Intercompartmental Transport in the Golgi Complex Is a Dissociative Process: Facile Transfer of Membrane Protein between Two Golgi Populations

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ABSTRACT The transfer of the vesicular stomatitis virus-encoded glycoprotein (G protein) between Golgi populations in fused cells (Rothman, J. E., L. J. Urbani, and R. Brands. 1984. *J. Cell Biol.* 99:248–259) is exploited here to study and to help define the compartmental organization of the Golgi stack and to characterize the mechanism of intercompartmental transport. We find that G protein that has just received its peripheral *N*-acetylglucosamine in the Golgi complex of one cell is efficiently transferred to the Golgi complex of another cell to receive galactose (Gal). Remarkably, this transport occurs at the same rate between these two compartments whether they are present in the same or different Golgi populations. Therefore, a dissociative (presumably vesicular) transport step moves G protein from one part of the Golgi in which *N*-acetylglucosamine is added to another in which Gal is added. Minutes later, upon receiving Gal, the same G protein molecules are very poorly transferred to an exogenous Golgi population after cell fusion. Therefore, once this intercompartmental transfer has already taken place (before fusion), it cannot take place again (after fusion); i.e., transport across the compartment boundary in the Golgi complex that separates the sites of *N*-acetylglucosamine and Gal incorporation is a vectorial process. We conclude that transfers between Golgi cisternae occur by a stochastic process in which transport vesicles budding from cisternae dissociate, can diffuse away, and then attach to and fuse with the appropriate target cisterna residing in the same or in a different stack, based on a biochemical pairing after a random encounter. Under these circumstances, a transported protein would almost always randomize among stacks with each intercisternal transfer; it would not progress systematically through a single stack. Altogether, our studies define three sequential compartments in the Golgi stack.

The Golgi complex occupies a central position in the pathway of intracellular protein transport. A mixture of proteins exported from the endoplasmic reticulum seems to enter the Golgi stack at one end (the *cis* face) and to exit from the stack at the other (the *trans* face) after having been sorted (6, 20, 22, 24, 25). The mechanism of transfer between the cisternae, resulting in passage across the stack, is unknown but must be central for understanding the sorting of proteins that occurs in the Golgi complex. A major difficulty in elucidating the nature of intercisternal protein transport has been that these transfers occur between membrane compartments that are physically attached and not easily separated. In general, transport processes can only be studied effectively when compartments that provide and receive the transferred substrate can be separated or otherwise distinguished.

In the preceding article (21), we have described a cell-fusion technique with which to form hybrid cytoplasms containing two distinct Golgi populations. This experimental design facilitates the study of protein transport in the Golgi complex by allowing transfers to be detected in which a glycoprotein originating in a Golgi complex from one cell is received by an enzymatically distinct Golgi complex from another cell. The principal evidence for this was the transfer of freshly acylated vesicular stomatitis virus (VSV)[1]-encoded glycopro-

[1] *Abbreviations used in this paper:* Gal, galactose; GlcNAc, *N*-acetylglucosamine; $GlcNH_2$, glucosamine; G protein, glycoprotein; Met, methionine; VSV, vesicular stomatitis virus.

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tein (G protein) largely containing trimmed ($mannose_5$)-oligosaccharide chains, a pool believed to reside in Golgi membranes (11).

In this article we provide a further demonstration that the transferred G protein can originate in the Golgi complex, and locate one such source within this organelle more precisely. We show that G protein that has just received peripheral *N*-acetylglucosamine (GlcNAc) in one Golgi complex is readily transferred to another Golgi complex to receive galactose (Gal); moments later, having received Gal in the original Golgi complex, the same G-protein molecule is poorly transferred, if at all. The differential fate of G protein, depending upon its location within the Golgi complex at the time of fusion, offers new insights into the compartmental organization and the nature of intercompartmental transport in the Golgi stack.

MATERIALS AND METHODS

Proteins and Enzymes: Neuraminidase from *C. perfringens* was type X from Sigma Chemical Co. (St. Louis, MO) and was dissolved at a concentration of 100 U/ml in 50 mM sodium citrate buffer (pH 5.3) and stored at −20° in aliquots. *Ricinus communis* agglutinin I (ricin, RCA 120) was from Vector Laboratories, Inc. (Burlingame, CA) and came dissolved in 10 mM phosphate buffer (pH 7.1), 0.9% NaCl, 0.04% NaN_3 at 5 mg/ml. Wheat germ agglutium (Vector Laboratories, Inc.) was dissolved at 10 mg/ml in 0.15 M NaCl, 10 mM HEPES pH 7.4, 0.02% NaN_3. Pronase was from Calbiochem-Behring Corp. (San Diego, CA), and fetuin from Gibco Laboratories, Grand Island Biological Co. (Grand Island, NY). Slug lectin (*Limax flavus* agglutinin; LFA) was purified as described earlier (15), lyophilized from 0.1 M NaCl–50 mM Tris–HCl (pH 7.5), dissolved in one-fifth the original volume of water, dialyzed into 0.1 M NaCl–50 mM Tris–HCl (pH 7.5), frozen in liquid nitrogen, and stored in aliquots at −80°C. The final protein concentration was 3 mg/ml.

Cell Lines: Chinese hamster ovary (CHO) clones 13, 15B, and 1021 were kindly provided by Stuart Kornfeld (Washington University, St. Louis, MO). The CHO mutant Lec2 and its wild-type parent W5 were generously provided by Pamela Stanley (Albert Einstein College of Medicine, Bronx, NY). All were grown in monolayer in Eagle's minimum essential medium as described (21).

Preparation of Mixed Monolayers: These were formed by exactly the procedures described in the preceding article (21), except the CHO cell lines employed were different. For one set of experiments, the infected cells were of clone 13 and the uninfected cells were of clone 15B. For the other, the infected cells were of clone 1021 and the uninfected cells were again of clone 15B.

Labeling and Fusion of Mixed Monolayers: Mixed monolayers (6 cm) were rinsed with warm Tris-saline medium (21) plus 15 mM NH_4Cl, and then treated with 2 ml of a cycloheximide-containing medium for 7 min before a 3-min pulse-label of D-[6-^{3}H]glucosamine ($GlcNH_2$) (20 Ci/mmol) or D-[1-^{3}H]galactose (Gal) (11 Ci/mmol; (both from New England Nuclear, Boston, MA) in the presence of cycloheximide. Both of these steps employed the low-bicarbonate minimum essential medium described (reference 21, footnote 4), but containing 10% of the usual glucose (0.1 g/liter) and supplemented with 7% dialyzed fetal calf serum, 15 mM NH_4Cl, and 100 μg/ml cycloheximide. For the pulse-labeling, 1.5 ml per plate of this low-glucose medium containing 1 mCi/ml of the appropriate ^{3}H-sugar was used. This labeling medium was saved for repeated use, and was centrifuged (2,000 *g* for 2 min) in between each occasion to remove cellular debris.

After the 3-min pulse, a variable period of chase was carried out before fusion. Labeling medium was immediately replaced with 2 ml of a chase medium; this was growth medium supplemented with 100 μg/ml cycloheximide, 15 mM NH_4Cl, 15 mM HEPES, pH 7.4, and either 5 mM $GlcNH_2$ (when only [^{3}H]$GlcNH_2$ was used in the experiment) or 2.5 mM $GlcNH_2$ and 2.5 mM Gal (when both ^{3}H-sugars were used in the same experiment). When no period of chase was required before fusion, the plate was simply rinsed with the chase medium and then fused.

To fuse the mixed monolayer, the chase medium was aspirated and immediately replaced with 2 ml of warm pH 5 fusion medium (reference 21, footnote 3) containing 15 mM NH_4Cl and 100 μg/ml cycloheximide. After 1 min at 37°C, this medium was replaced with 4 ml of chase medium. Unless otherwise specified, after a further 1-h incubation to allow inter-Golgi transport and glycosylation, the monolayer was harvested for analysis. Every plate was examined by light microscopy within 10 min after fusion to confirm that extensive fusion had in fact occurred.

Purification of Protein from Mixed Monolayers by Immunoprecipitation and SDS-Gel Electrophoresis: The cells were solubilized in a 0.5-ml vol and all of this used for immunoprecipitation with anti-G serum onto *Staphylococcus aureus* cells as described in the preceding paper (21) for the [^{3}H]palmitate experiments. Then, the washed *S. aureus* cell pellet was suspended with 75 μl of SDS–gel electrophoresis sample buffer (13) and boiled. The supernatant was combined with 20 μl of sample buffer containing a 1:1 mixture of [^{35}S]methionine (Met)-labeled VSV virions grown on clone 13 and on wild-type CHO cells, ~8,000 cpm of ^{35}S in total protein. These samples were then electrophoresed in alternate lanes (to prevent their cross-contamination) of 10% polyacrylamide gels (13), which were then fixed for 2 h in 25% (vol/vol) isopropanol, with one change of fixative. After a 5-min rinse with water, the gels were dried onto Whatman 3 MM paper (Whatman Chemical Separation Inc., Clifton, NJ) and autoradiographed for 3 d. The ^{35}S-labeled VSV enabled the G-protein band to be located by autoradiography. In that the mobility of G varies with the extent of its glycosylation, a mixture of ^{35}S-G protein (from clone 13 and wild-type CHO cells) that would bracket the entire mobility range of ^{3}H-G proteins was co-electrophoresed. The resulting double band of ^{35}S-G protein was located in each lane of the dried gel and cut out together with an extra 2 mm above and below it. The remainder of the gel was reexposed to x-ray film to confirm that all of the G-protein region had been excised.

