Src-dependent aProtein Kinase C ι/λ (aPKCι/λ) Tyrosine Phosphorylation Is Required for aPKCι/λ Association with Rab2 and Glyceraldehyde-3-phosphate Dehydrogenase on Pre-Golgi Intermediates*

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The small GTPase Rab2 is required for membrane transport between the endoplasmic reticulum (ER) and the Golgi complex. Rab2 associates with pre-Golgi intermediates (also termed vesicular tubular clusters; VTCs) that sort cargo to the anterograde pathway from recycling proteins retrieved to the ER. Our previous studies have shown that Rab2 stimulates atypical protein kinase C ι/λ (aPKCι/λ) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) recruitment to VTCs. Both aPKCι/λ and GAPDH bind directly to Rab2 and aPKCι/λ and GAPDH interact. Based on the reports demonstrating aPKCι-Src interaction and Src activity in the retrograde pathway (Golgi-ER), studies were initiated to learn whether Rab2 also promoted Src recruitment to VTCs. Using a quantitative membrane binding assay, we found that Rab2-stimulated Src membrane association in a dose-dependent manner. The recruited Src binds to aPKCι/λ and GAPDH on the membrane; however, Src does not interact with Rab2. The membrane-associated Src tyrosine phosphorylates aPKCι/λ on the VTC. To determine the consequence of aPKCι/λ tyrosine phosphorylation, the membrane binding assay was supplemented with the Src-specific tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo(3,4-d)pyrimidine (PP2). Although Rab2, Src, and GAPDH recruitment was not affected, the Rab2-PP2-treated membranes contained a negligible amount of aPKCι/λ. Since Rab2 requires aPKCι/λ for the downstream recruitment of β-coat protein (β-COP) to VTCs, the Rab2-PP2-treated membranes were evaluated for the presence of β-COP. Like aPKCι/λ, the membranes contained a negligible amount of β-COP that was reflected by the drastic reduction in Rab2-dependent vesicle formation. These data suggest that Src-mediated tyrosine phosphorylation of aPKCι/λ facilitates aPKCι/λ association with Rab2-Src-GAPDH on VTCs, which is ultimately necessary for the downstream recruitment of β-COP and release of Rab2-mediated retrograde-directed vesicles.

The Rab family of small GTPases includes more than 60 members that participate in compartment-specific transport events of both the endocytic and the exocytic pathways through recruitment and interaction with unique effector molecules that promote 1) vesicle formation, 2) cytoskeletal interaction and motility, and 3) vesicle docking and fusion (1–3). In the early secretory pathway, membrane traffic between the endoplasmic reticulum (ER)2 and the Golgi complex requires Rab1 and Rab2 (4). Rab2 preferentially associates with pleiomorphic structures called pre-Golgi intermediates or vesicular tubular clusters (VTCs) that sort anterograde-directed cargo that is shuttled to the Golgi complex from the trafficking machinery that recycles to the ER for reutilization in ER export (5–8). We have shown that Rab2 bound to VTCs stimulates the recruitment of atypical protein kinase C ι/λ (aPKCι/λ), which binds directly to the Rab2 amino terminus (9, 10). aPKCι/λ kinase activity is required for Rab2-mediated vesicle formation. The Rab2-generated vesicles are devoid of secretory cargo but contain aPKCι/λ, β-coat protein (β-COP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the recycling protein p53/p58, suggesting that these carriers traffic in the retrograde pathway (11, 12). GAPDH also interacts directly with Rab2 as well as aPKCι/λ and additionally serves as a substrate for the kinase (13, 14). Although GAPDH is essential for membrane trafficking in the early secretory pathway independent of catalytic activity, the precise role of this glycolytic enzyme in transport is unclear (14).

