asn-linked oligosaccharides of transported glycoproteins in the *trans* compartment; N-acetylglucosamine (GlcNAc) residues in an earlier compartment now known to consist of several *medial* cisternae (Dunphy et al., 1985). Fatty acids are attached covalently either just before or after entry into the Golgi, which occurs at the *cis* face (Bergmann and Singer, 1983).

Transport between the successive compartments of the Golgi stack is a vectorial process that is dissociative in nature. Thus, when transport vesicles are allowed to choose between targets in the Golgi population from which they had budded and those in a second, exogenous population of Golgi (introduced by cell fusion), the ensuing fusion occurs on an entirely random basis (Rothman et al., 1984a, 1984b). This implies that, even in the cell, transport vesicles are sorted on the basis of a biochemical specificity and not physical proximity. Preexisting cytoplasmic organization is not important for accurate transport between Golgi compartments.

In this and previous work (Fries and Rothman, 1980, 1981; Rothman and Fries, 1981; Dunphy et al., 1981; Rothman et al., 1984b) we have taken advantage of both the dissociative nature of transport in the Golgi and a mutant defective in a step in glycosylation (but not in transport) to design an assay that measures transfers between successive Golgi compartments *in vitro*. The mutant is clone 15B of CHO cells (Gottlieb et al., 1975; Tabas and Kornfeld, 1978), missing the Golgi enzyme GlcNAc transferase I. This enzyme is normally present in the *medial* cisternae of the stack (Dunphy et al., 1985), and is needed to initiate the steps resulting in the addition of peripheral GlcNAc, Gal, and sialic acid residues to form complex-type Asn-linked oligosaccharides in the Golgi (Hubbard and Ivatt, 1981; Schachter et al., 1983). We study the transport of the vesicular stomatitis virus (VSV)-encoded glycoprotein (G protein) because this single membrane protein replaces the entire population of newly synthesized proteins in the Golgi during a viral infection (Zilberstein et al., 1981). G protein is synthesized in the

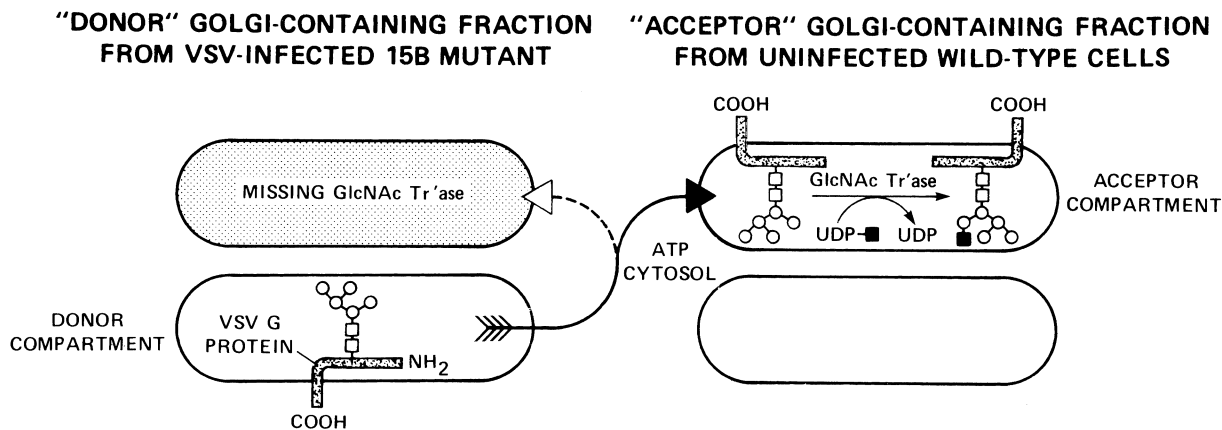


Figure 1. Assay for Transport of a Protein between Successive Golgi Compartments in Vitro, Based on a Transport-Coupled Glycosylation

A donor fraction containing Golgi membranes is prepared from VSV-infected clone 15B cells (a mutant cell line lacking UDP-GlcNAc glycosyltransferase I—GlcNAc Tr'ase). This fraction is incubated with an acceptor fraction also containing Golgi, but prepared from uninfected wild-type cells. Incorporation of ^3H -GlcNAc (from UDP- ^3H -GlcNAc) into G protein occurs when G protein is transferred in a dissociative fashion between the two Golgi. Specifically, the assay measures transfers of G protein into the acceptor compartment in wild-type Golgi housing the GlcNAc transferase (corresponding to *medial* cisternae) from the immediately prior donor compartment (currently believed to be *cis* cisternae or vesicles derived from these) present in the 15B Golgi. This dissociative transfer (solid arrows) results from the random choice of target cisternae from among the two Golgi populations. Corresponding transfers should also occur within the 15B Golgi (dashed arrow) but are not recorded in the assay because of the lack of the GlcNAc transferase in 15B Golgi. Incorporation into G protein is measured directly by immunoprecipitation. The polypeptide of G protein will span the Golgi membrane before and after the transfer. The trimmed core oligosaccharide containing two N-acetylglucosamine (GlcNAc) (□) and 5 Man residues (○), is the immediate substrate for GlcNAc transferase I. It is not clear whether this trimming occurs upon arrival in the acceptor compartment or before departure from the prior donor compartment, but the process is diagrammed as if the latter were the case. ^3H -GlcNAc (■).

rough endoplasmic reticulum (ER), and it passes through the Golgi en route to the plasma membrane, from which it is incorporated into the envelope of budding virions.

For the assay, a Golgi-containing fraction is prepared from VSV-infected 15B cells. When this fraction is incubated, transport of G protein into the *medial* compartment may well occur, but of course GlcNAc cannot be added to G protein, since the needed enzyme is missing from the 15B cell Golgi. However, when a Golgi fraction from uninfected wild-type CHO cells is included (Figure 1), GlcNAc can be added to G protein following a dissociative form of this transfer, into the *medial* ("acceptor") compartment of the wild-type Golgi. Provided the *in vivo* specificity of membrane traffic and fusion is preserved *in vitro*, the incorporation of GlcNAc into G protein will be an exclusive measure of transfers of G protein between successive compartments in the Golgi (these compartments residing in different Golgi populations), even when these Golgi membranes are present as a minor fraction within a crude homogenate. In fact, the cell-free system seems to respect the same compartment boundaries that exist in the cell, exhibiting an appropriate specificity (Fries and Rothman, 1981; Dunphy et al., 1981; Rothman et al., 1984b). The G protein transported to the acceptor compartment thus originates within the immediately prior "donor" compartment present in the Golgi population from the infected 15B cells, currently believed to be the *cis* portion of the stack. Constructing an assay that measures the activities of donor and acceptor compartments in different Golgi fractions permits their separate biochemical manipulation prior to incubations. Since donor activities (e.g. the budding of vesicles) and acceptor activities (e.g. the fusion of vesicles)

are certain to be distinct at a molecular level, this is an important advantage.

In this paper we report a much more rapid, quantitative, and flexible form of this assay, as well as the extensive purification of active and stable donor- and acceptor-containing Golgi fractions, opening the way to an analysis of the mechanisms of transfer. In the second paper of this series (Braell et al., 1984), we will report the use of this improved assay to localize the transferred G protein by electron microscopic autoradiography to morphologically intact Golgi stacks, derived from the wild-type and not from the 15B Golgi population. This finding implies that the transport *in vitro* is highly specific, and involves intact Golgi stacks. In the third paper (Balch et al., 1984), we exploit two-stage incubations and the selective inhibitory effects of N-ethylmaleimide to reveal two successive kinetic intermediates in the transport process. On the basis of electron microscopy, these seem to represent intermediate stages in the budding and fusion of transport vesicles.

