Reconstitution of Constitutive Secretion Using Semi-intact Cells: Regulation by GTP but Not Calcium

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Abstract. Regulated exocytosis in many permeabilized cells can be triggered by calcium and nonhydrolyzable GTP analogues. Here we examine the role of these effectors in exocytosis of constitutive vesicles using a system that reconstitutes transport between the trans-Golgi region and the plasma membrane. Transport is assayed by two independent methods: the movement of a transmembrane glycoprotein (vesicular stomatitis virus glycoprotein [VSV G protein]) to the cell surface; and the release of a soluble marker, sulfated glycosaminoglycan (GAG) chains, that have been synthesized and radiolabeled in the trans-Golgi. The plasma membrane of CHO cells was selectively perforated with the bacterial cytolysin streptolysin-O. These perforated cells allow exchange of ions and cytosolic proteins but retain intracellular organelles and transport vesicles. Incubation of the semi-intact cells with ATP and a cytosolic fraction results in transport of VSV G protein and GAG chains to the cell surface. The transport reaction is temperature dependent, requires hydrolyzable ATP, and is inhibited by N-ethylmaleimide. Non-hydrolyzable GTP analogs such as GTPγS, which stimulate the fusion of regulated secretory granules, completely abolish constitutive secretion. The rate and extent of constitutive transport between the trans-Golgi and the plasma membrane is independent of free Ca\(^{2+}\) concentrations. This is in marked contrast to fusion of regulated secretory granules with the plasma membrane, and transport between the ER and the cis-Golgi (Beckers, C. J. M., and W. E. Balch. 1989. J. Cell Biol. 108:1245–1256; Baker, D., L. Wuestebuehe, R. Schekman, and D. Botstein. 1990. Proc. Natl. Acad. Sci. USA. 87:355–359).

Eucaryotic cells deliver newly synthesized proteins to the cell surface by sequentially transferring them through a series of membrane-bound compartments. In many differentiated cells, proteins are secreted via distinct pathways: they may be packaged into storage granules for regulated release in response to physiological stimuli, enter vesicles for constitutive secretion, or become incorporated into apical- or basolateral-bound vesicles for delivery to distinct domains of epithelial surface (for reviews see Burgess and Kelly, 1987; Simons and Fuller, 1985; Rodriguez-Boulan, 1989). The trans-Golgi region performs an essential function in sorting proteins into these different types of secretory vesicles. To date, many important questions concerning post-Golgi traffic remain unanswered. How does the trans-Golgi network generate such a diversity of transport vesicles? Are the mechanisms controlling budding, targeting, and fusion of regulated granules similar to or different from those for constitutive vesicles? How are these components sorted during assembly of vesicles at the trans-Golgi region? Answering these questions will require functional dissection of the molecular components involved in both constitutive and regulated secretion.

The mechanism of regulated secretion has been studied extensively using permeabilized cell systems and patch-clamp techniques (Knight and Baker, 1982; Dunn and Holz, 1983; Vallar et al., 1987; Howell et al., 1987; for reviews see Comperth, 1990; Almers, 1990; Burgoyne, 1990). These studies have identified calcium and GTP as two key components in this process; calcium and the nonhydrolyzable GTP analogue guanosine 5'-O-(3-thiotriphosphate) (GTP\(^\gamma\)S)\(^1\) often act synergistically to trigger secretion from storage granules. Interestingly, both calcium and GTP-binding proteins have also been shown to operate in other steps of the secretory pathway. However, their exact roles in these steps appear to be different from regulated exocytosis. In both mammalian and yeast cells, transport from the ER to the cis-Golgi region in reconstituted systems requires calcium (Beckers and Balch, 1989; Baker et al., 1990). Optimal transport occurs at 50–200 nM free Ca\(^{2+}\), which is at least one order of magnitude lower than that required to trigger regulated secretion. The requirement for calcium is relatively late, suggesting that it may also be needed for vesicle fusion. Un-

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1. Abbreviations used in this paper: CPC, cetylpyridinium chloride; EndoH, endoglycosidase H; GAG, glycosaminoglycan; GMP-PNP, guanylylimidodiphosphate; GTP\(^\gamma\)S, guanosine 5'-O-(3-thiotriphosphate); NEM, N-ethylmaleimide; SL-O, streptolysin-O; VSV G protein, vesicular stomatitis virus glycoprotein; xyloside, 4-methylumbeUiferyl-\(\beta\)-D-xyloside.
like regulated exocytosis which is stimulated by GTPγS, transport between ER and Golgi complex (Beckers and Balch, 1989; Baker et al., 1990), as well as between Golgi cisternae (Melancon et al., 1987), is blocked by GTP analogues.

At present, the role of calcium and GTP-binding proteins in constitutive transport from the trans-Golgi region to the cell surface is less clear. Secretion from intact fibroblast cells is impaired when the cellular Ca2+ level is lowered by calcium ionophore A23187 and EGTA, suggesting that calcium may be required for constitutive transport. However, the same treatment has no effect on secretion from macrophages (Tartakoff et al., 1978). Using perforated baby hamster kidney (BHK) cells, deCurtis and Simons (1988) observed that transport of Semliki Forest virus glycoproteins to the cell surface requires added Ca2+. In this case, it is not yet clear whether calcium is directly involved in the transport process, or if it prevents the release of exocytic vesicles from the perforated cells (as was found with perforated MDCK cells; Bennett et al., 1988), thereby enhancing the efficiency of delivery to the plasma membrane. Another unresolved issue is the role of guanyl nucleotides. Studies of secretion mutants of the yeast Saccharomyces cerevisiae have elucidated the involvement of a GTP-binding protein, Sec4p, in constitutive transport between the Golgi complex and the cell surface (Salminen and Novick, 1987, 1989; Walworth et al., 1989). Woodman and Edwardson (1986) developed an in vitro transport system using postnuclear supernatants from BHK cells but the effect of nonhydrolyzable GTP analogues on constitutive transport in this system has not been reported. Using an in vitro budding assay, Tooze and Huttner (1990) found that GTPγS inhibited the formation of both regulated and constitutive post-Golgi vesicles by ~50%.

