Glyceraldehyde-3-phosphate Dehydrogenase Interacts with Rab2 and Plays an Essential Role in Endoplasmic Reticulum to Golgi Transport Exclusive of Its Glycolytic Activity*

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Rab2 requires atypical protein kinase C \(\alpha\) (aPKC\(\alpha\)) to promote vesicle formation from vesicular tubular clusters (VTCs). The Rab2-generated vesicles are enriched in recycling proteins suggesting that the carriers are retrograde-directed and retrieve transport machinery back to the endoplasmic reticulum. These vesicles also contained the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We have previously established that GAPDH is required for membrane transport between the endoplasmic reticulum and the Golgi complex. Moreover, GAPDH is phosphorylated by aPKC\(\alpha\) and binds to the aPKC\(\alpha\) regulatory domain. In this study, we employed a combination of in vivo and in vitro assays and determined that GAPDH also interacts with Rab2. The site of GAPDH interaction was mapped to Rab2 residues 20–50. In addition to its glycolytic function, GAPDH has multiple intracellular roles. However, the function of GAPDH in the early secretory pathway is unknown. One possibility is that GAPDH ultimately provides energy in the form of ATP. To determine whether GAPDH catalytic activity was critical for transport in the early secretory pathway, a conservative substitution was made at Cys-149 located at the active site, and the mutant was biochemically characterized in a battery of assays. Although GAPDH (C149G) has no catalytic activity, Rab2 recruited the mutant protein to membranes in a quantitative binding assay. GAPDH (C149G) is phosphorylated by aPKC\(\alpha\) and binds directly to Rab2 when evaluated in an overlay binding assay. Importantly, VSV-G transport between the ER and Golgi complex is restored when an in vitro trafficking assay is performed with GAPDH-depleted cytosol and GAPDH (C149G). These data suggest that GAPDH imparts a unique function necessary for membrane trafficking from VTCs that does not require GAPDH glycolytic activity.

Membrane traffic in the early secretory pathway requires the participation of Rab2 (1, 2). This Ras-related small GTPase associates with pleiomorphic structures that are composed of vesicles and tubules, termed vesicular tubular clusters (VTCs)†

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‡ The abbreviations used are: VTCs, vesicular tubular clusters; ER, endoplasmic reticulum; PKC, protein kinase C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G3P, glyceraldehyde-3-phosphate; NRK, normal rat kidney; GTP\(\gamma\S, guanosine 5'-3'-O-(thio)triphosphate, \(\beta\)-COPI, \(\beta\)-coat protein; CAT, chloramphenicol acetyltransferase; TBS, Tris-buffered saline; HRP, horseradish peroxidase; TSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay.
associated with VTCs could ultimately provide energy in the form of ATP, a factor essential for membrane trafficking. To address the question whether GAPDH catalytic activity was critical for transport in the early secretory pathway, a conservative substitution was made at the invariant residue (Cys-149) located at the active site. As expected, GAPDH (C149G) was inactive when evaluated in a glycolytic activity assay. GAPDH (C149G) was further characterized biochemically to be assured that the mutation did not affect other physiological properties in addition to loss of enzyme activity. By employing a quantitative micromolar binding assay we found that Rab2 recruited the mutant protein to membranes and that the membrane-associated form was the tetrameric species. Moreover, GAPDH (C149G) binds directly to Rab2 and a PKC/α. Importantly, purified recombinant GAPDH (C149G) rescued VSV-G transport between the ER and Golgi complex when an in vitro trafficking assay was supplemented with GAPDH-depleted cytosol. These combined results suggest that GAPDH provides a specific function essential for membrane trafficking from VTCs that does not require GAPDH glycolytic activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—The human expression-tested clone GAPDH pDual was purchased from Stratagene (La Jolla, CA). Rab2 N-terminal peptides were synthesized at the University of Michigan Protein and Carbohydrate Structure Facility (Ann Arbor, MI). The Mammalian Matchmaker two-hybrid assay kit was purchased from Clontech Laboratories, Inc. (Palo Alto, CA).

