Atypical Protein Kinase C Plays a Critical Role in Protein Transport from Pre-Golgi Intermediates*

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The small GTPase Rab2 requires atypical protein kinase C η/α (PKC η/α) kinase activity to promote vesicle budding from normal rat kidney cell microsomes (Tisdale, E. J. (2000) Traffic 1, 702–712). The released vesicles lack anterograde-directed cargo but contain coat protein I (COP1) and the recycling protein p53/p58, suggesting that the vesicles traffic in the retrograde pathway. In this study, we have directly characterized the role of PKC η/α in the early secretory pathway. A peptide corresponding to the unique PKC η/α pseudosubstrate domain was introduced into an in vitro assay that efficiently reconstitutes transport of vesicular stomatitis virus glycoprotein from the endoplasmic reticulum to the cis-medial Golgi compartments. This peptide blocked transport in a dose-dependent manner. Moreover, normal rat kidney cells incubated with Rab2 and the pseudosubstrate peptide displayed abundant swollen or dilated vesicles that contained Rab2, PKC η/α, β-COP, and p53/p58. Because Rab2, β-COP, and p53/p58 are markers proteins for pre-Golgi intermediates (vesicular tubular clusters, VTCs), most probably the swollen vesicles are derived from VTCs. Similar results were obtained when the assays were supplemented with kinase-dead PKC η/α (W274K). Both the pseudosubstrate peptide and kinase-dead PKC η/α in tandem with Rab2 caused sustained membrane association of PKC η/α, suggesting that reverse translocation was inhibited. Importantly, the inhibitory phenotype of kinase-dead PKC η/α was reversed by PKC η/α wild type. These combined results indicate that PKC η/α is essential for protein transport in the early secretory pathway and suggest that PKC η/α kinase activity is required to promote Rab2-mediated vesicle budding at a VTC subcompartment enriched in recycling cargo.

Protein kinase C (PKC) is a family of serine/threonine kinases that are classified into three major subfamilies: classical, novel, and atypical (1). The different isoenzymes probably possess unique functional properties since activation results in their translocation from the cytosol to distinct intracellular sites including the endoplasmic reticulum (ER) and the Golgi complex (2–4). PKC association with these organelles suggests that the kinase is a potential regulator of membrane traffic. In that regard, we have recently reported that PKC η/α is essential for Rab2-stimulated β-COP protein (β-COP) recruitment to membranes (5). Furthermore, Rab2 required PKC η/α kinase activity to generate vesicles enriched in PKC η/α, β-COP, glyceroldehyde-3-phosphate dehydrogenase (GAPDH), and p53/p58 from normal rat kidney cell (NRK) microsomes (5).

PKC η/α is a member of the atypical subgroup of PKC that includes PKCζ. Unlike the other PKC isoforms, the atypical members do not contain the calcium binding domain and possess only one cysteine-rich region. The atypical subgroups of PKC are activated by the lipid mediator phosphatidylinositol 3,4,5-triphosphate and phosphatidic acid as well as by protein-protein interactions that direct the PKC in proximity of its cognate substrate (6). These specific interactions are necessary for the selective regulation of the different PKC isoforms. This subfamily of kinases plays a critical role in controlling cell growth and cell survival. The atypical subgroup of PKC binds proteins, which function as scaffolds that ultimately link signaling cascades with cytoskeletal rearrangement (7–9).

Since it was important to determine the PKC η/α substrate(s) that potentiated Rab2 activity in vesicle formation, studies were initiated to identify the phosphorylated protein(s). We found that GAPDH was efficiently phosphorylated by PKC η/α and that GAPDH interacts directly with PKC η/α through its regulatory domain (10). PKC η/α-mediated phosphorylation of GAPDH is prevented by the peptide encoding for the unique pseudosubstrate region of PKC η/α (10). The pseudosubstrate or autoinhibitory domain is present in the regulatory domain of all of the PKC isoforms and maintains phosphoinositide-dependent kinase 1-phosphorylated and autophosphorylated cytosolic PKC in an inactive conformation by blocking the protein-substrate binding site (11). Translocation of this catalytically competent but inactive PKC to membrane results in the removal of the pseudosubstrate domain, allowing substrate access to the active site and its subsequent phosphorylation. The same consequence of kinase inactivation occurs in the presence of peptidomimetics of the pseudosubstrate sequence. In fact, pseudosubstrate peptides have been used successfully to determine the substrate specificity of PKC as well as to define the biological response of the phosphorylated substrate (12–14). Unlike most PKC inhibitors that interfere with the function of multiple kinases, these peptides have the advantage of specifically interfering with the activity of a particular PKC isozyme because of the fact that the pseudosubstrate amino acid sequence differs considerably among the subfamilies (15).