Preparation of Glycopeptides from Purified G Protein: The excised portions of dried gel containing G protein were swollen for ~15 min in 0.1 M ammonium bicarbonate containing 1 mM $CaCl_2$ and 0.02% NaN_3. Pieces of gel, separated from the paper support, were chopped with a razor blade into ~1 mm^3 fragments. To digest G protein in situ, the gel pieces were vigorously shaken with 0.5 ml of 1 mg/ml pronase in the bicarbonate buffer in a tightly sealed tube for 1 d at 50°C. (The pronase solution used had been previously heat-treated for 1 h at 50°C.) The aqueous phase, containing released peptides and glycopeptides, was saved and the gel pieces further incubated with another 0.9 ml of pronase solution for another day at 50°C. The combined digests were centrifuged to remove debris, and the supernatant was lyophilized, dissolved in 0.5 ml water, centrifuged again, and lyophilized again, and finally the residue was dissolved in 60 μl of 0.1 M Tris–HCl (pH 7.5), 10 mM EGTA, 0.02% NaN_3, and boiled for 3 min to inactivate any remaining pronase. The resulting concentration of free Ca^{++} was calculated to be 13 mM (above the EGTA concentration). This glycopeptide preparation was used directly for binding to slug lectin (i.e., for experiments with clone 1021 mixed monolayers). When binding to ricin was to be measured (i.e., when clone 13 was used), terminal sialic acid residues were first removed. To accomplish this, the entire glycopeptide preparation was incubated with 2 μl of 100 U/ml neuraminidase for ~16 h at 37°C, and then boiled. Control experiments established that this treatment quantitatively removes sialic acid but does not release any Gal or GlcNAc (data not shown). The overall recovery of ^{3}H for all of the steps in going from immunoprecipitate to the glycopeptide preparation averaged 70%. The ^{35}S/^{3}H ratio in the preparations was in the range of 0.5–1.

Binding of Glycopeptides from Fusion Experiments to Lectins for Analysis: To assay the percent of the glycopeptide able to bind to ricin (1), 20-μl samples of each neuraminidase-digested glycopeptide preparation were incubated with 40 μl of ricin (5 mg/ml) for 15 min at 25°C, in duplicate. Afterwards, each incubation was mixed with 1 ml of ice-cold 4 M $(NH_4)_2SO_4$ (dissolved in 50 mM Tris and titrated to pH 7) containing 0.01% bovine serum albumin (added from a 1% solution). After centrifugation for 15 min in a microfuge in the cold, the pellet was resuspended in 1 ml of the ice-cold buffered 4 M $(NH_4)_2SO_4$ solution (not containing albumin) and centrifuged for 15 min. The washed pellet was dissolved in 1.5 ml of water and counted for ^{3}H (representing glycopeptide), correcting for the trace of ^{35}S-peptides present in the lectin precipitate. The duplicates agreed to within 5%. The total amount of ^{3}H in the glycopeptide preparation was measured by counting a 10-μl sample, correcting for the ^{35}S present. All of the ^{3}H in the preparations was in glycopeptides because ^{3}H was quantitatively precipitated by 40 μl of wheat germ agglutinin (10 mg/ml) when this lectin was used in place of ricin (data not shown). The percentage of ^{3}H-glycopeptide binding to ricin was then calculated. This quantity, being an internal ratio, is independent of variations from monolayer to monolayer in the yield of G protein and its glycopeptides.

Binding to slug lectin was assayed identically, using 40 μl of 3 mg/ml slug lectin, except the wash with albumin-free ammonium sulfate was omitted.

Preparation of Viral Particles and Glycopeptide Markers: To prepare ^{35}S-labeled virions, a confluent 10-cm plate of the desired cell type was infected with VSV at ~5 plaque-forming units/cell. At 3 h postinfection, 100 μCi of [^{35}S]Met per plate was added in 4 ml of Met-free minimum essential medium (21) containing 7% dialyzed fetal calf serum. After 3 h, the medium was clarified by centrifugation at 2,000 *g* for 10 min, and this supernatant was layered onto a cushion of 20 (wt/vol)% sucrose in 1 mM Na_2EDTA, 10 mM Tris–HCl (pH 7.4) and centrifuged in the SW50.1 rotor for

1 h at 50,000 rpm, and the viral pellet thus obtained.

To prepare virions in which G protein was labeled with isotopic sugars, a confluent 10-cm plate of the desired cell type was infected and 3 h later the monolayer was rinsed with Tris-saline medium (21) and 2.5 ml of low-glucose medium containing 200 μCi of ^{3}H-sugar (either $GlcNH_2$ or Gal) or 50 μCi of [^{14}C]Gal (New England Nuclear, uniformly labeled) was added. Low-glucose medium had the composition of Met-free minimum essential medium (reference 21, footnote 2) but contained glucose (0.1 g/liter), had 0.25 mM Met, and was supplemented with 7% dialyzed fetal calf serum and 15 mM HEPES (pH 7.4). After 2 h virions were prepared as above. SDS-gel electrophoresis (data not shown) confirmed that G was the only radioactive protein in these preparations.

To prepare labeled-VSV glycopeptides for use in testing the specificity of lectin precipitation assays (see Figs. 2 and 8), the viral pellet was boiled in 200 μl of 1% SDS, 15 mM dithiothreitol, 50 mM Tris–HCl (pH 6.8) and precipitated by adding 20 μl of 2% Triton X-100 as carrier and then 200 μl of cold 20% trichloroacetic acid. The precipitate was collected by centrifugation for 5 min in a microfuge, washed once with ice-cold acetone (0.3 ml), and air-dried. Then, 100 μl of a freshly prepared pronase solution (20 mg/ml in 0.1 M Tris–HCl pH 7.5, 10 mM $CaCl_2$, with 1 drop of toluene per ml) was added. After 1 d at 50°C, the digest was sonicated for 30 s using a water-bath sonicator, and another 100 μl of pronase solution was added. After another day at 50°C, the digest was boiled and stored at −20°C. The precipitates that formed upon thawing were removed by centrifugation before use and contained no radioactivity. 50–90% of the total radioactivity in these preparations was in glycopeptide, as judged by the percentage that could be precipitated by saturating levels of wheat germ agglutinin (data not shown). The remainder is presumably due to contaminating sugar precursors.

RESULTS

Transfer of G Protein between Golgi Complexes after Cell Fusion

To ascertain whether G protein present in the Golgi complex of one cell can move to the Golgi complex of another after fusion, mixed monolayers were formed by the procedure of the preceding paper (21). These consisted of VSV-infected CHO clone 13 cells and uninfected CHO clone 15B cells (schematized in Fig. 1). Clone 13 is a mutant line that is defective in glycosylation but apparently not in intracellular transport (2). Specifically, G protein synthesized in clone 13 cells will acquire GlcNAc termini in the Golgi complex, but will lack Gal and sialic acid residues. The idea (Fig. 1) is to label G protein via [^{3}H]GlcNAc in the Golgi complex of infected clone 13 cells, and then to fuse these cells to an uninfected cell population whose Golgi complex is capable of adding Gal residues. The question is, can G protein, labeled in its GlcNAc residues in the clone 13 Golgi complex, be transported to another Golgi population where it will be further glycosylated with Gal and possibly other sugars? The preceding paper (21) shows that two Golgi populations derived from two types of CHO cells remain distinct after cell fusion.

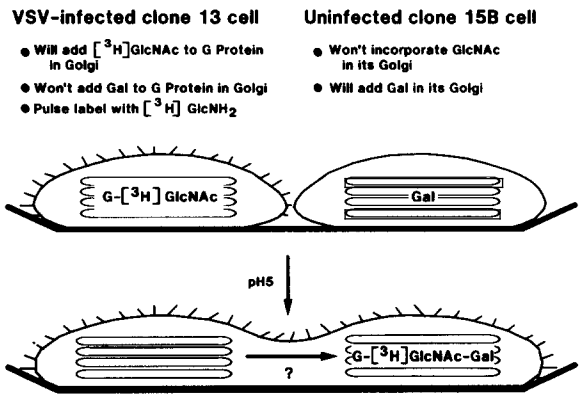

FIGURE 1 Design of a cell-fusion experiment to detect transfers between two Golgi populations. A mixed monolayer is formed containing VSV-infected CHO clone 13 cells and uninfected CHO clone 15B cells. VSV G protein is labeled in the Golgi complexes of clone 13 cells with [^{3}H]glucosamine ($GlcNH_2$), incorporated as peripheral *N*-acetylglucosamine (*GlcNAc*). The clone 13 Golgi is unable to add galactose (*Gal*). The clone 13 cells containing [^{3}H]GlcNAc-labeled G protein are then fused to neighboring clone 15B cells (via a brief exposure to pH 5) whose Golgi complexes are able to add Gal. Transfer of G protein from the clone 13 to the clone 15B Golgi complexes is monitored by the addition of Gal to [^{3}H]GlcNAc-labeled G protein after fusion.

