Myosin II Is Involved in the Production of Constitutive Transport Vesicles from the TGN

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Abstract. The participation of nonmuscle myosins in the transport of organelles and vesicular carriers along actin filaments has been documented. In contrast, there is no evidence for the involvement of myosins in the production of vesicles involved in membrane traffic. Here we show that the putative TGN coat protein p200 (Narula, N., I. McMorrow, G. Plopper, J. Doherty, K.S. Matlin, B. Burke, and J.L. Stow. 1992. J. Cell Biol. 114: 1113–1124) is myosin II. The recruitment of myosin II to Golgi membranes is dependent on actin and is regulated by G proteins. Using an assay that studies the release of transport vesicles from the TGN in vitro, we provide functional evidence that p200/myosin is involved in the assembly of basolateral transport vesicles carrying vesicular stomatitis virus G protein (VSVG) from the TGN of polarized MDCK cells. The 50% reduced efficiency in VSVG vesicle release from the TGN in vitro after depletion of p200/myosin II could be reestablished to control levels by the addition of purified nonmuscle myosin II. Several inhibitors of the actin-stimulated ATPase activity of myosin specifically inhibited the release of VSVG-containing vesicles from the TGN.

Transport of proteins synthesized in the ER is mediated by vesicular carriers that shuttle membrane components between successive stations along the secretory pathway (Palade, 1975). Distinct sets of cytosolic factors are involved in the release, transport, docking, and fusion of these transport vesicles (Rothman, 1994). The initial stage in transport, the release of nascent vesicles from the donor compartment, is thought to occur in two steps. The first step is the curvature of the donor membrane into a bud, mediated by coat protein complexes recruited by address-specific membrane receptors and, in concentrative transport, by signals in the cytoplasmic domains of cargo proteins (Müsch et al., 1996; Rothman and Wieland, 1996; Schekman and Orci, 1996). The second step is the fission of the nascent vesicle, an energy-requiring membrane fusion event initiated from the luminal side of the bud, which requires acetyl coenzyme A in the Golgi complex (Glick and Rothman, 1987; Rothman and Warren, 1994), and the microtubule-associated GTP-dependent motor protein dynamin in clathrin-coated vesicles budding from the plasma membrane (Takel et al., 1995).

The number of cellular transport steps mediated by vesicles largely exceeds the number of known families of coat–adaptor complexes. These are: (a) clathrin, with the adaptor complexes AP1 at the TGN, AP2 at the plasma membrane, and unknown adaptors at endosomes (Pearse and Robinson, 1990; Stoovogel et al., 1996); (b) COPI, at the Golgi apparatus (Letourneur et al., 1994; Orci et al., 1993a) and COPI-like molecules in endosomes (Whitney et al., 1995); (c) COPII, involved in transport between ER and Golgi (Aridor et al., 1995; Bednarek et al., 1995); and (d) β-NAP (Newman et al., 1995) and a novel adaptor complex (Simpson et al., 1996), which were postulated to mediate anterograde transport in neurons. From the TGN, the major sorting compartment in the exocytic route, newly synthesized proteins are sorted into vesicular pathways to lysosomes, regulated secretory granules, and the plasma membrane. At least two biochemically and pharmacologically distinct routes to the cell surface have been recently identified in polarized and nonpolarized cells (Ikonen et al., 1995; Müsch et al., 1996; Yoshimori et al., 1996), as well as in yeast (Harsay and Bretscher, 1995). Of all these post-TGN routes, only the clathrin/AP1 coat that mediates the receptor-mediated packaging of lysosomal hydrolases has been characterized; morphological evidence exists for an additional, lace-like coat in the TGN (Ladinsky et al., 1994), but its composition and function are unknown.

The recruitment of coat proteins from the cytosol into budding vesicles is mediated by small monomeric GTP binding proteins of the ADP ribosylation factor (ARF)1/2...
Sar1 family (Rothman, 1994; Schekman and Orci, 1996) and regulated by trimeric G proteins (Donaldson et al., 1991; Robinson and Kreis, 1992). Consequently, coat protein assembly onto membranes is enhanced by GTPγS and AlF₃, and inhibited by the fungal metabolite brefeldin A (BFA), which inhibits the GTP–GDP exchange in small monomeric G proteins (Helms and Rothman, 1992). Proteins that bind to isolated organellar membranes with similarly regulated features are considered good candidates to be components of novel coat complexes. One such protein is p200, which binds to isolated Golgi membranes with identical characteristics as COPI and was initially identified by an mAb (AD7) raised against peripheral Golgi proteins (Narula et al., 1992). p200 has been detected on TGN-associated nascent vesicles by immuno-EM (Narula and Stow, 1995).

In this report we demonstrate that p200 is involved in the release of vesicular stomatitis virus G protein (VSVG) but not hemagglutinin (HA)—containing vesicles from the TGN. To our surprise, sequence analysis of immunoprecipitated p200 revealed a stretch of 19 amino acids with 100% homology to human nonmuscle myosin II. In agreement with this result, polyclonal antibodies against platelet myosin II recognized in immunoblots p200 immunoprecipitated from rat liver cytosol with the AD7 mAb. Previous data had suggested roles for both myosin II (Mochida et al., 1994) and unconventional myosins (Fath and Burgess, 1993; for review see Hasson and Mooseker, 1995) in a late, postmicrotubular stage in the transport of apical or regulated secretory vesicles across the cortical actin meshwork (Breitscher, 1991; Muñlem et al., 1995). However, to our knowledge, there is no evidence for an early role of myosin in the assembly of any kind of transport vesicles. The data we present here demonstrate that myosin II is recruited to isolated Golgi membranes with features characteristic of coat proteins. Furthermore, a variety of myosin functional tests shows conclusively that myosin mediates the release of VSVG transport vesicles from the TGN of semiintact MDCK cells and from a purified Golgi fraction. Our data suggest that myosin II is part of the cytosolic machinery that regulates assembly or fission of a set of constitutive transport vesicles from the TGN.

**Materials and Methods**

**Cells, Viruses, and Antibodies**

MDCK II cells, VSV (Indiana strain), and influenza virus A (WSN) were grown as described (Rodriguez-Boulan and Sabatini, 1978). The protocols for viral infection of MDCK cell monolayers, for pulse labeling, and for TGN accumulation of the VSVG protein and the WSN HA, as well as the antibodies against the viral markers, were described previously (Musch et al., 1996).