aPKCι/λ is a member of the atypical subgroup of protein kinase C. PKCι is the human homolog of mouse PKCι, and both share homology with the third member of the family, PKCζ. The aPKCι subfamily differs in structure from the other PKC isoforms by the absence of a calcium binding domain and the presence of only one zinc finger that renders the kinase insensitive to activation by calcium, diacylglycerol, and phosphol esters (15, 16). Moreover, the regulatory domain sequence of the aPKCs is divergent from other PKC members, and this divergence has been exploited to identify unique aPKC-interacting molecules (16–19). In that regard, activated Src has been reported to bind directly with aPKCi through the Src homology 3 (SH3) domain and residues 98–114 in the aPKC regulatory domain (20). Src is a ubiquitous member of the nonreceptor tyrosine kinase family that is involved in signal transduction events that regulate cell growth, survival, motility, and differentiation (21, 22). Src phosphorylates aPKCι on multiple tyrosine residues located in both the regulatory and the catalytic domain, which influences aPKCι association with specific binding proteins (20). Recent studies have also implicated Src activity in facilitating KDEL-receptor recycling between the ER and Golgi complex and thereby suggest a potential role for Src in retrograde transport (23). The fact that Rab2-aPKCι/λ directly interact and aPKCι/λ associates

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2 The abbreviations used are: ER, endoplasmic reticulum; VTC, vesicular tubular cluster; aPKCι/λ, atypical protein kinase Cι/λ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NRK, normal rat kidney; VSV, vesicular stomatitis virus; VSV-G, VSV-glycoprotein; β-COP, β-coat protein; GST, glutathione S-transferase; HRP, horseradish peroxidase; GTPγS, guanosine 5′-O-(thiophosphoryl); TBS, Tris-buffered saline; NTA, nitritotriacetic acid; PP2, 4-amino-5-(4-chlorophenyl)7-(t-butyl)pyrazolo(3,4-d)pyrimidine; PP3, 4-amino-7-phenylpyrazolo(3,4-d)pyrimidine; P, pellet.
with Src suggested the possibility that Src was a Rab2 downstream effector. We employed a quantitative binding assay that measures Rab2-dependent recruitment of soluble components to membranes and found that Rab2 promoted Src membrane association. Unlike aPKCα and GAPDH, Src does not directly interact with Rab2. However, Src does bind to both aPKCα and GAPDH. The Rab2-recruited Src is activated and tyrosine phosphorylates the membrane-associated aPKCα. Importantly, Src-mediated phosphorylation of aPKCα is essential for interaction with Rab2-GAPDH-Src on the VTC and for the downstream recruitment of β-COP. These combined results suggest that Src kinase activity is required for Rab2-mediated retrograde vesicle formation from the VTC.

**EXPERIMENTAL PROCEDURES**

**Quantitative Membrane Binding Assay**—Normal rat kidney (NRK) cells were washed three times with ice-cold phosphate-buffered saline. The cells were scraped off the dish with a rubber policeman into 10 mM Hepes (pH 7.2) and 250 mM mannitol and then broken with 10 passes through a cell homogenizer using a 7.992-mm tungsten carbide ball (8-μm clearance) (Isobiotec, Heidelberg, Germany). The broken cells were pelleted at 500 × g for 10 min at 4 °C, and the supernatant was recentrifuged at 100,000 × g for 60 min at 4 °C to obtain a pellet (P1). For some experiments, the supernatant was recentrifuged at 35,000 × g for 30 min at 4 °C in a Sorvall Discovery M120 ultracentrifuge. The membrane pellet was washed with 1 M KCl in 10 mM Hepes (pH 7.2) for 15 min on ice to remove peripheral associated proteins and then recentrifuged at 35,000 × g for 30 min at 4 °C. The resultant pellet was resuspended in 10 mM Hepes (pH 7.2) and 250 mM mannitol and employed in the binding reaction, as described previously (24). 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, 5 mM EGTA, 1.8 mM CaCl2, 1 mM ATP, 5 mM creatine phosphate, and 0.2 units of rabbit muscle creatine kinase. Preimmune serum, affinity-purified anti-Rab2 antibody, affinity column-purified recombinant His6-aPKCα (100 ng), or Rab2 was added at the concentrations indicated under “Results,” and the reaction mix was incubated on ice for 10 min. Rat liver cytosol (50 μg of total protein) and 2.0 μM GTPγS were then added, and the reactions were shifted to 32 °C and incubated for 12 min. The binding reaction was terminated by transferring the samples to ice and then centrifuged at 35,000 × g for 20 min at 4 °C to obtain a pellet (P1). For some experiments, the supernatant was recentrifuged at 100,000 × g for 60 min to recover released vesicles (P2). P1 and P2 were separated by SDS-PAGE and transferred to nitrocellulose in 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol. The membrane was blocked in Tris-buffered saline (TBS) that contained 5% nonfat dry milk and 0.5% Tween 20 and then incubated with a monoclonal antibody to SRC (GD11) (Upstate Biotechnology, Lake Placid, NY), a monoclonal antibody to GAPDH (Chemicon International, Temecula, CA), a monoclonal antibody to aPKCα (BD Biosciences), a polyclonal antibody to phospho-Src (Tyr-416) (Cell Signaling, Beverly, MA), a monoclonal antibody to phosphotyrosine (P-Tyr-100) (Cell Signaling), or an affinity-purified polyclonal antibody to β-COP (EAGE) (24), and washed, further incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibody, developed with enhanced chemiluminescence (ECL) (Amersham Biosciences), and then quantified by densitometry.