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§ The abbreviations used are: PKC, protein kinase C; ζ/α, endoplasmic reticulum; VTCs, vesicular tubular clusters; GAPDH, glyceroldehyde-3-phosphate dehydrogenase; NRK, normal rat kidney; PBS, phosphate-buffered saline; GTPγS, guanosine 5'-O-(thiotriphosphate); β-COP, β-cot protein; Endo H, endoglycosidase H; VSV-G, vesicular stomatitis virus G protein; ts, temperature-sensitive; ANOVA, analysis of variance.

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In this study, we directly characterized the role of PKC/α in the early secretory pathway by introducing a synthetic peptide corresponding to the PKC/α pseudosubstrate region into a battery of biochemical and morphological assays. We found that this peptide was a potent inhibitor of VSV-G transport from the ER to the Golgi complex. Permeabilized NRK cells treated with Rab2 and the PKC/α pseudosubstrate peptide accumulated dilated vesicular structures that labeled with an antibody to PKC/α, Rab2, p53/p58, and β-COP but contained a negligible amount of VSV-G. Based on our previous findings, this labeling profile is highly suggestive that the swollen vesicular structures are a Rab2-regulated VTC subcompartment. A quantitative microsomal binding assay indicated that membranes incubated with Rab2 and the pseudosubstrate peptide showed a constant level of membrane-associated PKC/α, indicating that reverse translocation of the kinase was delayed. Because the pseudosubstrate peptide inhibits PKC/α-dependent phosphorylation and Rab2-mediated retrograde-vesicle formation and therefore mimics the effect of kinase-dead PKC/α (W274K), we characterized the PKC/α mutant in the transport and morphological assay. The kinase-dead mutant blocked VSV-G transport to the Golgi complex. Furthermore, permeabilized NRK cells incubated with the PKC/α mutant contained vesicles similar to those observed in cells treated with the pseudosubstrate peptide. Importantly, PKC/α wild type markedly reversed the inhibition in VSV-G transport and the phenotype produced by kinase-dead PKC/α.

We propose that Rab2 stimulates PKC/α recruitment to a VTC subcompartment. The PKC/α pseudosubstrate peptide causes the sustained binding of PKC/α to pre-Golgi intermediates by preventing reverse translocation that requires functional PKC/α kinase activity (16, 17). The VTC subcompartment becomes “swollen” because Rab2-mediated retrograde-vesicle release is blocked in the absence of kinase activity by PKC/α (K274W) or inhibition by the pseudosubstrate peptide. This causes VTCs to be stalled in their forward movement to the Golgi complex because of the inability to recycle proteins to the ER and transfer anterograde-directed cargo to the late compartments of the secretory pathway.

**EXPERIMENTAL PROCEDURES**

**Materials and Methods**—PKC/α pseudosubstrate peptide (SIYRGR-ARWRWKLYCAN), scrambled PKC/α peptide (WRICGNKLRRYY-SAR), PKCα pseudosubstrate peptide (RFARKGALRQKNVHEVKD), and PKCγ pseudosubstrate peptide (LFCRGKHALQKVHEVKD) were synthesized at the University of Michigan Protein and Carbohydrate Structure facility (Ann Arbor, MI). The cDNA to PKC/α was a gift from Dr. Trevor Biden (Garvan Institute of Medical Research, Sydney, Australia). Dr. Alan Tartakoff (Case Western Reserve University, Cleveland, OH) provided the monoclonal antibody (6F4C5) to the Golgi structure facility (Ann Arbor, MI). The cDNA to PKC/α, Rab2, and PKCγ were cloned into the pGEX-4T-1 vector (GE Healthcare) and expressed in BL21 (DE3) competent E. coli cells. Recombinant proteins were purified using glutathione-Sepharose 4B (GE Healthcare) and quantitated by a Storm PhosphorImager (Amersham Biosciences).

