Rab2 Interacts Directly with Atypical Protein Kinase C (aPKC) \( \delta/\lambda \) and Inhibits aPKC/\( \delta/\lambda \)-dependent Glyceraldehyde-3-phosphate Dehydrogenase Phosphorylation*

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Atypical protein kinase C \( \delta/\lambda \) (PKC/\( \delta/\lambda \)) is essential for protein transport in the early secretory pathway. The small GTPase Rab2 selectively recruits the kinase to vesicular tubular clusters (VTCs) where PKC/\( \delta/\lambda \) phosphorylates glyceraldehyde-3-phosphate dehydrogenase (GAPDH). VTCs are composed of small vesicles and tubules and serve as transport intermediates that shuttle cargo from the endoplasmic reticulum to the Golgi complex. These structures are the first site of segregation of the anterograde and retrograde pathways. When Rab2 binds to a VTC subcompartment, the subsequent recruitment of PKC/\( \delta/\lambda \) and soluble components, including COPI (coatomer and ADP-ribosylation factor), results in the release of retrograde-directed vesicles. Because Rab2 stimulates PKC/\( \delta/\lambda \) membrane association in a dose-dependent manner, we investigated whether the two proteins physically interact. Using a combination of \textit{in vivo} and \textit{in vitro} assays, we found that Rab2 interacts directly with PKC/\( \delta/\lambda \) and that this interaction occurs through the Rab2 amino terminus (residues 1–19) and the PKC/\( \delta/\lambda \) regulatory domain. A mutant lacking the PKC/\( \delta/\lambda \) binding domain (Rab2N\( \Delta 19 \)) was functionally characterized. In contrast to Rab2, Rab2N\( \Delta 19 \) failed to recruit PKC/\( \delta/\lambda \) to normal rat kidney microsomes in a quantitative binding assay. To determine whether Rab2 modulates the ability of PKC/\( \delta/\lambda \) to phosphorylate GAPDH, an \textit{in vitro} kinase assay was supplemented with Rab2 or Rab2N\( \Delta 19 \). Rab2 inhibited PKC/\( \delta/\lambda \)-dependent GAPDH phosphorylation, whereas no effect was observed when the assay was performed with the amino-terminal truncation mutant. These results suggest that a downstream effector recruited to the VTC stimulates PKC/\( \delta/\lambda \)-mediated GAPDH phosphorylation by alleviating the inhibition imposed by Rab2-PKC/\( \delta/\lambda \) interaction.

The Rab family of small GTPases regulates membrane traffic in the exocytic and endocytic pathways (1, 2). These essential proteins alternate between a GTP- and GDP-bound conformation, which catalyzes a cycle of membrane association and release to the cytosol. Rab proteins bind to specific intracellular compartments and promote recruitment of soluble components that facilitate membrane fusion, cytoskeletal interaction, and vesicle tethering/docking (2–4). Multiple sequence alignments indicate that the amino- and carboxyl-terminal regions of Rab proteins are highly divergent and therefore potential sites for protein-protein interaction with unique effectors that regulate compartment-specific transport events. In that regard, the amino terminus of Rab5 was found to play a critical role in Rab5-dependent early endosome fusion (5, 6), whereas residues 19–22 in Rab3A are required for interaction with the effector Rabphilin-3A (7). The amino terminus of Rab2 is also required for function: Deletion of residues 1–14 attenuated the inhibitory properties of the Rab2 trans dominant mutant (N119I) (8). Moreover, a peptide corresponding to the deleted amino acids (residues 2–14) was a potent and irreversible inhibitor of ER\(^2\) to Golgi traffic when introduced into an \textit{in vitro} transport assay (8). Studies were initiated to elucidate the mechanism by which the Rab2 (13-mer) arrests transport and included employing a quantitative binding assay that measures recruitment of transport-related proteins to membranes incubated with the peptide. The Rab2 (13-mer) markedly stimulated recruitment of COPI (coatomer and ADP-ribosylation factor) to normal rat kidney (NRK) cell microsomes in a protein kinase C (PKC)-dependent manner (9, 10). We have since identified the participating isoform as PKC/\( \delta/\lambda \), a member of the atypical subgroup of PKC (11). This subfamily of kinases plays a critical role in controlling cell growth by interacting with proteins that ultimately link Cdc42-coordinated signaling cascades with cytoskeletal rearrangement and vesicle transport (12–15). Interestingly, the Rab2 (13-mer) not only stimulates COPI membrane association but also promotes membrane binding of PKC/\( \delta/\lambda \) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (10, 11). These combined results suggest that the Rab2 amino terminus is necessary for Rab2 activity and that the Rab2 (13-mer) functions as a \textit{bona fide} Rab2 domain that interacts with one or more components of the trafficking machinery. Rab2 is required for membrane transport in the early secretory pathway. This protein immunolocalizes to vesicular tubular clusters (VTCs) that function as transport intermediates between the endoplasmic reticulum and the Golgi complex (16, 17). These structures are the first site of segregation of the anterograde and retrograde pathways and thereby sort and recycle resident proteins from itinerate proteins destined for secretion (18, 19). In our ongoing studies, we found that Rab2 requires PKC/\( \delta/\lambda \) kinase activity to promote retrograde vesicle

