Glyceraldehyde-3-phosphate Dehydrogenase Is Phosphorylated by Protein Kinase C\(\mu/\lambda\) and Plays a Role in Microtubule Dynamics in the Early Secretory Pathway*

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The small GTPase Rab2 immunolocalizes to vesicular tubular clusters (VTCs) that function as transport complexes carrying cargo between the endoplasmic reticulum and the Golgi complex. Our previous studies showed that Rab2 promotes vesicle formation from VTCs and that the released vesicles are enriched in \(\beta\)-coat protein, protein kinase C \(\mu/\lambda\) (PKC\(\mu/\lambda\)), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the recycling protein p53/gp58. Because PKC\(\mu/\lambda\) kinase activity was necessary for vesicle formation, a search was initiated to identify the substrate(s) that potentiate Rab2 function within VTCs. In this study, we found that PKC\(\mu/\lambda\) phosphorylates GAPDH. Moreover, GAPDH interacts directly with the PKC\(\mu/\lambda\) regulatory domain. Based on numerous observations that show \(\beta\)-coat GDP/GTP GAPDH associates with cytoskeletal elements, we examined the role of phospho-GAPDH in promoting microtubule (MT) binding to membrane. Using a quantitative microsomal binding assay, we found that membrane association of \(\beta\)-tubulin was dependent on phospho-GAPDH and was blocked by reagents that interfere with Rab2-dependent GAPDH membrane recruitment or with PKC\(\mu/\lambda\) kinase activity. Furthermore, normal rat kidney cells transfected with a constitutively activated form of Rab2 (Q65L) or with our anti-GAPDH polyclonal antibody displayed a dramatic change in MT organization. These combined results suggest that Rab2 stimulated PKC\(\mu/\lambda\) and GAPDH recruitment to VTCs, and the subsequent PKC\(\mu/\lambda\) phosphorylation of GAPDH ultimately influences MT dynamics in the early secretory pathway.

Protein phosphorylation-dephosphorylation events play a critical role in the regulation of intracellular membrane traffic. Phosphatase inhibitors have been reported to arrest endoplasmic reticulum (ER)\(^1\) to Golgi transport (1), transport from the trans-Golgi network to the cell surface (2), as well as fluid phase endocytosis (3). For some transport-related proteins, phosphorylation modulates their activity as well as influences the phosphoproteins cellular location. For example, phosphorylation of membrane-associated Rab1 and Rab4 promotes their redistribution to the cytosol (4, 5). Rab1 and Rab4 are two members of the Rab GTPase family that includes more than 40 members that regulate vesicular traffic between specific compartments of the endocytic and exocytic pathways (6, 7). Like Rab1, Rab2 is required for membrane traffic in the early secretory pathway (8). The Rab2 protein immunolocalizes to pre-Golgi intermediates composed of vesicles and tubular clusters (vesicular tubular clusters, VTCs) (9). VTCs serve as transport intermediates between the ER and the Golgi complex and are the first site of segregation of the anterograde and retrograde pathways (10, 11).

In our ongoing studies to characterize the role of Rab2 in secretion, we found that Rab2 requires protein kinase C \(\mu/\lambda\) (PKC\(\mu/\lambda\)) activity to generate retrograde-directed vesicles enriched in \(\beta\)-coat protein (\(\beta\)-coat, PKC\(\mu/\lambda\)), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the recycling protein p53/gp58 (12–14). PKC\(\mu\) is the human homolog of mouse PKC\(\alpha\), and both share homology with the third member of the atypical PKC family, PKC\(\zeta\). The atypical PKCs are activated by the lipid second messengers phosphatidylinositol 3,4,5-trisphosphate and ceramide as well as by protein-protein interactions that direct the PKC in proximity of its cognate substrate. Most likely different binding partners are required to integrate the multitude of signaling pathways in which PKC is a participant.

Our previous finding that kinase activity was necessary for vesicle formation prompted a search for the PKC\(\mu/\lambda\) sub-strate(s) that potentiates Rab2 function within VTCs. Interestingly, three proteins that associate with VTCs have been reported to undergo phosphorylation and include Rab2, \(\beta\)-coat, and GAPDH. In that regard, phospho-Rab2 was found in HeLa and COS-1 cells biosynthetically labeled with \(^{32}\)P\(^1\). Although these studies did not identify the responsible kinase, it is possible that PKC is involved because Rab2 contains six PKC serine phosphorylation sites that may regulate recruitment to the Golgi complex. Our previous studies reported that GAPDH undergoes autophosphorylation as well as phosphatidylinositol (PS)-dependent phosphorylation by PKC (18–20). Although GAPDH is commonly known as a key enzyme in glycolysis (GAPDH catalyzes the NAD-mediated oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate), a number of intriguing intracellular roles have been reported including modulation of the cytoskeleton, kinase activity, and the promotion of vesicle fusion (18, 21–24). These activities are all essential to the maintenance of mem-