Other antibodies (sources) were as follows: polyclonal antibody against human blood platelet myosin II (Biomedical Technologies, Inc., Stoughton, MA); polyclonal antibody against chicken brush border myosin II (kindly provided by K. Fath and D. Burgess, University of Pittsburgh, PA); affinity purified on myosin II from chicken brush border (kindly provided by K. Fath and D. Burgess); AD7 mAb against p200 (Narula et al., 1992); prepared by a hybridoma kindly provided by K. Matlin (Harvard University, Boston, MA); polyclonal antibody against human cathepsin D (Biodiagnost Inc., Kennebunkport, ME); mAb against gp114 (hybridoma Y-652 from K. Matlin, Harvard University); β-COP mAb M3A4 (kindly provided by T. Kreis, University of Geneva, Switzerland); affinity-purified rab 8 polyclonal antibody, raised against a peptide corresponding to the amino acids 182–197 of human rab 8 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**p200 and Actin/Myosin Depletion**

100 µl rat liver cytosol (50 mg/ml) was incubated three times with 30 µg immobilized antibodies against platelet myosin (300 µg IgG cross-linked to 100 µg protein A-Sepharose; Pharmacia Fine Chemicals, Piscataway, NJ), or p200 (300 µg IgG cross-linked to 50 µl protein G–agarose, Gamma-Bind; Pharmacia Fine Chemicals). 3 mM Mg-ATP, pH 7.0, was added during the last 10 min of each immunosolation step to prevent coagulation of actin. About 90% of cytosolic myosin or p200 was removed with a loss of 15% of the total protein, as tested by Western blotting and a Lowry assay, respectively. The amount of cytosol added in the recruitment and vesicle release assays was corrected for the loss of total protein. Mock-depleted cytosol was incubated under the same conditions with protein A–Sepharose or protein G–agarose alone.

**Actin Depletion**

Polymerization of actin in rat liver cytosol was stimulated with 50 µg/ml phallolidin (Sigma Chemical Co., St. Louis, MO) and the actin filaments were pelleted for 20 min at 27 pounds per square inch (psi) (150,000 g) in an airfuge (A100/18 rotor; Beckman Instruments Inc., Fullerton, CA). 5 mM Mg-ATP, pH 7.0, was added immediately before the spin when myosin depletion was to be avoided. The actin or actin/myosin pellet was resuspended in the original volume of a buffer composed of 50 mM KCl, 5 mM MgCl₂, 1 mM PMSF, 1 mM DTT, and 10 mM Tris/HCl, pH 7.0. To elute myosin, the protein complex was incubated with 5 mM ATP, pH 7.0, and actin fibers were repelleted for 20 min at 27 psi.

**Vesicle Release in Semiintact Cells**

The release of HA and VSVG transport vesicles from the TGN semiintact cells was studied in the absence or presence of 2 mg/ml rat liver cytosol (1 and 2 mg/ml for the caldesmon experiments), as described (Musch et al., 1996). Before the assay, the semiintact cells were stripped off peripheral membrane proteins with a high salt buffer (0.5 M KCl, 20 mM Hepes/KOH, pH 7.4) as described (Musch et al., 1996). The SI fragments prepared from chicken muscle myosin by either chymotryptic cleavage (Ch-S) or papain cleavage (Mg-S1) were kindly provided by Dr. S. Margossian (University of Albany, NY) and the 20-kD COOH-terminal chymotryptic fragment of caldesmon was kindly provided by J. Chalovich (East Carolina University, Greenville, NC). Both probes were directly incubated with the cytosol in the assay at the concentrations indicated in the text.

**Vesicle Release from Purified Golgi Fractions**

**Golgi Membranes.** Cells were homogenized with a Balch homogenizer (Balch et al., 1984) after the accumulation of labeled VSVG in the TGN. A fraction enriched in Golgi was isolated from a light mitochondrial fraction by flotation on a Nycodenz step gradient (25, 17.5, and 10%), according to the manufacturer’s protocol (Cenrifugation Techniques IV; Nycomed, Oslo, Norway). Organelle markers (see Results; Table I) were chosen as follows: for mitochondria, succinate dehydrogenase (Centrifugation Techniques IV; Nycomed); for ER, α-glucosidase II fluoroscent assay, using 4-methyl umbelliferyl (4MU)–α-glucoside (Sigma Chemical Co.) as substrate; for Golgi, α-mannosidase II fluorescence assay using 4MU–α-mannopyranoside (Sigma Chemical Co.) as substrate (Storrie and Madden, 1990); for TGN, sialyltransferase (Brandli et al., 1988; Simon et al., 1996); for lysosomes, immunoblot analysis of mature form of cathepsin D; for plasma membrane, immunoblot analysis of gp114 or dot blot analysis of biotinylated membranes with 125I–streptavidin.

**VSVG Vesicle Release from Purified Golgi Membranes.** Aliquots of the golgi fraction (protein concentration 0.5 mg/ml) that were stored at −80°C for up to 2 mo were thawed rapidly, pelleted at 10,000 g for 10 min, incubated for 20 min in a high salt buffer (0.5 M KCl, 20 mM Hepes/KOH, pH 7.4), repelleted, and resuspended in transport buffer (Musch et al., 1996) with 0.2 M sucrose. 2.5 µg Golgi and 20 µg rat liver cytosol were incubated in 20 µl 1× transport buffer and supplemented with 1 mM ATP, pH 7.0, 1 mM GTP (or GTP-γ-S in Fig. 2), 75 µM creatine phosphate, 0.5 µM creatine kinase. Where indicated, 1 µl VSVG peptide (0.5 mM in transport buffer; Musch et al., 1996) or 1 µl platelet myosin II (2 mg/ml in 0.6 M KCl, 10 mM imidazole, pH 7.0), or 2.5 µl chicken brush border myosin (70 µg/ml in 7 mM imidazole, 2.8 mM EDTA, 0.7 mM EGTA, 0.6 M KCl, 10 mM imidazole, pH 7.0), and 2.5 µl chicken brush border myosin II were added 15 min before the addition of 100 µl 1× transport buffer. After 10 min of incubation, 20 µl of the reaction mixture were taken for analysis of biotinylated membranes with 125I–streptavidin.
0.014% NaNO₃, 0.7 mM DTT, 30% glycerol, pH 7.3) was added to the assay mix. Control samples received 1 or 2.5 μl of the respective myosin buffers. The samples were incubated for 60 min at 37°C and the Golgi membranes were subsequently pelleted for 10 min at 10,000 g; both the supernatant and pellet fractions were analyzed by SDS-PAGE.

Controls (not shown) performed to validate Golgi vesicle release assays were as described in Müsch et al. (1996) and demonstrated: (a) protease protection of VSVG in the vesicle fraction, and (b) endoglycosidase H resistant N-glycans in the Golgi membranes but not in the vesicle fractions.

**Controls for TGN Fragmentation.** The levels of nonspecific TGN fragmentation were determined by measuring the release of sialyltransferase activity. Controls (not shown) had established that the enzymatic reaction was in the linear range with the amount of Golgi and substrate used. The amount of VSVG in the radioactive Golgi and vesicle pellets was quantified as described before (Müsch et al., 1996).

**Immunolocalization of Vesicles.** After release from semintact cells, the vesicles were sedimented in an airfuge for 20 min at 27 psi; vesicles released from isolated Golgi membranes were isolated directly from the supernatant fraction of a vesicle release assay. When a high salt wash preceded the isolation, samples were incubated in 500 mM KCl, 20 mM Tris/HCl, pH 7.4, for 30 min, and the KCl concentration was adjusted to 150 mM before the antibodies were added. For analysis of rab 8 and β-COP in nonradiolabeled vesicles isolated with p200 antibodies, assays (1 ml) were performed in the presence of GTPγS; the vesicles were pelleted for 5 h at 150,000 g in a TLA 100.3 rotor. One control was an identically processed supernatant of a vesicle release assay lacking Golgi membranes. One-fifth of each of the cold Golgi pellet fractions and the total cold vesicle pellets was resuspended in 200 μl of 10 mg/ml asialofetuin, 1% Triton X-100, 0.1 M cacydatoyl/NaOH, pH 6.6, and incubated with 0.1 μCi cytidine 5'-monophosphate–sialic acid (9 H-sialic acid) for 2 h at 37°C. Subsequently, protein was TCA precipitated as described (Brandli et al., 1988; Simon et al., 1996) and the incorporation of H-sialic acid into asialofetuin was measured in a liquid scintillation counter. Previous experiments had established that the enzymatic reaction was in the linear range with the addition of Golgi and substrate used. The amount of VSVG in the radioactive Golgi and vesicle pellets was quantified as described before (Müsch et al., 1996).