Results

Transport-Coupled Incorporation of ^3H -GlcNAc into G Protein

In previous work we monitored the transport-coupled addition of GlcNAc to G protein indirectly, as judged by the ensuing resistance of the oligosaccharide to endoglycosidase H (Fries and Rothman, 1981). This procedure required that the polypeptide backbone of G be radioactively labeled *in vivo*, and it necessitated the use of SDS gels and autoradiography for analysis, taking several days. A more rapid, sensitive, and flexible assay was needed to

enable a further analysis and fractionation. We reasoned that by measuring the transport-coupled incorporation of ^3H -GlcNAc into G protein directly (from UDP- ^3H -GlcNAc), these needs could be fulfilled (Figure 1). The donor fraction can be prepared from unlabeled VSV-infected 15B cells, and the ^3H -labeled G protein can be retained on filters as a rapidly formed immunoprecipitate and counted.

Postnuclear supernatants of VSV-infected CHO clone 15B and of uninfected wild-type cells were prepared as described in Experimental Procedures. These crude fractions were incubated together in the presence of ATP, an ATP regenerating system, and UDP- ^3H -GlcNAc. Assays were terminated by the addition of detergent to solubilize the membranes, followed by anti-G protein antiserum. The immunoprecipitates (formed within 45 min at 37°C or overnight at 4°C) were collected on a Millipore filter, washed, and counted. As shown in Figure 2, a time-dependent incorporation of ^3H -GlcNAc into G protein was observed during the incubation. Only trace levels of ^3H were retained on the filter when the anti-G protein serum was replaced with a preimmune serum, implying that all of the ^3H retained had been in G protein. The time course of incorporation of ^3H -GlcNAc into G protein was very similar to that previously reported for the transport-coupled processing of G protein to the EndoH-resistant form *in vitro* (Fries and Rothman, 1981).

Analysis of a 60 min incubation using SDS-gel electrophoresis revealed that G protein is the major protein species labeled (Figure 2 inset, lane a), accounting for about 50% of the total radiolabel incorporated. The immunoprecipitate contained G protein as the only radiolabeled protein species (Figure 2, inset, lane b); G protein was quantitatively immunoprecipitated. No radiolabeled proteins were observed with preimmune serum (Figure 2 inset, lane c). The incorporation of ^3H -GlcNAc in incubations employing crude postnuclear supernatants exhibits all of the properties detailed in the following sections for incubations with sucrose-gradient-purified donor and acceptor Golgi fractions (data not shown). For example, no ^3H was incorporated into G protein when the postnuclear supernatant of uninfected 15B cells replaced the corresponding fraction from infected cells.

Donor Activity Copurifies with the Golgi Membranes of VSV-Infected 15B Cells

Previous work using crude postnuclear supernatants (PNS) as donor and acceptor suggested that the assay employed measures only the reconstitution of a single stage within total ER-Golgi-plasma membrane transport pathway: the transfer of G protein between two successive locations in the Golgi complex (Fries and Rothman, 1981; Dunphy et al., 1981; Rothman et al., 1984b). The improved assay should measure the same process, except that the transport-coupled incorporation of GlcNAc is quantitated differently and more directly. To confirm this, and to offer novel lines of evidence for the compartmental specificity of the assay, it is necessary to show that the two assays measure the transfer of the same population of G protein molecules

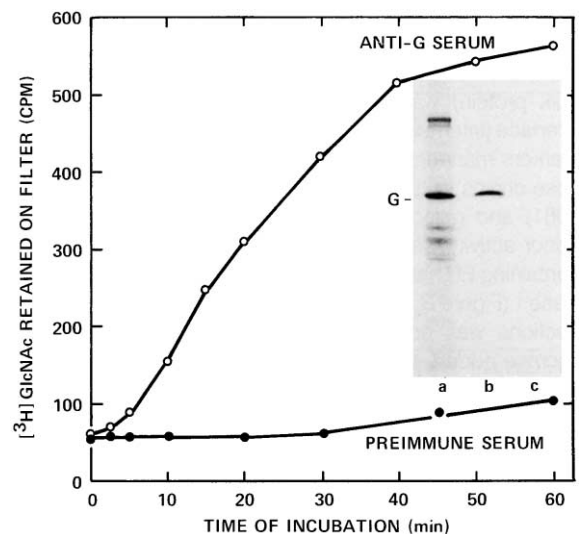


Figure 2. Incorporation of ^3H -GlcNAc into G Protein during Incubations of the PNS of VSV-Infected 15B Cells with the PNS of Uninfected Wild-Type Cells

Crude homogenates of VSV-infected clone 15B cells and wild-type cells were prepared as described in Experimental Procedures. The homogenates were centrifuged at $600 \times g$ for 5 min, yielding a PNS fraction (about 15 mg/ml protein). Then, 35 μg of the PNS from the VSV-infected clone 15B cells and 70 μg of the PNS from uninfected wild-type cells were incubated for increasing times under standard assay conditions (see Experimental Procedures) except no cytosol was added. The incorporation of ^3H -GlcNAc into G protein was measured by collecting the immunoprecipitate on Millipore filters, using anti-G protein serum (\circ) or a preimmune serum (\bullet), as in Experimental Procedures. For the inset autoradiograph, incubations were as described above for 60 min, followed by SDS gel electrophoresis. (Lane a) The incubation was terminated by the addition of 50 μl of gel sample buffer (GSB; 0.1 M Tris-HCl, pH 6.8; 2% sodium dodecylsulfate; 30 mM dithiothreitol; 10% glycerol; 1 $\mu\text{g}/\text{ml}$ bromophenol blue), and boiled prior to SDS gel electrophoresis. (Lanes b and c) Incubations were terminated by addition of 50 μl detergent buffer (DB, see Experimental Procedures) and 15 μl of anti-G protein serum (lane b) or 15 μl preimmune serum (lane c). After an overnight incubation on ice, 25 μl of a 10% suspension of Staph A cells (Pansorbin, Calbiochem Corp., previously washed twice with washing buffer, (see Experimental Procedures) was added. After 1 hr on ice, the cells were pelleted in a microfuge in 15 sec, washed twice with 1 ml each of washing buffer, resuspended in 50 μl of water prior to addition of 50 μl GBS, and boiled. The cells were then pelleted for 3 min, and the supernatants were electrophoresed in a 10% polyacrylamide gel according to the method of Laemmli (1970). The dried gel was autoradiographed with fluorographic enhancement for 7 days.

in Golgi, with identical properties and requirements. The following sections document these and related points.

Since the G protein should originate within the Golgi membranes of the homogenate of the VSV-infected 15B cells, fractionation of the homogenate on a density gradient should yield a Golgi-rich fraction containing all of the donor activity. The crude homogenate, prepared by breaking cells in the presence of 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4), was adjusted to 1.4 M sucrose and 1 mM EDTA and loaded as a layer of a discontinuous sucrose gradient underneath layers of 1.2 M and 0.8 M sucrose (in 10 mM Tris-HCl, pH 7.4). After centrifugation, fractions were assayed for ER and Golgi marker enzymes, and for

their donor activity; i.e., for their ability to provide G protein for transport-coupled glycosylation. As shown in Figure 3 (top), a single peak of donor activity (well separated from bulk protein) was observed at the 0.8/1.2 M sucrose interface (interface III). This activity copurified with the Golgi markers mannosidase I (the enzyme that trims high-mannose chains to the Man₅ intermediate; Hubbard and Ivatt, 1981) and galactosyltransferase (Figure 3, bottom). No donor activity was observed in the more dense fractions containing ER membranes, marked by the enzyme glucosidase I (Figure 3, bottom). The donor activity of the Golgi fractions was not significantly inhibited by the level of sucrose added when assaying the ER fractions from the bottom of the gradient (not shown). A minor peak of

glucosidase I was detected in the fractions preceding the donor activity peak.