*This work was supported by Grant 0030385Z from the American Heart Association-Midwest Affiliate. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\(^1\)The abbreviations used are: ER, endoplasmic reticulum; VTC(s), vesicular tubular clusters; PKC, protein kinase C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PS, phosphatidylinerine; MTs, microtubules; NRK, normal rat kidney; PBS, phosphate-buffered saline; N\(^{\alpha}\)-NTA, nickel nitrolotriacetic acid; GTP\(_yS\), guanosine 5’-3-O-(thio)triphosphate; STICKS, substrates that interact with C-kinase.
brane trafficking. Furthermore, we found recently that GAPDH is required for transport in the early secretory pathway. Our affinity-purified anti-GAPDH polyclonal antibody was a potent inhibitor of ER to Golgi traffic (14).

We began our search for the PKCα substrate(s) that participates in this trafficking event by first focusing our attention on these three proteins. In this study we found that unlike Rab2 and β-COP, GAPDH was phosphorylated efficiently by PKCα in an in vitro kinase assay. GAPDH is not only a substrate for PKCα, but also interacts directly with PKCα via its regulatory domain. Moreover, a quantitative microsomal binding assay showed that Rab2-dependent GAPDH recruitment to membranes results in GAPDH phosphorylation by membrane-associated PKCα. Because there were numerous reports showing that GAPDH binds to microtubules (MTs) resulting in the formation of cross-linked structures or bundles, we examined the effect of GAPDH on MT membrane association and intracellular distribution in the early secretory pathway. Interestingly, phospho-GAPDH promoted tubulin binding to microsomes which was inhibited by reagents that interfere with Rab2-dependent GAPDH membrane recruitment or with PKCα kinase activity. Furthermore, NRK cells protein transfected with constitutively activated Rab2 (Q65L) or with our affinity-purified anti-GAPDH polyclonal antibody displayed a striking and opposite consequence on MT organization.

These combined results implicate a mechanistic link among Rab2-stimulated PKCα recruitment, GAPDH membrane binding, and subsequent phosphorylation. We propose that phospho-GAPDH is necessary for MT binding to the VTC sub-compartment that contains Rab2. The MTs associated with this VTC subcompartment may then provide tracks that direct movement of the retrograde vesicle back to the ER.

**EXPERIMENTAL PROCEDURES**

**Materials—PKCα/pseudosubstrate peptide (SIYRRGARWRKRL-Can) and scrambled PKCα peptide (WRICGNKLRRYYSAR) were synthesized at the University of Michigan Protein and Carbohydrate Structure Facility (Ann Arbor). The cDNA to PKCα was a gift from Dr. Trevor Biden (Garvan Institute of Medical Research, Sydney, Australia). Monoclonal antibodies to phosphoserine and phosphothreonine were purchased from Calbiochem-Novabiochem. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody was synthesized at the University of Michigan Protein and Carbohydrate Structure Facility (Ann Arbor). These combined results implicate a mechanistic link among Rab2-stimulated PKCα recruitment, GAPDH membrane binding, and subsequent phosphorylation. We propose that phospho-GAPDH is necessary for MT binding to the VTC sub-compartment that contains Rab2. The MTs associated with this VTC subcompartment may then provide tracks that direct movement of the retrograde vesicle back to the ER.

**Purification of Recombinant PKCα/ and Measurement of PKCα Kinase Activity—**The kinase-deficient PKCα mutant (K274W) was made as described previously (13). The PKCα regulatory domain was generated by PCR using a 5′-oligonucleotide primer that contained an EcoRI site, 5′-GCGCAATTCTAGGGCACTACCGGCGGCGGAG-3′, in tandem with the 3′-antisense oligonucleotide, 5′-TCAAAATTCAATCTGGAAAGCCT-3′. The PKCα catalytic domain was generated by PCR using the primer 5′-TTGTCCTGGGTAATAGGAAAGAGGAAGT-3′ and the antisense primer 5′-TTCCAGAACACGACCACTTCTTTCTC-3′. The amplified products were subcloned into pcDNA/HisMax-Topo (Invitrogen), and the sequence was verified by DNA analysis.