**Preparation of Rat Liver Cytosol.**

Rat livers were perfused with homogenization buffer (0.25 M sucrose, 20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml pepstatin, 10 mg/ml antipain) and homogenized in a motor-driven potter homogenizer with 4 ml homogenization buffer. The postmitochondrial supernatant was pelleted for 2 h at 150,000 g. The resulting supernatant was precipitated with saturated ammonium sulfate, resuspended in one-fifth of the original volume in 20 mM Tris/HCl, pH 7.4, 50 mM KCl, and dialyzed for 12 h with three changes against the same buffer (100× the volume). Aliquots were snap frozen and stored at −80°C.

**Preparation of Human Platelet Myosin II**

Platelets from 700 ml of human blood were isolated as described in Williams et al. (1993). Nonmuscle myosin II from 0.5-g platelets was prepared as described (Isenberg, 1995) according to Pollard (1982), and the protein was stored at a concentration of 2 mg/ml in 0.6 M KCl, 10 mM imidazole, pH 7.0, for 5 d on ice. Aliquots were stored at −20°C in 50% glycerol for up to 1 mo.

**Immunofluorescence.**

For these experiments, confluent monolayers of MDCK cells were grown on coverslips. Where indicated, the cells were incubated with 30 mM NaF/30 mM AlCl₃ for 30 min at 37°C before fixation/extraction. In some experiments, to stimulate the release of cytosolic proteins and simultaneously to allow the GTPγS-induced membrane recruitment of cytosolic coat proteins, cells were incubated in 5 μl digitonin for 10 min at 20°C (conditions that permeabilized the plasma membrane, but not the Golgi; Esparraguera and Ogando et al., 1994), and further incubated on ice for 20 min in the absence of VSVG GTPγS in PBS. Cells were fixed and extracted in three different ways: (a) 2% paraformaldehyde (PFA)/PBS, with subsequent dehydration in ice-cold acetone for 1–3 min; (b) extraction in cytoskeletal buffer (Cramer and Mitchison, 1996) (138 mM KCl, 3 mM MgCl₂, 2 mM EGTA, 0.32 M sucrose, 0.1% Triton X-100, 10 mM MES, pH 6.1) for 45 s, and subsequent fixation in 4% PFA in cytoskeletal buffer without Triton X-100; and (c) fixation in 2% PFA/PBS, followed by permeabilization with 0.1% Triton X-100. Indirect immunolabeling was carried out in 0.1% Triton in PBS, using Texas red–coupled anti–rabbit secondary antibodies and FITC-coupled anti–mouse secondary antibodies for double immunofluorescence and FITC-phallolidin when indicated. The samples were analyzed by laser scanning confocal microscopy.

**Immuo-EM.** Membrane recruitment assays with 15 μg Golgi membrane preparations were performed in the presence of cytosol and GTPγS as described above. The membranes were spread on nickel grids that had been previously coated with Formvar and subsequently with poly-lysine. Membranes were fixed with PLP (0.01 M periodate, 0.075 M lysine, 0.037 M sodium phosphate buffer, 2% PFA), quenched, and permeabilized with 10 μM

**In Vivo Transport Assays.**

Pulse-labeled VSVG was accumulated in the TGN of VSV-infected MDCK cells as described above, except that the cells were grown on poly-carbonate filters. Alternatively, filter-grown monolayers were labeled for 10 min at 37°C with 0.5 μM [35]Sphingosine/cysteine and subsequently chased for 2 h at 20°C. After the 20°C block, cells were washed with ice-cold Hank’s solution (GIBCO BRL, Gaithersburg, MD), and either kept on ice or subsequently incubated at 37°C with prewarmed Hank’s solution in the presence or absence of 20 mM butanedione monoxime (BDM) (Sigma Chemical Co.) added to both apical and basalolateral chambers for the time periods indicated in the text. Apical and basolateral media were collected separately. In uninfected cells, total radioactivity was counted directly in EcoLume (ICN Biomedicals Inc., Irvine, CA), in a liquid scintillation counter, and the radioactivity was expressed as percentage of total (intracellular plus secreted) protein. Total cellular protein was extracted in 1% Triton X-100 for 30 min, TCA precipitated, and solubilized in Solvable (Packard Instrument Co., Meriden, CT). VSVG-infected cells were subsequently pelleted for 10 min at 37°C. The levels of nonspecific TGN fragmentation were determined by measuring the release of sialyltransferase activity. Controls (not shown) performed to validate Golgi vesicle release assays as described above. The vesicles were sedimented in an airfuge for 20 min at 27 psi; vesicles released from isolated Golgi membranes were isolated directly from the supernatant fraction of a vesicle release assay.
digitonin for 5 min at room temperature. The detergent was washed out and the grids were blocked with 2% BSA/5% goat serum in PBS. Immunogold labeling was performed in the same buffer with antibodies to p200 and to the ectodomain of VSVG. Negative staining was with 2% ammonium molybdate for 1 min. Grids were observed in a JEOL-100 EX II electron microscope (JEOL USA Inc., Peabody, MA) at 80 mV. The specificity of the labeling was confirmed by the omission of the first antibodies, or of the detergent extraction (when antibodies against luminal epitopes of VSVG were used).

Miscellaneous Techniques

Quantification of bands in fluorograms or autoradiograms was carried out as described in Müsch et al. (1996). The percentage of VSVG vesicle release refers to the amount of VSVG in the supernatant fraction divided by the sum of VSVG in the pellet and supernatant fractions. Protein concentrations were determined with the bicinchoninic acid assay kit from Pierce Chemical Co.

