Glyceraldehyde-3-phosphate Dehydrogenase Is Required for Vesicular Transport in the Early Secretory Pathway*

Ellen J. Tisdale‡

From the Department of Pharmacology, Wayne State University School of Medicine, Detroit, Michigan 48201

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Protein transport in the early secretory pathway requires Rab2 GTPase. This protein promotes the recruitment of soluble components that participate in protein sorting and recycling from pre-Golgi intermediates (vesicular tubular clusters (VTCs)). We previously reported that a constitutively activated form of Rab2 (Q65L) as well as Rab2 wild type promoted vesicle formation from VTCs. These vesicles contained Rab2, β-COP, p53/gp58, and protein kinase C but lacked anterograde-directed cargo. To identify other candidate Rab2 effectors, the polypeptide composition of the vesicles was further analyzed. We found that vesicles released in response to Rab2 also contained the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To study the relationship of this enzyme to Rab2 function, we performed a quantitative binding assay to measure recruitment of GAPDH to membrane when incubated with Rab2. Rab2-treated microsomes showed a 5–10-fold increase in the level of membrane-associated GAPDH. We generated an affinity-purified anti-GAPDH polyclonal to study the biochemical role of GAPDH in the early secretory pathway. The antibody arrests transport of a reporter molecule in an assay that reconstitutes ER to Golgi traffic. Furthermore, the affinity-purified antibody blocked the ability of Rab2 to recruit GAPDH to membrane. However, the antibody did not interfere with Rab2-stimulated vesicle release. These data suggest that GAPDH is required for ER to Golgi transport. We propose that membranes incubated with anti-GAPDH and Rab2 form “dead end” vesicles that are unable to transport and fuse with the acceptor compartment.

In recent years, a large number of polypeptides that participate in intracellular membrane trafficking have been characterized. These proteins ensure temporal and spatial specificity of vesicular traffic and include members of the Rab family (1, 2). The Rab proteins are small monomeric GTPases that participate in intracellular membrane trafficking (1, 2). The Rab proteins are small monomeric GTPases that function in both the endocytic and exocytic pathways. These essential proteins interconvert between a GDP- and GTP-bound form that results in a cycle of membrane association and release to the cytosol. When these proteins are membrane-associated, they show a unique subcellular location, which suggests that Rab proteins regulate compartment-specific transport events. Most likely, this is due to their ability to recruit in a step-specific manner soluble factors that facilitate protein-protein, membrane-membrane, or membrane-cytoskeletal interactions (2).

Protein transport in the early secretory pathway requires Rab2 (3). This protein immunolocalizes to pre-Golgi intermediates that contain clusters of vesicles and tubules, termed vesicular tubular clusters (VTCs) (4), and function as transport intermediates between the endoplasmic reticulum (ER) and the Golgi complex. Moreover, VTCs are the first site of segregation of the anterior and retrograde pathways and thereby sort and recycle resident proteins from itinerant proteins destined for secretion (5, 6). In our ongoing studies to define the role of Rab2 within VTCs, we have found that a constitutively activated form of Rab2 (Q65L) as well as Rab2 wild type promoted vesicle formation from pre-Golgi intermediates (7). The released vesicles contained β-COP and were enriched in a protein that recycles to the ER, suggesting that Rab2 promoted formation of retrograde-directed carriers. Since protein transport between the ER and Golgi complex is bidirectional, it is likely that vesicles containing Rab2 possess a select set of molecules that labels the carrier as a retrograde-directed shuttle. With this in mind, the polypeptide content of the released vesicles was further analyzed with the goal of identifying candidate Rab2 effectors. We found that the Rab2-generated vesicles contained a ~38-kDa protein.