The transient expression of PKCα, PKCα, and the PKCα regulatory and catalytic domains was performed as described previously (8). Briefly, HeLa cells (3 × 10^5/10-cm dish × 5) were inoculated with a vaccine T7 RNA polymerase recombinant virus (TVFpT-3) for 30 min and then transfected with 20 μg of pcDNA/HisMax-Topo encoding for one of the various PKCα constructs that had been previously mixed in LipofectACE (Invitrogen). 8 h post-transfection, the cells were detached from the dish with phosphate-buffered saline (PBS)/EDTA and washed once with 10/18 (10 μg KOAc, 18 μg Hepes, pH 7.2). The cells were resuspended in an 18 containing protease inhibitors (10 μM aprotinin, 10 μM 1-chloro-3-tosylamido-7-amino-2-heptanone, 1 μM/ml chymotrypsin, 0.1 μM pepstatin), swollen on ice for 10 min, and then homogenized by 20 passes through a 27-gauge needle after which the concentration was adjusted to 25 mM KOAc, 125 mM Hepes. The nuclei were recovered by centrifugation for 5 min at 500 × g, and the post-nuclear supernatant was centrifuged at 100,000 × g for 60 min at 4 °C in a micro-ultracentrifuge. The supernatant that contained overexpressed His6Xpress-tagged PKCα, PKCα (K274W), or PKCα regulatory and catalytic domain, was then applied to Ni-NTA-agarose (Qiagen, Valencia, CA) equilibrated in buffer A (10 mM Hepes, 7.9 mM MgCl2, 0.1 mM EDTA, 50 mM NaCl, and 0.8 mM imidazole). The column was washed with 10 volumes of buffer A containing 8 mM imidazole. The tagged protein was eluted with buffer A supplemented with 80 mM imidazole. A 25-μl aliquot of the collected eluates was run on SDS-PAGE and then incubated with anti-Xpress monoclonal antibody. His6Xpress-tagged PKCα, PKCα (K274W), and PKCα regulatory and catalytic domain-enriched fractions were pooled, concentrated, and the protein concentration was determined by Micro BCA protein assay reagent (Pierce Chemical Co.).

2 μg of recombinant PKCα or PKCα (K274W) was incubated in kinase buffer containing 0.5 mM EGTA, 10 mM MgCl2, and 35 mM Tris, pH 7.4, supplemented with PS (100 μg/ml as sonicated vesicles), and then incubated and incubated for 10 minutes with 1 μCi[^35]S PIATP (PerkinElmer Life Sciences) for 15 min at 30 °C in the presence or absence of 1 μg of rabbit muscle GAPDH, myelin basic protein, GAPDH purified from rat liver cytosol, recombinant Rab2, or Rab2 mutant proteins. The reaction was stopped by the addition of 5× sample buffer and then separated by SDS-PAGE followed by autoradiography and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Purification of recombinant Rab2 wild type and mutants and in vitro prenylation was performed as described previously (25). GAPDH from rat liver cytosol was purified sequentially by batch absorption to GTP-agarose (Sigma) and elution with 10 mM GTP, followed by batch absorption to Blue-Sepharose (Amersham Biosciences, Inc.) and elution with 10 mM NAD+ in 0.5 mM Tris-HCl, pH 8.6.

**Quantitative Microsomal Binding Reaction—**NRK cells were washed three times with ice-cold PBS. The cells were scraped off the dish with a rubber policeman into 10 mM Hepes, pH 7.2, and 250 mM mannitol, then broken with 15 passes of a 27-gauge syringe. 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 20 min at 4 °C. The resultant pellet containing ER, pre-Golgi, and Golgi membranes was resuspended in 10 mM Hepes, pH 7.2, and 250 mM mannitol and employed in the binding reaction as described previously (12, 25). For some experiments, the membranes were washed with 1 mM KCl in 10 mM 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 2.5 mg/ml Hepes, 2.5 mM MgCl2, 65 mM KCl, 1.8 mM EGTA, 1.8 mM CaCl2, 1 mM ATP, or 10 μM [gamma-35]S PIATP (PerkinElmer Life Sciences), 5 mM creatine phosphate, and 0.2 unit of rabbit muscle creatine kinase. Recombinant Rab2, PKCα, and PKCα pseudosubstrate peptide were added at the concentrations indicated under “Results,” and the reaction mix was incubated on ice for 10–20 min. 50 μg of rat liver cytosol and 2.0 μM GTPγS were then added and the reaction was incubated for 30 min at 30 °C. 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 recentered at 100,000 × g for 60 min in a micro-ultracentrifuge to recover released vesicles (P2). P1 and P2 were separated by SDS-PAGE, and the gel was either processed for autoradiography, or the proteins were transferred to nitrocellulose in 25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol. The blot was blocked in Tris-buffered saline that contained 5% nonfat dry milk and 0.5% Tween 20, incubated with affinity-purified polyclonal antibody made to GAPDH (14), or a monoclonal antibody to β-tubulin, or a monoclonal antibody to phosphoserine (16βA), or a monoclonal antibody to phosphothreonine (4D11), then washed, incubated further with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody and developed with enhanced chemiluminescence (ECL) (Amersham Biosciences, Inc.), then quantified by densitometry.