**Kinase Activity Is Essential for ER to Golgi Transport**

**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 trans-[35S]methionine (specific activity 1192 Ci/mmol, ICN Biomedicals, Irvine, CA) for 10 min at the restrictive temperature (39.5 °C) to accumulate the VSV-G mutant protein in the ER. The cells were then perforated by swelling and scraping and were employed in the ER to cis-medial Golgi transport assay as described previously (18). 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, and 0.2 IU of rabbit muscle creatine phosphokinase), 5 μl of rat liver cytosol (25–30 μg of total protein), and 5 μl of semi-intact cells (~5 × 10⁷ cells/ml) reconstituted in 50 mM Hepes-KOH, and 90 mM KOAc, pH 7.2. The reactions were incubated at 32 °C for 60 min, and then transferred to ice to terminate transport. Membrane samples were pelleted and solubilized in buffer, digested with endoglycosidase H (endo H), and then analyzed by SDS-PAGE and fluorography. The fraction of ts045 VSV-G protein processed to the endo H-resistant forms was quantitated by a Storm PhosphorImager (Amersham Biosciences).

**Morphological Assay, Indirect Immunofluorescence—**NRK cells plated on coverslips were infected with ts045 at 39.5 °C for 2–3 h and then shifted to ice and permeabilized with digitonin (20 μg/ml) as outlined previously (19, 20). Coverslips with permeabilized cells were inverted and placed in tissue culture wells that contained the transport mixture described above, preincubated on ice for 15 min without or with Rab2 and PKC/α pseudosubstrate peptide, His₆-PKC/α wild type, or His₆-PKC/α (K274W), and then incubated for 40 min at 32 °C. To terminate transport, the cells were transferred to ice and fixed in 3% formaldehyde-phosphate-buffered saline (PBS) for 10 min. To detect intracellular VSV-G, cells were re-permeabilized with 0.5% saponin in PBS, 5% normal goat serum for 10 min, washed with PBS, and then incubated for 30 min with a monoclonal antibody specific for the VSV-G protein cytoplasmic tail (PSD4) (Sigma) or with an affinity-purified polyclonal antibody to β-COP (EAGE) (21) or a polyclonal antibody to p53/p58 (22) or a polyclonal antibody to PKC/α (Transduction Laboratories, Lexington, KY) or an anti-His-Tag (27E8) monoclonal antibody (Cell Signaling Technology, Beverly, MA) or an affinity-purified polyclonal antibody to Rab2. Cells were then washed with PBS, stained for 30 min with Alexa Fluor 549 chicken anti-rabbit antibody or Alexa...
Fluor 488 chicken anti-mouse antibody, washed, mounted in Prolong antifade (Molecular Probes, Eugene, OR), and then viewed by Confocal light microscopy with a Zeiss LSM 510 confocal microscope (LSM 510) located at the John D. Dingell Veterans Administration Medical Center (Detroit, MI). The lasers and available lines are as follows: Enterprise (UV, 351 and 364 nm), Argon (485, 488, and 514 nm), HeNe1 (543 nm), and HeNe2 (633 nm). The confocal microscope was operated with pinhole settings between 1.1 and 1.4 Airy units in each of the fluorescence channels. Images were recorded at 2048 × 2048 pixels × 12 bits/channel and then viewed and converted to TIFF using the Zeiss LSM Vision software and processed for printing with Adobe Photoshop 6.0.