**Construction of Rab2 N-terminal Deletion Mutants, Rab2-Rab1 Chimeras, and GAPDH Catalytically Inactive Mutant—**N-terminal deletions of human Rab2 were generated as previously described (9). Reciprocal exchanges between human Rab2 and rat Rab1b were made by a two-step procedure involving complementary overlapping oligonucleotides in combination with flanking 5′- and 3′-primers for either Rab2 or Rab1. To generate the Rab1-(1-52)/Rab2-(50-211) chimera, the first reaction was performed with overlapping 5′- and 3′-fragments using Rab1 as the template. The 5′ sense primer (5′-GGCGCAATGCAACCCCAATGATGATC-3′) and the 3′ antisense primer (5′-TTTTATCTGGTTTCTTTTTTTATTGTTAAGTTCTGTTTATTCT-3′) were used to amplify the first 156 base pairs of Rab1. The 5′-portion of Rab2-(50-211) was generated using Rab2 as the template and the overlapping primer; 5′-AGAATGCAACATTGAAAGTTGATGAT-3′. The two PCR products were combined to generate the full-length mutant in a second reaction using the Rab1 sense primer and Rab2 antisense primer. To generate Rab2-(1-52)/Rab1-(50-211) chimeras, the first reaction was performed with overlapping 5′- and 3′-fragments using Rab1 as the template. The 5′ sense primer (5′-GGCAGAATGCGGTACGCCTATCTCTTCAAGTAC-3′) and the 3′ antisense primer (5′-GGGAAACAGATAAAGAAACCTT-3′) were used to amplify the first 156 base pairs of Rab2. The 5′-portion of Rab1-(50-211) was generated using Rab1 as the template and the overlapping primer; 5′-AAGGAGGTGCTCGAATGATA-3′. The two PCR products were combined to generate the full-length mutant in a second reaction using the Rab1 sense primer and Rab2 antisense primer. The chimeras were subcloned into the GAL4 DNA binding domain in the pM vector and GAPDH cDNA cloned in-frame to the EcoRI site of the activation domain of pVP16 (Clontech Laboratories, Inc.). The two constructs (5 μg each) were co-transfected with the reporter vector pG5CAT (5 μg) that contains the chloramphenicol acetyltransferase (CAT) gene into HeLa cells (107) using a calcium phosphate transfection protocol (15). Control cells were transfected with the reporter vector alone as described above. The cells were collected 72 h post-transfection, lysed in sample buffer, separated by SDS-PAGE, and then transferred to nitrocellulose. The blot was blocked as described above, incubated with an anti-CAT polyclonal antibody (Invitrogen Life Technologies), washed, further incubated with an HRP-conjugated secondary antibody, and then developed with ECL.

**Overlay Binding Assay—**Purified recombinant Rab2 (5 μg) was separated by SDS-PAGE and transferred to nitrocellulose. The blot was incubated in 50 mM Hepes/KOH (pH 7.2), 5 mM MgOAc, 100 mM KOAc, 10 mg/ml BSA, 0.1% Triton X-100, and 0.3% Tween 20 overnight at 4 °C to renature the protein. The blot was then incubated in 12.5 mM Hepes/KOH (pH 7.2), 1.5 mM MgOAc, 75 mM KOAc, 0.1% BSA, 10 μM GTPγS, 200 μM NaCl, and 10 μM purified recombinant His6-GAPDH or His6-GAPDH (C149G) for 4 h at room temperature (7, 9). After incubation, the blot was washed with TBS, and then probed with a rabbit anti-GAPDH monoclonal antibody (Chemicon Intl.), washed, further incubated with an HRP-conjugated secondary antibody, and then developed with ECL.