**Blot Overlay Assay—**The P1 fraction obtained from microsomes treated with recombinant Rab2 was separated by SDS-PAGE and transferred to nitrocellulose, as described above. The blot was incubated with 2.5 μg/ml of 35S-labeled serum albumin, 0.2 mM NaCl, and 10 μg/ml purified recombinant PKCα, PKCα, or PKCα regulatory and catalytic domain-enriched fractions of 20 μg/ml PS for 4 h at room temperature (26, 27). After incubation, the blot was washed briefly with PBS and then fixed in 0.5% formaldehyde in PBS for 20 min at room temperature, followed by incubation in 2% glycine in PBS to block reactive aldehydes (28). The blot was washed in Tris-buffered saline and probed with an anti-PKCα monoclonal antibody, then washed, incubated further with a horseradish...
A kinase assay was employed which made use of Ni\(^{2+}\)-NTA-agarose affinity-purified PKC\(\alpha\) from vaccinia infected HeLa cells transfected with pcDNA4/HisMax-PKC\(\alpha\). Although PKC\(\alpha\) efficiently phosphorylated myelin basic protein, indicating that the purified enzyme was active, PKC\(\alpha\) did not show any kinase activity toward recombinant Rab2 wild type (Fig. 1A). It was conceivable that PKC\(\alpha\) only recognized a specific guanine nucleotide-bound form of Rab2. We addressed this question by performing the kinase reaction with Rab2 mutant proteins. Rab2 (S20N) is equivalent to the Ras 17N mutant that shows reduced affinity for GTP, high affinity for guanine nucleotide exchange factor, and is likely to be in the GDP-bound form (29, 30). The Q65L substitution in Rab2 generates a constitutively activated protein with impaired intrinsic and GTPase activating protein stimulated GTPase activity (25, 31). PKC\(\alpha\) did not phosphorylate either of the Rab2 mutants, demonstrating further that Rab2 is not a substrate.

We used rat liver cytosol as the source of \(\beta\)-COP to determine whether this coamplifier subunit was a PKC\(\alpha\)/substrate. As was observed for Rab2, \(\beta\)-COP was not phosphorylated by PKC\(\alpha\) (Fig. 1B, lane a). However, it was possible that we did not detect phospho-\(\beta\)-COP because of phosphatase activity in the cytosol. To address this concern, \(\beta\)-COP was immunoprecipitated from rat liver cytosol with affinity-purified anti-EAGE polyclonal antibody and then analyzed in the kinase assay (12). The presence of \(\beta\)-COP in both protocols (b and d) was determined by Western blotting using anti-EAGE polyclonal antibody. Panel C, rabbit muscle GAPDH was incubated with purified PKC\(\alpha\) (a), or GAPDH purified from rat liver cytosol was incubated with (b) or without (d) purified PKC\(\alpha\) or kinase-dead PKC\(\alpha\) (R274W) (c), in buffer supplemented with PS, as above. The reactions were separated by SDS-PAGE and the gel processed for autoradiography. GAPDH was phosphorylated by PKC\(\alpha\). Panel D, PKC\(\alpha\)/peptide pseudosubstrate peptide was added in increasing concentrations to the kinase reaction supplemented with PKC\(\alpha\), as above. Control reactions included two concentrations of the scrambled peptide. PKC\(\alpha\)/dependent phosphorylation of GAPDH was inhibited by the pseudosubstrate peptide. A representative experiment of three independent assays is shown.

**Indirect Immunofluorescence**—NRK cells were plated overnight on coverslips and then transfected with 1 \(\mu\)g of recombinant Rab2 (Q65L) or 5 \(\mu\)g of affinity-purified anti-GAPDH polyclonal antibody using Chariot protein transfection reagent, as outlined by the manufacturer. Briefly, Rab2 (Q65L) or anti-GAPDH polyclonal antibody was diluted in PBS (total volume 100 \(\mu\)l), then added to the Chariot reagent prediluted 1:100 in \(\mathrm{H}_2\mathrm{O}\) (total volume 100 \(\mu\)l) and incubated for 30 min at room temperature to allow the Chariot-Rab2 (Q65L) or Chariot-anti-GAPDH antibody complex to form. The cells were washed three times with PBS, then the coverslips containing the cells were inverted and placed over a drop containing the macromolecular complex. The transfected cells were incubated for 4 h at 37 °C in a 5% CO\(_2\) incubator, fixed in 3% formaldehyde and PBS for 10 min, permeabilized with 0.05% saponin in PBS and normal goat serum for 10 min, washed with PBS, and then incubated for 30 min with an affinity-purified polyclonal antibody to GAPDH (14) (control and Rab2/Q65L transfected cells) and an anti-\(\beta\)-tubulin monoclonal antibody. Cells were then washed with PBS, stained for 30 min with a fluorescein isothiocyanate-conjugated anti-mouse antibody and a Texas Red anti-rabbit antibody, washed, mounted, and then viewed under a Zeiss Axiovert fluorescence microscope. The transfection efficiency ranged from 30 to 60% of the cells, and in 40% of the cells the input protein induced a phenotypic change.