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1 The abbreviations used are: ER, endoplasmic reticulum; VTC, vesicular tubular cluster; PKC, protein kinase C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NRK, normal rat kidney; FBS, phos- phate-buffered saline; GTP\( \gamma \S\), guanosine 5\'-\( \gamma \)-thiotriphosphate; \( \beta \)-COP, \( \beta \)-coat protein; CAT, chloramphenicol acetyltransferase; TBS, Tris-buffered saline; CDR, complementarity-determining region; HRP, horseradish peroxidase; COPI, coatomer and ADP-ribosylation factor; BSA, bovine serum albumin; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay; RT, room temperature; aPKC, atypical PKC.
Rab2 Amino Terminus Binds to the PKC\(/\alpha\) Regulatory Domain

PCR using a 5'-GAGTCGTTGTTGCGACACCGTGCGCCGC, in tandem with the 3'-antisense oligonucleotide, 5'-GGCATTCTCTCAAAAATCTAGAAACCC- TAG, and then cloned in-frame to the BamHI and EcoRI sites of pGEX-2T. The PKC\(/\alpha\) catalytic domain was generated by PCR using a 5'- primer that included a BamHI site, 5'-GGGAGATGCTTCTAGAGCATGCGCAAC, and then cloned in-frame to the BamHI and EcoRI sites of pGEX-2T.

In this study, we examined that the PKC\(/\alpha\) regulatory domain is not only binds to GAPDH, but also directly interacts with Rab2 via the Rab2 amino terminus. A Rab2 truncation mutant missing the first 19 residues was unable to bind the kinase. Unlike intact Rab2, Rab2\(N\Delta 19\) failed to recruit PKC\(/\alpha\) and GAPDH to NRK microsomes in a quantitative binding assay. Because PKC-binding proteins are potential regulators of kinase activity, we determined whether Rab2 modulates PKC\(/\alpha\) enzymatic activity, in vitro. Rab2 caused a dramatic reduction of PKC\(/\alpha\)-mediated GAPDH phosphorylation. A similar reduction in phosphorylation activity occurred in the presence of the Rab2\(N\Delta 19\) mutant. However, PKC\(/\alpha\)-dependent phosphorylation of GAPDH was restored when the in vitro kinase assay was performed in the presence of Rab2\(N\Delta 19\). We propose that Rab2 directly interacts with PKC\(/\alpha\) and inhibits PKC\(/\alpha\) kinase activity at the VTC. The subsequent recruitment of one or more Rab2 downstream effectors relieves the negative regulation imposed by Rab2 and stimulates PKC\(/\alpha\) to phosphorylate VTC-associated GAPDH and promote microtubule nucleation.