For the uninfected cell population, whose Golgi complex acts to galactosylate G protein in the experiment, we have chosen CHO clone 15B. This cell line is specifically missing GlcNAc transferase I (14) and so is unable to initiate the branch in the asparaginyl-linked oligosaccharide processing pathway that leads to the synthesis of complex-type oligosaccharides containing peripheral GlcNAc, Gal, and sialic acid (11). However, the Golgi complex of 15B cells should be able to complete the synthesis of a complex chain by adding additional GlcNAc, Gal, and sialic acid if GlcNAc transferase I has already acted. We have used clone 15B cells (rather than wild-type cells) as the uninfected acceptor population to minimize the incorporation of [^{3}H]GlcNAc into glycoproteins in these cells. This choice greatly lowers the background in immunoprecipitations, facilitating the analysis of the [^{3}H]-GlcNAc incorporated into G protein in the other minor cell population (clone 13) that is infected.

Briefly, the protocol for the experiment is to pulse-label the mixed monolayer with [^{3}H]$GlcNH_2$ for 3 min, and then to fuse the cells by exposing them to a pH of 5 for 1 min. Monolayers are then incubated in a chase medium to prevent further incorporation of ^{3}H, and stopped at various times to determine what fraction of the G protein labeled with [^{3}H]-GlcNAc while in the clone 13 Golgi complex had gone on to receive Gal in the clone 15B Golgi.

The pulse of [^{3}H]$GlcNH_2$ is added after a 7-min pretreatment with cycloheximide, and this drug is maintained in the media used thereafter. Cycloheximide, which does not affect intracellular protein transport (16), serves two essential purposes. First, it prevents the co-translational incorporation of [^{3}H]$GlcNH_2$ (via the dolichol-linked oligosaccharide) into the pair of GlcNAc residues in the inner core of the G-protein oligosaccharide. This ensures that all ^{3}H is incorporated into G in the Golgi complex, none in the endoplasmic reticulum. Second, cycloheximide greatly improves the efficiency of the chase. In CHO cells, G protein takes ~10 min to be transported to the Golgi complex after protein synthesis in the endoplasmic reticulum (7), so very little G protein remains available as potential substrate for incorporation of [^{3}H]-GlcNAc after a 7-min treatment with cycloheximide and a 3-min pulse label.

To measure the addition of Gal to [^{3}H]GlcNAc-labeled G protein, G protein was immunoprecipitated from detergent extracts of the mixed monolayers and further purified by SDS–gel electrophoresis, and glycopeptides were prepared by exhaustive digestion with pronase. All of the ^{3}H at this stage was derived from G protein, as mock-infected monolayers had no detectable ^{3}H in the glycopeptide fraction (data not shown). The fraction of these ^{3}H-glycopeptides that were able to bind to ricin, a lectin specific for Gal, was then determined. Following essentially the procedure of Baenziger and Fiete (1), the G-protein glycopeptides were incubated with excess

ricin, and the bound [^{3}H]GlcNAc-labeled glycopeptides were precipitated (together with ricin) by ammonium sulphate and counted. Only those glycopeptides from G-protein molecules that had received [^{3}H]GlcNAc in the clone 13 Golgi complex and then Gal in the clone 15B Golgi complex would score in this assay.

Fig. 2 demonstrates that the specificity of the ricin-binding assay for Gal-containing glycopeptides is as expected (1). The glycopeptides of G protein synthesized in wild-type CHO cells (terminating in Gal and Gal–sialic acid) bind to ricin (●) but the G protein glycopeptides made in clone 13 cells (terminating in GlcNAc) do not (△). The G-protein glycopeptides made in a mutant unable to add sialic acid (terminating exclusively in Gal) binds even more efficiently than the wild-type glycopeptide (○). Therefore, the VSV G glycopeptides from fusion experiments were routinely digested with neuraminidase before the ricin-binding assay.

Fig. 3 shows the kinetics of galactosylation of [^{3}H]-GlcNAc-labeled G protein after fusion of the mixed monolayers. Plotted is percent of [^{3}H]GlcNAc-labeled G protein glycopeptide that is bound by ricin as a function of time of harvest after fusion. After a lag of ~5 min, [^{3}H]GlcNAc-labeled G protein receives Gal in the ensuing 10 min until a maximum of ~35% has been galactosylated. This process is completed within ~20 min after the initiation of fusion. The lag of ~5 min is probably due to the time required for cells to fuse with neighbors (of the order of a few minutes as judged by light microscopy) but may also be due, in part, to the time needed for transit between the two Golgi types. The inset in Fig. 3 shows that the total amount of [^{3}H]GlcNAc incorporated into G protein does not change significantly during the period of chase after fusion. It is essential for the interpretation of the experiment that all of the [^{3}H]GlcNAc incorporated into G protein have been added in clone 13 Golgi complex before fusion and none thereafter. The efficiency of the chase, demonstrated in Fig. 3, assures that this is the case.

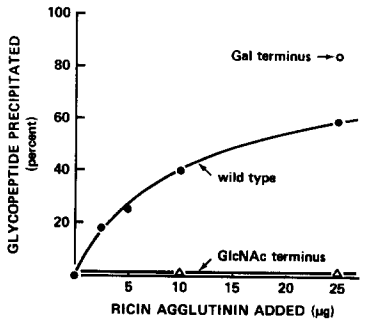

FIGURE 2 Specificity of ricin agglutinin for Gal-containing VSV glycopeptides. Pronase glycopeptide markers were prepared from [^{3}H]GlcNH$_2$-labeled G protein synthesized in wild-type W5 CHO cells (●), cells of a CHO mutant (Lec2) that does not add sialic acid (○), and cells of CHO clone 13 that does not add Gal (△). Incubations for 20 min at 25°C contained 2 μl of glycopeptide (~1,000 cpm) and varying amounts of ricin agglutinin I (5 mg/ml). Bound glycopeptide was precipitated with 4 M ammonium sulfate containing serum albumin carrier and washed once, and counted as described in Materials and Methods. Parallel incubations with saturating amounts of wheat germ agglutinin (able to bind all three forms of VSV glycopeptide) were carried out to determine what fraction of the total ^{3}H was present as glycopeptide. For this purpose, glycopeptide (2 μl) was incubated with between 2 and 5 μl of 10 mg/ml wheat germ agglutinin and processed as above. Shown is the ratio of ^{3}H precipitated by ricin to that precipitated by wheat germ agglutinin, expressed as percent.

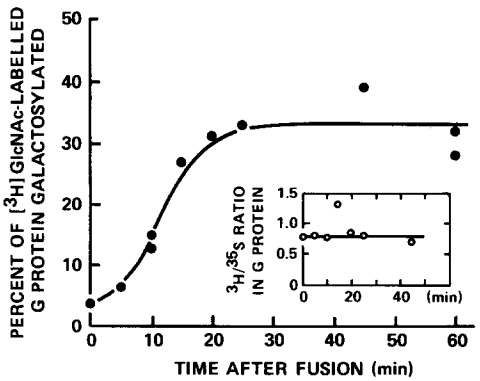

FIGURE 3 Kinetics of galactosylation of G protein labeled with [^{3}H]GlcNAc in VSV-infected clone 13 cells after fusion to uninfected clone 15B cells, according to the scheme in Fig. 1. Mixed monolayers were treated with cycloheximide for 7 min, pulse-labeled with [^{3}H]GlcNH$_2$ for 3 min, fused immediately, and chased for the indicated period of time before harvest, exactly as described in Materials and Methods. Then G protein was purified from detergent extracts by immunoprecipitation and SDS gel electrophoresis, and pronase glycopeptides were prepared. These were incubated with excess ricin agglutinin and the percent of ^{3}H-glycopeptide bound (●) was measured, as described in Materials and Methods. These glycopeptides derive from those ^{3}H-G protein molecules that had received Gal residues following fusion. *Inset:* Efficiency of the chase of [^{3}H]GlcNH$_2$, ascertained from the relative amounts of [^{3}H]-GlcNAc incorporated into G protein at different times of chase. To take account of plate-to-plate variation in overall yield of glycopeptides, the ^{35}S in the glycopeptide preparations (derived from identical amounts of co-electrophoresed ^{35}S-G protein marker) was used as an internal standard. Shown (○) is the ratio of ^{3}H to ^{35}S in glycopeptide preparations from monolayers harvested at different times of chase.