In view of the lack of a clear picture of the requirements for constitutive secretion, we have sought to use an independent method to study the components involved in this process. In this paper, we developed a semiintact cell system that efficiently reconstitutes budding from the trans-Golgi complex and fusion with the plasma membrane. We chose a method of permeabilization in which the cell structures remained relatively intact and the post-Golgi vesicles were retained within the cell. We used two independent markers to ensure measurement of vesicle fusion rather than membrane lysis. Using this system, we compare the requirements for Ca2+ for constitutive secretion to the fusion of regulated secretory granules. We also describe the effects of GTPγS on this step in biosynthetic transport. This system will be useful for defining components involved in constitutive secretion in mammalian cells.

Materials and Methods

Materials

Streptolysin-O (SL-O), purchased from Burroughs-Wellcome (Research Triangle Park, NC), was reconstituted as a 20 U/ml stock solution in distilled H2O and stored frozen in small aliquots at ~80°C. Each aliquot was thawed rapidly at 37°C immediately before use and aliquots were only used once. EGTA was from Fluka AG (Buchs, Switzerland). Hexokinase, creatine kinase, and creatine phosphate were from Sigma Chemical Co. (St. Louis, MO). ATP was from Calbiochem-Behring Corp. (San Diego, CA). GTPγS, guanylyl-imidodiphosphate (GMP-PNP), and endogycosidase H (EndoH) were from Boehringer Mannheim Biochemicals (Indianapolis, IN). [35S]SO4 and [35S]-methionine were obtained from Amersham Corp. (Arlington Heights, IL). Fixed Staphylococcus aureus cells (Immunoprecipitin) were from Bethesda Research Laboratories (Gaithersburg, MD). A stock solution of 0.5 M 4-aminopyridinium-9-α-trityl-β-D-glucoside (Sigma Chemical Co.) was prepared in DMSO and stored at ~20°C. All other reagents were of the highest quality available and obtained from the usual sources.

Preparation of Bovine Brain Cytosol

Bovine brains were obtained fresh from the slaughterhouse or stored frozen at ~80°C. 10 g of bovine brain was homogenized in 20 ml of 25 mM Hepes-KOH (pH 7.2), 0.1 M K-glutamate, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml leupeptin, 1 μM pepstatin, and 0.5 mM 1,10-phenanthroline by eight strokes in a motorized glass/Teflon homogenizer. This crude homogenate was centrifuged by centrifugation at 800 rpm for 20 min in a rotor (model SS-34; Sorvall Instruments Div., Newton, CT) and the resulting supernatant was centrifuged at 50,000 rpm for 90 min in a rotor (model 50.2 Ti; Beckman Instruments Inc., Palo Alto, CA). The high speed supernatant (cytosol) was frozen immediately in liquid nitrogen in small aliquots. Frozen cytosol fractions were stored at ~80°C with no detectable loss of activity over several months. The protein concentration of cytosol preparations ranged from 6.5 to 9.5 mg/ml. Protein concentrations were determined using protein assay (Bio-Rad Laboratories, Richmond, CA) with BSA as a standard.

SL-O Permeabilization

CHO cells grown on poly-l-lysine-coated coverslips were washed free of media and incubated for 4 min at 37°C in varying concentrations of SL-O in 20 m Hepes-KOH (pH 7.2), 125 mM K-glutamate, 5 mM EGTA, 5 mM free Mg2+ (as MgCl2), 15 mM KCl, 5 mM NaCl (buffer A) containing 5 μg/ml propidium iodide. The coverslips were rinsed with buffer A, inverted onto glass slides, and random fields were immediately photographed for quantitation using a fluorescence microscope. Under these conditions <5% of CHO cells incubated without SL-O display staining with propidium iodide. 100% of CHO cells are permeabilized after 3-4 min in 0.1-0.2 U/ml of SL-O (see Results). Equivalent concentrations of SL-O also resulted in the quantitative permeabilization of cells grown on plastic tissue culture dishes (not shown). For the experiments described here we routinely used 0.2 U/ml SL-O for permeabilization. For determination of SL-O permeabilization of cells in suspension, CHO cells grown on 10-cm dishes were detached by incubation in Ca2+/Mg2+-free PBS and washed three times with buffer A by centrifugation/resuspension. The cells were resuspended at 106 cells/μl in ice cold buffer A, mixed with an equal volume of buffer A containing various concentrations of SL-O, and then incubated for 4 min at 37°C. An aliquot was immediately withdrawn, spotted onto a glass cover-slip, and photographed for quantitation using a fluorescence microscope. Under these conditions <5% of CHO cells were stained by propidium iodide in the absence of SL-O and 100% staining was obtained at 0.05-0.10 U/ml SL-O.