EXPERIMENTAL PROCEDURES

Materials and Methods—Rab2 amino-terminal peptides were synthesized at the University of Michigan Protein and Carbohydrate Structure Facility (Ann Arbor, MI). The cDNA to PKC\(/\alpha\) was a gift from Dr. Trevor Biden (Garvan Institute of Medical Research, Sydney, Australia). The mammalian Matchmaker two-hybrid assay kit was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Horseradish peroxidase- and alkaline phosphatase-conjugated secondary antibodies were purchased from Pierce (Rockford, IL).

Immunoprecipitation—HeLa cells (3 \times 10\(^6\)) were lysed in 50 mM Tris-buffered saline (TBS) (pH 8.0) and 1% Triton X-100 for 10 min at 4°C. The soluble fraction was preclared with Protein G plus/Protein A agarose (Novagen, Madison, WI) and subjected to immunoprecipitation for 4 h at RT with an affinity-purified anti-Rab2 polyclonal antibody or with preimmune serum and Protein G plus/Protein A-agarose. The nonimmune complexes were collected by centrifugation at 5,000 rpm for 5 min, washed, and then incubated with horseradish peroxidase-labeled antibody. The blots were blocked in TBS that contained 5% BSA and 0.5% Tween 20, incubated with an anti-Rab2 polyclonal antibody and a monoclonal antibody to PKC\(/\alpha\) (BD Biosciences, San Diego, CA), washed, further incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit or an anti-mouse antibody and then developed with enhanced chemiluminescence (ECL) (Amersham Biosciences, Piscataway, NJ).

Mammalian Two-hybrid Assay—Human Rab2 cDNA was cloned in-frame to the EcoRI site of the GAL4 DNA-binding domain in the pM vector and PKC\(/\alpha\) cDNA cloned in-frame to the EcoRI site of the activation domain in pVP16 (Clontech Laboratories, Inc.). The two constructs (5 \mu g each) were co-transfected with the reporter vector pGAT (5 \mu g) that contains the chloramphenicol transferase (CAT) gene into HeLa cells (10\(^6\)) using a calcium phosphate transfection protocol (23). Control cells were transfected with a combination of vectors as described under “Results.” The cells were collected 72 h post-transfection, lysed in sample buffer, separated by SDSPAGE, and then transferred to nitrocellulose. The blot was blocked in TBS that contained 5% BSA and 0.5% Tween 20, incubated with an anti-CAT polyclonal antibody (Invitrogen), washed, further incubated with an HRP-conjugated secondary antibody, and then developed with ECL (Amersham Biosciences).

GST-PKC\(/\alpha\) Pull-down—The cDNA encoding PKC\(/\alpha\) was amplified by PCR using a 5'- primer that included a BamHI site and sequence corresponding to PKC\(/\alpha\) (5'-GGGAGATGCTTCTAGAGCATGCGCAAC, in tandem with the 3'-antisense primer that included an EcoRI site (5'-CCGAGATCTGGATCACACAGCTCTTG), and then cloned in-frame to the BamHI and EcoRI sites of pGEX-2T (Amersham Biosciences). The PKC\(/\alpha\) regulatory domain was generated by PCR using a 5'-oligonucleotide primer that contained a BamHI site, 5'-GGTGCGCTGCAAGCGGTCCGCCGC, in tandem with the 3'-antisense primer that included an EcoRI site, 5'-GGGAGATGCTTCTAGAGCATGCGCAAC, and then cloned in-frame to the BamHI and EcoRI sites in pGEX-2T.