**RESULTS**

**PKC\(\alpha\) Phosphorylates GAPDH.**—To determine whether Rab2, \(\beta\)-COP, and GAPDH were PKC\(\alpha\) substrates, an in vitro kinase assay was employed which made use of Ni\(^{2+}\)-NTA-agarose affinity-purified PKC\(\alpha\) from vaccinia infected HeLa cells transfected with pcDNA4/HisMax-PKC\(\alpha\). Although
liver cytosol were phosphorylated efficiently by PKCα/λ in the in vitro kinase assay (Fig. 1C, lanes a and b). Because GAPDH was reported to undergo autophosphorylation, it was important to demonstrate that phosho-GAPDH was the result of PKCα/λ modification. Therefore, we performed a series of experiments. First, purified GAPDH from rat liver cytosol was incubated in buffer containing [γ-32P]ATP in the absence of PKCα/λ. Under these conditions, phosho-GAPDH was not detected. The reaction was then performed with a kinase-dead mutant of PKCα/λ (K274W) (13). As expected, no phosho-GAPDH was produced after incubation with the kinase-deficient mutant (Fig. 1C, lane c). Finally, the kinase reaction was performed in the presence of a peptide made to the unique pseudosubstrate region of PKCα/λ which inhibits kinase activity (13, 32). The peptide blocked PKCα/λ phosphorylation of GAPDH in a dose-dependent manner, whereas a randomized form of this peptide had no effect on kinase activity (Fig. 1D). These combined results support our claim that GAPDH is a bona fide substrate for PKCα/λ.

**GAPDH Interacts with the Regulatory Domain of PKCα/λ**—Numerous studies have identified proteins that are not only PKC substrates, but also interact directly with a PKC isoform (33, 34). All of these substrates are PS-binding proteins. However, not all PS-binding proteins are PKC-binding partners (28, 35, 36). Interestingly, GAPDH binds to PS (37). To determine whether GAPDH interacts with PKCα/λ, a blot overlay assay was employed which is used routinely to identify PKC-interacting proteins (27, 28, 36). We first performed the microsomal binding assay to generate membranes for analysis. For this assay, microsomes were prepared from NBK cell homogenates, salt washed to remove the bulk of peripheral associated proteins, and then preincubated in the absence or presence of 100 ng of recombinant Rab2. This concentration of Rab2 stimulates both PKCα/λ and GAPDH membrane association, but not vesicle release (13, 14). The reaction was then supplemented with GTPγS and rat liver cytosol, which serves as the source of soluble components. To terminate the reaction, membranes were centrifuged, separated by SDS-PAGE, and transferred to nitrocellulose. The blot was incubated in an overlay buffer containing 20 µg of affinity-purified PKCα/λ in the presence or absence of PS, then probed with an anti-PKCα/λ monoclonal antibody. Any PKCα/λ bound to microsomes was detected after incubation with a secondary horseradish peroxidase-conjugated antibody followed by development with ECL (Fig. 2A). As predicted, we failed to detect any PKCα/λ binding to control membranes that lack GAPDH. However, in the presence of PS, Rab2-treated membranes showed an ~38 kDa immunoreactive band. When the blot was stripped and reprobed with an anti-GAPDH polyclonal antibody, the reactive band precisely aligned with the 38-kDa protein detected with anti-PKCα/λ antibody, indicating that purified PKCα/λ interacted with membrane-bound GAPDH.

The PS-dependent binding of PKCα/λ with GAPDH suggested that the PKCα/λ regulatory domain that contains the recognition site for acid phospholipids was involved in facilitating the interaction (38). To address this possibility, the PKCα/λ regulatory domain (amino acids 1–247) and the catalytic domain (amino acids 248–588) were expressed as fusion proteins in vaccinia-infected/transfected HeLa cells and purified from the cell lysate on Ni²⁺-NTA-agarose. Equal amounts of the regulatory and catalytic domains were bound to nitrocellulose using a slot blot apparatus. The blot was incubated in overlay buffer in the presence or absence of PS and 20 µg of rabbit muscle GAPDH and probed with an affinity-purified anti-GAPDH polyclonal antibody. Fig. 2B shows that GAPDH bound only to the PKCα/λ regulatory domain. This result is not surprising because the different PKC families are distinguished based on the variability in their regulatory domains which consequentially results in different cofactor dependence.

The pseudosubstrate domain located within the PKC regulatory domain associates with PS-binding proteins and is important for interactions with other proteins (36). To determine
whether the PKC/α pseudosubstrate domain was involved in GAPDH binding to the kinase, the microsomal binding assay was supplemented with PKC/α pseudosubstrate peptide at two concentrations that interfere with PKC/α kinase activity (see Fig. 1D). This peptide not only blocked phosphorylation of GAPDH but also caused a decrease in Rab2-stimulated GAPDH recruitment to membrane (Fig. 2C). Therefore, it appears that GAPDH and PKC/α association involves both protein-protein and protein-lipid interactions.