**GST Pull-down—**BL21 (DE3) pLysS cells (Novagen) that contained the recombinant plasmids GST-GAPDH, GST-Rab2/Rab1, GST-Rab2/ His6-GAPDH, or GST-Rab1 were grown at 32°C to an OD600 of 0.6 and then induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. The liquid culture was centrifuged at 6,000 rpm for 30 min, and the pellet resuspended in cold TBS, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, sonicated, centrifuged at 12,000 rpm for 20 min, and then the supernatant applied to a glutathione-Sepharose 4B column (Amersham Biosciences). The column was washed with 10-bed volumes of TBS, and the fusion protein eluted with 5 mM reduced glutathione. GST-GAPDH, GST-Rab2-(1–50), GST-Rab2-(51–211), or GST (5 μg) were preincubated with 20 μl of glutathione Sepharose 4B for 1 h at room temperature, collected by centrifugation at 4,000 rpm for 5 min, and then washed 3× with TBS and 1% Triton X-100 to remove any unbound protein. The beads were resuspended in 50 mM Tris (pH 7.5), 5 mM MgCl2, 10 mM DTT, 10 μM GTPγS, 0.2 μM GST-tag monoclonal antibody (Biochemics Inc., Irvine, CA), and then 5 μg of either Rab2 or His6-GAPDH added and incubated for an additional 2 h at room temperature. The beads were washed 4× with 1 ml of 50 mM Tris (pH 7.5), 10 mM MgCl2, and 100 mM NaCl, and the bound proteins boiled in sample buffer, separated by SDS-PAGE, and then transferred to nitrocellulose. The blot was blocked as above and the membrane probed with an anti-Rab2 polyclonal antibody, an anti-Rab1 polyclonal antibody, a GST-specific antibody, a mouse monoclonal antibody specific for anti-Rab2 polyclonal antibody, or an anti-Rab1 polyclonal antibody, and then developed with enhanced chemiluminescence (ECL) (Amersham Biosciences).

**Mammanlian Two-hybrid Assay—**Human Rab2, Rab2/Rab1, and Rab2/Rab1/ExoRI site was cloned in-frame to the EcoRI site of the GAL4 DNA binding domain in the pM vector and GAPDH cDNA cloned in-frame to the EcoRI site of the activation domain of pVP16 (Clontech Laboratories, Inc.). The two constructs (5 μg each) were co-transfected with the reporter vector pG5CAT (5 μg) that contains the chloramphenicol acetyltransferase (CAT) gene into HeLa cells (107) using a calcium phosphate transfection protocol (15). Control cells were transfected with the reporter vector alone as described above. The cells were collected 72 h post-transfection, lysed in sample buffer, separated by SDS-PAGE, and then transferred to nitrocellulose. The blot was blocked as described above, incubated with an anti-CAT polyclonal antibody (Invitrogen Life Technologies), washed, further incubated with an HRP-conjugated secondary antibody, and then developed with ECL.

**GST Pull-down—**BL21 (DE3) pLysS cells (Novagen) that contained the recombinant plasmids GST-GAPDH, GST-Rab2/Rab1, GST-Rab2/ His6-GAPDH, or GST-Rab1 were grown at 32°C to an OD600 of 0.6 and then induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. The liquid culture was centrifuged at 6,000 rpm for 30 min, and the pellet resuspended in cold TBS, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, sonicated, centrifuged at 12,000 rpm for 20 min, and then the supernatant applied to a glutathione-Sepharose 4B column (Amersham Biosciences). The column was washed with 10-bed volumes of TBS, and the fusion protein eluted with 5 mM reduced glutathione. GST-GAPDH, GST-Rab2-(1–50), GST-Rab2-(51–211), or GST (5 μg) were preincubated with 20 μl of glutathione Sepharose 4B for 1 h at room temperature, collected by centrifugation at 4,000 rpm for 5 min, and then washed 3× with TBS and 1% Triton X-100 to remove any unbound protein. The beads were resuspended in 50 mM Tris (pH 7.5), 5 mM MgCl2, 10 mM DTT, 10 μM GTPγS, 0.2 μM GST-tag monoclonal antibody (Biochemics Inc., Irvine, CA), and then 5 μg of either Rab2 or His6-GAPDH added and incubated for an additional 2 h at room temperature. The beads were washed 4× with 1 ml of 50 mM Tris (pH 7.5), 10 mM MgCl2, and 100 mM NaCl, and the bound proteins boiled in sample buffer, separated by SDS-PAGE, and then transferred to nitrocellulose. The blot was blocked as above and the membrane probed with an anti-Rab2 polyclonal antibody, an anti-Rab1 polyclonal antibody, a GST-specific antibody, a mouse monoclonal antibody specific for anti-Rab2 polyclonal antibody, or an anti-Rab1 polyclonal antibody, and then developed with ECL.