Table I offers a summary of data on the extent of inter-Golgi transport measured by the extent of galactosylation at 1 h after fusion, when this process had gone to completion. In two independent experiments (line A) 32 ± 9% (SD) and 25 ± 3% of [^{3}H]GlcNAc-labeled G protein received Gal. The difference between the experiments is due to a small but systematic difference in the composition of mixed monolayers (i.e., the ratio of the two types of Golgi) between experiments. This difference can be corrected for by employing a "prefusion" control (line D) in which the monolayer is fused first and then, 30 min later, labeled with [^{3}H]GlcNH$_2$, chased for 1 h, and then analyzed. This prefusion protocol enables G protein to completely randomize among the two Golgi populations before labeling, providing an internal standard that measures the maximum amount of galactosylation that can occur given the ratio of the two cell types in the mixed monolayers used. It is this value that would result if all of the G protein labeled before fusion were totally randomized among Golgi complexes after fusion. Prefusion gave 60% and 51% galactosylation, respectively, for Expts. I and II. The ratio of the extent of galactosylation after fusion (line A) to the prefusion control (line D) then measures the fraction of [^{3}H]GlcNAc-labeled G protein present in clone 13 cells before fusion that becomes randomized among the total Golgi population after fusion, and was very similar for the two experiments (0.53 vs. 0.49). Indeed, the overall efficiency of inter-Golgi transfer, resulting in ~50% randomization, is striking. As pointed out in the Discussion section, even this is an underestimate due to intercompartmental transfer occurring during the pulse and before fusion, the actual efficiency of

TABLE I

Addition of Gal to G Protein after Fusion of VSV-infected Clone 13 Cells to Uninfected Clone 15B Cells

Protocol	[^{3}H]GlcNAc-labeled G protein containing Gal (percent of total)*			
	Expt. I		Expt. II	
	%	(*n*)	%	(*n*)
A. Pulse, fuse	32 ± 9	(3)	25 ± 3	(3)
B. Pulse, don't fuse	5.9	(1)	5.4 ± 0.7	(2)
C. Pulse, fuse to clone 13	4.6 ± 1.1	(2)	5.3 ± 0.4	(2)
D. Fuse, then pulse ("prefuse")	60	(1)	51	(1)

* The percent of ^{3}H-glycopeptides that bound to ricin agglutinin. Glycopeptides were prepared from [^{3}H]GlcNAc-labeled G protein, synthesized in clone 13 cells as schematized in Fig. 1, following several different protocols: (A) Pulse-labeled with [^{3}H]$GlcNH_2$, then immediately fused. (B) Not fused. (C) Same as A but uninfected clone 13 cells replaced clone 15B cells in forming the mixed monolayer. (D) Prefusion control. Fused, allowed 30 min for G to randomize, and then pulse-labeled. In all cases, cells were harvested 1 h after fusion and/or chase was begun. See text and Materials and Methods for details. Shown, for two experiments, are the average values, standard deviations, and number of independent determinations in parentheses (each a separate plate). The ricin binding of each glycopeptide preparation was determined in duplicate, and these agreed to within 5%.

randomization among Golgi complexes being closer to 100%.

When the fusion step (the pH 5 treatment) was omitted, very little galactosylation (~5%) occurred (line B). When uninfected clone 13 cells replaced clone 15B cells in the fused mixed monolayers (line C), a similarly low level of galactosylation resulted. Together, these controls show that fusion to a complementing mutant is necessary for the galactosylation to occur, and that neither the act of fusion nor the pH 5 treatment somehow correct the defect in clone 13 cells. The low level of addition of Gal in clone 13 may reflect some leakiness in this mutation.

Transport in Golgi Complex within a Single Cell and Transport between Golgi Populations from Different Cells Occur at Similar Rates

The rapidity and efficiency with which G protein is transported between two Golgi populations after cell fusion (Fig. 3) prompted us to examine the kinetics of the corresponding process of transport within a single Golgi population in unfused cells, and also to see whether the act of fusion has any effect on this rate.

For this purpose we have used homogeneous monolayers of VSV-infected CHO clone 1021 cells. Clone 1021 is a line that will incorporate GlcNAc and Gal into proteins transported through its Golgi complex, but will not add sialic acid (2). Monolayers were pulse-labeled with [^{3}H]$GlcNH_2$ after cycloheximide pretreatment and harvested after varying periods of chase.

The percentage of [^{3}H]GlcNAc-labeled G protein terminating in Gal as a function of the time of chase was measured by the ricin binding of the glycopeptides (Fig. 4*a*). This process occurred with a half-time of ~5 min. These kinetics (Fig. 4*a*), within a single Golgi complex or population of Golgi complexes, are virtually superimposable upon those for the same transport segment purposefully measured between two Golgi populations (Fig. 3), apart from the initial lag of ~5 min (the time required for fusion) in the latter case.

The similarity of these rates makes it plausible to consider that the inter-Golgi transfers detected upon fusion of mixed

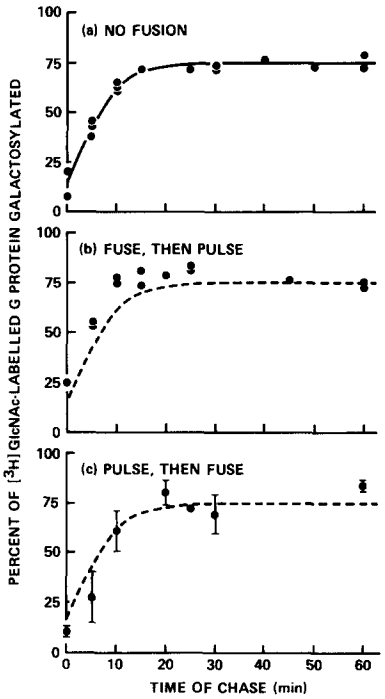

FIGURE 4 Kinetics of addition of Gal after the incorporation of [^{3}H]*N*-acetylglucosamine into G protein in the Golgi complex when cells are (*a*) not fused (i.e., transport occurs without fusion), (*b*) labeled immediately after fusing (i.e., transport occurs after fusion), or (*c*) labeled and then immediately fused (i.e., transport occurs during fusion). Confluent 6-cm monolayers of CHO clone 1021 cells were infected with 5 plaque-forming units/cell of VSV and used 3 h after infection. No NH_4Cl was present at any stage in these experiments. (*a*) Monolayers were treated with cycloheximide for 7 min and then labeled with [^{3}H]$GlcNH_2$ in the presence of cycloheximide for 3 min, and chased for the indicated period of time before harvest, exactly as described in Materials and Methods for mixed monolayers except NH_4Cl was omitted from all media. Then, monolayers were solubilized in 0.5 ml vol and all of this immunoprecipitated with anti-G serum onto *S. aureus* cells as described for [^{3}H]palmitate experiments (21). SDS-gel electrophoresis confirmed that G was the only labeled protein in these immunoprecipitates (data not shown). G protein was released from *S. aureus* cells by boiling in 200 μl of 1% SDS, 15 mM dithiothreitol, 50 mM Tris·HCl (pH 6.8) and precipitated (with 25 μl of 2% Triton X-100 as carrier) by adding 200 μl of ice-cold 20% trichloracetic acid, and the precipitate was washed with 0.3 ml cold acetone. This pellet was air-dried; 50 μl of pronase (20 mg/ml in 0.1 M Tris·HCl [pH 7.5], 10 mM $CaCl_2$, with 1 drop of toluene per milliliter, preheated for 1 h at 50°C) was added, and incubated for 1 d at 50°, and then boiled. The percent of [^{3}H]GlcNAc-glycopeptide having Gal (●) was determined as the ratio of ^{3}H precipitated by ricin to that precipitated by wheat germ agglutinin, exactly as described in Fig. 2 and Materials and Methods. Almost all of the ^{3}H was precipitated by wheat germ agglutinin. Data are from two independent experiments. (*b*) Same as in *a*, except the cells were fused after the 7-min cycloheximide pretreatment and before the 3-min pulse. Chase was initiated immediately after the pulse. (*c*) Same as in *a*, except that the cells were exposed to pH 5 in between the 3-min pulse and the initiation of chase. Data are from two independent experiments. The error bars indicate ±1 SD for duplicate or triplicate determinations; the data point in the center is the mean. The markedly poorer agreement among replicates in *c*, as compared with *a* or *b*, is due to plate-to-plate variations in the timing of the fusion step that are difficult to precisely control and that separate pulse from chase in these experiments but not *a* and *b*. The error is most pronounced at the earlier time points. The dashed lines in *b* and *c* are not drawn to fit the data presented therein, but are copies of the solid line from *a* redrawn for comparison.

monolayers occur to a similar extent within single cells. Consistent with this is the fact that fusion of a homogeneous monolayer of VSV-infected clone 1021 cells has no significant effect upon the kinetics of addition of Gal after the incorporation of GlcNAc in the Golgi. Fig. 4*b* shows the kinetics for this process when measured in cells that have already been fused, and Fig. 4*c* shows the kinetics in cells in the process of fusing. The dashed line shows the kinetics for the unfused cells, redrawn from Fig. 4*a* for comparison. The poor agreement between the replicate plates in Fig. 4*c* at the early time points (the bars show 1 SD about the mean) is due to the variability in the time needed for the manipulations required to fuse the cells in between the pulse and the chase.

A potential complication in the interpretation of these experiments arises from the use of NH_4Cl to prevent the infection of one of the cell populations in mixed monolayers. It was therefore important to ascertain whether or not NH_4Cl, under our experimental conditions, has a quantitatively significant effect on the transport of VSV G protein. Fig. 5*a* shows that the overall rate of intracellular transport of G protein—the kinetics of appearance in virions of G protein labeled in the rough endoplasmic reticulum with [^{35}S]Met—is not affected by 15 mM NH_4Cl. Similarly, the rate of the particular transport segment upon which this study is focused—the movement of G protein for the site of GlcNAc addition to the site of Gal addition in the Golgi complex—is not affected by NH_4Cl (Fig. 5*b*). All of the experiments in Fig. 4 were carried out in the absence of NH_4Cl.