De Matteis et al. (8) reported that a 38-kDa protein identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) was ADP-ribosylated in a brefeldin A-dependent manner. Brefeldin A blocks transport in both the exocytic and endocytic pathways and causes the Golgi complex to redistribute to the ER (9). Chemical inhibitors of ADP-ribosylation antagonized brefeldin A-induced effects, suggesting that mono-ADP-ribosylated proteins may play a role in preserving Golgi structure and therefore a role in membrane trafficking (10). In addition, Robbins et al. (11) reported that a Chinese hamster ovary cell line harboring a mutant form of GAPDH was defective in endocytosis. Based on these observations, we used reagents specific to the enzyme and identified the 38-kDa vesicle-associated protein as GAPDH.

GAPDH catalyzes the NAD-mediated oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate (12). The active enzyme exists as a tetramer containing identical 37-kDa subunits. Although this abundant protein (10–20% of total cellular protein) is commonly known as a key enzyme in glycolysis, a number of intriguing intracellular roles have been reported for GAPDH, including modulation of the cytoskeleton, phosphotransferase/kinase activity, and the pro-
motion of vesicle fusion (13–16). Interestingly, all of these activities are essential to the maintenance of normal secretory function.

In this study, we found that Rab2 stimulates GAPDH membrane binding. To establish whether GAPDH is required for ER to Golgi transport, an affinity-purified anti-GAPDH polyclonal antibody was generated. This antibody potently inhibits transport of VSV-G from the ER to the Golgi complex and inhibits Rab2-stimulated recruitment of GAPDH to membrane. Although vesicles released in response to Rab2 contain GAPDH, the antibody does not block vesicle budding. These data are the first to show that GAPDH is required for trafficking in the early secretory pathway. Our results suggest that the GAPDH-negative vesicles formed in the presence of Rab2 and anti-GAPDH antibody are “dead end retrograde carriers” that are unable to transport to and fuse with the target compartment, resulting in the arrest of ER to Golgi transport.

**EXPERIMENTAL PROCEDURES**

**Membrane-binding Reaction—**NRK cells were washed three times with ice-cold phosphate-buffered saline (PBS). The cells were scraped off the dish with a rubber policeman into 10 ml Hepes (pH 7.2) and 250 mM mannitol and then broken with 15 passes of a 27-gauge needle. The broken cells were pelleted at 500 × g for 10 min at 4 °C, and the supernatant was removed and recentrifuged at 20,000 × g for 10 min at 4 °C. The resulting pellet containing ER, pre-Golgi, and Golgi membranes was resuspended in 10 ml Hepes (pH 7.2) and 250 mM mannitol and employed in the binding reaction, as described previously (5, 17). For some experiments, the membranes were washed with 1 ml KCl in 10 ml Hepes, pH 7.2, for 15 min on ice to remove peripherally associated proteins. Microsomes were recovered by centrifugation at 20,000 × g for 20 min at 4 °C. Membranes (30 μg of total protein) were added to a reaction mixture that contained 27.5 mM Hepes (pH 7.2), 2.75 mM MgOAc, 65 mM KOAc, 1.8 mM EGTA, 1 mM ATP, 5 mM creatine phosphate, and 0.2 units of rabbit muscle creatine kinase (Boehringer Mannheim Corp., Indianapolis, IN). Recombinant Rab2, Rab2 13-mer, and affinity-purified anti-GAPDH were added at the concentrations indicated under “Results,” and the reaction mix was incubated on ice for 10–20 min. Rat liver cytosol (50 μg) and 2 μM GTP-γ-S were added, and the reactions were shifted to 37 °C and incubated for 5–10 min. The binding reaction was terminated by transferring the samples to ice and then centrifuged at 20,000 × g for 10 min at 4 °C to obtain a pellet (P1). The supernatant (20,000 × g) was recentrifuged at 30,000 × g (95,000 rpm) for 30 min in an air centrifuge to recover released vesicles (P2). In some cases, P2 was subjected to equilibrium density centrifugation, as described previously (7, 18). P2 was mixed with 1 ml of 70% sucrose and overlaid with 5 ml of a 30–50% sucrose. The gradient was centrifuged at 50,000 rpm for 4 h at 4 °C, and 300-μl fractions were collected from the bottom. The recovered fractions were run on 7.5% SDS-PAGE, which contained 2.5 mM MgOAc, 5 mM EGTA, 1.8 mM CaCl₂, and 1 mM N-acetylcysteine, an ATP-regeneration system (1 mM ATP, 5 mM creatine phosphate, and 0.2 IU of rabbit muscle creatine phosphokinase), 5 μl of rat liver cytosol, and 5 μl of semi-intact cells (~5 × 10⁶ cells/ml, ~25–30 μg of total protein) resuspended in 50 ml Hepes-KOH, 90 mM KOAc (pH 7.2). The cells were incubated on ice and fixed in 3% formaldehyde/PBS for 10 min. Intracellular ts045 VSV-G was detected by repermeabilization of the fixed cells with 0.05% saponin in PBS/normal goat serum for 10 min, washed with PBS, and then incubated for 30 min with a monoclonal antibody to VSV-G (P5D4) and Texas Red-labeled donkey anti-mouse IgG (Molecular Probes, Eugene, OR).