**Immunoprecipitation**—NRK cells (4 × 106) were incubated without or with 10 μM PP2 or 10 μM PP3 (EMB Biosciences Inc., San Diego, CA) for 12 h, lysed in 50 mM TBS (pH 8.0), 1% Triton X-100, and 1.0 mM phenylmethylsulfonyl fluoride for 10 min on ice and then clarified by centrifugation at 20,000 × g for 10 min at 4 °C. The soluble fraction was preclarified with protein G plus/protein A-agarose (Novagen, Madison, WI) and then subjected to immunoprecipitation for 12 h at 4 °C with an affinity-purified anti-Rab2 polyclonal antibody or with preimmune serum and protein G plus/protein A-agarose. The immune complexes were collected by centrifugation at 5,000 rpm for 5 min, washed 3 × with TBS, 1% Triton X-100, and 100 mM NaCl, boiled in sample buffer, and then separated by SDS-PAGE and transferred to nitrocellulose. The blot was incubated with an affinity-purified anti-Rab2 polyclonal antibody, a monoclonal antibody to aPKCα (BD Biosciences), a monoclonal antibody to SRC (Upstate Biotechnology), and a monoclonal antibody to GAPDH (Chemicon), washed, further incubated with a HRP-conjugated anti-rabbit or an anti-mouse antibody, and then developed with ECL (Amersham Biosciences).

**His6-GST Pull-down**—The transient expression of His6-aPKCα was performed as described (4, 13). The supernatant that contained overexpressed His6-aPKCα was applied to a 2-mL column of Ni2+-NTA-agarose (Qiagen, Valencia, CA). Equilibrated in buffer A (10 mM Hepes, pH 7.9, 5 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. His6-aPKCα (2.5 μg) was preincubated with 20 μl of Ni2+-NTA-agarose for 1 h at room temperature in buffer A, and then 1 μg of recombinant SRC (Upstate Biotechnology) was added and the sample was incubated for an additional 2 h at room temperature. The beads were washed 4 × with 1 ml of buffer A, and the bound proteins were boiled in sample buffer, separated by SDS-PAGE, and then transferred to nitrocellulose. The GST pull-down experiments were performed, as described previously (14). Briefly, BL21 (DE3) pLysS cells (Novagen) that contained the recombinant plasmids GST-GAPDH, GST-Rab2, or GST-Rab1 were grown at 37 °C to 0.6 A600 units and then induced with 0.4 mM isopropyl-1-β-D-thiogalactopyranoside for 3 h at 37 °C. The liquid culture was centrifuged at 6,000 rpm for 30 min, and the pellet was resuspended in cold TBS, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, sonicated, and centrifuged at 12,000 rpm for 20 min, and then the supernatant was applied to a glutathione-Sepharose 4B column (Amersham Biosciences). The column was washed with 10 bed volumes of TBS, and the fusion protein was eluted with 5 mM reduced glutathione. The GST-tagged proteins (2.5 μg) were preincubated with 30 μl of glutathione-Sepharose 4B for 1 h at room temperature in 50 mM Tris (pH 7.5), 5 mM MgCl2, 100 mM NaCl, and 10 mM GTPγS, and then 1 μg of recombinant SRC (Upstate Biotechnology) was added alone or in combination with rabbit muscle GAPDH (Sigma), and His6-PKCα (1 μg) and then 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, 0.1% Triton X-100, and 100 mM NaCl, and the bound proteins were boiled in sample buffer, separated by SDS-PAGE, and then transferred to nitrocellulose. The blot was blocked as above, and the membrane was probed with the antibodies indicated previously, washed, and developed, as described above.