Quantitative Membrane Binding Assay—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 and then broken with 15 passes through a 27-gauge syringe. The broken cells were pelleted at 500 × g for 10 min at 4 °C, and the supernatant was re-centrifuged at 20,000 × g for 20 min at 4 °C. The pellet containing ER, pre-Golgi, and Golgi membranes was washed with 1 M KCl in 10 mM Hepes, pH 7.2, for 15 min on ice and then centrifuged at 20,000 × g for 20 min at 4 °C. The membranes were resuspended in 10 mM Hepes, pH 7.2, and 250 mM mannitol and then broken with 15 passes through a 27-gauge syringe. The binding reaction was described previously (21). 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. PKCα/pseudosubstrate peptide and Rab2 protein were added to obtain the final concentration as indicated under “Results,” and the reaction mixture was incubated on ice for 15 min. Rat liver PTPs and 2.0 μM GTPγS were then added, and the reactions shifted to 37 °C and incubated for 10 min. The binding reaction was terminated by transferring the samples to ice and then centrifuged at 20,000 × g for 10 min at 4 °C. The pellet was resuspended in sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membrane in 25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol. The membrane was blocked in Tri-buffered saline, which contained 5% nonfat dry milk and 0.5% Tween 20, incubated with an affinity-purified polyclonal antibody made to the EAGE peptide of β-COP (21) or a monoclonal antibody to GAPDH (Chemicon, Temecula, CA) or a monoclonal antibody to PKCα (Transduction Laboratories) or an anti-phosphoserine monoclonal antibody (Calbiochem), washed, further incubated with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Pierce), developed with ECL (Amersham Biosciences), and then quantitated by densitometry.

RESULTS

PKCα Pseudosubstrate Peptide Inhibits ER to Golgi Transport—Although PKC activators and inhibitors are routinely...
used to demonstrate the requirement for PKC, few studies have shown that PKC is directly involved in intracellular transport. To address this issue, we have introduced a synthetic peptide specific to the PKC\(\alpha\) pseudosubstrate domain into an in vitro transport assay (18). This peptide interferes with PKC\(\alpha\) activity without modulating the kinase activity of other PKC isoenzymes (26, 27). Tissue culture cells were first infected with ts045 VSV, a virus that encodes for a glycoprotein (G protein) with a thermoreversible defect that results in ER retention at 39.5 °C (28). The plasma membrane of these cells was perforated to release soluble content and then incubated at 32 °C in the presence of cytosol and ATP to initiate export of ts045 VSV-G from the ER. This semi-intact cell assay measures transport of ts045 VSV-G protein by following the processing of the two N-linked oligosaccharides to endo H-resistant forms indicative of transport to the cis-medial Golgi stacks. Fig. 1 shows that ts045 VSV-G transport from the ER to the Golgi complex was blocked in cells treated with increasing concentrations of the PKC\(\alpha\) pseudosubstrate peptide. Transport was reduced by 50% at a pseudosubstrate peptide concentration of 25 \(\mu\)M, whereas a randomized peptide had no effect on transport. These results indicate that PKC\(\alpha\) kinase activity is essential for ER to Golgi transport.

We then determined the kinetics of inhibition of the PKC\(\alpha\) pseudosubstrate peptide relative to the transport block imposed by the addition of Rab2 (13-mer). This peptide made to the Rab2 amino terminus arrests VSV-G transport from the VTC (20). ts045 VSV-G-infected NRK cells were incubated at 32 °C and at the indicated time (\(\Delta t\)), transferred to ice (control) or supplemented with 50 \(\mu\)M PKC\(\alpha\) pseudosubstrate peptide or with 75 \(\mu\)M Rab2 (13-mer), and then incubated for a total of 60 min. This protocol allows any VSV-G that has migrated past the Rab2 and the PKC\(\alpha\)-sensitive step to continue migration to the cis-Golgi compartment. Therefore, the fraction of VSV-G that has transported beyond the Rab2 or PKC\(\alpha\)-requiring step at the time of their addition. In control cells, VSV-G endo H-resistant forms were detected after a 15-min lag (Fig. 1B). Within ~20 min of incubation, 50% of the VSV-G protein was processed, indicating migration to the cis-Golgi compartment. In contrast, cells treated with the pseudosubstrate peptide were blocked in transport ~5 min before the processing of VSV-G to endo H-resistant forms, and by 20 min of incubation, 65% VSV-G had transported through the pseudosubstrate-sensitive step. This temporal site of inhibition by PKC\(\alpha\) peptide occurs immediately following the block that
occurs in the presence of Rab2 (13-mer). In both cases, the block in transport by pseudosubstrate peptide and Rab2 preceded the processing of VSV-G to endo H-sensitive forms by 5–10 min. This result indicates that PKC\(\lambda\) acts downstream of Rab2 but is required prior to cargo delivery to the Golgi complex.