**Indirect Immunofluorescence—**NRK cells plated on coverslips were permeabilized with digitonin (20 μg/ml), as outlined previously (20). Coverslips with permeabilized cells were inverted and placed in tissue culture wells that contained the transport mixture described above, preincubated on ice for 20 min with or without 10 μg of anti-GAPDH, and then incubated for 30 min at 32 °C. To terminate transport, the coverslips were washed twice in PBS (3 × 5 min) and fixed in 3% paraformaldehyde/PBS for 10 min. Intracellular ts045 VSV-G was detected by repermeabilization of the fixed cells with 0.05% saponin in PBS/normal goat serum for 10 min, washed with PBS, and then incubated for 30 min with a monoclonal antibody to VSV-G (P5D4) and Texas Red anti-mouse antibody, mounted, and viewed under a Zeiss Axiosvert fluorescence microscope.

**Generation of Anti-GAPDH Antibody—**Antibodies directed against rabbit muscle GAPDH (Sigma) were produced in a New Zealand White rabbit by subcutaneous injection with 1 mg of antigen emulsified in complete Freund’s adjuvant. Three biweekly immunizations of the antigen were made in Freund’s incomplete adjuvant. The serum was applied to cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech) to which rabbit muscle GAPDH was coupled for affinity purification (21). The column was washed with five bed volumes of PBS, eluted with 0.1 M glycine, pH 2.8, and then neutralized to pH 7.2. The eluate was dialyzed against 25 mM Hepes, pH 7.2, 125 mM KCl, and 1 mM NaCl. The protein concentration was determined by the Micro BCA Protein Assay Reagent (Pierce).

**RESULTS**

**GAPDH Is Found on Vesicles Containing β-COP—**We previously demonstrated that Rab2 promotes vesicle formation from VTCs and that the released carriers contain Rab2, β-COP, p53/p58, and protein kinase Cδ (7, 22). To determine whether GAPDH was also associated with these vesicles, we then eluted with a linear NaCl gradient (0–400 mM) in buffer A. 3-ml fractions were collected, and an aliquot of each fraction was separated by SDS–PAGE and immunoblotted with a Rab2 polyclonal antibody. Rab2-enriched fractions were pooled, concentrated, and applied to a 250-ml column containing Sephacryl S-100 (Amersham Pharmacia Biotech). The eluted protein was then affinity-purified by SDS–PAGE and immunoblotting and then pooled and concentrated. Typically, the protein prepared from this procedure was ~90% pure. For use in the assays, the purified protein was first preincubated in an *in vitro* reaction. The isoprenylation reaction was performed in a total volume of 500 μl that contained 5.0 μg of recombinant Rab2, 100 μg of geranylgeranyl pyrophosphate (Sigma), 1 mM dithiothreitol, 250 μl of rat liver cytosol, 10 mM MgCl₂, 1 mM ATP, 5 mM creatine phosphate, and 0.2 units of rabbit muscle creatine kinase. The reaction was incubated for 1 h at 37 °C and then desalted through a 10-ml column of Sephadex G-25 (Amersham Pharmacia Biotech) to remove incompatible reagents that may inhibit the *in vitro* assays. The fraction containing prenylated Rab2 was collected and concentrated, and the protein concentration was determined by Micro BCA Protein Assay Reagent (Pierce). Routinely, 40–45% of the total Rab2 was prenylated as determined by phase separation in Triton X-114 and 70–75% active as determined by examining guanine nucleotide binding exchange properties (7).