**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 of Express85S3S (specific activity, 1,175 Ci/mmol, PerkinElmer Life Sciences) for 10 min at the restrictive 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 (25). Transport reactions were performed in a final volume of 40 μl in a buffer that 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, 0.2 IU of rabbit muscle creatine phosphokinase), and 5 μl of semi-intact cells (5 × 107).
Tyrosine Phosphorylated aPKC/λ Interacts with Rab2

**RESULTS**

**Rab2 Promotes Src Binding to NRK Membranes**—Based on the observations that Rab2 directly interacts with aPKC/λ (10) and aPKC/λ associates with c-Src in PC12 cells (20), a quantitative membrane binding assay was employed to determine whether Rab2 also promoted Src membrane association. Salt-washed NRK cell membranes were incubated with rat liver cytosol and GTPyS, in the absence or presence of increasing concentrations of purified recombinant Rab2. The control membranes and membranes containing Rab2-reconstituted proteins were then analyzed by SDS-PAGE and Western blot. As shown in Fig. 1, Rab2-stimulated Src binding to NRK cell membranes in a dose-dependent manner. The amount of membrane-bound Src increased ~3-fold when incubated with 50 ng of Rab2. The membrane-associated Src is activated since the recruited form is detected on the Western blot by antibody specific to anti-phospho-Src (Tyr-416) (Fig. 1).

The Rab2-dependent Src recruitment to membranes was prevented when the membrane binding assay was supplemented with an affinity-purified anti-Rab2 polyclonal antibody (Fig. 2A). Similarly, Rab2-dependent recruitment of aPKC/λ and GAPDH to membranes was inhibited by the addition of the anti-Rab2 antibody to the assay (Fig. 2B). The Rab2 antibody is a potent inhibitor of VSV-G transport from the ER to the Golgi complex when introduced into an *in vitro* quantitative transport assay (Fig. 3A) and was previously reported to co-immunoprecipitate Rab2, aPKC/λ, and GAPDH from HeLa cell detergent lysate (10, 14). When the immunoprecipitation experiment was performed in this

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**FIGURE 1.** Rab2 promotes Src-membrane association. Salt-washed NRK microsomes prepared as described under “Experimental Procedures” were preincubated with purified recombinant Rab2 at the concentrations indicated, and the reaction mix was incubated on ice for 10 min. Rat liver cytosol and GTPyS were then added, and the reaction was shifted to 32 °C and incubated for 12 min. To terminate the reaction, the membranes were collected by centrifugation, and the membrane pellet was separated by SDS-PAGE and then transferred to nitrocellulose. The blot was probed with an anti-Src monoclonal antibody (GD11), washed, further incubated with HRP-conjugated secondary antibody, and then developed with ECL. The blot was stripped and reprobed with a polyclonal antibody to phospho-Src (Tyr-416) and processed as above. The amount of recruited activated Src was quantitated by densitometry. A representative Western blot with each antibody is shown in panels **A** and **B**. The results are the mean ± S.D. of three independent experiments performed in duplicate. cont, control.

**FIGURE 2.** Anti-Rab2 antibody blocks Rab2-dependent aPKC/λ-Src-GAPDH recruitment to membranes. A, salt-washed NRK microsomes prepared as described under “Experimental Procedures” were preincubated with 100 ng of purified recombinant Rab2 in the absence or presence of affinity-purified anti-Rab2 antibody at the concentrations indicated under “Results,” and the reaction mix was incubated on ice for 10 min. Rat liver cytosol and GTPyS were then added, and the reactions were shifted to 32 °C and incubated for 12 min. To terminate the reaction, the membranes were collected by centrifugation, and the membrane pellet was separated by SDS-PAGE and then transferred to nitrocellulose. The blot was probed with an anti-Src monoclonal antibody (GD11) and then developed and quantitated as described under “Experimental Procedures.” cont, control. B, the membrane binding assay was performed as above. The immunoblot was probed with either a monoclonal antibody to aPKC/λ or a monoclonal antibody to GAPDH. A representative Western blot (WB) with each antibody is shown in panels **A** and **B**. The results are the mean ± S.D. of three independent experiments performed in duplicate.
study, Src was found present in the immune complex with Rab2, GAPDH, and aPKCα/β, whereas no interaction of these four proteins was detected with preimmune serum as assessed by Western blot analysis (Fig. 3B).