Results

p200 Participates in the Release of VSVG Transport Vesicles from the TGN

The regulated association of p200 with the TGN of MDCK cells (de Almeida et al., 1993) and its presence on nascent vesicles (Narula and Stow, 1993) suggest a role in vesicle production. To study whether p200 is functionally involved in the formation of secretory vesicles, we used a novel in vitro assay that measures the cytosol-dependent vesicular release of the apical marker influenza HA or the basolateral marker VSVG from the TGN of semiintact MDCK cells (Müsch et al., 1996). After infection with either VSV or influenza, the viral glycoproteins were pulse labeled (15 min) with [35S]methionine/cysteine and subsequently accumulated in the TGN of MDCK cells using a 20°C temperature block (Matlin and Simons, 1983). MDCK cells were then mechanically perforated, depleted of their cytosolic and peripheral membrane proteins by a high salt wash, and stimulated for vesicle release by incubation at 37°C, in the presence of rat liver cytosol and an energy regenerating system. Typically, cytosol induces a three- to fivefold increase in marker release into the supernatant. Vesicles released from the TGN were separated from the semiintact cells by a brief spin, and the amount of marker protein in pellet and supernatant fractions was analyzed.

mAb AD7 depleted >90% of p200 from cytosol, as judged by immunoblots (Fig. 1 A). We confirmed previous findings (de Almeida et al., 1993) that GTPγS stimulates the recruitment of p200 from rat liver cytosol onto MDCK membranes. High salt–washed membranes displayed no p200 (Fig. 1 B, lane 2), but addition of control (lane 3) or mock-depleted (lane 4) cytosol resulted in efficient recruitment of this protein. No p200 recruitment was observed upon addition of p200 immunodepleted cytosol (Fig. 1 B, lane 5). Immunodepletion of p200 from cytosol had specific effects on the production of post-TGN transport vesicles (Fig. 1 C). Cytosol immunodepleted of p200 had a 50% reduced ability to stimulate the release of VSVG containing vesicles, as compared with control and mock-depleted cytosol, but it was as effective as mock-

![Figure 1](https://jcb.rupress.org/)

Figure 1. Reduced ability of p200-depleted cytosol to stimulate the release of VSVG transport vesicles from the TGN in semiintact cells. (A) Immunodepletion of p200. Equivalent aliquots of control rat liver cytosol or p200-immunodepleted cytosol (as described in Materials and Methods) were analyzed by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with p200 antibodies. (Lanes Coomassie) Eluant from immunobeads, control cytosol, and p200-immunodepleted cytosol samples, analyzed by SDS-PAGE and stained with Coomassie blue. (B) Western blot of membrane-bound p200 recruited from rat liver cytosol. (Lane 1) cytosol; (lane 6) p200 eluant from immunobeads; (lanes 2–5) high salt–washed semiintact cells were incubated in the presence of 1 mM GTPγS with no cytosol (lane 2), control cytosol (lane 3), mock-depleted cytosol (lane 4), or p200-depleted cytosol (lane 5). (C) Supernatant fractions of a VSVG (left) or HA (right) vesicle release assay in the presence of no cytosol (lane 1) or control cytosol (lane 2), p200-depleted cytosol (lane 3), or mock-depleted cytosol (lane 4). (D) VSVG and HA released from semiintact cells in an in vitro vesicle release assay, expressed as a percentage of the total marker in supernatant and pellet fractions. Mean and standard errors from five experiments.
depleted cytosol in stimulating the release of HA-containing vesicles (Fig. 1 C, compare lane 3 with lanes 2 and 4, for both VSVG and HA). The results of the vesicle release experiments, represented graphically in Fig. 1 D, suggest a functional role of p200 in the production of VSVG transport vesicles.

**p200 Is Present in VSVG Transport Vesicles Released from Semiintact Cells**

If p200 is involved in the formation of VSVG transport vesicles, it should be detected at the vesicle surface immediately after release. Indeed, immunoprecipitation experiments initially carried out with vesicles released from semiintact MDCK cells (Fig. 2 A) showed that p200 antibodies immunoisolated intact VSVG transport vesicles, but not HA transport vesicles (Fig. 2 A, VSVG and HA panels, lanes 4 and 5). Control experiments confirmed our previous observations (Müsch et al., 1996) that luminal epitopes of VSVG and HA were accessible to specific antibodies only after solubilization of the vesicular membrane with Triton X-100 (Fig. 2 A, compare lane 1 with lane 3 in both VSVG and HA panels). In contrast, antibodies against cytoplasmic epitopes of these proteins were effective in immunoisolating VSVG and HA intact vesicle populations in the absence of detergent; however, a high salt wash was required for efficient precipitation, presumably to remove proteins that masked these cytoplasmic epitopes (Fig. 2 A, lane 2).

**VSVG Transport Vesicles Released from Purified Golgi Fractions Contain p200**

To facilitate the manipulation of Golgi membranes before or during the in vitro vesicle release assay, we carried out experiments using a purified Golgi fraction, obtained from cells that had accumulated viral glycoprotein in the TGN during an incubation at 20°C (see Materials and Methods). When used for the in vitro assay, the high salt–washed Golgi fraction supported cytosol-induced release of vesicles carrying VSVG (Fig. 2 B, compare lanes 2 and 3). To study the presence of p200 in the coat of VSVG transport vesicles, all experiments shown in Fig. 2 B were carried out in the presence of the nonhydrolyzable GTP analogue GTPγS, which promotes coat retention without affecting vesicular release from Golgi complex (Orci et al., 1993b).
or TGN (Simon et al., 1996; see Fig. 4). GTPγS prevents coat release by locking the small GTP binding proteins Sar1/Arf1 in the GTP state (Rothman, 1994). VSVG in vesicles released under these conditions was latent; i.e., it was immunoprecipitated by ectodomain antibodies only after addition of detergent (Fig. 2 B, compare lanes 3 and 4). Strikingly, VSVG transport vesicles released in the presence of GTPγS were more efficiently immunoprecipitated by p200 antibodies than vesicles released in the presence of GTP (compare Fig. 2 B, lanes 3 and 7, with Fig. 2 A, lanes 3 and 5). Precipitation with p200 antibodies was reduced by prior treatment of the vesicles with 500 mM KCl or 1% Triton X-100 (Fig. 2 B, compare lane 7 with lanes 8 and 9). These experiments demonstrated that p200 behaves like a peripheral vesicular coat protein, and that VSVG and p200 do not interact directly but likely through the membrane of a common transport vesicle. Additional experiments provided support to the hypothesis that VSVG transport vesicles purified by p200 antibody precipitation are involved in anterograde transport (Fig. 2 C).

Rab 8, a component of basolateral transport vesicles that carry VSVG protein (Huber et al., 1993), was detected by immunoblot in the isolated vesicle fraction. On the other hand, β-COP, reportedly involved in anterograde and retrograde transport between the ER and Golgi, was not detected under the same conditions.

Electron microscopic examination of purified Golgi fractions incubated with cytosol and GTPγS, in which VSVG and p200 had been labeled with two different sizes of colloidal gold particles, revealed the dual presence of VSVG and p200 in vesicular structures of ~80 nm (Fig. 3). In these experiments, we labeled VSVG with ectodomain (luminal) antibodies in the presence of a brief digitonin treatment, since our vesicle precipitation experiments (Fig. 2 A) predicted that epitopes in the cytoplasmic tail of VSVG would not be readily accessible after GTPγS treatment, presumably because of coat protein recruitment. Specificity for VSVG and p200 labeling was supported by various controls, including the absence of label with antibodies against luminal epitopes when digitonin was not present. Taken together, our data suggest that p200 participates specifically in the release of basolateral vesicles from the TGN.