**ELISA—**ELISA was performed as described previously (9, 16). Briefly, Nunc immunomodules were coated with peptides and purified Rab2 N-terminal-truncated recombinant proteins (1 μg/100 μl of 50 μM NaHCO3, pH 9.6) listed in Table 1 at 4 °C overnight. The wells were
washed in TBS, blocked in TBS and 5% fetal bovine serum for 1 h at 37 °C, additionally washed in TBS, and then incubated with 5 μg of purified recombinant His6-GAPDH for 3 h at 37 °C. After each wash was washed for 10 min in 10 mM Hepes (pH 7.2), 250 mM mannitol and employed in the binding reaction, as described previously (5, 8). Membranes (30 μg of total protein) were added to a reaction mixture, which contained 27.5 μM Hepes (pH 7.2), 2.75 mM MgOAc, 65 mM KOAc, 5 mM EGTA, 1.6 mM CaCl2, 1 mM ATP, 5 mM creatine phosphate, and 0.2 units of rabbit muscle creatine kinase. Recombinant Rab2 and His6-GAPDH (C149G) were added at the concentrations indicated under “Results,” and the reaction mix incubated on ice for 10 min. The resultant pellet containing ER, pre-Golgi, and Golgi membranes was washed with 50 mM Tris (pH 7.5), 5 mM MgCl2, 100 mM NaCl, 10 μM GTP-S was added for 2 h at 37 °C, washed, and further incubated with an anti-mouse alkaline phosphatase-conjugated secondary reagent for 1 h at 37 °C. The wells were again washed with TBS, developed with Sigma FAST β-nitrophenyl nitrocellulose (Sigma-Alrich), and then read at 405 nm on a microplate reader.

**Assay for GAPDH Activity—** Purified recombinant His6-GAPDH or His6-GAPDH (C149G) (1 μg) were incubated in 10 mM sodium pyrophosphate (pH 8.5), 20 mM sodium phosphate, 0.25 mM β-NAD (ICN Biomedicals, Inc., Aurora, OH), and 3 μg dithiothreitol at 25 °C for 5 min to establish a baseline. The reaction was initiated by the addition of 0.4 μg gearlydehyde-3-phosphate (Sigma-Aldrich), and the catalytic reaction of NAD to NADH was monitored at the indicated times described under “Results” by an increase in absorbance at 340 nm. The GAPDH units correspond to the mass (μM) of GAPDH converted per min per mg. The mass of NADH generated was calculated using ε = 0.622.

**Analysis of Transport In Vitro—** NRK cells were infected for 4 h with the temperature-sensitive VSV strain ts045, and then biosynthetically radiolabeled with 100 μCi [35S]S-sulfate (specific activity 1175 Ci/mmol, PerkinElmer Life Sciences Products) for 1 h at 39 °C. At the periphery of the temperature (39.5 °C) to maintain 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 (17). Transport reactions were performed in a final volume of 40 μl in a buffer which contains 25 mM Hepes-KOH, pH 7.2, 75 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA, 1.8 mM CaCl2, 1 mM N-acetylglucosamine, an ATP regeneration system (1 mM ATP, 5 mM creatine phosphate, and 0.2 international units of rabbit muscle creatine phosphokinase), 5 μl of semi-intact cells (∼5 × 10⁹ cells/ml, ∼25–30 μg of total protein) resuspended in 50 mM Hepes-KOH, 90 mM KOAc (pH 7.2), and 5 μl of rat liver cytosol depleted of GAPDH by batch absorbed on Blue Sepharose (Amersham Biosciences) followed by immunoprecipitation for 4 h at 4 °C with 10 μg of anti-GAPDH monoclonal antibody (Chemicon International, Inc., Beverly, MA), washed, further incubated with an HRP-conjugated anti-mouse antibody that co-precipitates Rab2 and aPKC/A (9).

**To further verify Rab2-GAPDH interaction in vivo, we employed a mammalian two-hybrid assay that was used previously to demonstrate Rab2-aPKCα interaction (9).** This system allows detection of transient and weak protein–protein interactions. For this assay, Rab2 cDNA was used as bait and cloned into pM to generate a GAL4 DNA binding domain-Rab2 fusion protein whereas GAPDH cDNA that serves as prey was cloned into pVP16 to generate a VP16 activation domain-GAPDH fusion protein. HeLa cells were co-transfected with these two constructs and with a reporter plasmid that contains the CAT gene. If the two fusion proteins interact in vivo, transcription of the CAT reporter gene is activated. Three days post-transfection, the cells were lysed in sample buffer and the lysate separated on SDS-PAGE and immunoblotted with an anti-CAT polyclonal antibody. Cells co-transfected with pM-Rab2 and pVP16-GAPDH expressed ~20-fold higher level of CAT protein compared with controls indicating that Rab2 and GAPDH interacted in vivo (Fig. 1B, lane d).