GAPDH Recruited to Membrane by Rab2 Is Phosphorylated by PKC/α—To determine whether GAPDH recruited to microsomes by Rab2 is phosphorylated by membrane-associated PKC/α, the microsomal binding assay was supplemented with 10 μCi of [γ-32P]ATP in the presence or absence of 300 ng of Rab2 to promote vesicle formation. The membranes (P1) were collected by centrifugation (20,000 × g), and the supernatant was recentrifuged (100,000 × g) to recover released vesicles (P2) (25). Both P1 and P2 from the control reaction lacking recombinant Rab2 contained an insignificant amount of radio-labeled 38-kDa protein (Fig. 3a). However, phospho-p38 was detected in P1 and P2 obtained from microsomes preincubated with recombinant Rab2 (Fig. 3b) and is most likely GAPDH because no phospho-p38 was found on membranes incubated with rat liver cytosol depleted of GAPDH (Fig. 3c). To identify further the labeled protein as GAPDH, the blot was probed with affinity-purified anti-GAPDH polyclonal antibody. The GAPDH protein detected by immunoblotting was superimposed with pp38 (Fig. 3b). We reprobed the Western blot with phosphoserine-specific and phosphothreonine-specific antibodies. The modified residue(s) appears to be serine. A strong chemiluminescent signal was observed with the anti-phosphoserine antibody that aligned with GAPDH (Fig. 3b).

To demonstrate further that the membrane-associated GAPDH was a specific substrate for PKC/α, the microsomal binding assay was supplemented with PKC/α (K274W). We reasoned that if GAPDH was specifically phosphorylated by PKC/α, then no phospho-GAPDH should be detected on membranes defective in PKC/α kinase activity. In this binding assay, the mutant enzyme competes with the cytosolic PKC/α for recruitment to membrane. Fig. 3d shows that salt-washed microsomes incubated with recombinant Rab2 and PKC/α (K275W) showed a negligible amount of phospho-GAPDH. These combined results indicate that Rab2 recruitment of GAPDH to membrane results in the subsequent PKC/α phosphorylation of GAPDH.

GAPDH Is Required for Microtubule Binding to NRK Microsomes—There are numerous PKC-binding proteins that are also PKC substrates and are referred to as STICKS (substrates that interact with C-kinase) (33). Frequently, these proteins provide a direct link between the membrane and cytoskeletal elements (26–28). Because GAPDH fulfills the criteria for a STICK and has been shown to modulate the cytoskeleton by promoting actin polymerization and MT bundling, we investigated whether phospho-GAPDH played a role in tubulin binding to VTCs. To address this question, we determined whether β-tubulin was recruited to membranes in the microsomal binding assay. After incubation at 32°C for 10 min, the microsomes were sedimented at 20,000 × g through a 15% sucrose cushion to separate MTs associated with membranes from cytosolic components, and the resultant membrane pellet was separated on SDS-PAGE and immunoblotted. Fig. 4A shows that recombinant Rab2 stimulated the colinear recruitment of GAPDH and β-tubulin to membranes.

To determine whether GAPDH is required for β-tubulin binding to membrane, the microsomal binding assay was supplemented with 5 μg and 10 μg of affinity-purified anti-GAPDH polyclonal antibody. As we observed previously, the antibody blocked Rab2-dependent GAPDH recruitment to membrane, whereas no effect was observed with membranes treated with preimmune serum (Fig. 4B). A comparative decrease in membrane-associated β-tubulin also occurred in microsomes treated with the antibody. Similar results were obtained when the assay was supplemented with PKC/α pseudosubstrate peptide (Fig. 4B). The amount of membrane-bound β-tubulin decreased proportionately to the level of membrane-associated GAPDH.

The apparent relationship between membrane-associated phospho-GAPDH and MT recruitment prompted us to treat membranes recovered after incubation with or without Rab2.
FIG. 4. Phospho-GAPDH is necessary for β-tubulin binding to NRK microsomes. Panel A, salt-washed NRK microsomes were incubated in the absence or presence of 50 or 100 ng of recombinant Rab2, cytosol, and GTPγS for 10 min at 37 °C. The membranes were collected by centrifugation, separated by SDS-PAGE, and transferred to nitrocellulose. The blot was probed with an affinity-purified anti-GAPDH polyclonal antibody and an anti-β-tubulin polyclonal antibody, washed, and then incubated with a secondary horseradish peroxidase-conjugated antibody, developed with ECL, and quantitated by densitometry. Panel B, the microsomal binding assay was supplemented with 100 ng of recombinant Rab2 and performed in the presence or absence of the indicated concentration of affinity-purified anti-GAPDH polyclonal antibody, preimmune (PI) serum, or PKC/α pseudosubstrate peptide. Membranes were collected by centrifugation, separated by SDS-PAGE, and transferred to nitrocellulose. The blot was probed with a polyclonal antibody to β-tubulin and a monoclonal antibody to GAPDH, washed, and incubated with a secondary horseradish peroxidase-conjugated antibody, and then developed with ECL. The blot was stripped and reprobed with a monoclonal antibody to phosphoserine and processed as above. Panel C, the microsomal binding assay was performed with salt-washed NRK microsomes supplemented with 100 ng of recombinant Rab2 (a) or with 100 ng of recombinant Rab2 and 100 ng PKC/α (K274W) (b). One set of membranes (a) was treated with 1.0 unit of acid phosphatase for 60 min at 37 °C. The membranes were collected by centrifugation and then separated by SDS-PAGE and subjected to Western blotting. The blot was probed and processed as above.