**VSVG Vesicle Release Is Not Caused by Fragmentation of the TGN**

The biochemical and morphological data presented above strongly support the presence of p200 in VSVG-containing transport vesicles. However, to demonstrate conclusively that the vesicle release observed in our in vitro assays is the result of a specific vesicle assembly mechanism rather than fragmentation of the Golgi membranes, we studied the release of sialyltransferase, a TGN marker enzyme. Fig. 4 shows that the percentage of sialyltransferase released from the Golgi in standard assays in the presence of GTP or GTPγS was small compared to that of VSVG (3% vs 22% in the presence of GTP; 1.7 vs 24% in the presence of GTPγS). Furthermore, whereas depletion of p200 reduced the release of VSVG by >50% (to 10.3%), it did not significantly change the release of sialyltransferase (2.8%). These results indicate that our assay measures a
Myosin II from Three Different Sources Restores the Activity of Rat Cytosol Depleted of p200 to Promote Vesicle Release

If p200 is myosin II, depletion of myosin II with specific antibodies should result in specific inhibition of the production of VSVG transport vesicles in the in vitro assay, as shown for p200, and purified myosin II should restore the activity of p200-depleted cytosol. Indeed, cytosol that was immunodepleted of myosin II with polyclonal antibodies against human platelet myosin II (Fig. 7 B, compare lanes 1 and 2) had a ~50% reduced ability to stimulate the release of VSVG transport vesicles from semiintact cells, but stimulated normally the release of HA transport vesicles (Fig. 7, C and D). These results are identical to those described before (Fig. 1) for the p200 antibody AD7. The full activity of myosin-depleted cytosol in stimulating vesicle release was recovered (Fig. 7 C, lane 4), or even increased above control levels (Fig. 7 C, lane 6), by the addition of a myosin II–enriched cytosol fraction, purified from rat liver cytosol by binding to phallolidin-stabilized actin filaments in the absence of ATP and released in the presence of ATP (Fig. 7 A). These data strongly support a myosin role in vesicle release from the TGN and provide additional evidence that p200 is myosin II.

Because the myosin II used in the experiment just described was purified from rat cytosol, the data above are also consistent with a scenario in which p200 and myosin are two different proteins that are independently involved in vesicle release from the TGN. To critically test this possibility, we carried out complementation experiments with muscle myosin II, isolated from human platelets by an independent chromatographic procedure (Fig. 8 A). Whereas rat cytosol stimulates ~2.5-fold the release of VSVG vesi-
Figure 5. The p200 antibody AD7 recognizes a myosin II. (A) p200, immunoprecipitated from rat liver cytosol with the AD7 antibody, was transferred to polyvinylidene difluoride membranes and cleaved with endoproteinase Lys-C. A 19-amino acid peptide was sequenced and aligned with the sequences available from the National Institutes of Health gene bank and EST database (Sequencing Facility, Rockefeller University, New York). Note sequence homology with three nonmuscle myosins II from three different origins. (B) Total rat liver cytosol (lane cytosol) or the AD7 immunoprecipitate from rat liver cytosol (lane p200) was probed by immunoblot analysis with antibodies against human platelet myosin II.

Figure 6. Colocalization of myosin II and p200 in the Golgi of MDCK cells treated with AlF₄⁻. MDCK monolayers were incubated in the presence (A, B, D, and F) or absence (C, E, and G–L) of AlF₄⁻ (30 mM NaF/50 μM AlCl₃) for 30 min before they were processed as follows: (A–F) fixation in 2% PFA in PBS, extracted in 0.1% Triton X-100; (G–J) extracted in cytoskeleton buffer, fixed in 4% PFA in cytoskeleton buffer (see Materials and Methods); (K and L) fixed in 2% PFA, acetone extracted. Indirect immunofluorescence was performed with antibodies against either platelet myosin (pl) (E, F, J, and K), chicken brush border myosin II (bb) (C and D), or the p200 antibody AD7 (H and L). Double fluorescence of MDCK cells with FITC-phalloidin and platelet myosin (I and J), or p200 (G and H) antibodies that were incubated with Texas red–conjugated secondary antibody. Double immunofluorescence with affinity-purified antibody to myosin II from chicken brush border and p200 was performed in A and B. The samples were analyzed by laser scanning confocal microscopy.
of vesicle formation and release. Rather, the data support the hypothesis that myosin II in the presence of the VSVG peptide results in a stimulation of vesicle release, without, however, reaching the levels observed with control cytosol in the absence of ATP (lane 2); depletion was prevented by addition of 5 mM Mg-ATP (lane 3); the resuspended actin–myosin pellet obtained in the absence of ATP (lane 5) was incubated with 5 mM Mg-ATP to release myosin, and actin filaments were removed by sedimentation (lane 4). (Lower panel) Coomassie-stained gel showing control cytosol and the myosin-enriched fraction prepared from it (corresponding to lanes 1 and 4 of the upper panel, respectively). (B) Immunoblot of cytosol samples with myosin and actin antibodies. Cytosol depleted with polyclonal antibodies against nonmuscle myosin coupled to protein A–Sepharose (lane 1); control cytosol (lane 2); cytosol mock-depleted with protein A–Sepharose alone (lane 3); and myosin eluted from the immunobeads. (C) Vesicle fractions of VSVG (upper panel) or HA (lower panel) vesicle release assays performed in the absence of cytosol (lane 1), with control cytosol (lane 2), mock-depleted cytosol (lane 3), myosin-depleted cytosol (lane 5), myosin-depleted cytosol supplemented with myosin eluted from the actin/myosin pellet in A (lane 4), or control cytosol supplemented with myosin released from the actin pellet (lane 6). (D) Data from four independent experiments similar to C. VSVG and HA released into the supernatant fractions were expressed as a percentage of the total marker in supernatant plus pellet fractions. Results were expressed as mean ± SEM.

We and others (Müsch et al., 1996; Pimplikar et al., 1994) have recently shown that a synthetic peptide identical to the cytoplasmic domain of VSVG inhibits the release of VSVG-containing vesicles from the TGN in vitro. When incubated at the half-maximal inhibitory concentration with p200-depleted cytosol in the assay, the already low efficiency of vesicle release is further decreased to background levels (Fig. 8 B, lanes 9 and 10). The addition of myosin II in the presence of the VSVG peptide results in a stimulation of vesicle release, without, however, reaching the levels observed with control cytosol in the absence of the peptide (Fig. 8 B, lanes 11 and 12). This result indicates that depletion and readdition of myosin to the system do not change the mechanism of VSVG vesicle release. Rather, the data support the hypothesis that myosin and the VSVG peptide act on one and the same pathway of vesicle formation and release.

**Functional Evidence for the Involvement of Myosin in the Release of VSVG Transport Vesicles**

To provide independent functional evidence for the involvement of a myosin in the release of VSVG transport vesicles from the TGN, we tested experimental conditions that either inhibit the actin-dependent myosin ATPase activity or uncouple energy hydrolysis from force generation in the acto–myosin complex. The chymotryptic NH2-terminal fragment of both muscle and nonmuscle myosin (S1 fragment) contains the myosin “heads” with both the actin and the ATP binding site, and shows a similar actin-dependent ATPase activity as the intact molecule (Hynes et al., 1987; Kishino and Yanagida, 1988). S1 lacks the ability of intact myosin to dimerize (controlled by the myosin tail), and therefore cannot transduce nucleotide hydrolysis into force generation, which is achieved by myosin dimers sliding actin filaments against each other; however, since S1 competes with myosin for the binding of actin, it is therefore expected to inhibit any process that involves the force-generating ATPase activity of myosin. Fig. 9 A shows the effect of S1 prepared from muscle myosin on the recruitment of myosin from rat liver cytosol to the membrane fraction of semiintact MDCK cells. High salt-washed, semiintact MDCK cells were incubated with two
Human platelet myosin II can substitute for p200 in the production of VSVG transport vesicles. (A) Coomassie stain (lane 1) of myosin II isolated from human platelets. The 100-kD band represents a degradation product as confirmed in a myosin immunoblot (lane 2). (B) Supernatants of an assay studying the release of VSVG-containing vesicles from a Golgi-enriched membrane fraction. Golgi membranes were incubated with an energy-regenerating mix in the presence of: no cytosol (lane 1), control cytosol (lanes 2 and 3), p200-immunodepleted cytosol (lanes 4 and 5), p200-depleted cytosol and myosin II (lanes 6 and 7), p200-depleted cytosol and 25 μM VSVG peptide (lanes 8 and 9), or p200-depleted cytosol, VSVG peptide, and myosin II (lanes 10 and 11) as described in Materials and Methods. After the incubation, the Golgi membranes were separated from the vesicle fraction and both fractions were analyzed directly by SDS-PAGE. (C) Quantification of the data from four individual experiments similar to B.