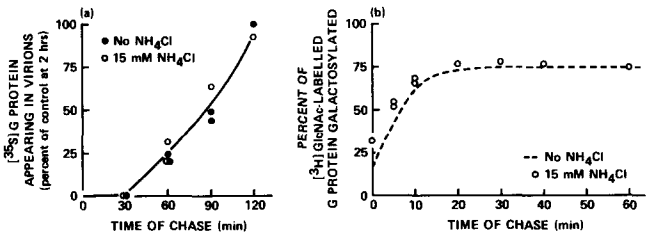

FIGURE 5 (*a*) Lack of effect of ammonium chloride on the kinetics of transport of G protein from rough endoplasmic reticulum to budded virions. Confluent 6-cm monolayers of VSV-infected CHO clone 1021 cells were pulse-labeled with 25 μCi per plate of [^{35}S]-Met in 2 ml of Met-free MEM for 10 min, starting at 3 h postinfection. Then, a variable period of chase in growth medium plus 2.5 mM unlabeled Met was begun. At the appropriate time, the medium was removed from a plate and virions pelleted as described in Materials and Methods. The pellet was dissolved in sample buffer, electrophoresed in a 10% polyacrylamide SDS gel which was dried and autoradiographed. The relative amounts of ^{35}S-labeled G protein in the pellets were then determined as the area under the G band by densitometry of the X-ray film. Two such experiments were conducted in parallel. In one (●) no NH_4Cl was present at any stage, as just outlined. In the other (○), 15 mM NH_4Cl was added at 1 h postinfection and maintained at every stage thereafter. Plotted is the amount of ^{35}S-G protein in the viral pellet as a function of the time of chase at which the medium was harvested, expressed as a percent of the value obtained for the NH_4Cl-free experiment at 2-h chase. The amount of ^{35}S-G protein synthesized in the 10 min pulse was the same with and without NH_4Cl (data not shown). (*b*) Lack of effect of NH_4Cl on the kinetics of addition of Gal after the incorporation of [^{3}H]GlcNAc into G protein in the Golgi complex of VSV-infected CHO clone 1021 cells. The same experiment as in Fig. 4*a* except 15 mM NH_4Cl was added at 1 h postinfection and maintained at every step thereafter. The dashed line is not drawn to fit the data from this experiment (○), but instead is redrawn from Fig. 4*a* to allow comparison.

Availability of G protein for Transfer Depends upon Its Location in the Golgi Complex at the Time of Fusion

Experiments presented in the preceding article (21) show that freshly acylated G protein, pulse-labeled with [^{3}H]palmitate just before fusion, will transfer to an exogenous Golgi population (to receive peripheral GlcNAc). But, when a period of chase is allowed to permit further transport before cell fusion, G protein rapidly becomes unavailable for this same transfer, with a half time of ~5 min.

A very similar result is obtained when a period of chase separates the labeling of G protein with [^{3}H]GlcNAc (in the Golgi complex of clone 13 cell) and fusion (Fig. 6). The fraction of [^{3}H]GlcNAc-labeled G protein that eventually reaches the 15B Golgi complex to receive Gal falls precipitously as the period of transport before fusion is increased, declining with a half time of ~5 min.

There are two general kinds of explanations for these kinds of behaviors. On the one hand, G protein could be transported during the chase into a new location in the cell from which it does not transfer after cell fusion; the time course (Fig. 6) would then measure the rate of its entry into this new compartment. Alternatively, G protein might stay in the same physical location during this brief period of chase but be modified or associated with other component(s) so as to prevent its transfer. The latter kind of possibility seems to be ruled out because during this same period after the addition of GlcNAc in the Golgi complex, G protein can move between Golgi complexes (Fig. 3) and therefore is undergoing transfers to new locations.

Given that G protein is relocating during the time course in Fig. 6, where is it going to? On the one hand, G protein could be leaving the Golgi complex entirely, on its way to the plasma membrane (which would readily explain why this pool of G protein would not reenter a second Golgi population after fusion). On the other hand, G protein could be entering a new and later compartment within the Golgi complex. Given that [^{3}H]GlcNAc-labeled G protein becomes unavailable for transfer (Fig. 6), with the same kinetics that it normally receives Gal (Figs. 3 and 4) in the Golgi complex, it

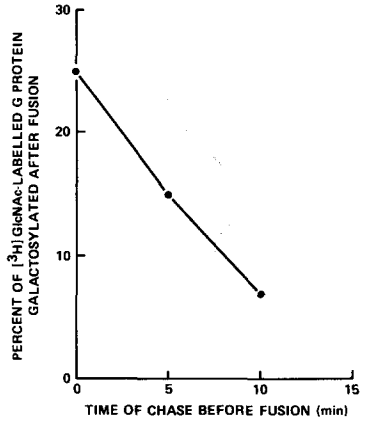

FIGURE 6 Effect of a period of chase before fusion of [^{3}H]GlcNH$_2$-labeled VSV-infected clone 13 cells with uninfected clone 15B cells. Plotted is the extent of galactosylation of [^{3}H]GlcNAc-labeled G protein 1 h after fusion versus the time at which fusion was initiated. Protocol as in Fig. 3, except a variable period of chase (0, 5, or 10 min) intervened between the 3-min pulse and the initiation of fusion.

would seem likely that the compartment it is entering is still within the Golgi complex. Indeed, the simplest possibility would be that GlcNAc and Gal are added in distinct and sequential compartments in the Golgi complex, between which G protein is transported unidirectionally. Transfer between these compartments would normally occur by a dissociative process, which in our experiments is detected as transfers between the two compartments residing in different Golgi populations. Once the intercompartmental transfer has already occurred (before fusion), it could not take place again (after fusion).

Compartmental Specificity of the Transfer Process

This hypothesis makes a strong prediction. G protein, freshly labeled with [^{3}H]GlcNAc in the earlier (GlcNAc) Golgi compartment, should be efficiently transferred to the later (Gal) compartment of a second Golgi population after fusion. But G protein already within the later (Gal) compartment, freshly labeled with [^{3}H] Gal, should be poorly transferred to the same sites after fusion.

To test this, we have constructed mixed monolayers consisting of VSV-infected CHO clone 1021 cells and uninfected clone 15B cells (Fig. 7). Clone 1021 will add both GlcNAc and Gal to G protein in its Golgi complex, but will not incorporate sialic acid (2). Clone 15B cells, missing only GlcNAc transferase I, can potentially incorporate both Gal and sialic acid in their Golgi complexes. Therefore, G protein can be pulse-labeled with either [^{3}H]Gal or [^{3}H]GlcNAc in the Golgi complex of clone 1021 cells, which can be fused to clone 15B cells to see whether the two forms of G protein will be transferred to an exogenous Golgi population with differing efficiencies. This transfer can be monitored by the incorporation of sialic acid that occurs after arrival (G protein labeled with [^{3}H]GlcNAc would also have to receive Gal in the 15B Golgi complex).

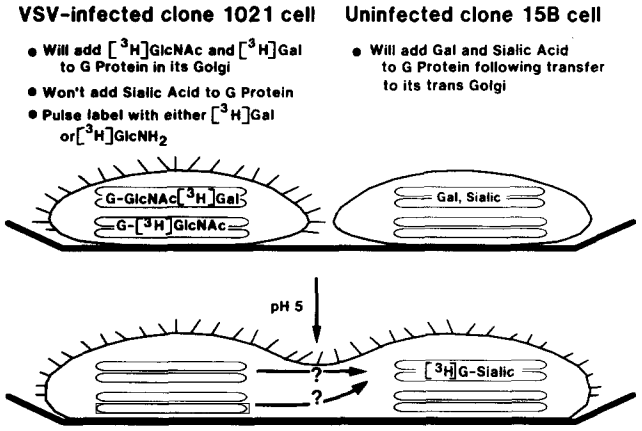

FIGURE 7 Design of a cell fusion experiment to measure the relative efficiency with which G protein present in two different Golgi complex subcompartments (in which GlcNAc and Gal, respectively, are added) is transferred to an exogenous Golgi population. A mixed monolayer is formed containing VSV-infected CHO clone 1021 cells and uninfected clone 15B cells. VSV G protein is labeled in the Golgi complex of clone 1021 cells either with [^{3}H]GlcNH$_2$ (incorporated as peripheral GlcNAc) or with [^{3}H]Gal. Clone 1021 is able to incorporate both of these sugars, but not sialic acid. The clone 1021 cells, now harboring ^{3}H-G protein in their Golgi complexes, are then fused to neighboring clone 15B cells (via a brief exposure to pH 5) whose Golgi complex are able to add sialic acid. Transfer of G protein from the clone 1021 to the clone 15B Golgi complex is monitored by the addition of sialic acid to the ^{3}H-G protein upon arrival. In the case of the [^{3}H]GlcNAc-labeled G protein, the addition of Gal (presumably in the 15B Golgi complex) must occur before sialic acid can be added.

To assay the addition of sialic acid to [^{3}H]GlcNAc- and [^{3}H]Gal-labeled G protein, the G protein was purified from detergent extracts of fused monolayers by immunoprecipitation and SDS–gel electrophoresis, and pronase glycopeptides were prepared as before. To distinguish those glycopeptides that contained terminal sialic acid, we employed the recently described (15) *Limax flavus* agglutinin. This slug lectin is specific for sialic acid (15), and like ricin we have found that it can be used in a binding assay, in which complexes of sialylated glycopeptides with slug lectin are separated from unbound glycopeptide by an ammonium sulfate precipitation. Fig. 8 demonstrates the specificity of the slug lectin binding assay for sialic acid-containing VSV G protein glycopeptides. A maximum of ~30% of the sialylated glycopeptides made in wild-type CHO cells is bound. Binding is prevented by competition with excess fetuin, is abolished by neuraminidase digestion, and is not detectable when Gal-terminating VSV G protein glycopeptides (made in a mutant CHO cell unable to add sialic acid) are used as substrate.