**Analysis of Transport in Vitro—**NRK cells were infected for 4 h with the temperature-sensitive VSV strain ts045 and then harvested synthetically radiolabeled with 100 μCi of Trans-32P-S (specific activity of 1192 Ci/mmol, ICN Biomedicals, Irvine, CA) for 10 min at the restrictive temperature (39.5 °C) to accumulate the VSV-G mutant protein in the ER. The cells were then perforated by swelling and scraping and employed in the ER to cis/medial Golgi transport assay as described (19). Briefly, transport reactions were performed in a final volume of 40 μl in a buffer that contained 25 mM Hepes-KOH (pH 7.2), 75 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA, 1.8 mM CaCl₂, and 1 mM N-acetylcysteine, an ATP-regeneration system (1 mM ATP, 5 mM creatine phosphate, and 0.2 IU of rabbit muscle creatine phosphokinase), 5 μl of rat liver cytosol, and 5 μl of semi-intact cells (~5 × 10⁶ cells/ml, ~25–30 μg of total protein) resuspended in 50 ml Hepes-KOH, 90 mM KOAc (pH 7.2). The cells were incubated on ice and fixed in 3% formaldehyde/PBS for 10 min. Intracellular ts045 VSV-G was detected by repermeabilization of the fixed cells with 0.05% saponin in PBS/normal goat serum for 10 min, washed with PBS, and then incubated for 30 min with a monoclonal antibody to VSV-G (P5D4) and Texas Red anti-mouse antibody, mounted, and viewed under a Zeiss Axiosvert fluorescence microscope.

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made use of a quantitative binding assay. For this assay, microsomes were prepared from whole cell homogenates and washed with 1 M KCl to remove peripherally associated proteins. These membranes were preincubated in buffer for 20 min on ice in the presence of 300 ng of recombinant Rab2. This concentration of Rab2 efficiently stimulates vesicle formation. The reaction was then supplemented with rat liver cytosol and GTPγS and incubated at 37 °C for 10 min to promote binding of soluble components. To stop the reaction, membranes were pelleted by centrifugation at 20,000 × g. The supernatant was recovered from the reaction and then centrifuged at high speed (~95,000 rpm) to recover any vesicles released into the supernatant. This slowly sedimenting pellet was subjected to equilibrium density gradient centrifugation (7, 18). The gradient fractions were separated by SDS-PAGE, and the presence of β-COP was determined by Western blotting (Fig. 1). Consistent with our previous results, membrane-associated β-COP peaked at ~42–43% (w/w) sucrose, which is the expected density for coated vesicles. After determining the distribution of β-COP, the blot was reprobed with a monoclonal antibody to GAPDH (Fig. 1). This protein showed the same gradient distribution as β-COP, suggesting that the enzyme is a component of COPI-coated vesicles.

Rab2 Increases Membrane-associated GAPDH—To learn whether GAPDH was recruited from the cytosol to membrane in response to Rab2, we measured membrane association of GAPDH in the microsomal binding assay, described above. Salt-washed membranes were preincubated in buffer for 20 min on ice in the presence or absence of increasing concentrations of recombinant Rab2, which stimulate β-COP and protein kinase Cα/λ membrane association (17, 22). The reaction was then supplemented with rat liver cytosol and GTPγS and incubated at 37 °C for 10 min. Membranes were collected by ultracentrifugation (75,000 rpm for 30 min at 4 °C) and then separated by SDS-PAGE and immunoblotted for β-COP (closed circles) and for GAPDH (open circles). Vesicles released in response to incubation with Rab2 contained both β-COP and GAPDH. The results are representative of three independent experiments.