To further explore the potential interaction of Src with Rab2-aPKCα/β and GAPDH, a His6/GST pull-down experiment was performed. The bacterial produced His6/GST-tagged fusion proteins shown in Fig. 3C were prebound to Ni2⁺-NTA-agarose or glutathione-Sepharose 4B and then incubated with purified Src. As expected based on a previous report, Src bound to aPKCα/β (20). Interestingly, Src also interacted with GAPDH. In contrast, Src did not bind directly to Rab2. However, GST-Rab2 binds Src when co-incubated with His6-aPKCα/β and rabbit muscle GAPDH (Fig. 3C). The interaction of Src with Rab2-aPKCα/β-GAPDH is specific since there is no binding to Rab1. Like Rab2, Rab1 is required for membrane trafficking in the early secretory pathway (4). These combined results indicate that Src indirectly interacts with Rab2 through co-association with aPKCα/β and GAPDH.

Src-dependent aPKCα/β Tyrosine Phosphorylation Is Required for aPKCα/β Membrane Association—A variety of cellular proteins have been reported to be Src kinase substrates (26). In fact, PC12 cells treated with nerve growth factor results in Src-mediated tyrosine phosphorylation of aPKCs (20). The ability of Src to phosphorylate purified recombinant His6-aPKCα/β was confirmed in an in vitro kinase assay (Fig. 4A). As we anticipated, His6-aPKCα/β was efficiently tyrosine phosphorylated by Src, whereas the Src-dependent activity was inhibited when the assay was supplemented with the Src-specific tyrosine kinase inhibitor PP2 (Fig. 4A). In contrast, the inactive form and negative control for Src tyrosine kinase inhibitor PP3 had no effect on Src-aPKCα/β phosphorylation.

After learning that His6-aPKCα/β was a Src substrate, and therefore, the His tag did not influence Src modification of the recombinant protein, we then determined whether the membrane-associated His6-aPKCα/β recruited in response to Rab2 in the binding assay was tyrosine phosphorylated. Rab2 promoted His6-aPKCα/β recruitment to membrane, and the membrane-bound His6-aPKCα/β was tyrosine phosphorylated as determined by probing the Western blot with a phosphotyrosine-specific monoclonal antibody (Fig. 4B). A strong chemiluminescent signal was observed with the anti-phosphotyrosine antibody that aligned with anti-His, aPKCα/β. However, when the membrane binding assay was performed in the presence of 1.0 μM PP2, Rab2-stimulated His6-aPKCα/β recruitment to membranes was reduced by ~50%, whereas no effect was obtained in the presence of PP3 (Fig. 5A). In contrast, PP2 addition to the assay had no effect on Rab2-stimulated Src and GAPDH membrane binding (Fig. 5A). Because aPKCα/β is required for Rab2-mediated downstream β-COP recruitment to VTCs, the Western blot was probed with an affinity-purified polyclonal antibody to β-COP to assess the effect of PP2 on COPII binding. Fig. 5A shows that PP2 treatment caused a concomitant decrease in β-COP membrane association.
Tyrosine Phosphorylated aPKC/λ Interacts with Rab2

**FIGURE 4. Rab2-recruited aPKC/λ is tyrosine phosphorylated on membranes by Src.** A, Src (0.1 μg) was incubated with His6-aPKC/λ (0.5 μg) in 50 mM Tris, pH 7.4, 10 mM MgCl2, 3 mM MnCl2, 50 μM ATP, and 10 μM γ-32P]ATP for 15 min at 30 °C in the absence or presence of 5 μM PP2 or 5 μM PP3. The reaction was stopped by the addition of 5× sample buffer and then separated by SDS-PAGE followed by autoradiography. cont, control. B, salt-washed NRK microsomes prepared as described under “Experimental Procedures” were preincubated with purified recombinant Rab2 at the concentrations indicated under “Results,” and the reaction mix was incubated on ice for 10 min. Rat liver cytosol and GTP-γ-S were then added, and the reactions were shifted to 37 °C and incubated for 12 min. To terminate the reaction, the membranes were collected by centrifugation, and the membrane pellet was separated by SDS-PAGE and transferred to nitrocellulose. The blot was probed with an anti-phosphotyrosine monoclonal antibody (P-Tyr-100) and then developed as described under “Experimental Procedures.” The blot was then stripped and reprobed with a monoclonal antibody to the His-tag and then processed and quantitated as described under “Experimental Procedures.” A representative Western blot is shown. The results are the mean ± S.D. of three independent experiments performed in duplicate.