Microheterogeneity in sialic acid combined with a strong preference of slug lectin for glycopeptides containing two or more sialic acid residues accounts for the relatively small fraction of wild-type glycopeptide that are bound. This is illustrated in Fig. 9, which presents a gel filtration analysis of the total VSV G glycopeptides made in wild-type CHO cells (labeled with ^{3}H, ●) and of the subfraction that can be bound to slug lectin (labeled with ^{14}C, ○). The Bio-Gel P-4 column separates VSV glycopeptides principally according to the

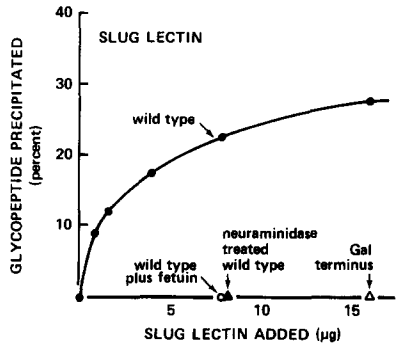

FIGURE 8 Specificity of slug lectin (*Limax flavus* agglutinin) for sialic acid-containing VSV glycopeptides. Pronase glycopeptide markers were prepared from [^{3}H]GlcNH$_2$-labeled G protein synthesized in a wild-type line of CHO cells, W5 (●), and a mutant isolated from this line that is unable to incorporate sialic acid but will incorporate Gal, Lec2 (△). Incubations for 15 min at 25°C contained 2 μl of glycopeptide (about 1,000 cpm) and the indicated amounts of slug lectin (0.8 mg/ml in 0.1 M NaCl, 50 mM Tris·HCl [pH 7.5], 0.02% NaN_3). In one case (▲), neuraminidase-digested wild-type glycopeptide was used (prepared by adding to the pronase digest 10 u/ml of neuraminidase and incubating for 16 h at 37°C, then boiling). In another case (○), intact wild-type glycopeptide was used but fetuin (7 mg/ml final concentration) was also added. Bound glycopeptide was precipitated with 4 M $(NH_4)_2SO_4$ containing albumin, washed once, and counted as described in Materials and Methods for ricin binding. Parallel incubations of each glycopeptide preparation were carried out with wheat germ agglutinin and processed exactly as described in Fig. 2 to precipitate all of the ^{3}H-glycopeptide present. Shown is the ratio of ^{3}H precipitated by slug lectin to that precipitated by wheat germ agglutinin, expressed as percent.

number of sialic acid residues that they contain (12) resulting in four major peaks labeled S_0, S_1, S_2, and S_3 in Fig. 9, containing 0, 1, 2, and 3 sialic acids per oligosaccharide chain, respectively. (The double peak of S_0 is probably due to microheterogeneity in fucose; it is not related to sialic acid because the profile of clone 1021 VSV glycopeptides consists of a double S_0 peak and no S_1, S_2, or S_3.) The glycopeptides from CHO cells consist of S_0, S_1, and S_2, with very little S_3. However, the slug lectin precipitate consists almost exclusively of S_2 and S_3, with only a trace of S_1 and no S_0.

Table II summarizes data on the extent of transport of [^{3}H]GlcNAc- and [^{3}H]Gal-labeled G protein from the Golgi of clone 1021 cells to the Golgi complexes of clone 15B cells, as measured by the percent of labeled glycopeptide that can bind to slug lectin by 1 h after fusion. An average of 10.3 ± 0.5% of [^{3}H]GlcNAc-labeled glycopeptide (line A) bound slug lectin, as contrasted to 3.7 ± 0.1% of the [^{3}H]Gal-labeled glycopeptide (line B). Much less sialylation occurred when

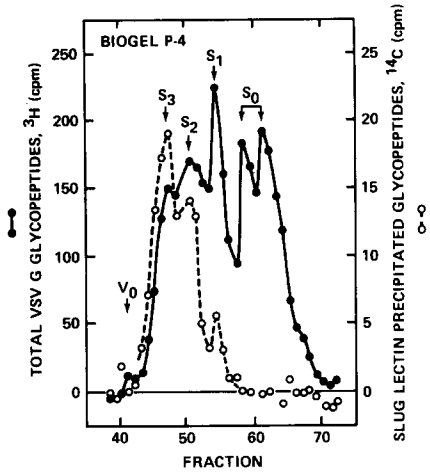

FIGURE 9 Separation of the glycopeptides that bind to slug lectin according to the number of sialic acid residues they contain, by gel filtration on Bio-Gel P-4. The slug lectin precipitate from ^{14}C-labeled glycopeptide (○) was co-chromatographed with total ^{3}H-glycopeptides (●). For this purpose, 10 μl of [^{14}C]Gal-labeled VSV glycopeptides (containing 1,100 cpm) synthesized in a wild-type line of CHO cells (W5) were incubated with 50 μl of slug lectin (0.8 mg/ml), precipitated with ammonium sulfate containing albumin, and washed with ammonium sulfate as in Materials and Methods. The precipitate was dissolved in 100 μl of 1% SDS–1% β-mercaptoethanol, boiled, and mixed with 10 μl of [^{3}H]GlcNAc-labeled VSV glycopeptides (containing 2,500 cpm) prepared from infected W5 CHO cells, and 90 μl of 33 mM EGTA–18 mg/ml bovine serum albumin–110 mM Galactose. This sample was loaded onto an 1-×-105-cm column of ~80 ml bed volume of Bio-Gel P-4 (minus 400-mesh, Bio-Rad) that was equilibrated and eluted with 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM NaN_3, using gravity flow. Fractions of 0.6 ml were collected and counted for 10 min to measure ^{14}C (○) and ^{3}H (●). The void volume, fraction 41, was determined as the peak fraction in which albumin eluted. The total included volume, judged by the elution of free [^{14}C]Gal, was in fraction 132. Only the fractions containing glycopeptides are shown. The positions of VSV glycopeptides containing 0, 1, 2, and 3 sialic acid residues (S_0, S_1, S_2, and S_3, respectively) were located by reference to glycopeptides from VSV-infected baby hamster kidney cells (in which S_3 is a major species [12, 17]) and CHO 1021 cells (in which S_0 is the only significant species due to the lack of sialic acid addition). Note that the sialic acid-free species (S_0) chromatographs as a double peak. This is also true of the 1021 cell VSV glycopeptide marker (not shown), and probably represents unsialylated species having or lacking a fucose residue.

the fusion step was omitted, 1.6 ± 0.2% for [^{3}H]GlcNAc (line C) and 1.9 ± 0.8% for [^{3}H]Gal (line B). A slightly, but not significantly, lower degree of sialylation was found in controls in which uninfected clone 1021 cells replaced the uninfected clone 15B cells in the formation of the mixed monolayers, 0.8 ± 0.3% (line E). This represents the background level of sialylation in the clone 1021 mutant cells. To rule out the possibility that any ^{3}H in the slug lectin precipitates was present as [^{3}H]sialic acid (derived by metabolism of [^{3}H]-$GlcNH_2$), glycopeptides were released from the precipitate by boiling and digested with neuraminidase. No ^{3}H was released from the glycopeptides; however, all of the sialic acid was released, as judged by the fact that the binding of the glycopeptides to slug lectin had been abolished (data not shown).

It is particularly important to recognize that in these fusion experiments only a fraction (roughly one-third) of the G-protein molecules (Figs. 8 and 9) transported will actually be scored in the slug lectin-binding assay, due to the microheterogeneity of the sialylation process and the requirement of multiple sialylation for binding. This factor accounts for the apparently low numbers in Table II as contrasted to Table I. In fact, the actual efficiency of transfer of [^{3}H]GlcNAc-labeled G protein is very similar in the two types of fusion experiments. If only one-third of the transfers in Table II were scored in the slug-lectin assay employed, then ~10% × 3 = 30% of [^{3}H]GlcNAc-labeled G-protein molecules would have been transferred to the exogenous Golgi complex (to receive Gal and be incompletely sialylated). This is to be compared with the efficiency when Gal addition is directly measured in the clone 13 experiment, ~28% (Table I, average of Expts. I and II). The prefusion experiment is also important in this regard. This control, in which the mixed monolayer is fused 30 min before labeling to enable G protein to randomize amongst the two Golgi populations, measures the maximum fraction of glycopeptide that could possibly be scored in the slug lectin-binding assay. This value was 32.3 ± 1.9% (Table II, line F) for [^{3}H]GlcNAc and 30.3% for [^{3}H]Gal (line G), respectively. Additionally, the fact that very similar values were obtained for the two sugar precursors assures that no systematic differences exist between them to account for the differential sialylation that is observed in the transfer experi-

TABLE II

Addition of Sialic Acid to G Protein after Fusion of VSV-infected Clone 1021 Cells to Uninfected Clone 15B Cells

Protocol	^{3}H incorporated into	[^{3}H]G protein sialylated (percent of total)*	
		%	*n*
A. Pulse, fuse	GlcNAc	10.3 ± 0.5	(7)
B. Pulse, fuse	Gal	3.7 ± 0.1	(4)
C. Pulse, don't fuse	GlcNAc	1.6 ± 0.2	(2)
D. Pulse, don't fuse	Gal	1.9 ± 0.8	(2)
E. Pulse, fuse to clone 1021	GlcNAc	0.8 ± 0.3	(2)
F. Fuse, then pulse ("prefuse")	GlcNAc	32.3 ± 1.9	(4)
G. Fuse, then pulse ("prefuse")	Gal	30.3	(1)

* The percent of ^{3}H-glycopeptides that bound to slug lectin (*Limax flavus* agglutinin). Prepared from [^{3}H]GlcNAc- or [^{3}H]Gal- (as indicated) labeled G protein synthesized in clone 1021 cells, as schematized in Fig. 7. The seven different protocols used were as in Table I, with the additional complication that either of two ^{3}H-sugars ([^{3}H]$GlcNH_2$ or [^{3}H]Gal) were employed as labels. See text and Materials and Methods for details. In all cases, cells were harvested 1 h after fusion and/or chase was begun. The slug-lectin binding of each glycopeptide preparation was determined in duplicate, and these agreed to within 5%.

ment itself (Table II, line A vs. line B).