In Vitro Transport Reaction

Attached Cells. Cells grown in 12-well plates were washed twice with PBS, and then incubated at 37°C in 20 mM Na-Hepes (pH 7.2), 110 mM NaCl, 5.4 mM KCl, 0.9 mM Na2HPO4, 10 mM MgCl2, 2 mM CaCl2, and 1 glu-l ter glucose (buffer B) containing 500 μM xylose. After 30 min the media was aspirated, 250 μl of buffer B containing 100-200 μl/cm2 [35S]SO4 (25-40 Ci/mg) was added, and the incubation continued for 2 min at 37°C. The labeling media was aspirated and the cells were washed quickly with two 1-ml washes of buffer B supplemented with 4 mM Na2SO4. SL-O permeabilization was immediately initiated by the addition of 250 μl of buffer A containing 0.2 U/ml SL-O and an ATP-depleting system (30 IU/ml hexokinase, 2 mM glucose). Permeabilization was effected by incubating the cells for 4 min at 37°C. Sham incubations were carried out by incubating pulse-labeled cells in 250 μl of buffer B containing 4 mM Na2SO4 for 4 min at 37°C. After permeabilization cells were incubated for 60 min at 4°C in 0.5 ml of buffer A (or buffer B for sham permeabilization) with one change after 30 min. The cells were then shifted to 37°C and incubated for varying times in 250 μl of transport buffer (20 mM Hepes-KOH, pH 7.2, 80 mM K-glutamate, 15 mM KCl, 5 mM NaCl, 5 mM EGTA, 5 mM free Mg2+ (as MgCl2), and various concentrations of Ca2+ in the absence of EGTA, as described above) or an ATP-regenerating system (40 IU/ml creatine kinase, 2 mM creatine phosphate, 500 μM ATP and 325-475 μg (1-3.1-9 mg/ml) cytosol). In experiments in which free Ca2+ was varied, the total concentration of Ca2+ was adjusted to give the indicated concentrations of free Ca2+; the total concentration of EGTA was...
maintained at 5 mM in all cases. In each case the total concentration of MgCl₂ was adjusted to maintain free Mg²⁺ at 5 mM. Unless otherwise indicated, transport buffer contained 100 mM free Ca²⁺. The free concentrations of Ca²⁺ and Mg²⁺ were determined with the program Mathematika (Wolfram Research Inc., Champaign, IL) using the stability constants of the EGTA complexes, corrected for temperature and pH, of Martell and Smith (1974). Transport reactions were terminated by cooling to 4°C. The media was removed and combined with a 250-µl PBS wash. The media samples were then centrifuged for 2 min in an Eppendorf microfuge to remove any cells that may have detached during the transport reaction and the supernatant was transferred to fresh tubes. Cells were extracted with 100 µl of 50 mM Tris (pH 8.0), 150 mM NaCl, 2 mM MgCl₂, 1% Triton X-100 for 5 min at 37°C and the wells were rinsed with 0.4 ml of PBS. The detergent extracts were combined with all cells pelleted from the media samples. Cells were grown to 80-90% confluence on 10-cm dishes and detached by incubation for 5 min at 37°C in Ca²⁺/Mg²⁺-free PBS containing 5 mM EDTA. The cells were pelleted for 4 min in a tabletop centrifuge, resuspended in 1 ml of buffer B, and then transferred to a polypropylene microcentrifuge tube. The cells were washed once with buffer B by centrifugation/resuspension, and then resuspended in 0.4 ml of buffer B containing 500 µM xyloside and incubated for 30 min at 37°C. The cells were labeled by adding 100 µl of buffer B containing 1 mCi of [³⁵S]SO₄ (25-40 Ci/mg) and incubated at 37°C for 90 s. Labeling was terminated by the addition of 0.5 ml of ice cold buffer B supplemented with 4 mM Na₂SO₄. The cells were pelleted in a microcentrifuge, washed three times at 0°C in buffer A containing 4 mM Na₂SO₄, and resuspended at 10⁶ cells/ml in ice cold buffer A. Permeabilization was carried out by adding an equal volume of ice cold buffer A containing 0.1% [wt/vol] cetylpyridinium chloride (CPC; 2% final) and 10 µl of 10 mg/ml pronase E and incubation for 4-16 h at 37°C. The cells were then washed free of pronase E and incubated for 10 min in 250 µl of transport buffer containing either an ATP-regenerating system, and various concentrations of added free Ca²⁺. Reaction mixtures were initiated by shifting to 37°C and terminated by the addition of 450 µl of ice cold transport buffer and rapid centrifugation. Pelleted cells were extracted as described above for attached cells and media and cell extracts were assayed for [³⁵S]-labeled glycosaminoglycans (GAG) as described below.

Quantitation of [³⁵S]SO₄ GAG Chains

[³⁵S]SO₄-labeled GAG chains were quantitated by a precipitation/filtration essentially as described by Luikart et al. (1985). Briefly, media samples or cell extracts (0.5 ml total volume) were proteolytically digested by the addition of 100 µl of 6 mg/ml pronase E and incubation for 4-16 h at 37°C. Sulfated GAG chains were then precipitated by the addition of 150 µl of 10% (w/v) cetylpyridinium chloride (CPC; 2% final) and 10 µl of 10 mg/ml chondroitin sulfate as a carrier. After incubation at 37°C for an additional 60 min the precipitated samples were collected by rapid vacuum filtration using Metricel GN-6 filters (2.4 mm, 0.45 µm) followed by four 5-ml washes with 1% CPC/25 mM Na₂SO₄. The filters were dried and counted using Metricel GN-6 filters (2.4 mm, 0.45 µm) followed by four 5-ml washes with 1% CPC/25 mM Na₂SO₄. The filters were dried and counted using Metricel GN-6 filters (2.4 mm, 0.45 µm) followed by four 5-ml washes with 1% CPC/25 mM Na₂SO₄. The filters were dried and counted using Metricel GN-6 filters (2.4 mm, 0.45 µm) followed by four 5-ml washes with 1% CPC/25 mM Na₂SO₄.

VSV ts045 Infection

CHO cells grown on poly-L-lysine-coated coverslips were infected with vesicular stomatitis virus (VSV) ts045 as described previously (Rivas and Moore, 1989). After infection the cells were washed twice in DME-H₂1, 2% FCS, 25 mM Hepes (pH 7.4), and incubated in the same buffer at 39.5°C for 3.5 h to accumulate VSV glycoprotein (G protein) in the ER (Zilberstein et al., 1980). The cells were then shifted to 19°C for 1 h to accumulate VSV G in the trans-Golgi apparatus (Griffiths et al., 1985). In vitro transport reactions were carried out using the assay described above for attached cells except that the xyloside preincubation and [³⁵S]SO₄ labeling were omitted; transport was performed at 32°C rather than 37°C; and the reactions were terminated by fixing the cells and processing for indirect immunofluorescence microscopy as described below. For cell surface immunoprecipitation and accumulation of VSV G protein in the ER were carried out as described above except that cells were grown on 10-cm dishes. VSV G was metabolically labeled by shifting the cells to a methionine-free medium for the final 30 min of the 3-h incubation at 39.5°C then adding 300 µCi of [³⁵S]-methionine and continuing the incubation for 10 min at 39.5°C. Unlabeled methionine was then added to a final concentration of 5 mM and the incubation continued for an additional 5 min at 39.5°C. The cells were then washed and incubated for 2 h at 39°C to accumulate VSV G in the trans-Golgi cisternae. In vitro transport reactions were carried out using the suspension assay described above except that the xyloside preincubation and [³⁵S]SO₄ labeling were omitted; SL-O permeabilization was done at 40°C; and the reactions were carried out at 32°C rather than 37°C. The transport reactions were terminated by adding an equal volume of ice cold buffer A and cell surface immunoprecipitation was carried out as described below.