Fig. 1. GAPDH is associated with vesicles containing β-COP. Microsomes prepared from NRK cell homogenates, as described under “Experimental Procedures,” were preincubated with 300 ng of Rab2 for 10 min on ice. Cytosol and GTPγS were added, and the incubations were transferred to 37 °C for 10 min to promote recruitment of soluble factors. Rapidly sedimenting membranes were collected by centrifugation (20,000 × g for 10 min) to obtain a pellet (P1). The supernatant was recentrifuged at 95,000 rpm for 30 min, and the resulting pellet (P2) was subjected to equilibrium density centrifugation, as described under “Experimental Procedures.” The gradient was fractionated from the bottom into 300-μl fractions, and the recovered fractions were pelleted by ultracentrifugation (75,000 rpm for 30 min at 4 °C) and then separated by SDS-PAGE and immunoblotted for β-COP (closed squares) and for GAPDH (open circles). Vesicles released in response to incubation with Rab2 contained both β-COP and GAPDH. The results are representative of three independent experiments.

Rab2 Recruits GAPDH to VTCs—To determine whether GAPDH is directly recruited to membrane by Rab2 or requires an additional cytosolic(s) component, the binding assay was supplemented with a peptide made to the amino terminus of Rab2 (residues 2–14) (23). Like Rab2, this peptide has previously been shown to require GTPγS, ADP-ribosylation factor, and protein kinase C to stimulate β-COP recruitment to membrane (17). By using the peptide (Rab2 13-mer), which binds directly to microsomes, it is not necessary to generate purified Rab2-GDP dissociation inhibitor complexes to deliver Rab2 to the membrane. Microsomes were preincubated with or without increasing concentrations of Rab2 (13-mer) for 15 min to allow binding of the peptide to membranes, in the absence of cytosol. Purified GAPDH and GTPγS were then added and incubated for 10 min at 37 °C, after which the membranes were analyzed by SDS-PAGE and immunoblotted to assess the level of membrane-bound.
GAPDH. As we observed with Rab2 protein, the Rab2 peptide efficiently recruited GAPDH to membrane in a dose-dependent manner (Fig. 2B). We made use of a battery of peptides employed in our previous studies to learn whether GAPDH recruitment was specific to the Rab2 (13-mer) (Table I). In contrast to Rab2 (13-mer), the control peptidomimetics failed to stimulate GAPDH membrane binding at a comparable concentration (Fig. 2C). These combined results suggest that the enhanced recruitment of GAPDH to membrane was specific to the Rab2 amino terminus.

**Anti-GAPDH Arrests ER to Golgi Transport**—To assess the biochemical role of GAPDH in transport of cargo between the ER and the Golgi complex, we generated a polyclonal antibody to the enzyme. The affinity-purified antibody detected purified GAPDH (Fig. 3A, lane a) and an ~38-kDa protein that comigrated with GAPDH from NRK cell lysates (Fig. 3A, lane b) and from rat liver cytosol (Fig. 3A, lane c). Since the antibody appeared to be specific to GAPDH, we tested the reagent in an *in vitro* transport assay to determine whether the antibody had an effect on the early secretory pathway. For this assay, tissue culture cells are first infected with ts045 VSV-G (VSV-G), a virus that synthesizes a protein with a thermoreversible defect resulting in ER retention at 39.5 °C (24). The plasma membrane of these cells is perforated to release soluble content but retain functional ER and Golgi stacks (19). Incubation of the perforated cells at the permissive temperature of 32 °C initiates export of VSV-G from the ER in the presence of cytosol and ATP. This semi-intact cell assay measures transport of VSV-G protein from the ER to the cis medial Golgi compartment by following the processing of the two N-linked oligosaccharides to endo H-resistant forms.