Table 1 shows that the pooled Golgi fraction (interface III) was about 40-fold purified over the PNS, as judged by the Golgi marker (mannosidase I) recovered in the greatest yield (82%). Donor activity was purified 20-fold, but this lower value is almost certainly an underestimate by about 2-fold since the recovery of donor activity (43%) was about half that of mannosidase I and no other peaks were found in the gradient. Galactosyltransferase was recovered (55%) and purified (25-fold) to about the same extent as donor activity. Less than 6% of glucosidase I activity was recovered in the active donor fraction. In general, different preparations yielded a 15 to 25 fold enrichment of donor activity, accompanied by a 40% to 70% yield.

An important aspect of this procedure is the collection of the Golgi fraction at an interface in a sucrose gradient, avoiding the formation of a pellet. Pelleting and washing steps, used in many purifications of Golgi membranes and needed for the physical separation of Golgi compartments (Dunphy et al., 1981), greatly reduce the yield of donor activity. The pooled Golgi fraction (interface III) will be referred to as the donor membrane fraction and is used routinely. It is frozen in aliquots in liquid nitrogen and is stable for months when stored at -80°C .

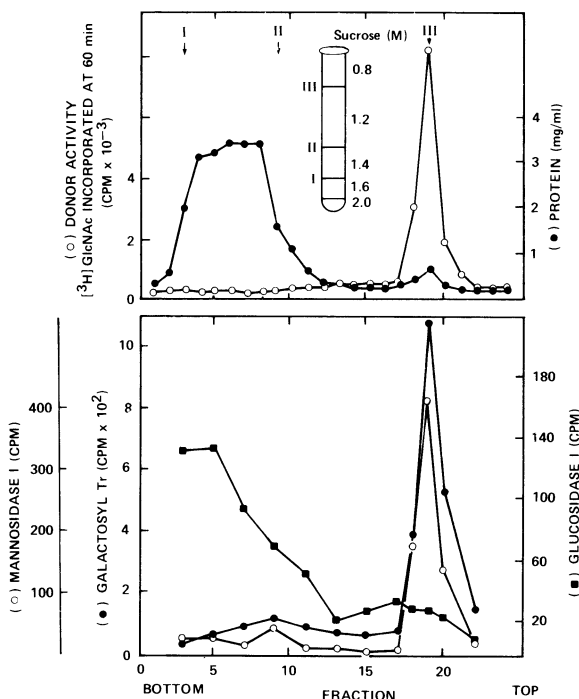


Figure 3. Copurification of Donor Activity with Golgi Markers following Sucrose Gradient Centrifugation of Crude Homogenates of VSV-Infected Clone 15B Cells

A crude homogenate prepared from VSV-infected clone 15B cells was fractionated using discontinuous sucrose density gradient centrifugation as described in Experimental Procedures, except that additional layers of sucrose below the homogenate were included. The gradient consisted of layers of 2.0 M sucrose (2 ml), 1.6 M sucrose (4 ml), the homogenate adjusted to 1.4 M sucrose (as described in Experimental Procedures, 8 ml), 1.2 M sucrose (12 ml), and 0.8 M sucrose (8 ml). All sucrose solutions were prepared with 10 mM Tris-HCl (pH 7.4). After centrifugation in the SW27 rotor for 2.5 hr at 25,000 rpm, fractions (about 1.3 ml) of the gradient were collected from the bottom. Then 2.5 μl of each fraction was assayed in the standard cocktail containing 5 μl of a gradient-purified membrane fraction prepared from wild-type cells (1 mg protein/ml) and 50 μg of gel-filtered CHO cytosol, using a 60 min incubation. Each gradient fraction was also assayed for the activity of mannosidase I, galactosyltransferase, and glucosidase I as described previously (Dunphy and Rothman, 1983). Protein was measured by the method of Lowry. (Top) Donor activity (○) and protein (●). (Bottom) Gal Transferase (●), Mannosidase I (○), and Glucosidase I (■). The arrows in the top panel labeled I, II, and III indicate the positions of the three significant interfaces, at 1.6/1.4 M (I), 1.4/1.2 M (II), and 1.2/0.8 M (III). The Golgi fraction is routinely harvested from interface III.

Acceptor Activity Copurifies with the Golgi Membranes of Wild-Type Cells

Using the density gradient centrifugation procedure described above, but now loading the homogenate of uninfected wild-type CHO cells, the major peak of acceptor activity was observed after 2.5 hr of centrifugation at the 0.8 M/1.2 M interface III (Figure 4, top), the same interface shown to contain the Golgi fraction active as donor (Figure 3, top). Acceptor activity cofractionated with GlcNAc transferase I, as well as another Golgi marker enzyme, galactosyltransferase (Figure 4, bottom). In addition, a broader and less purified peak of acceptor activity (typically representing 20% of the total recovered) was consistently observed just above interface II. This minor peak is no longer detected when the centrifugation is prolonged so as to allow a complete equilibrium to be reached (not shown). Most likely, the minor peak of the acceptor activity is present in smaller membranes that take longer to float to equilibrium at interface III. For routine purposes, the Golgi fraction was pooled from interface III after 2.5 hr of centrifugation. This fraction was well separated from ER membranes (marked by glucosidase I), and was about 10-fold purified over the PNS with respect to acceptor and Golgi marker activities, with yields of 30% to 40% (Table 2). Correcting for the yield, the actual degree of copurification of acceptor activity and Golgi markers in the Golgi fraction is likely to be about 30-fold, a value more consistent with that obtained for the donor Golgi fraction. In general, a 10 to 20 fold increase in specific acceptor activity accompanied by a yield of 25% to 50% was obtained for many independent preparations (data not shown), suggesting that the actual purification of Golgi

Table 1. Enrichment of Donor Activity and Golgi Marker Enzymes from VSV-Infected Clone 15B Cells upon Sucrose Density Gradient Centrifugation

Activity Assayed	Fraction Tested	Total Protein (mg)	Total Activity	Recovery of Activity (%)	Specific Activity	Enrichment of Activity (fold)
Donor ^a	PNS	67.5	4.2	[100]	62	[1]
	Golgi	1.5	1.8	43	1240	20
Mannosidase I ^b	PNS		8.0	[100]	0.12	[1]
	Golgi		6.6	82	4.46	37
Galactosyl transferase ^c	PNS		110	[100]	1.7	[1]
	Golgi		63	55	42.5	25
ER glucosidase I ^d	PNS		0.40	[100]	0.006	[1]
	Golgi		0.021	5.8	0.014	2.6

^aA postnuclear supernatant (PNS) was prepared from the homogenate of VSV-infected 15B cells by centrifugation at $600 \times g$ for 5 min. This PNS was fractionated using a discontinuous sucrose density gradient centrifugation as described in Experimental Procedures for homogenates. The Golgi fractions found at the 0.8/1.2 M sucrose interface (interface III, Figures 3 and 4) was collected. Assays of 1.5 μ l of the PNS, or the Golgi fraction, were carried out in the standard cocktail for 60 min containing 5 μ g of the acceptor Golgi membrane fraction, and 50 μ g gel-filtered CHO cytosol. To compensate for the competitive effects of the endogenous UDP-GlcNAc pool found in the cytosol within the PNS fraction, 1.5 μ l of cytosol from 15B cells (prepared from the 15B PNS and not gel-filtered) was added to each assay of donor Golgi fraction. Incorporation was linearly dependent upon the volume of fraction assayed in the range used, for both PNS and Golgi fractions. The total donor activity in the fraction was calculated based on incorporation of 3H per unit volume assayed. The numbers shown represent cpm $\times 10^{-6}$. Specific activity is reported as the total activity divided by the total protein in the fraction, and is shown as cpm/ μ g protein.

^bThe Golgi marker mannosidase I was assayed as described previously (Dunphy and Rothman, 1983). Activity is total units in the fraction. Specific activity reported in units/mg.