This potential in vivo interaction was further evaluated in a blot overlay assay that is routinely used to identify Rab-interacting proteins (18). Rab2 was separated on SDS-PAGE, transferred to nitrocellulose, and the membrane incubated in overlay buffer supplemented with purified recombinant His6-GAPDH. Any GAPDH bound to Rab2 was detected after incubation with a monoclonal antibody to GAPDH followed by a secondary HRP-conjugated antibody and development with ECL. Consistent with the in vivo results, we detected His6-GAPDH binding to Rab2 (Fig. 1C). This in vitro result was further confirmed in a GST pull-down experiment in which GST-GAPDH was first immobilized on glutathione Sepharose 4B, and then incubated with purified recombinant Rab2 in a buffer containing GTPγS or GDP. After extensive washing, agarose beads containing GST-GAPDH retained Rab2-GTPγS whereas minimal interaction was detected with Rab2-GDP (Fig. 1D). These collective results indicate that activated Rab2 interacts directly with GAPDH.

To map the GAPDH binding site in Rab2, we aligned the sequence of Rab2 with Rab1 and swapped the corresponding residues (1–50), and then evaluated the chimeras in the mammalian two-hybrid assay described above. Like Rab2, Rab1 is essential for trafficking in the early secretory pathway (1). Cells co-transfected with pM-Rab2/Rab1 and pVP16-GAPDH expressed ~15-fold higher level of CAT protein compared with the reverse construct and the control suggesting that GAPDH interacts with the first 50 residues in Rab2 (Fig. 2A).

The two chimeras were then evaluated in GST pull-down experiments. The bacterial produced fusion proteins were pre-bound to glutathione Sepharose 4B, and then incubated with His6-GAPDH. Fig. 2B shows that similar to the in vivo assay, GAPDH specifically associated with Rab2/Rab1. We made use of reagents present in the laboratory that were first employed to identify the aPKCα binding domain in Rab2 to eliminate sequence and to begin defining the minimal residues required for GAPDH association with Rab2 (9). Using an ELISA to evaluate protein interaction, we learned that GAPDH binds to Rab2 between amino acids 20 and 50 (Table 1). It is noteworthy that this Rab2 segment contains the putative effector domain.
GAPDH Catalytic Activity Not Required for Transport from VTCs

Although GAPDH is essential for ER to Golgi transport—although GAPDH is essential for ER to Golgi transport, we did not know whether GAPDH-catalytic activity was required for Rab2-mediated events at the VTC. To address this issue, a conservative substitution was made at the invariant residue (Cys-149) located at the active site and the key residue that binds substrate. The mutated and GAPDH wild-type cDNA was subcloned into a His<sub>6</sub>-tagged expression vector, and then expressed and purified from E. coli. The recombinant proteins purified by affinity chromatography on Ni<sup>2+</sup>-NTA-agarose beads. We first assessed the enzymatic activity of His<sub>6</sub>-GAPDH and His<sub>6</sub>-APGDP (C149G) by measuring NADH production. As we anticipated, substitution of Cys-149 resulted in an inactive GAPDH mutant protein that possessed no dehydrogenase activity (Fig. 3A).

To learn whether His<sub>6</sub>-GAPDH (C149G) was recruited to that mediates interactions with specific accessory proteins (19, 20).