(Fig. 5A). Since these results were highly suggestive that tyrosine phosphorylation of aPKC/λ was necessary to promote interaction with Rab2-GAPDH-Src in vitro, we assessed whether Src-dependent aPKC/λ phosphorylation facilitated interaction with Rab2-GAPDH-Src in vivo. NRK cells were incubated with either 10 μM PP3 or 10 μM PP2 to inhibit endogenous Src kinase activity. The detergent-lysed cells of these treated cells was subjected to immunoprecipitation with affinity-purified anti-Rab2 polyclonal antibody and then analyzed by SDS-PAGE and Western blot. Analysis of the anti-Rab2 co-immunoprecipitated proteins from cells incubated with PP3 show the presence of Rab2, Src, GAPDH, and aPKC/λ. In contrast, the immune complex obtained from PP2-treated cells contained Rab2, Src, and GAPDH but was devoid of aPKC/λ. These in vivo results are supportive of the in vitro data indicating that tyrosine phosphorylation of aPKC/λ facilitates interaction with Rab2 and Rab2 effectors.

The decrease in membrane-bound aPKC/λ and β-COP when the binding assay is supplemented with PP2 was reflected in the degree of vesicle release from VTCs. In this case, the membrane binding assay was supplemented with 300 ng of Rab2, a concentration that effectively stimulates retrograde vesicle formation in the absence or presence of PP2 (11). The membranes (P1) were collected after the reaction by centrifugation at 35,000 × g. and the supernatant was recenterfuged at 100,000 × g to recover any released vesicles (P2). Western blot analysis of P1 and P2 obtained from the assay showed that Rab2 promoted the release of vesicles containing β-COP, whereas membranes incubated with PP2 resulted in a ~76% decrease of the total β-COP signal in the vesicle fraction. It appears that Src activity at the VTC is essential for Rab2-dependent vesicle formation.

**DISCUSSION**

The traditional view that diacylglycerol and phosphatidyserine regulate PKC has been modified over the years due to numerous observations that demonstrate that PKC also associates with inositol phospholipids and with a variety of proteins. These interactions with phospholipids and proteins are necessary for the selective regulation of the different PKC isozymes. In that regard, the nonreceptor tyrosine kinase Src was shown to interact directly with aPKC, and this interaction resulted in tyrosine phosphorylation of multiple residues within the aPKC catalytic and regulatory domains (20, 27). Like PKC, the Rab proteins associate with multiple effectors and accessory proteins to regulate compartment-specific transport events (1, 3). aPKC/λ is a specific downstream effector for Rab2, and Rab2 requires aPKC/λ kinase activity to promote retrograde vesicle formation from a VTC subcompartment (9). Since Rab2 directly associates with aPKC/λ and aPKC binds to Src, it seemed reasonable to investigate whether Rab2 function at the VTC required Src tyrosine kinase activity. Indeed, we found that Rab2-stimulated Src recruitment to membranes in a quantitative binding assay and that the recruitment of Src as well as aPKC/λ and GAPDH could be inhibited by a Rab2-specific antibody. This affinity-purified anti-Rab2 antibody has proved to be a powerful agent to dissect Rab2 function in the early secretory pathway; that is, the polyclonal antibody rapidly and specifically blocks ER to Golgi transport and immunoprecpitates Rab2 and associated molecules. To that end, we are currently pursuing the identification of additional polyepitides that co-precipitate with Rab2 by employing this reagent.

Src does not directly bind to Rab2 but associates with aPKC/λ and GAPDH that interact with the Rab2 amino terminus. Both Src and GAPDH bind to the aPKC/λ regulatory domain (13, 20). The interaction between GAPDH and aPKC/λ can be partly blocked in the presence of a peptide made to the unique aPKC/λ pseudosubstrate region (13). A similar decrease in membrane-associated Src is observed when the binding assay is supplemented with the aPKC/λ pseudosubstrate peptide (data not shown). The pseudosubstrate domain is downstream but adjacent to the aPKC/λ proline-rich motif located within residues 98–114 that binds to the Src-SH3 domain. Our results indicated that the interaction of Src with aPKC/λ is not critical for Src association with Rab2 but that GAPDH interaction is necessary to stabilize Src association with Rab2 effectors on the VTC. The precise role of GAPDH in the early secretory pathway is unknown. Intriguingly, GAPDH catalytic activity is not required, and therefore, the enzyme is providing an activity necessary for membrane trafficking that is independent of ATP production, which could have been utilized by both Src and aPKC/λ. In contrast, aPKC/λ requires Src-dependent tyrosine phosphorylation for the kinase to associate with Rab2-Src-GAPDH in vitro and in vivo. The finding that the Src-specific tyrosine kinase inhibitor PP2 influences aPKC/λ membrane association but not Rab2, Src, or GAPDH membrane association supports this interpretation.