Data from cultured PtK2 cells suggest that BDM rapidly enters into cells, when added to the culture medium, and promotes redistribution of myosin away from stress fibers (Cramer and Mitchison, 1996). These data led us to test whether BDM affected the post-Golgi transport of VSVG in vivo. Indeed, we observed (Fig. 10 B) that addition of BDM immediately after the 20°C block that accumulated VSVG in the TGN resulted in a reproducible reduction of 37.5% (P < 0.05) of the delivery of VSVG to the basolateral surface, or of 30.5% (P < 0.03) of the release of VSVG in virions to the basolateral medium after 15 min of incubation at transport-permissive temperature. In agreement with this result, BDM affected total basolateral secretion from uninfected MDCK cells differently from apical secretion. Whereas total basolateral secretion was significantly inhibited (by 17%, P < 0.05) after 30 min of transport, total apical secretion was slightly stimulated (15%, P < 0.02). The effect of BDM, however, was transient. When transport to the surface was followed over 1 h in the presence of BDM, neither VSVG transport nor basolateral secretion was consistently affected by the drug. In fact, all reports of in vivo effects of the drug on myosin are studies of short-term effects (in the range of minutes). It is not clear whether the transient effect of BDM on basolateral transport reflects inactivation of the drug over different amounts of rat liver cytosol in the presence of GTPγS, and the amount of myosin recruited to the membrane fraction during 30 min at 37°C was determined by immunoblot, with the AD7 mAb against p200 (Fig. 9 A, lanes 1–3). Addition of muscle S1 in ~10× molar excess over cytosolic myosin effectively competed with the recruitment of this protein at both cytosol concentrations (Fig. 9 A, lanes 4–6; note that AD7 cross-reacts with S1). Furthermore, S1 fragments prepared by either papain (Mg-S1) or chymotryptic (Ch-S1) cleavage from muscle myosin II inhibited the release of VSVG transport vesicles from the TGN in the in vitro assay (Fig. 9 B), suggesting that the catalytic region of myosin is needed for this process. VSVG release from the TGN was also inhibited by a chymotryptic 20-kD COOH-terminal fragment of caldesmon, a protein that regulates actin–myosin binding in smooth muscle and nonmuscle cells (Fig. 9 C) (Velaz et al., 1993).

Additional functional evidence for a role of myosin in vesicle release from the TGN was provided by experiments with BDM, an effective and specific inhibitor of nonmuscle myosin ATPase activity in vitro (Cramer and Mitchison, 1996). Fig. 10 A shows that BDM strongly inhibits the release of VSVG transport vesicles in vitro but did not affect the release of HA transport vesicles.
immunoprecipitated with antibodies against VSVG in the presence of Triton X-100 (lanes 2, 4, 6, and 8) or with antibodies against p200 (lanes 1, 3, 5, and 7) in the absence of Triton X-100. Samples from lanes 3 and 6 and 7 and 8 were incubated with an estimated 10-fold excess of S1-Mg (generated by papain cleavage) or Ch-S1 (generated by chymotryptic cleavage) over myosin in the cytosol during the reaction (myosin was estimated to account for 5% of all cytosolic proteins). Data are from two independent experiments. (C) The 20-kd COOH-terminal fragment of caldesmon inhibits the release of VSVG-containing vesicles. (Upper panel) Supernatant fractions of a VSVG vesicle release assay performed in the absence (lane 1) or presence of 1 mg/ml (10 μg) (lanes 2 and 4) or 2 mg/ml (20 μg) (lanes 3 and 5) cytosol, and in the absence (lanes 1, 2, and 3) or presence of 0.5 μg (lane 4) and 1 μg (lane 5) caldesmon fragment. (Lower panel) Quantitation of data from four independent experiments. Mean ± SEM.

Fig. 11 B further reveals that myosin binding to isolated Golgi membranes is always accompanied by the recruitment of actin. The association of actin with Golgi membranes is also regulated by GTP binding proteins and is sensitive to BFA. The fact that ATP prevented the Golgi recruitment of myosin in the presence of GTPγS (Fig. 11 A, lane 5) is consistent with myosin binding to Golgi-associated actin. To test whether myosin recruitment to the Golgi requires actin, we reduced the actin concentration in the cytosol by 80% by sedimentation of phalloidin-stabilized actin filaments in the presence of ATP (to prevent myosin binding) at 150,000 g (Fig. 11 C). Although the resulting cytosol has normal myosin levels (Fig. 11 C, lane 7 vs lane 8), myosin recruitment to the Golgi is drastically reduced (Fig. 11 C, compare lane 2 and 3). The addition of a myosin fraction alone is not sufficient to increase the amount of myosin at the membrane (Fig. 11 D, lane 4), but the actin/myosin complex has to be incubated to reestablish the GTPγS-dependent binding of myosin to the Golgi (Fig. 11 D, lane 6). Additional cytosolic proteins seem to be involved in the recruitment process since actin was not able to recruit myosin to the Golgi in the absence of cyto-
the membranes, whereas HA transport vesicles were precipitated with antibodies against HA in the presence of Triton only. (Lower panel) Quantitation of the data from two independent experiments for HA and VSVG. (B) Effect of BDM on TGN to surface transport of VSVG and secretion of apical and basolateral proteins in vivo. Filter-grown MDCK cells were pulse labeled after VSV infection and radiolabeled VSVG accumulated in the TGN (time 0). Surface transport of VSVG (second column) and release of VSVG-containing viruses into the basolateral medium (third column) was measured after a 15-min incubation at 37°C in the absence (solid bars) or presence (striped bars) of 20 mM BDM. (Fourth through ninth columns) Uninfected cells were processed in the same way as the VSV-infected ones, and total radioactivity in the basolateral (fourth through sixth columns) and apical (seventh through ninth columns) medium was measured after a 15-min (fifth and eighth columns) or 30-min (sixth and ninth columns) incubation in the absence (solid bars) or presence (striped bars) of 20 mM BDM. Each bar with standard error results from nine data points established in three individual experiments. * Significant difference between ± BDM by t test (P < 0.05).