Preincubation of cytosol with affinity-purified antibody to GAPDH led to a dose-dependent inhibition of ER to Golgi transport (Fig. 3B). The processing of VSV-G to endo H-resistant forms was reduced by 50% when the assay was supplemented with 2 μg of antibody. Transport of VSV-G was almost completely arrested in the presence of 10–15 μg of antibody. We then determined the kinetics of inhibition of anti-GAPDH relative to the block imposed by the addition of Rab2 (13-mer). Semi-intact ts045-infected NRK cells were incubated at 32 °C and the indicated time (∆t), transferred to ice (control), or supplemented with 10 μg of anti-GAPDH or with 75 μM Rab2 (13-mer) and then incubated for a total of 60 min. This protocol allows any VSV-G that has migrated past the GAPDH- or Rab2-requiring step to continue migration to the cis Golgi compartment. Therefore, the fraction of VSV-G processed to endo H-resistant forms will depend on the amount of the reporter molecule that has transported beyond the GAPDH- and the Rab2-sensitive steps at the time of their addition. In control cells, VSV-G endo H-resistant forms were detected after a 15-min lag (Fig. 3C). Within ~20 min of incubation, 50% of the VSV-G protein was processed, indicating migration to the Golgi complex (Fig. 3C). In contrast, cells treated with anti-GAPDH were blocked in transport ~5–7 min before the processing of VSV-G to endo H-resistant forms, and by 20 min of incubation >60% of the VSV-G had transported through the anti-GAPDH-sensitive step. This temporal site of inhibition by anti-GAPDH occurs immediately following the block that occurs when cells are incubated with Rab2 (13-mer). In both cases, the block in transport by anti-GAPDH and Rab2 (13-mer) preceded the processing of VSV-G to the endo H-sensitive form by ~5–10 min. It appears that GAPDH acts downstream of Rab2 but is required prior to delivery of cargo to the cis-Golgi.

To define the morphological site of VSV-G accumulation in response to anti-GAPDH, NRK cells were infected with ts045 VSV-G for 2 h at the nonpermissive temperature to restrict VSV-G to the ER. The cells were then permeabilized and incubated in the presence or absence of anti-GAPDH for 40 min at 32 °C, and the distribution of VSV-G was determined by indirect immunofluorescence. Control cells efficiently transported the reporter molecule to the Golgi complex (Fig. 4B). Consistent with the biochemical data, cells incubated with anti-GAPDH failed to transport VSV-G to the Golgi complex. VSV-G accumulated in a collar-like structure composed of small vesicular elements that ringed the nucleus. This staining pattern is similar to that observed when cells are incubated with a constitutively activated form of Rab2 (Rab2 Q65L), which causes

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**Table I**

Peptides tested in microsomal binding assay

| Rab protein | Peptide                      |
|-------------|------------------------------|
| Rab1B       | NPEYDYLFKLLIGD               |
| Rab2 (7-mer)| AYAYLF                        |
| Rab2 (13-mer)| AYAYLFKKYIIGD               |
| Rab2 (random)| AYKAGYIDLYIFP               |
| Rab3A       | AASATDSDRYQGR                |
| Rab5        | ANBGATRNP4GNTGK              |