It has been proposed that the biological role of tyrosine phosphorylation is to promote protein-protein interaction (28). Indeed, it has been reported that Src-dependent aPKC tyrosine phosphorylation is required for aPKC association with the nerve growth factor receptor TrkA in PC12 cells (20). The fact that Src phosphorylates multiple residues in aPKC suggests that site-specific phosphorylation in aPKC may regulate temporal and spatial interaction with Rab2 and Rab2 effectors as well as other unique binding proteins including λ-interacting protein (LIP) (18), Par 4 (29), γ-interacting protein (ZIP/p62) (19, 29), and cell polarity protein Par 6 (28).

The involvement of Src in regulation of membrane traffic has been confirmed by studies indicating that the adaptor protein Cbl that func-
Tyrosine phosphorylated aPKC/λ interacts with Rab2

Tyrosine phosphorylation of aPKC/λ is required for membrane association. A, salt-washed NRK microsomes prepared as described under “Experimental Procedures” were incubated for 12 min at 37 °C with 100 ng of purified recombinant Rab2, rat liver cytosol, and GTP-γ-S in the absence or presence of 1.0 μM or 5.0 μM PP2. To terminate the reaction, the membranes were collected by centrifugation, and the membrane pellet was separated by SDS-PAGE and transferred to nitrocellulose. The blot was probed with the indicated primary antibodies and then developed and quantitated as described under “Results.” The results are the mean ± S.D. of three independent experiments performed in duplicate. cont, control. B, NRK cells were incubated for 12 h in the absence or presence of 10 μM PP2 or 10 μM PP3. The detergent cell lysate was incubated with an affinity-purified anti-Rab2 polyclonal antibody and protein G plus/protein A-agarose for 12 h at 4 °C. The immune complexes were collected by centrifugation, washed, and analyzed as described under “Experimental Procedures.” The blot was probed with the indicated primary antibodies, washed, incubated with HRP-conjugated secondary antibodies, and then developed with ECL. C, salt-washed NRK microsomes prepared as described under “Experimental Procedures” were incubated for 12 min at 32 °C with 100 ng of purified recombinant Rab2, rat liver cytosol, and GTP-γ-S in the absence or presence of 5 μM PP2 or 5 μM PP3. The reaction mix was centrifuged at 35,000 × g for 20 min to obtain a pellet (P1). The supernatant was recentrifuged at 100,000 × g for 60 min, and the resulting pellet (P2) and P1 were separated by SDS-PAGE and immunoblotted with anti-β-COP. The results are representative of three independent experiments.

Tyrosine phosphorylated aPKC/λ interacts with Rab2. It has also been reported that Rab24 is phosphorylated by Src-related tyrosine kinases and that the modification might influence Rab24 interactions with effector molecules (32). Our data showed that Src-mediated tyrosine phosphorylation of aPKC/λ is a key signal in maintaining aPKC/λ on the VTC in association with Rab2 and Rab2 effectors and for ensuring the downstream recruitment of β-COP. Indeed, we found...
that supplementing the binding assay with the Src-specific inhibitor PP2 significantly inhibited Rab2-mediated vesicle formation.

It may not be coincidental that three effectors we have identified for Rab2 influence microtubule dynamics since recent studies indicate that the cytoskeleton plays a central role in the organization and operation of the secretory pathway by providing tracks to direct vesicle movement. We propose that Rab2-Src-aPKC/\lambda-GAPDH are components of a signaling complex that defines and regulates the site of vesicle budding from VTCs in association with microtubule-directed vesicle movement to the appropriate acceptor compartment. Studies are in progress to validate this hypothesis.

REFERENCES