Immunofluorescence Microscopy

CHO cells were grown for 72 h on poly-L-lysine-coated coverslips as described above. Indirect immunofluorescence was carried out as described previously (Miller and Moore, 1989). The cells were fixed with 3.7% formaldehyde in PBS (pH 7.4) at room temperature. The cells were permeabilized for 4 min at 37°C in 0.2 U/ml SL-O, incubated for 30 min in 3.7% formaldehyde, and then washed, incubated for 10 min in transport buffer, and then fixed for 30 min in 3.7% paraformaldehyde in PBS (pH 7.4) at room temperature.

Electron Microscopy

CHO cells were grown for 72 h in six-well plates. After treatment as described above, the figure legends, cells were fixed for 1 h using 2.5% glutaraldehyde, 1% acrolein, 1% paraformaldehyde in 0.1 M Na cacodylate (pH 7.4). Cells were then rinsed in PBS, osmicated for 1 h using 1% osmium tetroxide, 1% K₃Fe(CN)₆ in PBS, rinsed, dehydrated in 30 and then 50% EtOH, and finally stained for 1 h with 2% uranyl acetate in 50% EtOH. The EtOH dehydration series was continued, and the cells were scraped from the wells and transferred to embedding capsules. After final dehydration in propylene oxide the cells were embedded in Epox 812/Araldite and sectioned.

Golgi Staining with NBD-Ceramide

CHO cells grown on poly-L-lysine-coated coverslips were rinsed free of media, incubated for 10 min at 37°C in 1 mM C6-NBD-ceramide in DME-H₂1 containing 20 µM Hepes (pH 7.4) and 0.68 mg/ml BSA. The cells were then washed, incubated for 10 min at 37°C in DME-H₂1/20% Hepes, and then for 10 min at 37°C in DME-H₂1/20 mM Hepes/0.68 mg/ml BSA. The cells were permeabilized for 4 min at 37°C in 0.2 µM SL-O, incubated for 10 min in transport buffer, and then fixed for 30 min in 3.7% paraformaldehyde in PBS (pH 7.4) at room temperature.

Cell Surface Immunoprecipitation

Cell surface immunoprecipitation of VSV G protein was performed essentially as described by Compton et al. (1989). Cells from 100-µl aliquots of the transport reactions were pelleted and resuspended in 100 µl of ice cold PBS containing 0.1 mM Ca²⁺ and a 1:3 dilution of an anti-VSV rabbit antiserum recognizing the luminal domain of VSV G and incubated for 60 min at 4°C. The cells were pelleted by centrifugation for 2 min in a microcentrifuge and washed three times with ice cold PBS. The final cell pellet was solubilized in 500 µl of lysis buffer (50 mM Tris, pH 80, 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) containing unlabeled VSV-infected CHO cell extract to block binding of unreacted antibody sites, and insoluble materials were removed by centrifugation for 5 min at 12,000 g. The supernatant was incubated with 100 µl of a suspension of fixed S. aureus cells for 60 min at 4°C. The cells were pelleted by centrifugation for 5 min at 12,000 g and washed three times with lysis buffer. The final cell pellet was boiled in 30 µl of SDS gel sample buffer
Figure 1. CHO cells are quantitatively permeabilized by SL-O. CHO cells were washed free of media and incubated for 4 min at 37°C in a buffer containing 5 μg/ml propidium iodide (a membrane-impermeant DNA-binding fluorescent probe) and either 0 (A and B) or 0.2 (C and D) U/ml SL-O. The unfixed cells were then viewed under phase (A and C) or fluorescence (B and D) to detect permeabilized cells (identified by bright nuclear staining with propidium iodide). Bar, 40 μm.

and the eluted proteins were analyzed by SDS-PAGE on 10% polyacrylamide gels. The gels were fixed, treated for fluorography with 1 M salicylate, dried, and exposed to film. Quantitation of the resulting autoradiographs was accomplished using a scanning laser densitometer and integrating the areas of peaks of interest. Total VSV G was determined by treating parallel samples as described above except that the cells were solubilized with lysis buffer before adding antibody. In control experiments we found that a 1:10 dilution of antibody resulted in precipitation of the same amount of VSV G; therefore the amount of antibody used in these experiments is at least a threefold excess. The extent of EndoH resistance was determined using parallel samples as described by Rose and Bergmann (1982).

Results

Choice of SL-O for Perforation of CHO Cells

We have chosen the bacterial cytolysin SL-O to prepare semi-intact cells for the following reasons. SL-O has been used in a number of studies to examine regulated secretion (for review see Ahnert-Hilger et al., 1989). Thus, membranes treated with SL-O retain capability for fusion. SL-O permeabilization is rapid (<3–5 min; Ahnert-Hilger et al., 1989) as compared with detergents such as digitonin (typically 10–20 min) (Diaz and Stahl, 1989; Dunn and Holz, 1983; Shafer et al., 1987), and is highly selective for the plasma membrane (Ahnert-Hilger et al., 1989; see below). The pores formed in the plasma membrane are uniform in size and have been characterized as highly stable 20–30-nm structures (Bhakdi et al., 1985). The dimension of the pores therefore allows exchange of large cytosolic components (>150 kD) as well as small molecules such as nucleotides and ions (Ahnert-Hilger et al., 1989). Since Golgi-derived exocytic vesicles have diameters typically in the 80–300-nm range (Griffiths et al., 1985; Orci et al., 1987; deCurtis and Simons, 1988), they should be retained within the cellular boundary (see below).