^cThe Golgi marker galactosyltransferase was assayed as described previously (Dunphy and Rothman, 1983). Activity is the total units (nmol/hr) of the fraction. Specific activity is reported as nmole/hr/mg protein.

^dThe endoplasmic reticulum (ER) marker glucosidase I was assayed as described previously (Dunphy and Rothman, 1983). Activity is the total units in the fraction. Specific activity is reported as units/mg.

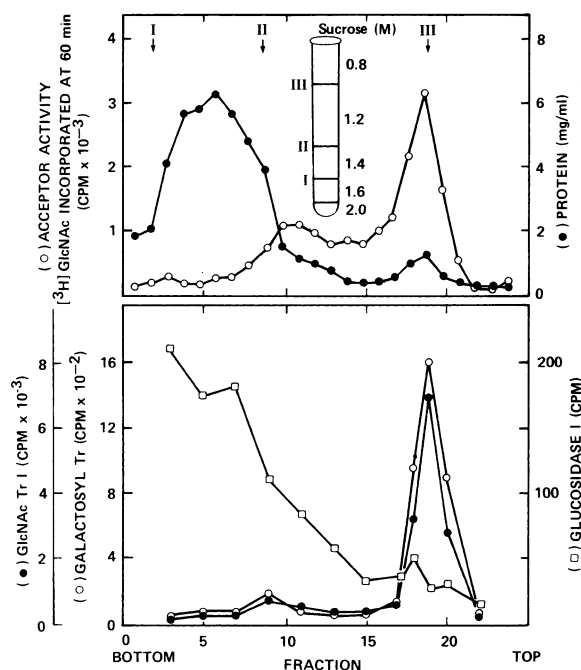


Figure 4. Copurification of Acceptor Activity with Golgi Markers following Sucrose Gradient Centrifugation of Crude Homogenates of Wild-Type Cells. The homogenate of uninfected wild-type cells was fractionated using discontinuous sucrose density gradient centrifugation as in Figure 3. Then, 2.5 μ l of each fraction was assayed for 60 min in the standard cocktail containing 5 μ l of the gradient-purified donor membrane fraction (1 mg/ml) prepared from VSV-infected clone 15B cells and 50 μ g of gel-filtered cytosol (prepared from uninfected clone 15B cells). Each gradient fraction was also assayed for the activity of N-acetylglucosamine transferase I (GlcNAc Tr),

membranes from wild-type cells was typically about 40-fold over the PNS. This Golgi fraction is used routinely, and is referred to as the acceptor membrane fraction. Like the donor membrane fraction, acceptor is routinely frozen in aliquots in liquid nitrogen and stored at $-80^{\circ}C$ for up to several months.

When the gradient-purified donor or acceptor Golgi fractions were added back to the crude PNS, their respective activities were neither inhibited nor enhanced (data not shown). This reinforces the validity of the quantitative data in Tables 1 and 2. Both donor and acceptor activities were eliminated by trypsin, suggesting that cytoplasmically disposed proteins are required for their function (Balch and Rothman, submitted).

Summary of Requirements for the Transport-Coupled Incorporation of GlcNAc

Earlier work using crude PNS fractions as donor and acceptor revealed that transport-coupled oligosaccharide processing required ATP as well as a soluble, cytosolic fraction (Fries and Rothman, 1981). The same requirements should be evident in the new assay, as both donor and acceptor membrane fractions should be effectively freed of soluble proteins and ATP during their preparation on sucrose gradients. As shown in Table 3, mixtures of gradient-purified donor and acceptor fractions are inert unless both ATP and the cytosol fraction are provided.

galactosyltransferase (Gal Tr), and glucosidase I as described previously (Dunphy and Rothman, 1983). (Top) acceptor activity (\circ) and protein (\bullet). (Bottom) Gal Tr (\circ), GlcNAc Tr (\bullet), and Glucosidase I (\square).

Table 2. Enrichment of Acceptor Activity and Golgi Marker Enzymes from Uninfected Wild-Type CHO Cells upon Sucrose Density Gradient Centrifugation

Activity Assayed	Fraction Tested	Total Protein (mg)	Total Activity	Recovery of Activity (%)	Specific Activity	Enrichment of Activity (fold)
Acceptor ^a	PNS	72	5.6	[100]	77	[1]
	Golgi	2.6	1.8	32	685	8.9
Mannosidase I ^b	PNS		19.9	[100]	0.28	[1]
	Golgi		7.3	37	2.82	10.
Gal transferase ^c	PNS		256.0	[100]	3.6	[1]
	Golgi		75.0	29	28.9	8.1
GlcNAc transferase I ^d	PNS		164.0	[100]	2.3	[1]
	Golgi		70.0	42	26.8	12.
ER glucosidase I ^e	PNS		0.60	[100]	0.008	[1]
	Golgi		0.026	4.3	0.010	1.2

^aThe PNS of uninfected wild-type cells was prepared and also further fractionated using the sucrose density gradient, and the Golgi fraction (interface III) was harvested, exactly as in Table 1 except wild-type cells were used. The acceptor activity of the PNS and Golgi fractions were assayed in a manner precisely analogous to Table 1, employing 5 μ g of donor Golgi fraction per 50 μ l incubation, but adding 1.5 μ l of non-gel-filtered cytosol from wild-type PNS to compensate for the endogenous UDP-GlcNAc pool. Total and specific activity are expressed as cpm $\times 10^{-6}$ and cpm/ μ g, as in Table 1.

^bActivity is total units of the fraction. Specific activity is units/mg. See Table 1.

^cActivity is total nmole/hr of the fraction. Specific activity is nmole/hr/mg. See Table 1.

^dThe Golgi marker GlcNAc transferase I was assayed as described previously (Dunphy and Rothman, 1983). Activity is the total units (nmol/hr) of the fraction.

^eActivity is total units in the fraction. Specific activity is units/mg. See Table 1.

Replacement of the Golgi fraction from wild-type CHO cells with a comparable Golgi fraction prepared from uninfected clone 15B (which lacks GlcNAc transferase I) eliminated incorporation into G protein. This proves that all of the ³H incorporated into G protein when wild-type membranes are provided as acceptor is via the *in vivo* enzymatic pathway, requiring GlcNAc transferase I. Addition of EDTA or omission of magnesium from the incubation cocktail eliminated incorporation.

A detailed analysis of conditions affecting the assay will be presented elsewhere (Balch and Rothman, submitted). Briefly, the apparent K_m for ATP is submicromolar; however, a regenerating system for ATP (creatine phosphate and creatine kinase) is needed to maintain ATP levels (Balch and Rothman, submitted). Inclusion of UTP helps protect ATP from hydrolysis. Transfer of G protein was optimal over a restricted range of close to physiological conditions including pH (6.9 to 7.2), temperature (35° to 39°C), salt (15 to 30 mM KCl), and osmolarity (0.15 to 0.3 M sucrose). Incorporation is specific for the *in vivo* substrate UDP-³H-GlcNAc ($K_m = 0.3 \mu$ M), which is stable throughout the time course of the incubation.