NRK Cells Treated with Pseudosubstrate Peptide Accumulate Swollen Vesicles—To define the morphological site of ts045 VSV-G accumulation in response to the pseudosubstrate peptide, NRK cells were infected with the virus for 3 h at the nonpermissive temperature to restrict VSV-G to the ER. The cells were then permeabilized and incubated in the absence or presence of Rab2 and PKC\(\lambda\)/pseudosubstrate peptide for 40 min at 32 °C, and the distribution of VSV-G was examined by confocal microscopy. Controls cells efficiently transported the reporter molecule to the Golgi complex (Fig. 2a) as did cells treated with the scrambled peptide (Fig. 2b). Consistent with the biochemical data, cells incubated with the pseudosubstrate peptide failed to transport VSV-G to the Golgi complex. Instead, VSV-G accumulated in a collar-like structure composed of small vesicular elements that ringed the nucleus (Fig. 2c). This staining pattern is similar to that observed when cells are incubated with constitutively activated Rab2 (Q65L) and with our affinity-purified anti-GAPDH polyclonal antibody (25, 29). When these pseudosubstrate peptide-treated cells were stained with an anti-p53/p58 polyclonal antibody, a nominal increase in membrane-associated PKC\(\lambda\) was employed (5, 21). For this assay, the degree of co-localization with anti-PKC\(\lambda\) was separated by SDS-PAGE and immunoblotted. A decrease in the amount of phospho-GAPDH bound to the membrane indicating that the pseudosubstrate peptide was inhibiting the kinase activity of the recruited PKC\(\lambda\) (Fig. 3D). The enhanced recruitment of PKC\(\lambda\) and \(\beta\)-COP was specific to the PKC\(\lambda\)/peptide. We did not observe any change in the level of these proteins when the assay was supplied with the scrambled peptide or with pseudosubstrate peptides made to PKC\(\alpha\) or to PKC\(\gamma\). These two isoforms were studied because both were found on NRK microsomes used in the binding assay (5).

A time course was performed to determine the rate of PKC\(\lambda\)/binding to membranes treated with the pseudosubstrate peptide and Rab2. We knew from our earlier studies that Rab2 stimulated rapid translocation of PKC\(\lambda\) to membranes within minutes of addition. Longer incubation times lead to a progressive loss of PKC\(\lambda\)/from the membrane due to the release of PKC\(\lambda\)/ (Fig. 3D). The enhanced recruitment of PKC\(\lambda\)/and \(\beta\)-COP was specific to the PKC\(\lambda\)/peptide. We did not observe any change in the level of these proteins when the assay was supplied with the scrambled peptide or with pseudosubstrate peptides made to PKC\(\alpha\) or to PKC\(\gamma\). These two isoforms were studied because both were found on NRK microsomes used in the binding assay (5).

To learn whether there was enhanced recruitment of those proteins detected on the swollen vesicles in response to treatment with Rab2 and PKC\(\lambda\)/pseudosubstrate peptide, a quantitative binding assay was employed (5, 21). For this assay, microsomes were prepared from NRK cell homogenates and washed with 1 M KCL to remove peripherally associated proteins. These membranes were preincubated with buffer for 15 min on ice in the absence or presence of 50 ng of Rab2 and with increasing concentrations of the PKC\(\lambda\)/pseudosubstrate peptide. The reaction was then supplemented with rat liver cytosol and GTP\(\gamma\)S and incubated at 37 °C for 10 min to promote binding of soluble components. To stop the reaction, membranes were pelleted by centrifugation at 20,000 \(\times\) g and the pellet was separated by SDS-PAGE and immunoblotted. A marked increase in membrane-associated PKC\(\lambda\)/was observed after incubation with Rab2 and dependent on the pseudosubstrate peptide concentration (Fig. 3A). As predicted, there was a dose-dependent increase in membrane-associated \(\beta\)-COP (Fig. 3B), whereas the amount of GAPDH bound to membranes decreased with increasing concentrations of the peptide (Fig. 3C). This result is consistent with our previous findings in which the pseudosubstrate peptide blocked Rab2-stimulated GAPDH recruitment to membrane by interfering with PKC\(\lambda\)/GAPDH interaction (10). Moreover, we found a concomitant decrease in the amount of phospho-GAPDH bound to the membranes indicating that the pseudosubstrate peptide was inhibiting the kinase activity of the recruited PKC\(\lambda\)/ (Fig. 3D). The enhanced recruitment of PKC\(\lambda\)/and \(\beta\)-COP was specific to the PKC\(\lambda\)/peptide. We did not observe any change in the level of these proteins when the assay was supplied with the scrambled peptide or with pseudosubstrate peptides made to PKC\(\alpha\) or to PKC\(\gamma\). These two isoforms were studied because both were found on NRK microsomes used in the binding assay (5).