CHO cells were chosen because these cells secrete newly synthesized proteins by the constitutive pathway and appear
to lack the ability to store proteins in regulated granules. This property allows us to study secretion by this pathway in isolation. For optimal reconstitution, only the plasma membrane but not internal organelles should be perforated. This could be accomplished in two ways. SL-O can be added to cells directly at 37°C; in this case the concentration and the time of exposure must be optimized. Alternatively, cells are first incubated with SL-O at 4°C to allow binding to the plasma membrane. Excess SL-O is then washed away, and the cells are warmed to 37°C to allow oligomerization and formation of the pores (Ahnert-Hilger et al., 1989). We found both procedures gave similar results. Fig. 1 shows that under the optimized conditions, 100% of CHO cells take up propidium iodide, a 668-D membrane-impermeant fluorescent dye that stains the nuclei of permeabilized cells. Less than 5% of control cells are stained with propidium iodide.

**Integrity of Intracellular Organelles in Perforated Cells**

Cells perforated by SL-O retained morphologically intact Golgi structures, as shown by staining with the fluorescent lipid C6-NBD-ceramide (Fig. 2; Pagano, 1989). Most intracellular organelles and cytoplasm appeared well preserved at the electron microscopic level, although after extended incubations the Golgi cisternae appeared more swollen than intact cells (Fig. 3). To determine if the plasma membrane is selectively perforated, we studied the accessibility of a cytoplasmically exposed antigen and a Golgi-lumenal antigen to exogenously added antibodies. CHO cells were infected with a temperature-sensitive mutant of the VSV, VSV-ts045, for 3 h at 39.5°C (Zilberstein et al., 1980). The cells were then shifted to 19°C for 1 h to accumulate the G protein in the trans-Golgi structures (Griffiths et al., 1985). After
permeabilization with SL-O, cells were incubated with monoclonal antibodies specific for either the lumenal or the cytoplasmic domain of G protein. After washing, bound antibodies were visualized with fluorescent secondary antibodies. Bright staining of the ER and Golgi structures was found in cells incubated with the anticytoplasmic tail antibody, but not with the anti-luminal antibody (Fig. 4). The lack of staining by the anti-luminal antibody was not due to its inability to recognize the expressed G proteins. Fig. 5 shows a parallel experiment in which sham-permeabilized cells were incubated with the same antibodies; under these conditions G protein was transported to the cell surface. The antiluminal antibodies showed considerable staining at the cell surface, whereas the anti-tail antibodies did not give rise to detectable staining as expected. Thus, under the conditions of our SL-O treatment the plasma membrane is selectively perfo-
rated to allow passage of antibodies into the cytoplasm. The internal membranes of the ER and Golgi apparatus remain impermeant to antibody molecules.

**Reconstitution of Golgi-to-Plasma Membrane Transport of VSV G**

We next tested if transport from the trans-Golgi structure to the plasma membrane could be reconstituted in the perforated cell preparation. CHO cells infected with VSV ts045 were incubated at 19°C to accumulate the G protein in the trans-Golgi apparatus (Griffiths et al., 1985). When the temperature was shifted to 32°C, G protein in intact cells was rapidly transported from the trans-Golgi apparatus to the plasma membrane (Fig. 6, A–D). In contrast, when SL-O–treated cells were incubated at 32°C in the presence of an ATP-depleting system, VSV G remained intracellular and no

**Table 1. Quantitation of In Vitro Transport of VSV-G by Cell Surface Immunoprecipitation**

| Condition          | Total | EndoH resistant | Surface | EndoH resistant at surface |
|--------------------|-------|-----------------|---------|---------------------------|
|                    |       | Arbitrary Units |         |                           |
| Intact (-ATP)      | 2,830 | 2,830           | 2,360 ± 120 | 83 ± 4                     |
| Intact (+ATP)      | 2,550 | 2,550           | 2,065 ± 255 | 81 ± 10                    |
| SL-O: –ATP/+Cytosol| 2,065 | 1,020           | 130 ± 16  | 13 ± 2                     |
| SL-O: +ATP/+Cytosol| 2,140 | 1,145           | 785 ± 140 | 68 ± 12                    |
| SL-O: +ATP/+Cytosol/GTPγS| 2,050| 965             | 115 ± 10  | 12 ± 1                     |

CHO cells were infected with VSV ts045, incubated for 3 h at 40°C, pulse labeled for 10 min at 40°C with [35S]methionine, and then incubated for 2 h at 19.5°C to accumulate VSV G protein in the trans-Golgi region (see Materials and Methods). The in vitro transport reaction was then carried out for 60 min at 32°C under the indicated conditions and the total amount of VSV G and the amount at the cell surface were assayed by immunoprecipitation as described in Materials and Methods. GTPγS was added to the reaction to a final concentration of 100 μM. Errors are the SD of triplicate determinations.
immunoreactivity could be detected at the cell surface (Fig. 6, E and F). However, when bovine brain cytosol and an ATP-regenerating system were added to the perforated cells, the G protein was efficiently transported to the plasma membrane (Fig. 6, G and H). To quantitate the amount of G proteins transported to the cell surface, we performed cell surface immunoprecipitation. CHO cells, infected with VSV ts045 and pulse labeled with [35S]methionine, were incubated for 2 h at 19.5°C to accumulate G protein in the trans-Golgi apparatus. Cells were then permeabilized and incubated either in an ATP-depleting system or in an ATP-regenerating system supplemented with bovine brain cytosol. At the end of the reaction, cells were incubated with antilumenal antibodies to detect exposed G proteins. An identical sample was detergent solubilized and then subjected to immunoprecipitation to determine the total amount of antigens present. A third sample was used to determine the amount of VSV G that had acquired EndoH resistance (Table I). ATP and cytosol stimulated exposure of EndoH-resistant VSV G proteins from 13 to 68% in SLO-treated cells. Intact cells showed 82% transport both in presence and absence of ATP. Control experiment showed that <5% of EndoH-resistant G proteins were exposed after incubation at 19.5°C (data not shown). Taken together, these experiments demonstrated that transport of VSV was indeed reconstituted in SLO-treated cells.