The Glycosylated G Protein Is a Transmembrane Protein Sequestered in Sealed Golgi Membrane Vesicles

To test whether the transferred G protein is present in a sealed membrane compartment with the proper asymmetric membrane orientation, trypsin was added after a 60 min incubation. Analysis of the products of tryptic digestion by SDS-gel electrophoresis and autoradiography revealed that proteolysis in the absence of detergent quantitatively converted G protein to a fragment of slightly lower molecular weight without a loss of its ³H-labeled oligosaccharide chains (Figure 5, lane 2 vs. lane 1). This shift in molecular

Table 3. Summary of Requirements of the Cell-Free System

Incubation Condition	³ H-GlcNAc Incorporated into G Protein ^a
1. Complete	[1]
2. — triphosphates and regenerating system	0.01
3. — cytosol	0.01
4. — VSV/15B donor fraction	0.01
5. — Wild-type Golgi fraction	0.03
6. — Wild-type Golgi fraction, + Golgi fraction from uninfected 15B	0.02
7. — Mg ⁺⁺	0.02
8. + EDTA	0.03

Two and a half micrograms of the gradient-purified donor membrane fraction, 5 μ g of the acceptor Golgi fraction, and 50 μ g of gel-filtered CHO cytosol were incubated in the standard cocktail (see Experimental Procedures) containing ATP, UTP, CP, CPK, and UDP-³H-GlcNAc for 60 min at 37°C for the complete incubation (1). (2–7) The indicated component(s) were selectively omitted from the assay. In 6, the acceptor Golgi fraction (from wild-type cells) was replaced with 5 μ g of the gradient-purified Golgi fraction prepared from uninfected clone 15B cells. In 7, Mg acetate was omitted. In 8, Na₂EDTA was added to a complete assay at a final concentration of 5 mM.

^aValues reported are normalized to the ³H incorporated into G protein in the complete incubation (1).

weight is due to proteolytic cleavage within the small carboxy-terminal domain of G protein that is normally exposed on the cytoplasmic face of cellular membranes (Zilberstein et al., 1981). Only low molecular weight fragments were detected when trypsin was added in the presence of detergent (Figure 5, lane 3). In fact, 95% of radioactive GlcNAc incorporated into the oligosaccharides of G protein remained attached to immunoprecipitable polypeptide after this trypsin treatment. However, only 2% of the incorporated ³H could be immunoprecipitated when the membranes were treated with trypsin in the presence

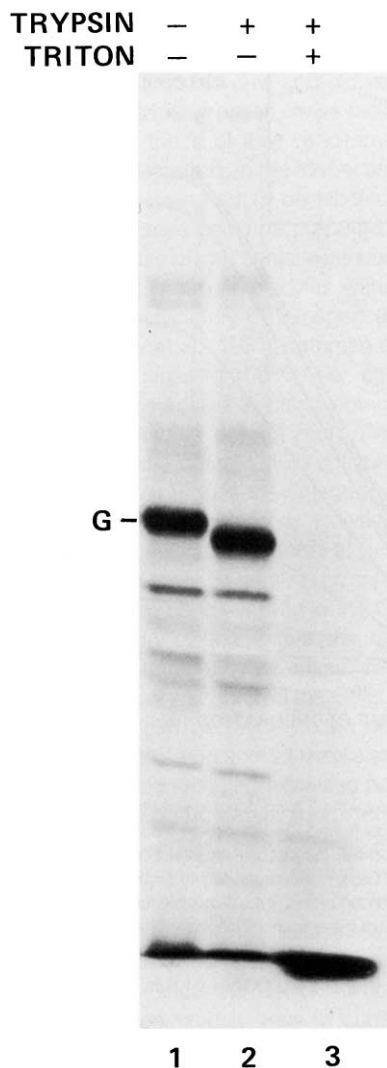


Figure 5. Transported G Protein Resides in Sealed Golgi Vesicles as a Transmembrane Protein with the Appropriate Orientation

A mixture of 2.5 μ g of the donor membrane fraction, 5 μ g of the acceptor Golgi, and 50 μ g of gel-filtered cytosol was incubated for 60 min in the standard cocktail. Assays were terminated by the addition of 2.5 μ l of 100 mM Na₂EDTA. Incubations shown in lanes 2 and 3 were treated with 5 μ l of 1 mg/ml trypsin for 15 min at 37°C in the absence (lane 2), or presence (lane 3), of 0.1% Triton X-100 (added from a concentrated stock). Trypsin was inhibited by the addition of 5 μ l of 1 mg/ml soybean trypsin inhibitor, and samples were mixed with an equal volume of gel sample buffer, boiled, electrophoresed in a 10% polyacrylamide gel, and autoradiographed, as in Figure 2.

of Triton X-100, added to disrupt the vesicles. These data establish that the transported G protein is retained in sealed Golgi vesicles with its carboxy-terminal domain on the outside and its oligosaccharide chains on the inside, as would be expected for membrane fission and fusion processes involving sealed compartments. This rules out the possibility that the GlcNAc transferase itself is artifactually released from wild-type Golgi membranes to act upon G protein in leaky or unsealed membranes from 15B cells.

We also found that addition of 0.1% Triton X-100 prior

to initiation of the assay completely eliminated incorporation of ³H-GlcNAc into G protein. GlcNAc transferase I is fully active in this concentration of Triton (Schachter et al., 1983). This detergent sensitivity distinguishes this transport-coupled glycosylation from an ordinary, uncoupled glycosyltransferase assay. The latter requires detergent to allow substrates and enzymes to mix; the former is profoundly inhibited because detergent dilutes G protein and the GlcNAc transferase with respect to each other. Evidently, these two proteins are concentrated together within the same vesicles as a result of specific transfer events, a prerequisite for efficient glycosylation.

The Donor Compartment Is Rapidly Depleted of G Protein When Protein Synthesis Is Inhibited In Vivo

Only G protein contained in the Golgi fraction of infected 15B cells is a substrate for transport-coupled glycosylation with GlcNAc (Figure 3). To test whether these G protein molecules within this fraction are in fact within the Golgi, we can investigate the time course with which the G protein population that comprises the substrate is depleted when protein synthesis is inhibited in vivo. Transport will continue normally in the absence of protein synthesis. So, when VSV-infected 15B cells are homogenized at various times after addition of cycloheximide, donor activity will progressively be reduced as the last molecules of G protein are transported out of the donor compartment. We found (Figure 6 inset, closed circles) that the donor activity of the whole PNS rapidly and completely disappeared after cycloheximide was added, with a half-time of about 10 min. At all times, the remaining donor activity banded with the Golgi at interface III in the sucrose gradient (Figure 6). As a control, a parallel experiment (Figure 6 inset, open circles) was carried out in which the wild-type cells, rather than the infected 15B cells, were treated with cycloheximide. No effect was observed on the acceptor activity of the PNS of wild-type cells.

These experiments show that it is a recently synthesized pool of G protein concentrated in the Golgi fraction of the 15B cell membranes that is subject to transport to receive GlcNAc. This pool is depleted with a half-time of ~10 min, a time course that is equal to that with which newly synthesized G protein enters the Golgi in CHO cells and far shorter than the time required to reach the cell surface (Fries and Rothman, 1981; Bergmann and Singer, 1983). This pool is not in the ER (Figure 3). Thus, independent kinetic evidence (Figure 6) and physical evidence (Figure 3) point to the conclusion that the transferred G protein originates in a Golgi compartment. These new results buttress the conclusion from earlier work that the assay defines a narrow segment of the transport pathway within the Golgi, and further imply that the new and old forms of the assay measure transport of the same molecules.