Purification of recombinant Rab2 and Rab2\(N\Delta 19\) were generated by PCR using 5'-oligonucleotides that included an NdeI site and sequence complementary to Rab2 that introduced a start codon at the respective deletion site, 38, 5'-GGCATATGATCAGCTGCAGGAGAC, in tandem with the 3'-antisense primer that included an EcoRI site, 5'-GGGAGATGCTTCTAGAGCATGCGCAAC, and then cloned in-frame to the BamHI and EcoRI sites of pGEX-2T. The PKC\(/\alpha\) catalytic domain was generated by PCR using a 5'- primer that included a BamHI site, 5'-GGGAGATGCTTCTAGAGCATGCGCAAC, and then cloned in-frame to the BamHI and EcoRI sites of pGEX-2T. The PKC\(/\alpha\) catalytic domain was generated by PCR using a 5'- primer that included a BamHI site, 5'-GGGAGATGCTTCTAGAGCATGCGCAAC, and then cloned in-frame to the BamHI and EcoRI sites of pGEX-2T.
Rab2 Amino Terminus Binds to the PKCα Regulatory Domain

Rab2 interacts directly with PKCα/λ. A, immunoprecipitation. HeLa cell lysate was incubated with preimmune serum or an affinity-purified anti-Rab2 polyclonal antibody and Protein G plus/Protein A-agarose for 4 h at RT. The immune complexes were collected by centrifugation, washed, and analyzed as described under “Experimental Procedures.” The blot was probed with an anti-PKCα/λ monoclonal antibody and an affinity-purified Rab2 polyclonal antibody, washed, incubated with HRP-conjugated secondary antibodies, and then developed with ECL. B, mammalian two-hybrid assay. The lysates from HeLa cells transiently transfected with α, pg5CAT, pM, and pVP16 (basal control); b, pM-Rab2, pVP16, and pg5CAT (pM control); c, pM, pVP16-PKCα/λ, and pg5CAT (pVP16 control); or d, pVP16-PKCα/λ, pM-Rab2, and pg5CAT (experiment) were separated on SDS-PAGE and transferred to nitrocellulose. The blot was probed with an anti-CAT polyclonal antibody, washed, further incubated with an HRP-conjugated secondary antibody, and then developed with ECL. C, overlay binding assay. Purified recombinant Rab2 (5 μg) was separated by SDS-PAGE and transferred to nitrocellulose. The blot was incubated in 50 mM Tris (pH 7.4), 0.1% BSA, 100 mM GTPγS, 200 mM NaCl, 20 μg/ml phosphatidylerine, and 10 μl/ml purified recombinant PKCα/λ for 4 h at RT, washed, probed with an anti-PKCα/λ monoclonal antibody, washed, incubated with an HRP-conjugated secondary antibody, and then developed with ECL. D, GST-pull-down. GST-PKCα/λ (5 μg) or GST (5 μg) were preincubated with 20 μl of glutathione-Sepharose 4B for 1 h at room temperature and then washed 3× with TBS 1% Triton-X-100 to remove any unbound protein. The beads were resuspended in 50 mM Tris (pH 7.5), 5 mM MgCl₂, 100 mM NaCl, 10 μM GTPγS, or 10 μM GDP, and then 5 μg of purified recombinant Rab2 was added, and the mixture was incubated for an additional 2 h at RT. The beads were washed 4× with 1 ml of 50 mM Tris (pH 7.5), 10 mM MgCl₂, and 100 mM NaCl, and bound proteins were boiled in sample buffer, separated by SDS-PAGE, and then transferred to nitrocellulose. The blot was probed with an affinity-purified Rab2 polyclonal antibody, washed, incubated with an HRP-conjugated secondary antibody, and then developed with ECL. All experiments were performed a minimum of three times.

RESULTS

Rab2 Interacts Directly with PKCα/λ—Because Rab2 recruits PKCα/λ to membranes in a dose-dependent manner, we used a combination of in vivo and in vitro approaches to determine whether Rab2-PKCα/λ physically associate. First, the detergent lysate from HeLa cells was incubated with preimmune serum or with an affinity-purified anti-Rab2 polyclonal antibody. Western blot analysis of the immune complex showed that Rab2 and PKCα/λ specifically co-immunoprecipitated, whereas no interaction was detected with preimmune serum (Fig. 1A).