To determine the relative efficiency with which [^{3}H]-GlcNAc and [^{3}H]Gal-labeled G proteins are transferred, it is necessary to calculate the amount of sialylation resulting from fusion in the two cases. For [^{3}H]GlcNAc this is 10.3% − 1.6% = 8.7% (line A − line C). For [^{3}H]Gal this is 3.7% − 1.9% = 1.8% (line B − line D). The relative efficiency of transfer is then 1.8/8.7 = 21%. This is shown graphically in Fig. 10 together with the cumulative errors in the form of vertical bars. This fivefold difference in efficiency is also about that expected from the pulse-chase experiment in Fig. 6, in which G protein labeled with [^{3}H]GlcNAc (and moving toward the site in which Gal would be added) is 28% as efficiently transferred after 10 min of chase as at the outset.

Altogether, these experiments suggest that the population of G protein that is being transferred to exogenous Golgi complex is one that has received peripheral GlcNAc but has not yet received Gal. In that both of these sugars are added in the Golgi complex (11), it seems clear that the transferred pool in between these two biochemical landmarks is in the Golgi complex as well.

The observed behavior also provides a clear internal control (in addition to the morphological ones presented in the preceding article) to indicate that the observed glycosylations result from transfer of G protein between two Golgi populations that remain distinct. As alternatives, had the two Golgi populations fused, or had the glycosylation defect in the G protein-containing Golgi population been corrected by any mechanism (such as replacement of the missing protein or transferase by material transferred from the 15B Golgi population, etc.), then the efficiency of sialic acid addition to [^{3}H]-Gal-labeled G protein would have been the same or greater than that for [^{3}H]GlcNAc-labeled G protein, the opposite of what is actually found. This is because the normal glycosylation pathway would be restored within the G protein-containing Golgi membranes by such mechanisms, and galactosylated G protein is the immediate substrate for sialylation.

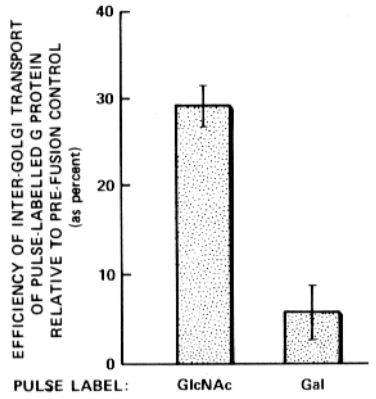

FIGURE 10 Efficiency of transfer of [^{3}H]GlcNAc and [^{3}H]Gal-labeled G protein from the Golgi complex of CHO clone 1021 cells. In each case, the percent of ^{3}H-G glycopeptides receiving sialic acids due to fusion with clone 15B cells (i.e., the increment in the percent of ^{3}H-glycopeptides bound to slug lectin due to fusion: 8.7 ± 0.7% for GlcNAc, 1.8 ± 0.9% for Gal; data from Table II) was divided by the corresponding value for the prefusion control (30%; Table II) and expressed as a percent. The error bars represent ±1 SD, calculated from the replicate determinations in Table II. The prefusion control in which G is allowed to randomize among the two Golgi populations before labeling measures the maximum amount of sialylation that can occur, and serves as an internal standard to gauge efficiency (see text).

DISCUSSION

Compartment Boundaries in the Golgi Complex

The properties of inter-Golgi complex transport in fused cells offer two new lines of evidence for a division of the Golgi complex into functionally distinct compartments.[2]

First, G protein labeled with GlcNAc in one Golgi complex is efficiently transported to another to receive Gal residues. This inter-Golgi transport occurs at essentially the same rate as transit within the Golgi complex in a single cell. Therefore, a dissociative movement is required to carry G protein from the site at which GlcNAc is added to that in which Gal is added, implying that these two sugars are added in physically distinct parts of the Golgi complex.

Second, transport between these parts of the Golgi complex is vectorial, implying that a compartment boundary intervenes. This follows from the observation that once this transfer has already occurred before cell fusion, it cannot take place again after cell fusion. This, in turn, implies that the inter-Golgi complex transfer being studied after cell fusion also occurs before cell fusion—protein transport in the Golgi complex must normally be a dissociative process. In general, an irreversible step is needed to effect a vectorial process. The addition of Gal per se does not constitute the irreversible step, since the same behavior is observed in experiments with the clone 13 mutant in which Gal is not added.

Addition of Gal (and possibly sialic acid) occurs in the *trans* cisternae of the Golgi stack (9, 18, 19, 22, 26). Presumably the compartment in which the GlcNAc is added consists of one or more earlier cisternae, but proof of this must await the immunocytochemical localization of GlcNAc transferases, now in progress.

How efficiently is G protein randomized among the Golgi populations during the intercompartmental transfer? About 50% (fraction galactosylated divided by prefusion control) of G protein labeled with [^{3}H]GlcNAc in clone 13 Golgi before

[2] Most of our knowledge of and concepts concerning subcellular compartments derive originally from electron microscopic studies which define compartments in morphological terms, as membrane-bound structures of distinct appearance. In general concept, a compartment is defined by the existence and properties of its boundaries. Therefore, a more widely applicable definition of subcellular compartments and one immediately pertinent to their molecular composition and their function in protein transport would be in terms of the selectivity of their boundaries. If a given molecule cannot move between two locations, or if it does so vectorially, then a compartment boundary can be said to exist to separate the two locations into distinct compartments. Such a boundary need not have an obvious morphological correlate. On the other hand, if a different molecule moves freely between these locations, then (from the point of view of this molecule) these two locations would be part of the same compartment or two copies of the same compartment. This definition encompasses the morphological concept of compartments, but also recognizes the complexity and specificity of biological transport processes by taking account of the possibility that the same compartment boundary can exist for, and be respected by, one type of molecule and be ignored by another. For example, each of several successive cisternae of the Golgi stack might contain different glycosyltransferases. So, boundaries would exist separating these cisternae into distinct compartments, and would be respected by these glycosyltransferases to prevent their intermixing. But a transported glycoprotein or a different set of glycosyltransferases might move randomly among these same cisternae. To such proteins, these cisternae would be indistinguishable and would represent multiple copies of a single compartment.

fusion was found to redistribute among the total Golgi population after cell fusion. In fact, this is a gross underestimate, due to the intercompartmental transport of [^{3}H]GlcNAc-labeled G protein that occurs (Fig. 4*a*) within the clone 13 Golgi population during the lag period of ~5 min before fusion actually takes place (Fig. 3). In that G protein undergoes this transfer with a 5-min half time (Fig. 6), only about half of the [^{3}H]GlcNAc-labeled G protein in clone 13 cells will still remain in the earlier (GlcNAc) compartment at the actual time of fusion, and potentially be available for transfer to the later (Gal) compartment after fusion. The true efficiency of randomization amongst Golgi complexes due to this intercompartmental transfer is thus closer to $2 \times 50\% \cong 100\%$. This fits well with the findings that the kinetics of transfer to the Gal compartment are the same when measured within a single Golgi population and between Golgi populations (Figs. 3 and 4).

The conclusion from our cell-fusion studies that GlcNAc and Gal are incorporated in different compartments of the Golgi complex fits well with earlier evidence from in vitro transport, cell fractionation, and electron microscopic studies. Originally, two successive pools of VSV G protein were discovered to reside in Golgi fractions—one subject to in vitro transport, the other not (7). We therefore inferred the existence of at least two functionally distinct compartments in the Golgi complex (4, 20), prompting an intensive effort by subcellular fractionation to see if physical and kinetic evidence for compartmentation could be obtained. We found that when CHO membranes were fractionated on a sucrose gradient under appropriate conditions, mannosidase I was concentrated in a different set of Golgi membranes from galactosyltransferase and sialyltransferase, as were the products of these enzymes' in vivo action (4). Work from other laboratories as well as our own then confirmed and extended these findings to include several other Golgi marker enzymes in other tissue and cell types (3, 5, 8). All of these cell fractionation studies are in agreement concerning the basic finding that the Golgi GlcNAc transferases (I, II, and IV) co-fractionate on sucrose density gradients, and distribute differently from galactosyl- and sialyltransferases, which are not separated from each other; i.e., the two sets of transferases reside in differing locations.

The immunocytochemical localization of galactosyltransferase to the *trans* cisterna (19) then pinpointed this end of the stack as the site of the later (Gal) compartment. In this light, the separation of GlcNAc transferases from Gal transferase revealed by cell fractionation implies *cis-trans* separation of glycosyltransferases in the Golgi complex as originally proposed (20). The *cis-trans* asymmetry in lectin-staining patterns (9, 18, 22, 26) provides independent evidence of a *cis-trans* asymmetry and also a morphological correlate of this conclusion concerning the glycosyltransferases, but is less direct. Here we provide a completely different kind of evidence pointing to the same compartmentation of these glycosyltransferases. Moreover, the new findings imply that this compartment boundary is respected by a glycoprotein in transit, the VSV G protein, which undergoes a vectorial transport across it.