Quantitation of Rates of Transport Using Sulfated Glycosaminoglycans As a Marker

To dissect the biochemical requirements for constitutive secretion, we needed a more sensitive and reliable method to quantitate the kinetics and extent of transport under different conditions. We took advantage of the fact that constitutive secretion is a bulk-flow process (Pfeffer and Rothman, 1987; Burgess and Kelly, 1987). Thus, the rate of secretion can be measured by a fluid-phase tracer generated in the Golgi lumen. GAG chains provide an ideal marker. Synthesis of free GAG chains can be induced by incubating cells in the presence of a membrane-permeant xyloside, which serves as initiator for GAG chain elongation (Schwartz et al., 1974). Since GAG chains are highly sulfated and sulfation occurs in the trans-Golgi apparatus (Kimura et al., 1984; Farquhar, 1985; Velasco et al., 1988), transport from the trans-Golgi apparatus to the surface can be followed by pulse labeling xyloside-treated cells with [35S]SO4.

Fig. 7 shows that SO4 GAG chains synthesized by CHO cells were rapidly secreted from the cells. To quantitate the amount of SO4 GAG chains, we have used a simple filtration assay (see Materials and Methods) that involves precipitation of GAG chains with CPC (Luikart et al., 1985). The method is so sensitive that it allows us to follow the secretion of GAG chains that are labeled during a short (1-2 min)
Figure 6. Reconstitution of transport of VSV G from the trans-Golgi complex to the plasma membrane in SL-O-permeabilized cells. CHO cells were infected with VSV ts045 for 40 min at 37°C, and incubated for 3 h at 39.5°C and then 1 h at 19°C to accumulate VSV G in the trans-Golgi complex. Cells were then incubated for 4 min in either 0 (A-D) or 0.2 (E-H) U/ml SL-O. For sham-permeabilized cells, the cells were either fixed immediately (A and B) or incubated for 60 min at 32°C in DME before fixation (C and D). For SL-O-treated cells, the cells were incubated in transport buffer containing cytosol with or without ATP. (E and F) ATP-depleting system; (G and H) ATP-regenerating system. The cells were fixed and VSV G that had been transported to the cell surface was detected by indirect immunofluorescence using an antilumenal antibody without Triton X-100 treatment. (A, C, E, and G) phase; (B, D, F, and H) fluorescence. Bar, 80 μm.

Biochemical Requirements for Constitutive Secretion from the trans-Golgi Apparatus

Using the CPC assay for GAG chains, we then determined the biochemical requirements for trans-Golgi-to-plasma membrane transport in the reconstituted system. Uptake of [35S]SO4, synthesis of the intermediate [35S]-3'-phosphoadenosine 5'-phosphosulfate and translocation of 3'-phosphoadenosine 5'-phosphosulfate into the Golgi lumen requires several minutes in mammalian cells (Baeuerle and Huttner, 1987). By using brief (1-2-min) pulse labeling with [35S]SO4, followed by rapid permeabilization with SL-O (permeabilization is complete within 2-3 min) in the presence of pulse. The kinetics of SO4 GAG released from intact cells CHO cells that had been starved for sulfate for 30 min and pulse labeled with [35S]SO4 for 2 min are shown in Fig. 7b. Approximately 80-90% of the labeled GAG chains were released during a 60-min chase. The time course of release exhibited a lag time of 7-8 min and a half-time for secretion of 12-14 min. The kinetics of secretion are consistent with a simple two-compartment transport model as described by Noe and Delenick (1989), suggesting that GAG chains are transported directly from the compartment in which they are sulfated to the plasma membrane. These findings are consistent with earlier studies indicating that GAG sulfation is a late Golgi event (Kimura et al., 1984; Velasco et al., 1988).
Figure 7. Sulfated glycosaminoglycans are secreted from CHO cells with kinetics characteristic of the constitutive pathway. (a) CHO cells grown on six-well plates were starved for 30 min in sulfate-free media containing 0.5 mM xyloside, and then labeled for 2 h at 37°C with [35S]SO4 in presence of xyloside. The cells were washed to remove excess [35S]SO4 and chased for 3 h at 37°C in DME. (Lane A) 0-1-h chase; (lane B) 1-2-h chase; (lane C) 2-3-h chase. Sulfated GAG chains were precipitated with acetone at -20°C, separated on an 18% polyacrylamide gel, and autoradiographed. The mobilities of molecular mass standards (in kD) are indicated. (b) CHO cells grown on 12-well plates were starved for 30 min in sulfate-free media containing 0.5 mM xyloside, and then pulse labeled for 2 min with [35S]SO4. After washing briefly to remove excess [35S]SO4, the cells were chased for varying times in DME. At each time point the media was collected and cells extracted. The amount of GAG chains in each sample was determined using a precipitation/filtration assay (see Materials and Methods). The plot shows the extent of secretion, expressed as the percentage of total 35S-sulfated GAG chains synthesized during the pulse, as a function of time. Data shown are the mean ±SD for triplicate data points.

Figure 8. Quantitation of transport efficiency in the reconstituted system using sulfated glycosaminoglycans. CHO cells grown on 12-well plates were incubated with xyloside and pulse labeled with [35S]SO4 for 2 min as described in the legend to Fig. 7 b. The cells were permeabilized for 4 min at 37°C with 0.2 U/ml SL-O and incubated for the indicated times in transport buffer containing either an ATP-depleting (○) or ATP-regenerating (★) system and cytosol. At each time point the media was collected and cells were extracted. The percentage of total 35S-labeled GAG released as a function of time was determined by a precipitation/filtration assay. Data shown are the mean ±SD for duplicate data points.
when the cells were held at 0-4°C for 1 h (Table II). This rather than direct leakage out of damaged Golgi apparatus.

lease of exocytic transport vesicles (Bennett et al., 1988), from vesicular transport between membrane compartments, at 100,000 g for 90 min (not shown).