To further verify Rab2-PKCα/λ interaction in vivo, we employed a mammalian two-hybrid assay. This system allows the 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 PKCα/λ cDNA that serves as prey was cloned into pVP16 to generate a VP16 activation domain-PKCα/λ 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

![Fig. 1. Rab2 binds directly to PKCα/λ. A, immunoprecipitation. HeLa cell lysate was incubated with preimmune serum or an affinity-purified anti-Rab2 polyclonal antibody and Protein G plus/Protein A-agarose for 4 h at RT. The immune complexes were collected by centrifugation, washed, and analyzed as described under “Experimental Procedures.” The blot was probed with an anti-PKCα/λ monoclonal antibody and an affinity-purified Rab2 polyclonal antibody, washed, incubated with HRP-conjugated secondary antibodies, and then developed with ECL. B, mammalian two-hybrid assay. The lysates from HeLa cells transiently transfected with α, pg5CAT, pM, and pVP16 (basal control); b, pM-Rab2, pVP16, and pg5CAT (pM control); c, pM, pVP16-PKCα/λ, and pg5CAT (pVP16 control); or d, pVP16-PKCα/λ, pM-Rab2, and pg5CAT (experiment) were separated on SDS-PAGE and transferred to nitrocellulose. The blot was probed with an anti-CAT polyclonal antibody, washed, further incubated with an HRP-conjugated secondary antibody, and then developed with ECL. C, overlay binding assay. Purified recombinant Rab2 (5 μg) was separated by SDS-PAGE and transferred to nitrocellulose. The blot was incubated in 50 mM Tris (pH 7.4), 0.1% BSA, 100 mM GTPγS, 200 mM NaCl, 20 μg/ml phosphatidylerine, and 10 μl/ml purified recombinant PKCα/λ for 4 h at RT, washed, probed with an anti-PKCα/λ monoclonal antibody, washed, incubated with an HRP-conjugated secondary antibody, and then developed with ECL. D, GST-pull-down. GST-PKCα/λ (5 μg) or GST (5 μg) were preincubated with 20 μl of glutathione-Sepharose 4B for 1 h at room temperature and then washed 3× with TBS 1% Triton-X-100 to remove any unbound protein. The beads were resuspended in 50 mM Tris (pH 7.5), 5 mM MgCl₂, 100 mM NaCl, 10 μM GTPγS, or 10 μM GDP, and then 5 μg of purified recombinant Rab2 was added, and the mixture was incubated for an additional 2 h at RT. The beads were washed 4× with 1 ml of 50 mM Tris (pH 7.5), 10 mM MgCl₂, and 100 mM NaCl, and bound proteins were boiled in sample buffer, separated by SDS-PAGE, and then transferred to nitrocellulose. The blot was probed with an affinity-purified Rab2 polyclonal antibody, washed, incubated with an HRP-conjugated secondary antibody, and then developed with ECL. All experiments were performed a minimum of three times.]{/Fig. 1. Rab2 binds directly to PKCα/λ. A, immunoprecipitation. HeLa cell lysate was incubated with preimmune serum or an affinity-purified anti-Rab2 polyclonal antibody and Protein G plus/Protein A-agarose for 4 h at RT. The immune complexes were collected by centrifugation, washed, and analyzed as described under “Experimental Procedures.” The blot was probed with an anti-PKCα/λ monoclonal antibody and an affinity-purified Rab2 polyclonal antibody, washed, incubated with HRP-conjugated secondary antibodies, and then developed with ECL. B, mammalian two-hybrid assay. The lysates from HeLa cells transiently transfected with α, pg5CAT, pM, and pVP16 (basal control); b, pM-Rab2, pVP16, and pg5CAT (pM control); c, pM, pVP16-PKCα/λ, and pg5CAT (pVP16 control); or d, pVP16-PKCα/λ, pM-Rab2, and pg5CAT (experiment) were separated on SDS-PAGE and transferred to nitrocellulose. The blot was probed with an anti-CAT polyclonal antibody, washed, further incubated with an HRP-conjugated secondary antibody, and then developed with ECL. C, overlay binding assay. Purified recombinant Rab2 (5 μg) was separated by SDS-PAGE and transferred to nitrocellulose. The blot was incubated in 50 mM Tris (pH 7.4), 0.1% BSA, 100 mM GTPγS, 200 mM NaCl, 20 μg/ml phosphatidylerine, and 10 μl/ml purified recombinant PKCα/λ for 4 h at RT, washed, probed with an anti-PKCα/λ monoclonal antibody, washed, incubated with an HRP-conjugated secondary antibody, and then developed with ECL. D, GST-pull-down. GST-PKCα/λ (5 μg) or GST (5 μg) were preincubated with 20 μl of glutathione-Sepharose 4B for 1 h at room temperature and then washed 3× with TBS 1% Triton-X-100 to remove any unbound protein. The beads were resuspended in 50 mM Tris (pH 7.5), 5 mM MgCl₂, 100 mM NaCl, 10 μM GTPγS, or 10 μM GDP, and then 5 μg of purified recombinant Rab2 was added, and the mixture was incubated for an additional 2 h at RT. The beads were washed 4× with 1 ml of 50 mM Tris (pH 7.5), 10 mM MgCl₂, and 100 mM NaCl, and bound proteins were boiled in sample buffer, separated by SDS-PAGE, and then transferred to nitrocellulose. The blot was probed with an affinity-purified Rab2 polyclonal antibody, washed, incubated with an HRP-conjugated secondary antibody, and then developed with ECL. All experiments were performed a minimum of three times.
the CAT reporter gene is activated. Three days post-transfection, the cells were lysed in sample buffer, and the lysate was separated on SDS-PAGE and immunoblotted with an anti-CAT antibody. The Amino Terminus of Rab2 Is Required to Stimulate PKC\(\alpha\) Recruitment to NRK Microsomes—Based on our previous studies, there was evidence to suggest that the Rab2 amino terminus interacted with PKC\(\alpha\) (11). Because a peptide corresponding to the first 7 amino acids of Rab2 following the initiator methionine (AYAYLFK) had minimal effect on membrane trafficking (8), we reasoned that the PKC\(\alpha\)–initiator methionine (AYAYLFK) had minimal effect on membrane association, addition of Rab2 N\(^{\Delta19}\) at either an equivalent or higher concentration had no effect on PKC\(\alpha\) recruitment to NRK microsomes (Fig. 2A). Based on the previous observation that PKC\(\alpha\) interacts with GAPDH, we determined whether removal of the Rab2 amino terminus also effected GAPDH recruitment to membrane. To address this question, the blot was re-probed with an anti-GAPDH monoclonal antibody. Unlike Rab2 that promoted GAPDH membrane binding, microsomes treated with Rab2N\(^{\Delta19}\) contained GAPDH at a level comparable to the control (Fig. 2A).