The Concentration of Cytosol Determines Both the Rate and Extent of Transport

The initial experiment with crude postnuclear supernatants to determine the feasibility of the new assay yielded a

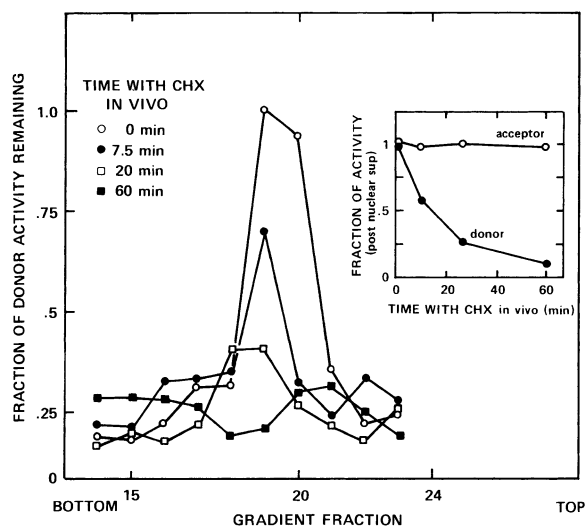


Figure 6. The Compartment in the Golgi Fraction That Donates G Protein In Vitro Is Rapidly Drained of G Protein In Vivo When Protein Synthesis Is Prevented

At 3.5 hr after infection, VSV-infected clone 15B cells were trypsinized into a suspension culture and incubated (starting at 4 hr after infection) for increasing periods of time with 100 μ g/ml of cycloheximide (CHX) to inhibit G protein synthesis. At each time point, a sample of the cells was rapidly pelleted at 4°C, and a crude homogenate was prepared and fractionated using density gradient centrifugation as described in Figure 3. Fractions were collected from the bottom and assayed with 5 μ g acceptor Golgi and 50 μ g gel-filtered CHO cytosol (prepared from uninfected clone 15B cells). Only the top portion of each gradient, consisting of fractions 14–23 (which contained all the detectable donor activity), is shown. The incorporation of 3 H has been normalized to the largest value obtained (0 min CHX, fraction 19) for convenience. Inset: Total donor activity in the crude PNS fraction of the homogenates of VSV-infected 15B cells, as a function of time after CHX (closed circles), using untreated acceptor (as in Figure 2) for 60 min. A parallel control experiment was performed with CHX-treated wild-type cells (open circles) assayed using untreated donor to test the effect of CHX on acceptor activity. For this purpose, wild-type cells were treated in suspension with CHX (100 μ g/ml) in vivo. At each time point, a crude PNS fraction was prepared. The values shown are the 3 H incorporated at a time point as a fraction of the untreated (i.e., no CHX) control.

complex time course for the incorporation of 3 H-GlcNAc into G protein (Figure 1). Similar kinetics were observed using the gradient-purified donor and acceptor Golgi fractions (Figure 7). The typical time course for incorporation in the presence of saturating amounts of cytosol (Figure 7, closed circles) shows a pronounced lag period of ~10 min after initiation of the incubation, followed by a phase of linear incorporation (extending from ~15 to 45 min), until eventually a plateau is reached by 60 min of incubation.

Both the observed rate of incorporation during the linear phase (Figure 7) and the level of the plateau attained (i.e. extent of incorporation measured at 60 min; see inset in Figure 7) are saturable functions of the concentration of cytosol, linear up to about 0.4 mg/ml cytosol protein. The lag period (indicated by extrapolation from the linear phase, dashed lines in Figure 7) is largely independent of the concentration of cytosol. The role of cytosol in the transfer of G protein between the donor and acceptor compart-

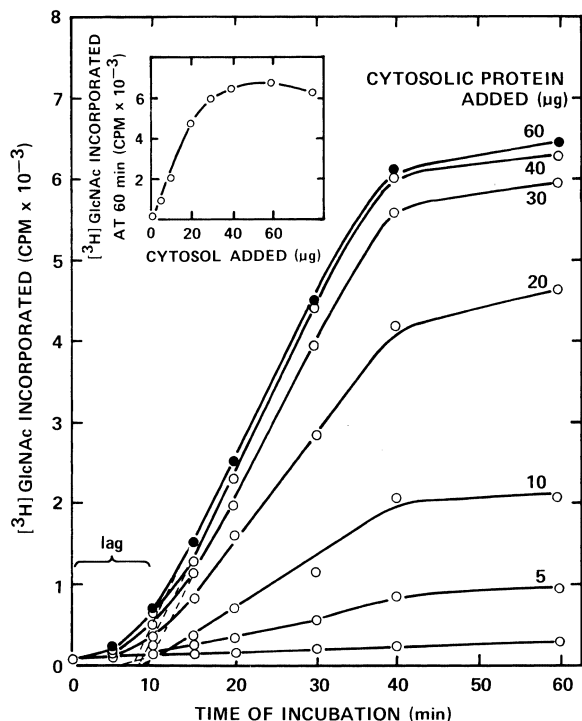


Figure 7. Effect of Cytosol Concentration on the Rate and Extent of Transport Each incubation (50 μ l) contained 2.5 μ g donor membrane fraction, 5 μ g of acceptor fraction, and gel-filtered CHO cytosol in the indicated amount in the standard assay cocktail. Incubations were stopped after various times at 37°C, and the 3 H-GlcNAc incorporated into G protein was determined. Inset: 3 H-GlcNAc incorporated into G protein at the plateau of incorporation as a function of the amount of cytosol protein added.

ments is considered in the third paper of this series (Balch et al., 1984).

Cytoplasmic component(s) active in the assay are found in a broad range of eucaryotic cell tissues, including 15B CHO cells, rat liver, and bovine brain, but not *E. coli*. The activity of the cytosol is sensitive to proteases and inhibited by N-ethylmaleimide, as will be detailed elsewhere (Balch and Rothman, submitted).

Discussion

We have developed an improved assay for measuring the transport of the VSV G protein between successive compartments of the Golgi via the coupled incorporation of GlcNAc. It is more direct than the former version of the assay (Fries and Rothman, 1981) and utilizes purified Golgi fractions, permitting the selective localization of the transferred G protein by electron microscopic autoradiography (Braell et al., 1984). Also, since radioactivity is incorporated only into transported molecules of G protein, there is a negligible background. This makes the new assay more sensitive and more quantitative, permitting a kinetic analysis that has revealed intermediates in the transfer process (Balch et al., 1984). Finally, the new procedure is rapid, so that it now takes less than a day to do what was previously

a week's work. This kind of pace is essential to enable the fractionation of such a complex multicomponent system.

We believe that the new and old versions of the assay measure the same process, since their basic design is the same, as are those of their requirements and properties that can be compared. In the former assay the polypeptide chain of G protein had to be labeled *in vivo* before the donor membranes could be prepared. This enabled us to use pulse-chase experiments to pinpoint the source of the transferred G protein molecules within the Golgi and to measure the efficiency of transport (Fries and Rothman, 1981; Dunphy et al., 1981; Rothman et al., 1984b), which seems to be close to 100%—i.e., as efficient in the cell-free system as *in vivo*. A disadvantage of the new assay is that it measures only G protein molecules that have already arrived, making it more difficult to ascertain exactly which 15B membranes they came from and the absolute efficiency of their transport. However, the unique and extensive copurification of donor activity with the 15B Golgi membranes (Figure 3) and the prompt effect of cycloheximide upon the donor activity in the Golgi fraction (Figure 6) offer strong evidence that the new and old assays measure the transfer of the same select population of G protein that has just entered the Golgi. A rough calculation of efficiency can be made based on the amount of ^3H -GlcNAc incorporated and the specific radioactivity of UDP- ^3H -GlcNAc, taking the endogenous pool into account. The result (Balch and Rothman, submitted) is that about 15 ng of G protein, added in a total of 2.5 μg of 15B Golgi fraction, receives GlcNAc residues *in vitro*. This amount would represent roughly 25% of the total G protein content of this fraction, using the data of Quinn et al. (1984) for the content of Semliki Forest virus glycoproteins in Golgi membranes. Since the donor compartment from which transfer is measured is likely to consist of only one or a few cisternae of the total stack, the actual efficiency is likely to be several times higher.

Specificity of the Cell-Free System

A priori, the addition of GlcNAc to G protein in our assays could result from a nonspecific fusion of the Golgi membranes from wild-type cells with almost any membrane from 15B cells. This is because G protein in ER, Golgi, plasma membrane, and other membranes of the mutant is incompletely glycosylated, and thus potentially a substrate for the missing enzyme, GlcNAc transferase I. Specifically, G protein in the Golgi and all later compartments of 15B (plasma membranes, endosomes, and lysosomes, the latter two filling with progeny virions) will carry Man_5 -containing oligosaccharides, the immediate substrates for GlcNAc transferase I (see Figure 1 and Hubbard and Ivatt, 1981). However, G protein in rough ER membranes carries Man_8 and Man_9 oligosaccharide chains (Zilberstein et al., 1981); thus prior action of Golgi mannosidase I to produce the Man_5 intermediate is needed before GlcNAc transferase I can act.