Since the radiolabeled chains recovered from the medium N-ethylmaleimide (NEM), completely abolished the trans-

Table II. Characteristics of In Vitro Transport of GAG Chains from the trans-Golgi to the Cell Surface

| Incubation | ATP-dependent transport % |
|------------|---------------------------|
| Complete   | 100                       |
| 0°C        | 2.1 ± 0.9                 |
| - Cytosol  | 9.2 ± 0.9                 |
| + NEM      | 3.9 ± 2.1                 |
| + GTPγS    | 7.5 ± 3.3                 |
| + GMP-PNP  | 28.8 ± 4.9                |

In vitro transport reaction was carried out for 60 min using suspended cells as described in Materials and Methods. The complete reaction contained the semi-intact cell preparation, bovine cytosol, and an ATP-regenerating system. For each condition, the extent of transport was determined for buffers containing either an ATP-regenerating or -depleting system. The difference between the two sets of reaction is defined as ATP-dependent transport. Except for the 0°C incubation, all reactions were carried out at 37°C. For NEM treatment, perforated cells were first incubated with 1 mM NEM at 0°C for 15 min; NEM was subsequently inactivated by incubation with 2 mM DTT at 0°C for 15 min. GTPγS was added to the reaction at a final concentration of 50 μM, and GMP-PNP at 100 μM. ATP-dependent transport in the complete reaction was defined as 100%.

Inhibition of Transport by GTPγS

To determine if GTPγS exerts any effect on constitutive secretion, we examined in vitro transport of VSV G from the trans-Golgi membrane to the plasma membrane in the presence of GTPγS. Inclusion of 50-100 μM GTPγS in the transport incubation blocked the appearance of VSV G at the cell surface as assessed by both immunofluorescence (Fig. 9) and cell surface immunoprecipitation (Table I). The inhibitory effect of GTPγS was also confirmed by CPC assays of sulfated GAGs (Table II; Fig. 10). Quantitation of transport rates in presence of increasing concentrations of GTPγS showed that half-maximal inhibition occurred at ~5 μM GTPγS. Including GTP in the transport reaction had little effect on its own (Fig. 10), but 1 mM GTP prevented the inhibition produced by 50 μM GTPγS (not shown). The inhibitory effect was not due to a general toxic effect of the GTP analogue on cells; treating intact cells with 100 μM GTPγS had no effect on the rate or extent of secretion of sulfated GAG chains (not shown). Transport was also inhibited by GMP-PNP; inclusion of 100 μM of GMP-PNP reduced transport to <30% of control levels (Table II). These results indicate that hydrolysis of GTP is necessary for efficient export via the constitutive pathway.

Comparison of Calcium Requirements for Constitutive and Regulated Secretion

The standard transport reactions described above were in buffers containing 100 nM free Ca2+. To test whether the concentration of free Ca2+ affected either the rate or extent of transport, we varied the Ca2+ concentrations of the in vitro reaction from <10 nM to 1 μM using a Ca2+-EGTA buffering system. As shown in Fig. 11, the extent of GAG chain secretion over a 60-min incubation period is not influenced by a wide concentration range of free Ca2+. The initial rate of secretion at <10 nM Ca2+ is identical to the rate measured at physiological Ca2+, 100 nM (Fig. 11). High rates of transport were also seen with 5 mM EGTA in the absence of added Ca2+ at pH 8.0 (not shown). Under these conditions free Ca2+ is calculated to be <1 nM, strongly suggesting that constitutive transport at this step does not have a requirement for free Ca2+. These results are further confirmed by transport of VSV G from the trans-Golgi region to the plasma membrane (Fig. 12): significant amounts of the G protein can be detected at the cell surface even when the in vitro transport reaction is carried out at <10 nM free Ca2+.

To directly compare the calcium requirement for regulated secretion, we accumulated [35S]-SO4 labeled GAG chains in regulated secretory granules from AtT-20 as previously described (Burgess and Kelly, 1984). After SL-O permeabilization, release of stored GAG chains from these cells required 1-2 μM free Ca2+ (buffered with the same Ca2+-EGTA-buffering system) (Fig. 13). The requirement for micromolar calcium for regulated exocytosis is consistent with previous studies in permeabilized cell systems (DeLisle and Williams, 1986). Taken together, these results indicate that constitutive exocytosis, in marked contrast to regulated exocytosis, does not require and is not modulated by calcium ions.

Discussion

In this paper, we have developed an in vitro system to address the requirements for vesicular traffic from the trans-Golgi region to the plasma membrane via the constitutive pathway. We used a transmembrane protein and a soluble marker to assay constitutive transport between the trans-Golgi region and the plasma membrane. Each assay alone has potential problems. For example, the release of soluble markers may result from lysis of the Golgi or intermediate compartments.
Figure 9. GTP\textsubscript{\gamma}S inhibits transport of VSV G from the trans-Golgi complex to the plasma membrane in vitro. VSV G was accumulated in the trans-Golgi complex as described in the legend to Fig. 4. The cells were permeabilized for 4 min at 37°C with 0.2 U/ml SL-O and incubated for 60 min at 32°C in transport buffer containing an ATP-regenerating system, cytosol, and either 0 (A-D) or 50 \(\mu\)M (E-H) GTP\textsubscript{\gamma}S. The cells were fixed and intracellular distribution of VSV G (A, B, E, and F) was determined by indirect immunofluorescence with an antilumenal antibody after Triton X-400 treatment. Alternatively, localization of VSV G transported to the cell surface (C, D, G, and H) was determined by using an antilumenal antibody but omitting the Triton X-100 treatment. (A, D, E, and H) phase; (B, C, F, and G) fluorescence. Bar, 60 \(\mu\)m.

Incorporation of a membrane protein into the plasma membrane is used to rule out this possibility. In addition, several lines of evidence suggest that GAG chain release is indeed a measure of transport between the Golgi region and the cell surface in our reconstituted system. (a) Like many other transport steps, release is both ATP and temperature dependent, and is sensitive to NEM and GTP\textsubscript{\gamma}S. This makes it unlikely that recovery of GAG chains in the medium is due to direct lysis of internal compartments. (b) The released GAG chains are freely soluble and are not associated with sedimentable membrane structures. Therefore, release cannot result from fragmented Golgi or exocytotic vesicles that have leaked into the medium through SL-O pores. (c) As mentioned above, under identical conditions VSV G protein is inserted into the plasma membrane. Thus, the transport reaction used here can support both the production and the consumption of exocytotic vesicles. (d) Using similar conditions, we also reconstituted the constitutive secretion of proopiomelanocortin from AtT-20 cells (Chou, S.-C., S. G. Miller, and H.-P. Moore, unpublished results). Thus, the results obtained with GAG chains are not limited to this bulk flow tracer but can be applied to endogenous secretory proteins.