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**FIG. 3.** Affinity-purified anti-GAPDH blocks ER to Golgi transport, *in vitro*. A, rabbit muscle GAPDH (2 μg) (lane a), NRK cell lysate (25 μg) (lane b), and rat liver cytosol (25 μg) (lane c) were separated by SDS-PAGE and then transferred to nitrocellulose for Western blot analysis. The blot was probed with affinity-purified anti-GAPDH prepared as described under “Experimental Procedures.” B, anti-GAPDH inhibits transport of ts045 VSV-G from the ER to the Golgi complex. Semi-intact NRK cells were incubated with the indicated concentration of affinity-purified anti-GAPDH for 20 min on ice in a transport mixture as described under “Experimental Procedures.” The cells were then transferred to 32 °C for a total of 60 min. The fraction of VSV-G processed to the endo H-resistant forms (% of Total) was determined after analysis by SDS-PAGE and fluorography. C, anti-GAPDH inhibits transport downstream of Rab2 function but prior to delivery of cargo to the cis Golgi complex. Semi-intact cells were incubated at 32 °C in a transport mixture described under “Experimental Procedures.” At the indicated time (∆t), the control (open circle) was transferred to ice, or 10 μg of anti-GAPDH (closed square) or 75 μM Rab2 13-mer (closed triangle) was added and incubated with the cells for a total of 60 min. The fraction of VSV-G processed to endo H-resistant forms was determined as described above. Results shown are representative of three independent experiments.
for transport. To address the first possibility, cytosol was pre-incubated with anti-GAPDH on ice for 20 min, and then GTPγS and salt washed microsomes were added in the presence or absence of recombinant Rab2 and incubated for 10 min at 37 °C. As observed previously, Rab2-treated membranes showed enhanced recruitment of GAPDH to membrane (Fig. 5A). However, the ability of Rab2 to stimulate membrane association of GAPDH was blocked when cytosol was pretreated with anti-GAPDH. Membrane-bound GAPDH was reduced to nearly the control level in the presence of 10 μg of anti-GAPDH (Fig. 5A). The effect of the antibody could be neutralized. We performed the binding assay as above, in the presence or absence of pure GAPDH. Fig. 5B shows that 5 and 10 μg of GAPDH acted as competitive inhibitors of the antibody and restored the ability of Rab2 to stimulate GAPDH recruitment. These results indicate that anti-GAPDH blocks GAPDH membrane binding and provide further evidence that the affinity-purified antibody specifically recognizes the enzyme.

**GAPDH Is Not Required for Vesicle Formation**—After finding that anti-GAPDH prohibited Rab2-stimulated GAPDH recruitment to membrane, we wanted to determine whether the enzyme was required for vesicle formation. To address this question, we performed the binding assay as above supplemented with an inhibitory concentration of anti-GAPDH (8 μg). The low speed supernatant was recovered and then recentrifuged at high speed to recover released vesicles, and the resulting pellet (P2) was subjected to SDS-PAGE and Western blotting. As we previously observed, Rab2 stimulated formation of vesicles containing β-COP (Fig. 6A) and GAPDH (Fig. 6B). We found that microsomes treated with anti-GAPDH released vesicles containing β-COP into the supernatant (Fig. 6A). However, these vesicles did not contain GAPDH (Fig. 6B). Although GAPDH is recruited to VTCs by Rab2 and is found on COPI carriers, the enzyme is not required for vesicle budding.

**DISCUSSION**

The ability to sort anterograde from retrograde transported proteins and retrieve escaped ER-resident proteins exists within pre-Golgi intermediates and throughout the Golgi stack (25, 26). However, the mechanism(s) for this segregation event(s) is unknown. Because protein traffic is bidirectional, it is likely that vesicles possess a distinct molecule or set of molecules that labels the vesicle as either a forward shuttle or a recycling intermediate. Membrane proteins that possess the dilsine motif (KKXX) at their carboxyl terminus are examples of targeting/sorting molecules that play a role in vesicle retrieval (27–29).

Our previous studies on Rab2 demonstrated a role for this protein in driving the formation of COPI vesicles enriched in the recycling protein p53/gp58 (KKXX motif) but lacking anterograde-directed cargo. In this study, the polypeptide composition of the vesicles was further analyzed with the thought that proteins associated with the carriers might be part of the retrieval machinery that functions in targeting/docking/fusion of recycling vesicles to the ER. GAPDH was detected on COPI vesicles and found to be actively recruited from the cytosol to membrane by Rab2.

GAPDH is a tetrameric enzyme consisting of four chemically identical subunits. The structure and enzymatic activity of this protein have been thoroughly investigated (12). In addition to its catalytic function, GAPDH is involved in diverse biological activities. One of these activities includes a role in endocytosis. Robbins et al. (11) reported that a single amino acid change in GAPDH (Pro in driving the formation of COPI vesicles enriched in the recycling protein p53/gp58 (KKXX motif) but lacking anterograde-directed cargo. In this study, the polypeptide composition of the vesicles was further analyzed with the thought that proteins associated with the carriers might be part of the retrieval machinery that functions in targeting/docking/fusion of recycling vesicles to the ER. GAPDH was detected on COPI vesicles and found to be actively recruited from the cytosol to membrane by Rab2.