In spite of this multitude of subcellular membranes containing potential G protein substrates for glycosylation *in*

vitro, the operative mechanism has proved highly specific for the Golgi membranes:

—Neither G protein present in the plasma membrane (or later derivatives, such as endosomes and lysosomes) nor G in the ER of 15B cells will receive GlcNAc in the *in vitro* system, even when the crudest membrane fractions are employed (Fries and Rothman, 1981; also, Figure 3). A nonspecific fusion of ER with Golgi membranes would presumably expose G protein to both Golgi mannosidase I and GlcNAc transferase I enzymes (60% of the mannosidase I activity of wild-type Golgi survives a 60 min incubation under standard assay conditions; data not shown).

—The donor activity distributes exclusively with Golgi membranes in sucrose gradients, and these are copurified some 40-fold (Figure 3). Enrichment is possible only when a select subset of subcellular membranes acts as donor.

—Only a freshly synthesized pool of G protein that has just entered the Golgi in pulse-chase experiments is subject to the glycosylation (Fries and Rothman, 1981; Dunphy et al., 1981; and Figure 7). These are the same molecules that can undergo dissociative transfers into *medial* cisternae *in vivo* (Rothman et al., 1984b).

—Once G protein undergoes this transfer within the 15B Golgi *in vivo*, it loses its ability to undergo what is apparently the same transport step *in vitro* (Rothman et al., 1984b). The cell-free system must therefore adhere to the compartment boundaries within the Golgi that exist in the cell.

There is also a high degree of specificity of the transport and/or fusion processes that occur *in vitro* for the Golgi membranes within the acceptor fraction from wild-type cells:

—The absolute efficiency with which G protein in the donor compartment goes on to receive GlcNAc *in vitro* is very high, perhaps approaching 100%, even when the crudest postnuclear supernatants of wild-type cells are used as acceptor. Since the Golgi membranes (which contain all of the GlcNAc transferase) are only a small fraction of the total membranes in such a crude fraction, nonspecific fusion with (or nonspecific transport to) the bulk membranes (which lack GlcNAc transferase) would result in a very low efficiency of glycosylation. A caveat is that massive, nonspecific fusions involving many organelles could occur such that each resultant giant vesicle would contain some GlcNAc transferase, permitting a high efficiency of glycosylation. This is ruled out by the electron microscope autoradiography experiments described in the next paper in the series (Braell et al., 1984) and also would be inconsistent with the high degree of specificity for compartment boundaries within the donor Golgi.

—The acceptor activity and GlcNAc transferase I activity copurify with similar yields. Such an enrichment implies a delivery process specific to the membranes of the Golgi fraction (Figure 4).

—Consistent with a high degree of specificity are the requirements for cytosol, ATP, and cytoplasmically exposed proteins on donor and acceptor membranes (Balch and Rothman, submitted)

Possible Mechanisms

It seems clear that G protein receives GlcNAc *in vitro* following a specific transport or fusion process involving membranes derived from successive Golgi compartments. That is, an authentic segment of the transport pathway between successive Golgi cisternal compartments has been reconstituted. But how much of the *in vivo* pathway exists *in vitro* and its exact correspondence to the Golgi structures that exist in the cell are not defined by the work described so far.

Several distinctions need to be made to help delineate the exact nature of the reconstitution and to characterize the mechanisms responsible. Does the G protein that is glycosylated *in vitro* start out in cisternae (or their remnants) or instead in transport vesicles that had already budded from cisternae at the time of homogenization? If G starts out in transport vesicles, then a specific fusion process has been reconstituted. If the glycosylated G protein starts out in Golgi cisternae, has the budding of a transport vesicle followed by its specific fusion been reconstituted? Or is there a *direct* but selective fusion between the appropriate cisternae of the 15B Golgi and those of the wild-type Golgi? The former possibility would represent a reconstitution of the entire process of vesicle-mediated intercompartmental transport; the latter something akin to a specific partial reaction. The experiments described in two subsequent papers (Braell et al., 1984; Balch et al., 1984) show that the bulk of Golgi membranes in both donor and acceptor fractions are in the form of stacks of cisternae, and suggest that G protein is transported between the stacks in the form of transport vesicles that form and fuse during the incubation.

Although the Golgi is particularly well suited for the study of protein transport, the principles embodied in the design of our assay system should be directly applicable to the reconstitution of other steps in secretion and in endocytosis. The general notion is to mix two homogenates, one of which contains the protein whose transport is monitored but lacks an enzyme that modifies the protein. The other homogenate contains the modifying enzyme in the compartment intended as target, but lacks the protein substrate. Constructions arranged by the use of wild-type cells and their mutants that have and lack the modifying enzyme can be used for reconstitution of steps in exocytic transport. Designs in which the enzyme is taken up by one cell population and the substrate by another cell population would be appropriate for reconstitution of endocytic transport in mixed homogenates.

Experimental Procedures

Materials

UDP-[6-³H]GlcNAc (24 Ci/mmol) was from New England Nuclear; creatine-phosphokinase (CPK, from rabbit muscle, Type 1, 120 units/mg), UDP-GlcNAc, and GlcNAc-1-phosphate were from Sigma; [2, 8-³H]-ATP (25 Ci/mmol) was from Amersham Corp. ATP and UTP were purchased from P-L Biochemicals.

Cells, Virus, and Antiserum

A wild-type line of Chinese hamster ovary (CHO) cells was maintained in suspension, and the CHO cell mutant, clone 15B (Gottlieb et al., 1975;

obtained from S. Kornfeld, Washington University, St. Louis, Mo.), was grown in monolayers as described (Balch et al., 1983). Stock of VSV (Indiana serotype, San Juan isolate) was grown in monolayers of BHK cells as described (Balch et al., 1983). The resulting titer of the infection medium was typically about 10⁹ plaque-forming units (pfu)/ml. Purified virions were used as a source from which G protein was purified to use as antigen in rabbits, following previously reported procedures (Balch et al., 1983). The activity of antiserum was followed by routinely using the *in vitro* transport assay.

Infection of 15B Cells to Yield Donor Fractions

Twenty plates (15 cm diameter) of densely confluent clone 15B cells (4–6 × 10⁷ cells per plate) were infected with 5–10 pfu of VSV per cell in serum-free growth medium (5 ml per plate) containing actinomycin D (5 µg/ml) and 20 mM HEPES-NaOH (pH 7.3). At 1 hr after the start of infection, 10 ml of complete growth medium was added to each plate. At 3.5 hr, cells were removed from the plates by trypsinization using the following procedure. The medium was aspirated, and each plate was rinsed with 10 ml of Tris-buffered saline (per liter: 0.4 g of KCl, 3.0 g of Tris base, 8.0 g NaCl, 0.1 g of Na₂HPO₄ · 12 H₂O, adjusted to pH 7.4 with HCl), and then rinsed quickly with 5 ml of Tris-saline containing 0.05% trypsin and 0.02% Na₂EDTA. After 5 min at room temperature, cells in each plate were suspended in 2 ml of ice-cold complete medium by pipetting and pelleted (600 g for 5 min at 4°C). The pellet was washed once in homogenate buffer (HB; 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) and resuspended with HB to achieve a final volume equal to five times the volume of the cell pellet (i.e. a 20% suspension). Mild trypsinization does not account for the inability of G protein at the cell surface to be transferred, since cells can be harvested by scraping (without trypsin) and these two methods give similar results (data not shown).