**GAPDH Glycolytic Activity Is Not Required for ER to Golgi Transport**—Although GAPDH is essential for ER to Golgi transport, we did not know whether GAPDH-catalytic activity was required for Rab2-mediated events at the VTC. To address this issue, a conservative substitution was made at the invariant residue (Cys-149) located at the active site and the key residue that binds substrate. The mutated and GAPDH wild-type cDNA was subcloned into a His<sub>6</sub>-tagged expression vector, and the recombinant proteins purified by affinity chromatography on Ni<sup>2+</sup>-NTA-agarose beads. We first assessed the enzymatic activity of His<sub>6</sub>-GAPDH and His<sub>6</sub>-APGDP (C149G) by measuring NADH production. As we anticipated, substitution of Cys-149 resulted in an inactive GAPDH mutant protein that possessed no dehydrogenase activity (Fig. 3A).
membranes in response to Rab2, we made use of a quantitative binding assay. For this assay, microsomes were prepared from HeLa cell homogenates and washed with 1 M KCl to remove peripherally associate proteins including GAPDH. These membranes were preincubated in buffer for 10 min on ice in the presence of increasing concentrations of recombinant Rab2 and 100 ng His$_6$-GAPDH (C149G). The reaction was supplemented with GAPDH-depleted rat liver cytosol and GTP$_7$S, and then incubated for 12 min at 32 °C to promote binding of GAPDH (C149G) and other soluble components. The membranes were collected by centrifugation at 20,000 g and then analyzed by SDS-PAGE and Western blot for the presence of His$_6$-GAPDH (C149G) and Rab2 efficiently recruited the GAPDH mutant to membranes in a dose-dependent manner (Fig. 3B). Moreover, GAPDH (C149G) interacts with Rab2 when evaluated in the overlay binding assay (Fig. 3C). A similar result was obtained when the overlay assay was performed with aPKC/λ and GAPDH (C149G) (Fig. 3C).
The molecular composition of recruited GAPDH was determined by subjecting the membranes obtained after performing the binding reaction to non-denaturing electrophoresis. Both His6-GAPDH and His6-GAPDH (C149G) had a mobility of ~150 kDa, which closely approximates the known molecular weight of native GAPDH as well as the contribution by His6 indicating that the recombinant GAPDH exist as tetramers on VTCs (Fig. 3D). The presence of the tetrameric species suggests that the oligomeric structure is required for GAPDH function on VTCs.

We previously reported that GAPDH is phosphorylated by aPKC/α and that phospho-GAPDH plays a role in microtubule dynamics in the early secretory pathway (7). To establish whether the active site mutation affected aPKC/α-dependent GAPDH phosphorylation, an in vitro kinase assay was employed. A comparable level of aPKC/α-dependent phosphorylation was observed when the assay was performed with His6-GAPDH or His6-GAPDH (C149G) demonstrating that the mutant GAPDH serves as substrate for the kinase (Fig. 3E).

To study the effect of GAPDH (C149G) on membrane trafficking in the early secretory pathway, we introduced the recombinant protein into an in vitro transport assay (17). For this assay, tissue culture cells are first infected with ts045 VSV-G, a virus that synthesizes a protein with a thermoreversible defect resulting in ER retention at 39.5 °C. The plasma membrane of these cells is then perforated to release soluble content, but retain functional ER and Golgi stacks. When the semi-intact cells are incubated at the permissive temperature of 32 °C, export of ts045 VSV-G from the ER is initiated and transport of VSV-G protein is measured by following the processing of the two N-linked oligosaccharides to endo H-resistant forms. Since this assay requires addition of rat liver cytosol to reconstitute intracellular transport, the cytosol was first depleted of GAPDH by a combination of affinity chromatography on Blue Sepharose, which binds NAD-requiring proteins, and by immunodepletion (Fig. 4A). When the transport assay was performed with the GAPDH-depleted cytosol, there was an ~76% reduction in the processing of VSV-G to endo H-resistant forms. Importantly, supplementing the GAPDH-depleted cytosol with increasing concentrations of His6-GAPDH or with His6-GAPDH (C149G) reversed the inhibition and restored transport to near 77% of the control level. These results are highly suggestive that GAPDH catalytic activity is not required for VSV-G transport from the VTC (Fig. 4B). Furthermore, these combined studies demonstrate that the biochemical properties of His6-GAPDH (C149G) are not compromised by the mutation. Therefore, the mutant has similar function on the VTC as GAPDH wild type.