Kinase-Dead PKC\(\lambda\)/Interferes with ER to Golgi Transport—To further demonstrate that PKC\(\lambda\)/was required for transport in the early secretory pathway, we introduced purified wild type His\(\gamma\)/PKC\(\lambda\)/and kinase-dead His\(\gamma\)/PKC\(\lambda\)/(K275W) into the in vitro transport assay. Kinase-dead PKC\(\lambda\)/
PKC\(\alpha\) Kinase Activity Is Essential for ER to Golgi Transport

**Fig. 5.** The block in ER to Golgi transport by kinase-dead PKC\(\alpha\) is reversed by the addition of PKC\(\alpha\) wild type (WT). A, semi-intact NRK cells were incubated with the indicated concentration of recombinant PKC\(\alpha\) (open circle) or with recombinant kinase-dead PKC\(\alpha\) (closed square) or with 75 ng of kinase-dead PKC\(\alpha\) and increasing concentrations of PKC\(\alpha\) wild type for 15 min on ice in a transport mixture as described under “Experimental Procedures.” The cells were then transferred to 32 °C for a total of 60 min. The fraction of VSV-G processed to endo H-resistant forms (% of Total) was determined after analysis by SDS-PAGE and fluorography. B, NRK cells grown on coverslips were permeabilized with digitonin and then incubated in a complete transport mixture in the presence of 100 mg of Rab2 and 150 ng of PKC\(\alpha\) wild type or 100 ng of kinase-dead PKC\(\alpha\) or 200 ng of PKC\(\alpha\) wild type for 60 min at 37 °C. The distribution of His\(_6\)-tagged PKC\(\alpha\) wild type and mutant (green) or p53/p58 (red) was determined as described under “Experimental Procedures.” Yellow region indicates co-localization of kinase-dead PKC\(\alpha\) with p53/p58.

Inhibited the processing of VSV-G to endo H-resistant forms in a concentration-dependent manner (Fig. 5A). Transport was reduced by 50% at a mutant protein concentration of 75 ng. This reduction is significant because the mutant protein must compete with endogenous PKC\(\alpha\) wild type for limited binding sites on VTCs. Importantly, PKC\(\alpha\) wild type reversed the block in transport by the mutant protein (Fig. 5A).

Kinase-dead PKC\(\alpha\) was then introduced into the morphological assay to determine whether the mutant protein caused a phenotype similar to cells treated with pseudosubstrate peptide. NRK cells were first permeabilized and then incubated in the absence or presence of Rab2 and His\(_6\)-PKC\(\alpha\) wild type or His\(_6\)-PKC\(\alpha\) (K275W) for 45 min at 32 °C. In cells incubated with His\(_6\)-PKC\(\alpha\), anti-His\(_6\), monoclonal antibody labeled tubular-like beaded structures largely concentrated around the nucleus and near the Golgi complex (Fig. 5B). These cells did not appear to contain the swollen vesicles that were present in pseudosubstrate peptide-treated cells. However, permeabilized cells incubated with kinase-dead His\(_6\)-PKC\(\alpha\) displayed enlarged vesicles co-stained with anti-His\(_6\) monoclonal antibody and anti-p53/p58 that were juxtanuclear in location and similar in size but less abundant compared with cells incubated with the pseudosubstrate peptide (Fig. 5B). This phenotype was lost when cells were co-incubated with PKC\(\alpha\) wild type and kinase-dead protein (Fig. 5B). In this case, PKC\(\alpha\) distribution was identical to cells treated with the wild type kinase. These combined results are highly suggestive that inactive PKC\(\alpha\) is a contributing factor to the formation and accumulation of swollen vesicles that most likely correspond to a VTC subcompartment.