Homogenization of Cells

For donor and acceptor fractions a crude homogenate was sometimes made from this suspension of VSV-infected clone 15B cells with 20 strokes of a very tight-fitting 7 ml Dounce homogenizer (Wheaton Co., Millville, NJ). Alternatively, we used a new device to break cells that made homogenization in 0.25 M sucrose much easier than with a Dounce homogenizer. Briefly, the cell suspension was forced repeatedly (via attached syringes) through a 0.5000 inch precision bore in a stainless steel block that contained a 0.4990 inch stainless steel ball (Industrial Tectonics Co., Ann Arbor, Mich.). Quantitative breakage of the cells required between 10 and 12 passes. The yield of activity and the properties of donor and acceptor fractions prepared with ball and Dounce homogenizers were very similar. However, the ball homogenizer had the advantage of permitting rapid and reproducible breakage of large volumes of cell suspension (20–30 ml) in less than 2 min. The ball homogenizer was used routinely. The details of construction of the homogenizer are given elsewhere (Balch and Rothman, submitted).

The crude homogenate obtained could be used at once or, more conveniently, frozen in liquid N₂ and stored at –80°C for later subcellular fractionation. Frozen crude homogenate was stable for several months. Immediately before subcellular fractionation, frozen homogenates should be thawed rapidly at 37°C and thereafter maintained on ice. This and later fractions rapidly lose activity at elevated temperatures.

For acceptor fractions, 2 liters of suspension of uninfected wild-type CHO cells were harvested at about 5 × 10⁵ cells/ml, washed, and suspended in HB as for the infected 15B cells, and homogenized in the same manner. The wild-type homogenate could be similarly frozen and stored.

Subcellular Fractionation: Preparation of Gradient-Purified Donor and Acceptor Golgi Membrane Fractions

The same procedure was used to prepare the Golgi fraction from VSV-infected clone 15B cells and from wild-type cells for use in routine assays. Six milliliter portions of the appropriate crude homogenate (in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) were adjusted to 1.4 M sucrose by the addition of 6 ml of ice-cold 2.3 M sucrose containing 10 mM Tris-HCl (pH 7.4). Then, 1 mM Na₂EDTA was added from a 100 mM stock solution, vortexed vigorously to ensure uniform mixing, loaded into an SW27 tube, and overlaid with 14 ml of 1.2 M sucrose–10 mM Tris-HCl (pH 7.4), and then 8 ml of 0.8 M sucrose–10 mM Tris-HCl (pH 7.4). The gradients were centrifuged for 2.5 hr at 25,000 rpm (90,000 × g) in the SW27 rotor. The

turbid band at the 0.8 M/1.2 M sucrose interface (interface III in Figures 3 and 4) was harvested in a minimum volume (~1.5 ml) by syringe puncture. This fraction was obtained at a protein concentration of 0.5 to 1.5 mg/ml protein, and is routinely used for the experiments reported, being referred to as either the donor or acceptor membrane fraction (when prepared from VSV-infected 15B cells or wild-type cells, respectively). The sucrose concentration in these fractions is always very close to 1.0 M. These fractions could be used immediately or, more conveniently, frozen in liquid N₂ in suitable aliquots and stored at -80°C. Frozen fractions are stable for several months and yield results identical to those obtained with freshly assayed fractions. The frozen membrane fractions should only be thawed shortly before the assay by a minimal exposure to 37°C, and maintained on ice prior to use. Protein was measured by the Lowry method.

Preparation of High Speed Supernatant Fractions (Cytosol)

Homogenate was prepared from uninfected wild-type CHO cells (or uninfected 15B cells) as described previously. This homogenate was centrifuged in an SW50.1 rotor for 60 min at 49,000 rpm. To remove inhibitory low molecular weight material (most likely the cytosolic pool of UDP-GlcNAc), 15 ml of the supernatant was filtered thru a Sephadex G-25 (Pharmacia Co.) column (2.5 × 50 cm) equilibrated with 25 mM Tris-HCl (pH 8.0)–50 mM KCl. The void volume fractions, containing all the excluded protein, were collected, pooled, and concentrated using an Amicon YM10 filter (Amicon Corp., Layton, Mass.) back to the volume originally loaded on the column. This gel-filtered cytosol could be routinely frozen in liquid N₂ in suitable aliquots and stored at -80°C. The cytosol retains full activity for several months under these conditions. Prior to use, frozen cytosol was thawed rapidly by a brief exposure to 37°C and maintained on ice.

Incubation Conditions to Achieve Transport In Vitro

In addition to donor membranes, acceptor membranes, and cytosol, standard incubations (50 µl) contained (final concentrations): 25 mM HEPES-KOH (pH 7.0), 25 mM KCl, 2.5 mM magnesium acetate (MgOAc), 50 µM ATP, 250 µM UTP, 2 mM creatine phosphate (CP), 7.3 IU/ml rabbit muscle creatine phosphokinase (CPK), and 0.4 µM (0.5 µCi) UDP-[³H-GlcNAc]. This was prepared by combining 5 µl of a 10-times-concentrated buffer-salt stock solution (containing 250 mM HEPES-KOH, pH 7.0; 250 mM KCl; and 25 mM MgOAc) with 5 µl of a triphosphate and regenerating system stock solution (prepared daily by mixing 5 µl of CPK, 1600 IU/ml, stored at -80°C; 25 µl of 200 mM CP; 5 µl of 10 mM ATP, Na form, neutralized with NaOH; 10 µl of 100 mM UTP, Na form, neutralized; and 65 µl H₂O with 5 µl of UDP-³H-GlcNAc, 100 µCi/ml, in H₂O, prepared daily by evaporation to dryness of an aliquot of an ethanolic stock of the sugar-nucleotide with a gentle stream of N₂, and then dissolving in H₂O, and 20 µl H₂O). To this mixture (on ice) was added, in the order listed, 5 µl of the gel-filtered cytosol (5–10 mg protein/ml), and 5 µl each of the donor and acceptor membrane fractions (0.5 to 1.5 mg protein/ml), which had been thawed as short a time as possible before use by a brief incubation (less than 15 sec) at 37°C and then chilled on ice. The assay was initiated by transfer to 37°C. Incubation was routinely for 60 min, and done in disposable glass tubes.

Quantitation of Transport by the Incorporation of ³H-GlcNAc into G Protein

The incubation (50 µl) was terminated by transfer to ice, and the membranes were solubilized by addition of 50 µl of detergent buffer (DB) containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM Na₂EDTA, 1% Triton X-100, and 1% sodium cholate. Then, rabbit anti-G protein antiserum (or the same volume of preimmune serum as a control) was added. The amount of antiserum needed (generally 10–20 µl) to obtain maximal binding of ³H-G protein was determined individually for each preparation of antiserum and of donor membrane. After 45 min at 37°C or an overnight incubation at 0–4°C, the immunoprecipitate was collected by its retention on Millipore filters (type HA, 0.45 µm; Millipore Corp., Bedford, Mass.). Each filter was first rinsed with 3 ml of a washing buffer (WB) (containing 50 mM Tris-HCl, pH 7.5; 250 mM NaCl; 5 mM Na₂EDTA; 1% Triton X-100). Before the filter can dry, the immunoprecipitated incubation mixture is diluted with 3 ml of WB and rapidly filtered. The tube is rinsed once with 3 ml WB, and this is filtered. The filter is then further washed in rapid succession with three portions of WB (3 ml each). It is important that incubations be filtered one at a time without any air drying of the filter between washes; otherwise

there is a high nonspecific sticking of ³H-sugar nucleotide to the filters. Filters are then dried under a heat lamp and counted in a scintillation cocktail.

Acknowledgments

This paper is dedicated to Professor Eugene P. Kennedy on the occasion of his 65th birthday. His former students thank him for his fine example.

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