We considered two possibilities to account for the lack of PKC\(\alpha\) and GAPDH recruitment by Rab2N\(^{\Delta19}\): 1) the truncated protein could not be in vitro prenylated and therefore unable to associate with membranes and stimulate PKC\(\alpha\) and GAPDH binding and 2) Rab2N\(^{\Delta19}\) had a profound effect on vesicle formation, which would give the appearance that there was no enhanced recruitment by the truncated mutant protein; that is, because the membranes analyzed are recovered at insufficient centrifugal force to pellet release vesicles. To address the first possibility, we determined whether Rab2N\(^{\Delta19}\) was prenylated in both in vitro and in vivo assays. First, the cell lysate from HeLa cells transfected transiently with pCR3.1-Rab2 was partitioned with Triton X-114, and the distribution of endogenous Rab2 and Rab2N\(^{\Delta19}\) in the two phases was analyzed by SDS-PAGE and immunoblotting (26). The endogenous pool of Rab2 is predominantly membrane-associated (~75%) and found in the detergent-rich phase (Fig. 2B). The phase separation of transiently expressed Rab2N\(^{\Delta19}\) showed that ~50% of the mutant distributed with the detergent phase (Fig. 2B). The increase in the distribution of the truncation mutant to the cytosolic pool may simply reflect saturation of the prenylation enzyme (geranylgeranyltransferase II) and limited cofactors required for the modification. Rab2N\(^{\Delta19}\) was further characterized in an in vitro prenylation reaction that employs \(^{3}H\)geranylgeranyl pyrophosphate. Fig. 2C shows that purified recombinant Rab2N\(^{\Delta19}\) can be prenylated as evidenced by the incorporation of radiolabeled lipid. Despite the finding that Rab2N\(^{\Delta19}\) was prenylated in vivo and in vitro, there was the slight possibility that the mutant protein could not bind to membranes used in the binding assay. To address this question, the assay was performed with increasing concentrations of purified recombinant Rab2N\(^{\Delta19}\). The truncated mutant protein bound to membranes in a concentration-dependent manner (Fig. 2D). It appears that Rab2N\(^{\Delta19}\) binds to NRK microsomes and is post-translationally modified at its carboxyl terminus.