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also required in the secretory pathway, an affinity-purified polyclonal antibody to GAPDH was introduced into an assay that reconstitutes ER to Golgi transport. The reagent efficiently inhibited transport, indicating that GAPDH was an essential component of the trafficking machinery. GAPDH activity is required after Rab2 recruitment to VTCs but prior to cargo delivery to the cis Golgi compartment. Because anterograde and retrograde transport are coupled, the block in ER to Golgi transport is most likely due to the formation of “dead end” retrograde carriers (GAPDH negative) that cannot transport and fuse to the ER. In this situation, components are not retrieved from VTCs that are necessary for cargo transport from the ER, resulting in the arrest of VSV-G transport.

Interestingly, the GAPDH from the Chinese hamster ovary mutant cells described above, also showed altered microtubule (MT) binding properties (11). GAPDH is known to modulate the cytoskeleton by promoting actin polymerization and MT bundling (13, 30). This change in the cell architecture could have profound consequences, since MTs control both organelle positioning and transport of vesicular and tubular elements, an activity that requires the motor proteins kinesin and dynein. Kinesin has been found on pre-Golgi intermediates and constitutively cycles between the ER and Golgi complex, implicating cytoskeletal involvement in retrograde trafficking (31). In the secretory pathway, GAPDH may function to promote the interaction of Rab2-generated retrograde vesicles with MTs. This would allow the vesicle to be sorted from anterograde traffic and redirected to the ER. GAPDH might work in unison with MT by providing energy and coordinating motile processes for vesicle movement. Although MT and actin assembly do not appear to be directly regulated by small GTPases, Rab proteins may provide a molecular link for vesicle movement to the appropriate target. In that regard, Peranen et al. (32) showed a striking change in actin and microtubule organization when cells overexpressed a constitutively activated form of Rab8.

We found that membrane-associated GAPDH was not essential to vesicle budding, yet protein trafficking was arrested in the presence of anti-GAPDH. This result suggests that the GAPDH associated with Rab2-generated vesicles may be required for fusion of the retrograde carrier to the target compartment. In support of this interpretation, Lopez Vinals et al. reported that rabbit muscle GAPDH was a potent fusogen of negatively charged liposomes (33). Later studies by Glaser and Gross (34) found that a form of GAPDH isolated from rabbit brain cytosol promoted fusion of vesicles containing plasmeneethanolamine, cholesterol, and phosphatidylserine. The absolute requirement for plasmeneethanolamine in the liposome was thought to be due to the ability of this lipid to adopt an inverted hexagonal phase, which promotes membrane fusion. Although fusion activity in this system was calcium-independent, the investigators pointed out that calcium might serve to modulate GAPDH activity. Subsequent studies from this laboratory have recently shown that GAPDH catalyzes membrane fusion between isolated pancreatic secretory gran-
ules and fractionated plasma membrane in vitro (35). In contrast to the above studies, Hesseler et al. using human neutrophil cytosol have found a calcium-dependent fusogenic activity toward vesicles containing phosphatidylethanolamine/phosphatidic acid (15). The fusogen in this system was also identified as GAPDH. It may be that the ability of GAPDH to promote fusion in a calcium-dependent versus calcium-independent manner simply reflects the participation of a unique GAPDH isoform. The different isoforms might function within intracellular compartments that provide the appropriate lipid content. This argument is consistent with the finding that multiple GAPDH isoforms exist within a cell/tissue (11, 34).

We propose that Rab2 functions in the early secretory pathway to regulate vesicular traffic through a subcompartment within pre-Golgi intermediates where recycling proteins have been sorted and concentrated away from anterograde-directed cargo. In this model, Rab2 initiates a cascade of events that results in recruitment of soluble factors including GAPDH to ultimately release vesicles enriched in recycling components. The GAPDH associated with the retrograde vesicle may interact with microtubules to direct movement or, alternatively, promote fusion of the vesicle to the ER. Experiments are in progress to distinguish between these possibilities.

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Ellen J. Tisdale

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