The properties of the in vitro transport reaction are similar in many ways to those described for earlier steps in the exocytic pathway. The reaction requires hydrolyzable ATP, exogenous cytolsic components provided by a bovine brain cytosol fraction, and is inhibited by the NEM. It is presently unknown how many factors are inhibited by NEM, and whether the NEM-sensitive factor, which has been shown to participate in the fusion reactions of ER to Golgi (Beckers et al., 1989), intra-Golgi (Block et al., 1988), and endosomal transport (Diaz et al., 1989), is involved in this step of transport. Future experiments using our system should resolve this question.

To date, our understanding of the exocytic apparatus involved in constitutive and regulated secretion remains quite rudimentary. It is not yet known whether the two forms of
exocytosis represent modifications of the same basic machinery, or they differ fundamentally. An apparent difference is the distinct effects of nonhydrolyzable analogs of GTP on exocytosis. Constitutive transport from the trans-Golgi region to the plasma membrane, as shown in this study, is blocked by 50 μM GTPγS. The same concentrations of GTPγS, however, does not appear to block, and in many cases even stimulates, regulated exocytosis (Knight and Baker, 1985; Howell et al., 1987). This apparent lack of inhibition of regulated exocytosis by GTPγS is curious, since hydrolysis of GTP is thought to be a general regulatory mechanism in vesicular transport to ensure that vesicle fusion only occurs with the correct target membrane (for reviews see Bourne, 1988; Balch, 1989). Thus, either the control of membrane recognition for regulated secretion differs from other transport steps, or another explanation is necessary. A possible explanation is that GTP exchange occurs early, at the trans-Golgi region or soon thereafter, such that mature regulated granules already contain stably bound GTP that is not displaced by added GTPγS. Consistent with this notion, we found that GTPγS exerts its inhibitory effect on constitutive secretion only when added early during transport reaction (Miller, S., and H.-P. Moore, unpublished observations), as was found for transport between ER and Golgi (Beckers and Balch, 1989) and between cis- and medial-Golgi region (Melancon et al., 1987). Further experiments will be necessary to decipher the exact role(s) of G proteins in constitutive and regulated secretion.

Physiologically, the two secretory pathways differ in one important aspect; i.e., regulated secretion is triggered by external signals whereas constitutive secretion is not. At the molecular level, this difference may be attributed to different sensitivities of the exocytotic machinery to free cytoplasmic Ca2+ levels. Although the exact agents capable of eliciting a regulated exocytic response may differ between cell types, the proximal trigger is generally a rise in intracellular Ca2+. By comparison, constitutive secretion must be operational at resting levels of free Ca2+ (50–200 nM) since it is ongoing.
even in the absence of external signals. Such differences in the Ca$^{2+}$ sensitivity may arise in one of two ways. First, a unique Ca$^{2+}$-responsive component may be specifically sorted to regulated secretory granules but not constitutive secretory vesicles; this would make the regulated granules uniquely sensitive to calcium. Alternatively, the same Ca$^{2+}$-responsive component may be present on both types of vesicles, but modified in such a way that they differ in their affinities for Ca$^{2+}$—constitutive vesicles requiring physiological Ca$^{2+}$ and regulated granules requiring higher levels for optimal secretory activities. Several considerations make the latter possibility an attractive hypothesis. (a) Previous studies have shown that the Ca$^{2+}$ sensitivity of the regulated secretory apparatus can indeed be modulated; phorbol esters, for instance, can stimulate exocytic release from storage granules without a rise in intracellular Ca$^{2+}$, most likely by increasing the affinity of the exocytic apparatus to Ca$^{2+}$ (Rink et al., 1983; Knight and Baker, 1985). (b) Cell lines that package peptide hormones into regulated granules often release a fraction of the newly synthesized peptides in an unregulated fashion. This phenomenon is consistent with the notion that regulated granules may first be made with similar or identical properties to constitutive vesicles, and then modified as the granules mature. (c) At least one step in the constitutive secretory pathway, namely, ER to Golgi complex, has been shown to require physiological levels of Ca$^{2+}$ for optimal transport both in CHO cells (Beckers and Balch, 1989) and in S. cerevisiae (Baker et al., 1990). The

Figure 12. Transport of VSV G from the trans-Golgi region to the plasma membrane does not require Ca$^{2+}$. VSV G was accumulated in the trans-Golgi region of CHO cells as described in the legend to Fig. 6. The cells were then permeabilized, and one set of cells was fixed immediately (A and B). The remaining coverslips were incubated for 60 min at 32°C in transport buffer containing cytosol and either an ATP-depleting system (C and D) or an ATP-regenerating system (E–H). The transport buffer contained either 0 (C–F) or 100 nM (G and H) free Ca$^{2+}$. The cells were then fixed, and VSV G that had been transported to the cell surface was determined by using an antilumenal antibody and omitting Triton X-100 treatment (Materials and Methods). (A, C, E, and G) phase; (B, D, F, and H) fluorescence. Bar, 80 μm.
studies presented in this paper, however, suggest that constitutive secretion from the trans-Golgi region to the plasma membrane does not require physiological Ca\(^{2+}\). Thus, constitutive and regulated secretion appear to differ fundamentally with regard to their requirements for calcium.

In summary, the trans-Golgi is the main compartment in which sorting occurs during biosynthetic transport in the exocytic pathway. The reconstitution of constitutive secretion described in this paper, and the eventual reconstitution of other post-Golgi transport steps should greatly facilitate our understanding of the molecular mechanisms underlying the sorting and delivery events.

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