We then performed experiments to determine whether the truncated protein could generate vesicles containing \(\beta\)-COP. In this case, the micosomal binding assay was supplemented with 300 ng of Rab2 or Rab2N\(^{\Delta19}\). This concentration of Rab2 effectively stimulates retrograde-vesicle formation (21). The
membranes (P1) from the binding reaction were collected by centrifugation at 20,000 \( \times \) g, and the low speed supernatant was re-centrifuged at 100,000 \( \times \) g to recover any released vesicles (P2). Western blot analysis of P1 and P2 from the binding assay supplemented with Rab2 showed that Rab2 stimulated the release of vesicles containing \( \beta \)-COP (\(-76\%\) of total \( \beta \)-COP signal) (Fig. 2E). In contrast, \(-34\%\) of the total \( \beta \)-COP signal was detected in the P2 fraction from membranes treated with Rab2N\( \Delta 19\). Although Rab2N\( \Delta 19\) associates with NRK microsomes, the truncated mutant does not stimulate vesicle formation. Therefore, the failure of Rab2N\( \Delta 19\) to recruit PKC\( \alpha /\lambda \) and GAPDH to NRK microsomes is most likely due to missing residues in the Rab2 amino terminus that interact with the kinase.
Rab2 Amino Terminus Binds to the PKCα/λ Regulatory Domain

Fig. 3. Rab2 binds to the regulatory domain of PKCα/λ and inhibits kinase activity. A, the PKCα/λ regulatory domain (residues 1–147) and the catalytic domain (residues 248–588) were expressed as GST-tagged fusion proteins and immobilized on glutathione-Sepharose 4B. Purified recombinant Rab2 (5 µg) in 50 mM Tris (pH 7.5), 5 mM MgCl₂, 100 mM NaCl, 10 µM GTPyS was added, and the mixture was incubated for an additional 2 h at RT. The beads were washed 4× with 1 ml of 50 mM Tris (pH 7.5), 10 mM MgCl₂, and 100 mM NaCl, boiled in sample buffer, separated by SDS-PAGE, and then transferred to nitrocellulose. The blot was probed with an affinity-purified Rab2 polyclonal antibody, washed, incubated with an HRP-conjugated secondary antibody, and then developed with ECL. Shown are representative results of three independent experiments. B, rabbit muscle GAPDH (1 µg) was incubated with purified PKCα/λ in the absence or presence of 50 ng of purified recombinant Rab2, or 75 µM Rab2 (13-mer), or 50 ng of purified recombinant Rab2NΔ19 in a kinase buffer supplemented with phosphatidyserine (100 µg/ml as sonicated vesicles) and 10 µCi of [γ-32P]ATP, and then incubated for 20 min at 32 °C. Reactions were separated by SDS-PAGE and the gel processed for autoradiography. Phospho-GAPDH was quantified by using a PhosphorImager. The results are the mean ± S.D. of five independent experiments.