with alkaline phosphatase. This treatment would remove any PKC/α-modified ester phosphate(s) on GAPDH. We were unable to detect phospho-GAPDH with the anti-phosphoserine monoclonal antibody, indicating that the phosphatase had efficiently hydrolyzed the linkage (Fig. 4C). Interestingly, dephosphorylation of GAPDH caused a significant decrease in membrane-associated β-tubulin (Fig. 4C). We then evaluated membranes from the microsomal binding reaction membranes from the microsomal binding reaction which had been incubated with PKC/α (K274W) for GAPDH and β-tubulin content. Although membranes supplemented with PKC/α (K274W) contained GAPDH, the glycolytic enzyme showed a trivial amount of phosphorylation. Moreover, these membranes displayed a significant decrease in the level of β-tubulin compared with controls. These results are highly suggestive that phospho-GAPDH influences MT binding to membranes.

Because Rab2 has a profound effect on GAPDH recruitment to VTCs, NRK cells were transfected with recombinant Rab2 (Q65L) protein, and the distribution of MTs was evaluated by indirect immunofluorescence. In control cells, MTs are concentrated near the nucleus and radiate out toward the cell periphery from a centrally located microtubule-organizing center, whereas GAPDH is distributed diffuse throughout the cytoplasm (Fig. 5). A striking change in MT organization occurred in cells transfected with constitutively activated Rab2 protein. The MTs redistributed into cross-linked networks of large filaments-like structures that resemble bundled MTs. When these cells were costained for GAPDH, the anti-GAPDH antibody labeled the filamentous structures that overlapped with the anti-tubulin-labeled elements (Fig. 5). An opposite phenotype was observed in NRK cells transfected with our affinity-
purified anti-GAPDH polyclonal antibody. The cells did not exhibit the characteristic MT distribution but instead contained prominent β-tubulin labeled elements that appeared to codistribute with structures that stained with anti-GAPDH antibody. These in vivo results are consistent with numerous in vitro studies that show that GAPDH binds to MTs and promotes formation of MT bundles. More importantly, these results suggest that GAPDH plays a critical role in MT organization.

**DISCUSSION**

We learned from our previous studies that Rab2 requires PKCα to recruit β-COP to membranes (12). Although kinase activity was necessary for this event, the PKCα substrate(s) that potentiated Rab2 activity in vesicle formation was not known (13). In this study, we have identified GAPDH as a substrate for PKCα in an in vitro kinase assay. Moreover, PKCα phosphorylation of GAPDH is specific based on the fact that no phospho-GAPDH was detected when the assay was supplemented with either the unique pseudosubstrate peptide or the kinase-deficient enzyme.

It has been reported that GAPDH undergoes autophosphorylation with MgATP and that the resulting phosphoprotein contains an acyl phosphate that has phosphotransferase properties (18). Under our experimental conditions, we did not detect autophosphorylated GAPDH. Likewise, Reiss and coworkers (19, 20) found no GAPDH autophosphorylation. Consistent with our findings, these investigators reported that GAPDH was phosphorylated by PKC and that PS stimulated the phosphorylation rate. It is possible that GAPDH undergoes a minor degree of autophosphorylation which was not detected because of the strong radioactive signal obtained from PKCα phosphorylation of GAPDH which allowed us to process the autoradiogram for several hours. However, treatment with acid phosphatase released all of the radiolabel from GAPDH, indicating that the incorporated $^{34}$PO$_4$ was in the form of an ester linkage. Because protein kinase catalyzes the formation of an ester phosphate on proteins, phospho-GAPDH is the result of PKCα kinase activity. This type of linkage also indicates that the phospho-GAPDH produced in the kinase assay is most likely not involved in phosphotransferase activity because ester phosphates (seryl and threonyl) are not high energy phosphate transport intermediates. Our preliminary Western blot results using phosphoserine/phosphothreonine-specific monoclonal antibodies suggest that the modified residue is a serine. There are four potential serine PKC phosphorylation sites in GAPDH, and we are currently pursuing the identification of the phosphorylated residue(s).