In the previous paper (21) we described experiments along the same lines as those reported here, but different in an essential way. Here, we measure transers of G protein from a Golgi compartment in which GlcNAc is added to a compartment in which Gal is added. Before (21), we measured transfers of [^{3}H]palmitoyl–G protein (that had just entered the Golgi complex as judged by cell fractionation and by the trimming by Golgi mannosidase I [4]) into a Golgi compartment in which GlcNAc is added; i.e., the destination of G protein in the preceding paper is the origin of G protein in the present paper.

We take this to mean that during transport through the Golgi complex, dissociative transfers move G protein into the GlcNAc compartment and out of it as well. The efficiency of both of these transfers, measured after fusion, promptly diminished with increasing chase before fusion, and with the same kinetics as the corresponding glycosylation in wild-type cells (4). This suggests in both cases that the transfers cannot occur again after fusion if they have already taken place before fusion, i.e., that these dissociative transfers into and out of the GlcNAc compartment are both vectorial.

Altogether, we are most likely dealing with at least three sequential compartments in the Golgi stack: the previous report (21) measured transfers from the first compartment (in which freshly acylated G protein resides upon entering the Golgi complex) to a second one (housing GlcNAc transferase I and mannosidase II, conferring endoglycosidase H resistance). This paper studies transfers from the second compartment to a third and terminal one (containing Gal and presumably sialic acid transferases, and consisting of the *trans* cisternae). Our data thus fit well with the three-compartment (*cis, medial, trans*) division originally proposed by Warren and colleagues on the basis of lectin-staining patterns (10). It is the transfer from the first to the second compartment that is most likely being measured in our cell-free system (4, 7, 21). However, it is not inconceivable that other compartments for which we currently lack markers may intervene among (or consist of further subdivisions of) these three. Indeed, provided that mutants missing the appropriate Golgi markers are available (23), it should be possible to apply the strategy developed here of studying inter-Golgi transfers to deduce the complete number and sequence of vectorial transfers (and therefore the number of compartments functioning) in the Golgi stack.

A New View of Transport in the Golgi Stack

It is extraordinary that intercompartmental transport in the Golgi complex occurs at essentially the same rate and efficiency when purposefully measured between two distinct Golgi populations (Fig. 3), as when measured within single cells (Fig. 4). This finding suggests that intercompartmental transport in the Golgi complex occurs by a dissociative process whose specificity is based upon a biochemical pairing and not physical proximity, which cannot distinguish whether the target is in the same or another Golgi stack. The most plausible mechanism would be one in which vesicles would bud off from the rim of a cisterna of one compartment, dissociate from the stack, and then attach to and fuse with a cisterna of the next compartment. The dissociated vesicles could then diffuse away,[3] binding to the appropriate target cisterna in the

[3] Physical diffusion of transport vesicles between two Golgi stacks need not be rate limiting even when the stacks are separated by a cell diameter (as in the fusion experiments). We can estimate how viscous (η) the cytoplasm would have to be to slow the diffusion of a 2.5×10^{-6} cm radius (R) transport vesicle to the point at which 300 s (t) would be needed to diffuse one cell diameter ($x = 1 \times 10^{-3}$ cm) at

same or another Golgi stack based on random encounter. This would happen unavoidably unless a special mechanism existed to prevent the budded vesicles from escaping. The rims of the Golgi complex are, of course, associated with numerous small vesicles.

Our experiments and their implications can help to distinguish between this kind of mechanism and several other widely discussed possibilities for protein transport in the Golgi stack (6, 20, 22, 25) diagrammed in Fig. 11. Cisternal progression (Fig. 11 *a*) is a model in which new cisternae form at one end and are consumed at the other, and so the cisternae themselves move across the stack as intact units. In this scheme there are no transfers between cisternae; hence, inter-Golgi protein transport would not be possible. Cisternal progression is also difficult to rationalize with the existence of compartment boundaries in the Golgi stack. Another proposal (Fig. 11 *b*) is that protein passes through the stack by lateral diffusion between transiently or permanently fused cisternae. Such fusions can occasionally be seen by electron microscopy, but it has not been clear whether they are real or, instead, fixation artifacts. Again, there are no dissociative transfers in the lateral diffusion scheme that would permit a facile inter-Golgi transport, and compartment boundaries would be hard to envision. Vesicular transport between the extensive apposing surfaces of adjacent cisternae (Fig. 11 *c*) would not allow escape from the stack to result in inter-Golgi transport. Therefore, the majority of intercisternal transfers are not likely to be *en face*.

The properties of inter-Golgi transport so far elucidated do not distinguish among many possible schemes for vesicular transport at the rim of the stack. At one extreme (Fig. 11 *d*), each cisterna could be a distinct compartment whose boundaries are respected by proteins in transit. If so, each vesicular transfer step could be a vectorial one, and a protein would pass across the stack from one cisterna to the next, undirectionally. This scheme would allow only one chance for inter-Golgi movement with each intercompartmental transfer. At the other extreme (Fig. 11 *e*) transport could occur within a block of cisternae (comprising multiple copies of the same compartment). Transit among these cisternae could proceed akin to a random walk. Such a scheme might allow many chances for inter-Golgi movement during a net passage across a stack.

In summary, the finding of a facile process for inter-Golgi transfer in CHO cells suggests a new view of the operation of the Golgi stack. The structure of the Golgi complex as a stack has led to the natural assumption that protein transport through it is processive; that is, a protein would necessarily progress in systematic fashion across a given stack. Our observations suggest the opposite. Protein transport in the Golgi stack is fundamentally a stochastic and dissociative process. Transport vesicles budding from cisternae dissociate, can diffuse away, and are capable of fusing with the appropriate target present in the same or a different stack, based on a random encounter.

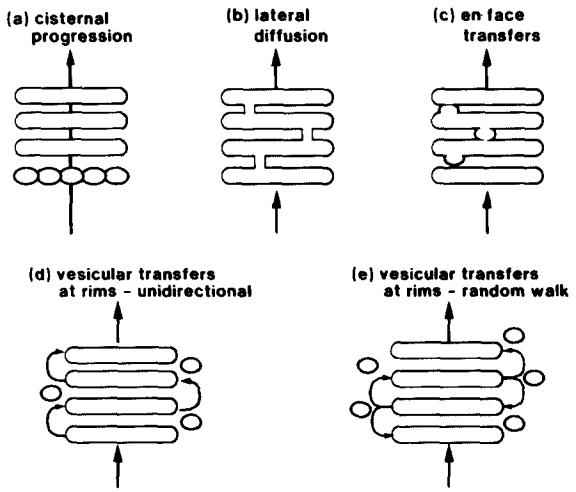

FIGURE 11 Illustrations of several possible mechanisms for transport of protein and other macromolecules within the Golgi stack. (a) Cisternal progression. New cisterna form from vesicles at the *cis* face, the oldest cisterna at the *trans* face is consumed by shedding or vesiculation. As a result, the cisternae themselves move across the stack; intercisternal transfers are not needed to effect transport across the stack. (*b*) Lateral diffusion. Proteins diffuse through continuities between membranes of adjacent cisterna, transient or permanent. (c) *En face* vesicular transfer. Vesicles budding from cisternae and fusing with neighboring cisternae are confined to the intercisternal spaces, the extensive zones of apposition of the large flatered cisternae. (*d*) Unidirectional (vectorial) transfers by vesicles at cisternal rims. Vesicles bud and fuse exclusively at the edge of the stack. A vesicle that buds from the rim of cisterna 1 can only fuse with cisterna 2, etc. In general, this would require at least as many biochemically distinct transport systems as there are cisternae, each of which would be a distinct compartment. (e) Random walk. Same as *d*, except the transfers are no longer unidirectional. A vesicle budding from the rim of any given cisterna can fuse any other cisterna in a stochastic process governed only by a probability distribution. This kind of scheme would only require a small number of biochemically distinct transport systems, but many transfers would be required to traverse a stack.

Why, then, does the Golgi complex need to exist as a stack? Our experiments suggest that protein transport in the Golgi complex could occur equally well if the compartments involved were separated and located at random. It may well be that, in many cells (but apparently not CHO cells), the cytoskeleton will so retard the vesicles that sufficiently rapid diffusion between the Golgi compartments would require their close proximity, as in a stack. In such instances, vesicles would still dissociate from the Golgi stack after their budding, but might be restrained from escaping by a meshwork of surrounding cytoskeleton, acting like a dialysis bag to keep the vesicles nearby.

We thank Dr. Stuart Kornfeld and Dr. Pamela Stanley for kindly providing mutant CHO cell lines.

This work was supported by American Cancer Society grant BC-394 to Dr. Rothman and was greatly aided by a Young Investigator award to Dr. Rothman from the DuPont Corporation.

Received for publication 3 January 1984, and in revised form 28 March 1984.

300 °K (T), using the relation $\eta = (kTt/3\pi Rx^2)$, where k is Boltzmann's constant. The answer is $\eta = 0.5$ poise (~50 times the viscosity of water), meaning that the effective viscosity of cytoplasm would have to be at least this great for diffusion to become a limiting factor in inter-Golgi transfers. The effective viscosity of cytoplasm is not known and probably depends greatly on the cell type and on the size of the diffusing species, but current estimates are in the range of 0.05–0.3 poise (27) from translational diffusion measurements in cytoplasm.

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