binding domain in PKCα/λ we created by PCR two GST-PKCα fusion proteins that encode for the regulatory (reg) domain (residues 1–245) and the catalytic (cat) domain (residues 246–587). These two fragments were screened for interaction with Rab2 by GST-pull down experiments. As we anticipated, Rab2 bound to the PKCα/λ regulatory domain (Fig. 3A). To date, all proteins that have been reported to bind to aPKCs do so via this domain. Because PKC-interacting proteins can serve as substrates and or regulate kinase activity (30), we determined whether Rab2 had any effect on PKCα/λ-mediated GAPDH phosphorylation. Although Rab2 interacts directly with PKCα/λ, we knew from our earlier studies that Rab2 is not a binding protein mediate PKC localization by placing the isozyme in proximity of its substrate, we performed in vivo and in vitro assays to demonstrate that PKCα/λ interacted directly with Rab2-GTP. PKCα/λ binding to Rab2 would explain why this particular isozyme was recruited to the VTC and would ensure that a signaling molecule is associated with Rab2 to regulate a transport-related event through phosphorylation.

To map the PKCα/λ binding domain in Rab2, we focused our attention on the Rab2 amino terminus based on the previous observations that: 1) the Rab2 (13-mer) stimulated PKCα/λ membrane association; 2) truncation of the Rab2 (N119I) amino terminus attenuated its inhibitory property; and 3) the amino terminus of several Rab proteins is required for their function and interaction with effector molecules. When a family of Rab2 peptides and Rab2 amino-terminal truncation proteins were evaluated for their ability to bind PKCα/λ, only reagents containing residues between 1 and 19 interacted with the kinase. Most likely, the PKCα/λ binding domain in Rab2 resides within those amino acids. Interestingly, this segment includes one of the five Rab-specific sequence motifs that are predicted to mediate interaction with different effectors (7, 31).

The requirement for the Rab2 amino terminus to promote interaction with specific components involved in ER to Golgi transport is in agreement with results reported for Rab5 in which its amino terminus was essential for early endosome fusion (5, 6). Recently, Li and Liang (32) have shown that the Rab5 phosphate-binding loop (P-loop, residues 24–36) is necessary for interaction with specific factors on endosomal membranes. The P-loop also contains a Rab complementarity-determining region (CDR). The combination of the variable Rab CDR and the conserved switch mechanism enables Rab proteins to interact with a wide variety of effectors in a specific and activation state-dependent manner (7). For example, Rabphilin-3A interacts with residues 19–22 of the Rab3A CDR. Deletion of those residues abolishes binding to Rabphilin-3A (7). It is also noteworthy that the Rabphilin-3A effector domain possesses two conserved zinc binding motifs and that the cysteines are sensitive to mutation. Similar motifs are also found in other Rab effectors, such as Rim and early-endosomal autoantigen 1 (33, 34). Likewise, PKCα/λ contains a zinc finger.

To determine the Rab2 binding site in the kinase, we generated two GST-PKCα fusion proteins that encode for the regulatory and catalytic domain. We expected that Rab2 would bind to the PKCα/λ regulatory domain based on the observations reported for other proteins that interact with the aPKCs. This part of the kinase not only contains motifs involved in 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 PKCα/λ (35, 36). Additionally, Ras binds to the PKCα regulatory domain (37). Because these proteins regulate aPKC kinase activity,
we evaluated the effect of Rab2 on PKC/α-mediated GAPDH phosphorylation.

Contrary to our prediction that Rab2-PKC/α interaction would promote GAPDH phosphorylation, no phosphorylated product was detected. The inhibition was reversed when the assay was performed in the presence of Rab2N, which lacks the PKC binding domain. This finding implies that the interaction between the Rab2 amino terminus and the PKC/α regulatory domain modulates enzyme activity by either directly interfering with kinase function or blocking access to GAPDH. Because GAPDH is phosphorylated by PKC directly interfering with kinase function or blocking access to GAPDH. Instead, phospho-GAPDH plays an essential role in promoting MT nucleation at the VTC. Studies are in progress to identify the downstream regulator of PKC/α kinase activity.

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