Discussion

p200 Is a Myosin II and Is Involved in the Release of VSVG Vesicles from the TGN

In this report we present microsequencing, immunological, and functional data with an in vitro vesicle release assay that clearly demonstrate that the TGN-associated protein p200 (Narula et al., 1992) is a myosin II involved in the release of VSVG transport vesicles from the TGN. A 19-amino acid fragment of p200 was 100% identical to a tail region of human nonmuscle myosin, and gel-purified p200 was recognized by platelet myosin II antibodies. Rat liver cytosol immunodepleted of p200 had a 50–80% reduced ability to stimulate the release of VSVG transport vesicles from the TGN of semiintact MDCK cells or purified Golgi fractions, and its activity in stimulating vesicle release was efficiently reconstituted by human platelet and chicken intestinal brush border myosin II, purified by nonimmune methods. Furthermore, immunodepletion of myosin II with specific antibodies resulted in comparable inhibition of VSVG transport vesicle release, which could be overcome by addition of a myosin-enriched fraction.

The extent of the inhibitory effect of myosin depletion from cytosol was dependent on the source of TGN transport vesicles used in the in vitro assay. Whereas, in semiintact cells, the inhibition of VSVG release by myosin-depleted cytosol averaged ~50%, in purified Golgi fractions it reached up to 80%. The detection of VSVG release from both semiintact cells and purified Golgi fractions required a high salt wash, presumably to remove membrane bound myosin II and other myosins or motors that could replace it. Overlapping roles of myosin and kinesin have been demonstrated by gene knockout experiments in yeast, *Aspergillus*, and *Dictyostelium* (Ostap and Pollard, 1996; Vale, 1993). Under the conditions used in our assay studying vesicle release from purified Golgi, addition of cytosol caused a release of only 2–3% of the total sialyltransferase, whereas the release of VSVG protein was in the range of 20–30% of Golgi protein.

Specific myosin function data provided important additional support for a role of myosin II in vesicle release from the TGN: (a) the S1 fragment of muscle myosin II competed effectively with the membrane recruitment of nonmuscle myosin II and inhibited the release of VSVG transport vesicles; (b) the 20-kD COOH-terminal frag-
ment of caldesmon, an inhibitor of actin/myosin binding, also inhibited VSVG protein release; (c) butanedione monoxime, an inhibitor of myosin ATPase activity in muscle and nonmuscle cells, reduced in vitro release of VSVG from compensation by the cells using alternative routes and/or from inactivation of the drug. BDM also inhibited basolateral delivery of VSVG and basolateral secretion in vivo, but not apical delivery, suggesting that myosin is involved specifically in basolateral transport (Fig. 10). The smaller and transient BDM effects observed in vivo may result from compensation by the cells using alternative routes and/or from inactivation of the drug.

Several lines of evidence demonstrate that the release of VSVG observed in our in vitro assay is indeed in TGN-derived basolateral transport vesicles and cannot be accounted for by nonspecific Golgi fragmentation. First, p200 or myosin II depletion did not affect the release of HA from the TGN, suggesting that a different apical sorting machinery is involved in the release of this protein and ruling out a nonspecific effect of myosin depletion on vesicle release from the TGN. Second, the release of an intrinsic TGN marker, sialyltransferase, under our assay conditions was insignificant when compared with that of VSVG protein (Fig. 4). Third, immuno-EM analysis of TGN fractions incubated under conditions that stimulate vesicle release demonstrated the colocalization of VSVG protein and p200 in vesicles of 70–80 nm (Fig. 3). Fourth, rab 8, a marker for basolateral vesicles (Huber et al., 1993), was detected on the p200 immunosolated vesicles, but not β-COP, a coat protein found in the Golgi and to a lesser extent in the TGN.

**Table 1. Distribution and Specific Activities of Marker Proteins for Different Organelles in a Golgi Preparation**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sialyltransferase (TGN)</th>
<th>Mannosidase II (Golgi)</th>
<th>Glucosidase II (ER)</th>
<th>Cathepsin D (lysosomes)</th>
<th>Succinate dehydrogenase (mitochondria)</th>
<th>Streptavidin (plasma membrane)</th>
<th>gp114 (plasma membrane)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear pellet</td>
<td>–</td>
<td>1.07</td>
<td>0.15</td>
<td>0.81</td>
<td>0.46</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Postnuclear supernatant</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>–</td>
<td>0.15</td>
<td>0.18</td>
<td>1.17</td>
<td>0.36</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Postmitochondrial supernatant</td>
<td>–</td>
<td>1.4</td>
<td>0.69</td>
<td>0.46</td>
<td>0.13</td>
<td>1.17</td>
<td>0.36</td>
</tr>
<tr>
<td>Golgi</td>
<td>4.7</td>
<td>3.8</td>
<td>0.19</td>
<td>0.12</td>
<td>0.01</td>
<td>0.05</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Specific enrichment of marker proteins for subcellular organelles in different steps of a Golgi preparation. Golgi-enriched MDCK membranes were prepared, and the enzymatic activities of sialyltransferase, mannosidase II, glucosidase II, and succinate dehydrogenase were measured as described in Materials and Methods for equal amounts of total protein. Equal amounts of protein were also analyzed in immunoblots for the plasma membrane marker gp114 and the mature form of the lysosomal marker cathepsin D. Dot blots were probed for streptavidin that bound to cell surface proteins that had been biotinylated before the preparation. Blots were probed with 125I–protein A or 125I-streptavidin, and the bands in the corresponding autoradiographs were quantified as described in Materials and Methods. All data were normalized for the postnuclear supernatant.
Our results contradict a recent report by Ikonen et al. (1996), which confirmed the TGN localization of p200 to vesicles and tubular structures by immuno-EM but reported (a) no effect of p200 depletion on VSVG transport from TGN to plasma membrane in streptolysin O-permeabilized cells; (b) no colocalization of p200 with VSVG protein; and (c) no p200 in VSVG vesicles floated in sucrose density gradients. We will discuss these discrepancies in order.

(a) We suggest that the failure to observe an effect of p200 depletion on VSVG transport can be explained by the requirement (discussed above) for a high salt wash to observe an effect of myosin depletion in semiintact cells. In their assay for transport, originally developed by Gravotta et al. (1996), transport of VSVG or influenza HA to their respective cell surfaces is studied after addition of streptolysin O to the opposite surface to allow the exit of cytoso. As the Simons group has previously reported, transport of HA or VSVG to the cell surface can be observed only if cytosol removal is $\approx 70\%$; under these conditions, a weak, usually less than twofold stimulation is observed (Pimplikar et al., 1994). Given that cytosolic myosin is one of the most abundant cellular proteins (~1–5% of total cellular protein), and that our data indicate that a high salt wash is required to observe cytosol-dependent release of VSVG in semiintact cells (Müsch et al., 1996, and this paper), it is therefore not surprising that p200 depletion from cytosol had no effect of VSVG transport in streptolysin O-permeabilized MDCK cells.

(b) In their studies, Ikonen et al. (1996) use an anti-tail antibody and immunogold to localize VSVG in MDCK cells ripped by the addition of filter paper to the apical surface; under these conditions, VSVG localizes to structures that look different from the TGN-like tubules and vesicular structures rich in p200. On the other hand, our results demonstrate colocalization of VSVG and p200 in tubules and vesicles (Fig. 3) if antibodies against VSVG ectodomain are used on samples that have been previously fixed and disrupted with detergent. These results suggest that the absence of VSVG tail antibody labeling in p200-rich structures may be due to steric interference by coat proteins and p200/myosin recruited to the TGN. Furthermore, Griffiths et al. (1985) failed to detect VSVG protein accumulated in the TGN by a 20°C transport block with the anti-tail antibodies in regions of the TGN that contained coated buds.