PKC activation involves translocation from the cytosol to subcellular sites that include the ER, Golgi complex, nucleus, as well as the cytoskeleton. Specific binding proteins mediate localization by placing the PKC in proximity of its substrate (34). A PKC-binding protein may serve both as substrate and to anchor the PKC to the appropriate target membrane. The polypeptides that provide those two functions are PS-binding proteins (28, 33, 35). We found that PKCα interacted with membrane-associated GAPDH using a gel overlay assay and that this binding required PS. It has been proposed that PS serves to bridge PKC to other PS-binding proteins and that the phospholipid-dependent interactions are stabilized additionally by protein-protein interactions (28, 33). Because the association of PKC to PS-binding proteins occurs in part at the pseudosubstrate site located in the regulatory domain, residues 1–247 of PKCα were assessed for the ability to interact with GAPDH (36). We detected GAPDH bound to the PKCα regulatory domain, whereas no binding was found to the catalytic half of the molecule. Furthermore, the interaction between GAPDH and PKCα was partly blocked in the presence of the pseudosubstrate peptide. These combined results indicate that the interaction of PKCα with GAPDH is mediated partially through the PKCα pseudosubstrate domain. Therefore, PKCα and GAPDH associate via lipid protein interaction as well as protein-protein interaction.

The pseudosubstrate or autoinhibitory domain is present in the regulatory domain of all PKC isozymes. The regulatory domain not only contains motifs involved in the binding of phospholipid cofactors and calcium but also participates in protein-protein interactions. For example, the novel protein modulators lambda-interacting protein and Par-4 have been reported to interact with the regulatory domain of PKCζ (39, 40). Additionally, Ras binds to the regulatory domain of PKCζ (41). Unlike these protein-protein interactions that either promote or inhibit PKC kinase activity, another class of proteins (STICKS) bind to the PKC regulatory domain and is a substrate for the isozyme. In several cases, PKC phosphorylation of a STICK has been shown to modify its activity (26, 27, 42). For instance, phosphorylation of the STICK called clone 72 results in the accumulation of this protein in membrane protrusions and ruffles, implicating a role in cytoskeletal alteration (27). Another protein identified as a STICK is adducin. PKC phosphorylation of this cytoskeletal protein inhibits actin capping and spectrin recruiting activity (42). Lastly, GAP-43 is a STICK that binds to actin and has been implicated in neurite outgrowth (43). PKCα phosphorylation of GAPDH may function in a similar manner and initiate cytoskeletal remodeling at the VTC. In that regard, PKCα has been reported to participate in Ras- and Cdc42-mediated reorganization of the actin cytoskeleton (44, 45).

GAPDH was the first glycolytic enzyme found associated with tubulin (22, 46, 47). In fact, a GAPDH-specific binding domain has been identified at the carboxyl terminus of α-tubulin (residues 409–451 designated T-GEBD-43mer) (48). GAPDH is known to modulate the cytoskeleton by promoting MT bundling (49). These dynamic complexes may be involved in motility or simply provide spatial organization within the cell because MTs control both organelle position and intracompartmental transport of vesicular and tubular elements. To determine whether GAPDH had any influence on MTs, microsomes recovered from the binding assay were analyzed for tubulin content. We found that β-tubulin recruitment to membrane was not only dependent on membrane-associated GAPDH but also required the phosphorylated form, an observation similar to that described above for STICKS. The role of GAPDH in the early secretory pathway may be to nucleate MT bundling at anchorage sites within a VTC subcompartment that binds Rab2. GAPDH could work in conjunction with MT bundles by providing energy and coordinating motile processes for retrograde vesicle movement to the ER. Although MT and actin assembly does not appear to be regulated directly by small GTPases, Rab proteins provide a molecular link for vesicle movement to the appropriate target. In support of this idea, Rab5 has been shown to regulate endosome interaction with the MT network (50). Both Rab6 and Rab8 are involved in cytoskeletal interaction and organization (51, 52). Furthermore, overexpression of Rab8 (Q67L) produces clusters of bundled MTs and actin in BHK cells (53). We found that Rab2 produces a similar phenotype. NRK cells transfected with a constitutively activated form of Rab2 showed a dramatic change in microtubule organization. The MTs appeared to organize into cross-linked structures or bundles located in the Golgi region. Interestingly, these structures also immunostained for GAPDH. These results imply a causal link between...
Phospho-GAPDH Promotes MT Binding to VTCs

Phospho-GAPDH binding and MT remodeling are supported by the observation that cells transfected with anti-GAPDH antibody showed altered MT distribution. The fact that MTs are dynamic polymers that respond to signaling pathways by changing their organization suggests that second messengers may also regulate the early secretory pathway.

Acknowledgments—We thank Dr. Assia Shisheva and Dr. Marie Wooten for critical reading of the manuscript. We are grateful to Dr. Klaus Hahn for advice on protein transfection.

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J. Biol. Chem. 2002, 277:3334-3341.
doi: 10.1074/jbc.M109744200 originally published online November 27, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109744200

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