**DISCUSSION**

Rab proteins associate with a variety of effectors to form multimolecular complexes (21, 22). This fact combined with our previous finding that aPKC/α interacts with the Rab2 N terminus and that the aPKC/α regulatory domain binds GAPDH prompted us to consider the possibility that GAPDH may also associate with Rab2. We performed in vitro and in vivo assays to establish that Rab2-GAPDH directly interact. The GAPDH binding site resides within residues 20–50 in Rab2 that includes the putative effector domain (residues 35–42). This region is one of two regions found by structural comparison of the GTP- and GDP-bound forms of small G proteins to undergo guanine nucleotide-dependent conformational changes that are recognized by regulatory factors and downstream effectors that modulate activity (19, 20). Rab1 and Rab2 share 45% identity within this segment. However, Rab1 does not recruit GAPDH to membranes (10). GAPDH interaction with this Rab2 sequence is compatible with the observation that activated Rab2 binds GAPDH. Based on results obtained from the gel overlay assay and GST pull-down studies that employed only purified Rab2 and GAPDH, the interaction does not require aPKC/α. However, we cannot rule out the possibility that aPKC/α enhances Rab2-GAPDH binding affinity and stabilizes their interaction on VTCs, in vivo. This interpretation would be consistent with our finding that a peptide made to the aPKC/α pseudosubstrate domain interferes with Rab2 recruitment of GAPDH to membranes (7). Our results are highly suggestive that Rab2-aPKC/α-GAPDH form a complex on VTCs. It is possible that GAPDH binding to aPKC/α and Rab2 on VTCs blocks the active site or induces a conformational change making the active site inaccessible to substrate. Indeed, the dehydrogenase activity of membrane-associated GAPDH is significantly inhibited in enzymatic activity when associated with NRK microsomes (data not shown). A similar result has been reported when GAPDH associates with isolated human erythrocyte membranes (23, 24). Although aPKC/α and GAPDH interact with other intracellular proteins and participate in various biochemical and signaling pathways independent of their roles at the VTC, the co-association with Rab2 “compartamentalizes” a specific activity required for retrograde transport. In that regard, aPKC/α phosphorylates GAPDH on VTCs and phospho-GAPDH influences microtubule nucleation at the budding site defined by Rab2 and associated effectors (7).

GAPDH is a well-characterized key enzyme in glycolysis that is responsible for the oxidative phosphorylation of G3P by...
NAD$^+$ and inorganic phosphate. The structure of GAPDH has been elucidated and studies have identified two significant regions that include the NAD$^+$ binding domain and the catalytic domain that binds substrate (25–28). The active enzyme exists as a tetramer containing identical 37 kDa subunits. We have found that both His$_6$-GAPDH and His$_6$-GAPDH (C149G) are tetrameric when bound to VTCs, and therefore GAPDH has the potential to be enzymatically active. GAPDH catalytic mechanism involves the formation of a hemithioacetal between the substrate and the active site thiol located at position 149. Cys-149 is essential for activity and the target of numerous thiol agents that inactive the enzyme (25, 29). The experiments within show that substitution of Cys-149 eliminates dehydrogenase activity. To our knowledge, this is the first study to characterize biochemically and functionally an active site GAPDH mutant. A battery of assays were performed with the mutant to be assured that the substitution had no influence on other biochemical properties inherent to the role of GAPDH in the early secretory pathway including; 1) Rab2-dependent recruitment to membranes, 2) interaction with Rab2 and aPKC$\alpha$, 3) aPKC$\alpha$-dependent phosphorylation, and 4) an essential role in membrane trafficking between the ER and Golgi complex. All assay results were identical when performed with His$_6$-GAPDH or His$_6$-GAPDH (C149G).

The involvement of GAPDH in multiple intracellular activities inclusive/exclusive of its role in gluconeogenesis is consistent with the observation that numerous proteins have two or more different functions. These multifunction proteins are referred to as “moonlighting proteins” and their activity can vary dependent upon subcellular location, oligomeric state, cell type, or ligand, substrate, and cofactor concentration (29). Interestingly, phosphoglucose isomerase is also a key enzyme in glycolysis but functions extracellularly as either a nerve growth factor, cytokine, a motility factor or a differentiation and maturation mediator (30–34). Since add-back of GAPDH has been elucidated and studies have identified two significant mechanisms for the retrograde-directed vesicle with the ER. Lopez Vinals et al. (35) found that rabbit muscle GAPDH was a potent fusogen of negatively charged liposomes whereas studies by Glaser and Gross (14) demonstrated that a GAPDH isofrom catalyzes fusion between pancreatic islet secretory granules and the plasma membrane. Moreover, Peters et al. (36) reported GAPDH as part of the fusion complex involving V-type ATPase and Ca$^{2+}$/calmodulin. These two activities are not mutually exclusive and GAPDH may have dual function at the VTC.

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