(c) Ikonen et al. (1996) failed to find p200 immunoreactivity in VSVG-rich vesicles purified by flotation in a sucrose gradient, from assays performed in the presence of guanylimidodiphosphate to promote membrane association of p200. Our results indicate, on the other hand, that vesicles isolated with p200 antibodies from the supernatant of our in vitro assay contain a large fraction of VSVG, ~80%, in the presence of GTPyS (Fig. 2B), and that precipitation of VSVG by p200 antibody is less efficient in the absence of GTPyS (~30%), indicating that p200 and coat proteins may be quickly removed by uncoating in the presence of GTP. It is well demonstrated that GTPyS does not inhibit the release of VSVG vesicles but prevents uncoating (Simon et al., 1996, and this paper); however, the effects of guanylimidodiphosphate on both p200 recruit-ment and on the efficiency of VSVG vesicle release were characterized by neither Ikonen et al. (1996) nor others.

**Binding of Myosin to Golgi Membranes Involves G Proteins and Actin**

Myosin II found in association with the Golgi complex showed binding properties characteristic of coat proteins involved in vesicle assembly. The binding of myosin to Golgi membranes was dependent on GTP or its nonhydrolyzable homologue GTPyS and was stimulated by AlF$_3$, supporting a contribution of trimeric G proteins. Brefeldin A, which prevents the recruitment of the small GTP binding protein ARF to Golgi membranes (Helms and Rothman, 1992; Robinson and Kreis, 1992) and causes tubulation of the TGN in MDCK cells (Wagner et al., 1994), is also effective in inhibiting myosin recruitment to the Golgi. AlF$_3$ also significantly stimulated the recruitment of myosin to the perinuclear regions in intact MDCK cells (Fig. 6); 10 µg/ml BFA effectively dispersed this perinuclear p200/myosin staining (Cohen, D., A. Müsch, and E. Rodriguez-Boulan, unpublished results). The same BFA concentration also inhibits the release of VSVG-containing vesicles from the TGN in the semiintact cell assay (Müsch, A., and E. Rodriguez-Boulan, unpublished results). Identical features for recruitment to Golgi membranes have been reported for coatamers and AP1/clathrin, the binding of which to the Golgi requires ARF1 (Orci et al., 1993a,b; Stamnes and Rothman, 1993; Traub et al., 1993). Thus, our results suggest that myosin either is part of a coat complex itself or is recruited to the site of vesicle budding subsequent to coat assembly to participate in vesicle fission, as described for the fission of clathrin-coated endocytic vesicles by dynamin, a GTP-dependent motor (Damke et al., 1995; Hinshaw and Schmid, 1995; Takel et al., 1995). Indeed, in Saccharomyces cerevisiae, the formation of endocytic vesicles has been recently shown to be dependent on the expression of myosin I; two myosin I isoforms have been characterized that complement each other in promoting endocytosis (Geli and Riezman, 1996). In Dictyostelium, several myosin I isoforms seem to have overlapping functions in pinocytosis and phagocytosis, explaining the absence of a phenotype after knockout of just one of the several myosin I genes (Jung et al., 1996). The involvement of actin filaments in endocytic vesicle formation had been suggested indirectly by pharmacologic experiments. In yeast, disruption of actin filaments by cytochalasin D resulted in the accumulation of coated pits at the plasma membrane (Kuebler and Riezman, 1993). A similar phenomenon was reported for apical membranes of polarized epithelial cells (Gottlieb et al., 1993). The involvement of actin filaments in endocytic vesicle formation has been recently shown to be dependent on the expression of myosin I; two myosin I isoforms have been characterized that complement each other in promoting endocytosis (Geli and Riezman, 1996).

The inhibition of VSVG transport vesicle formation by S1, calsedomon, and BDM, which prevent the interaction of myosin with actin (Figs. 9 and 10), suggests that the actin-dependent ATPase activity of myosin is involved in this process. The involvement of actin in the release of VSVG transport vesicles is also supported by experiments in which depletion of phalloidin-polymerized actin from cyto-
tosol prevented myosin recruitment to Golgi membranes; only the addition of resuspended actin restored myosin recruitment (Fig. 11). Furthermore, addition of ATP together with GTPyS inhibited membrane recruitment of myosin, consistent with this process of being mediated by actin and therefore readily abolished by ATP hydrolysis. Interestingly, the binding of actin to Golgi membranes, like myosin binding, was dependent on GTP, regulated by trimeric G proteins, and BFA sensitive, in agreement with a close interaction of both molecules during binding. In this respect, it is noteworthy that a dynamic cytoskeleton has been recently detected at the Golgi, which contains β-spectrin (Beck et al., 1994). Independently, the presence of ankyrin at the Golgi has been reported (Devarajan et al., 1996). Although the structure and links of this new cytoskeletal network are still obscure, recent data suggest that it mediates the interaction of the Golgi with microtubules via the binding of Golgi spectrin to the actin-related protein, centrinactin (Holleran et al., 1996). Centrinactin is part of the dynactin complex that contains the microtubular motor dynein that has been detected earlier at the Golgi (Fath et al., 1994). Similary, the spectrin cytoskeleton might connect the Golgi with actin filaments. The assembly of the spectrin cytoskeleton at the Golgi is dynamic and sensitive to BFA (Beck et al., 1994). It is tempting to speculate that the binding of actin to Golgi membranes described in this report might be mediated by the spectrin/ankyrin cytoskeleton, as described for the plasma membrane (Bennett, 1989). Myosin, in this scenario, might move actin filaments to either pull a bud or to move away vesicles from the donor membrane. Alternatively, myosin might promote actin rearrangements and local dissolution of the Golgi cytoskeleton, thus allowing coat protein recruitment and vesicle budding. Actin rearrangements of this sort have been suggested to be essential for both exo- and endocytic events at the plasma membrane (Bretscher, 1991; Jung and Hammer, 1990; Maniak et al., 1995; Mualllem et al., 1995; Sandvig and van Deurs, 1990). In this scenario, myosin might regulate the dynamics of a spectrin-based Golgi skeleton that directly or indirectly participates in the formation of buds and vesicles, as suggested by Beck and Nelson (1996). Further studies are needed to determine whether a rearrangement of the actin cytoskeleton might also be necessary for other vesicle budding events in the TGN. The participation of myosin I in a late step in apical transport has been demonstrated (Fath and Burgess, 1993). Indeed, although our current data do not support the involvement of myosin in vesicular release of HA, this could be the result of the full preservation of the actin cytoskeleton in MDCK cells infected with influenza virus (Salas et al., 1986). The intense disruption of the actin cytoskeleton caused by VSV infection might allow the in vitro vesicle budding assay to be more sensitive to myosin depletion. The important advances made in the identification of novel myosins in recent years (Hasson and Mooseker, 1995; Mooseker and Cheney, 1995) predict a plethora of trafficking functions for these molecules that are waiting to be discovered.

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