**DISCUSSION**

The PKC enzyme family contains in the C1 domain an autoinhibitory pseudosubstrate sequence that maintains the enzyme in the inactive form. This allows the substrate to be phosphorylated by the kinase after translocation to a specific intracellular membrane where the pseudosubstrate is removed by lipid activators (11). A similar effect occurs in the presence of exogenous pseudosubstrate sequences. Unlike PKC inhibitors that are generally nonspecific, pseudosubstrate peptides are the most selective tools to interfere with kinase activity. These peptides permit modulation of cell activities through direct interaction with a PKC isoform.

We found that the PKC\(\alpha\) pseudosubstrate peptide was a potent inhibitor of VSV-G transport from the ER to the Golgi complex, whereas the addition of a randomized peptide had no effect. The arrest in VSV-G transport also occurred when the assay was supplemented with kinase-dead PKC\(\alpha\). The pseudosubstrate peptide and kinase-dead PKC\(\alpha\) have previously been shown to efficiently block PKC\(\alpha\) phosphorylation of GAPDH in an *in vitro* kinase assay and inhibit Rab2-mediated vesicle release from NRK microsomes (10). Therefore, the inhibition by the PKC\(\alpha\) pseudosubstrate peptide is specific and like PKC\(\alpha\) (K274W) is because of the absence of PKC\(\alpha\) phosphorylation activity that is required for transport in the early secretory pathway. Moreover, our results indicate that PKC\(\alpha\) kinase activity is required for reverse translocation. NRK microsomes treated with Rab2 and pseudosubstrate peptide or kinase-dead PKC\(\alpha\) showed both enhanced recruitment and sustained PKC\(\alpha\) membrane association. Similar results have been reported for PKC\(\beta\)III in which the kinase-inactive enzyme exhibits reduced dissociation from membranes (16, 32). Importantly, VSV-G transport inhibition mediated by kinase-dead PKC\(\alpha\) was reversed by PKC\(\alpha\) wild type.

A dramatic change in cell morphology was observed in PKC\(\alpha\) peptide and kinase-dead incubated cells. These cells displayed swollen vesicles that labeled with antibodies to PKC\(\alpha\), β-COP, Rab2, and p53/p58. This phenotype was in direct contrast to cells incubated with recombinant PKC\(\alpha\) wild
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type, which contained anti-His6-labeled tubular-like elements in proximity to the Golgi complex. In some cases, the entities had a beaded-like appearance and may define domains actively involved in protein transport and in the initial stage of vesicle budding (33). Importantly, PKCα wild type prevented the formation of swollen vesicles by kinase-dead PKCα. This observation further indicates that the swollen structures are the product of dysfunctional PKCα. Because pS53/pS58, β-COP, and Rab2 are marker proteins for pre-Golgii intermediates, the swollen entities are most probably derived from VTCs. VTCs are composed of small vesicles and tubules and represent transport intermediates between the ER and the Golgi complex (34). 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. This sorting process results in domains highly enriched in retrograde-directed cargo and domains enriched in secretory cargo (35). For this reason, a negligible amount of VSV-G, which traffics in the anterograde pathway, was detected in the swollen vesicles.

The time of addition experiments performed in this study indicate that the pseudosubstrate peptide blocks VSV-G transport after the site of Rab2 (13-mer) action and before cargo transit to the Golgi complex. Therefore, it is possible that the swollen vesicles observed in kinase-dead and pseudosubstrate peptide-treated cells result from inhibition of retrograde-vesicle release from a VTC subcompartment enriched in recycling proteins, an event that requires PKCα/λ kinase activity. Interestingly, many of the Rab2-PKCα pseudosubstrate peptide-induced swollen structures contain a membrane “patch” where Rab2 and Rab2 effectors co-localized (Fig. 2e, inset). Since anterograde and retrograde transport are coupled, the inability to retrieve essential trafficking components from the VTC to the ER caused the arrest in VSV-G transport. Since GAPDH is a substrate for PKCα and is required for ER to Golgi transport, it was conceivable that phospho-GAPDH was the essential phosphoprotein required for vesicle release from the VTC. However, our affinity-purified anti-GAPDH polyclonal antibody, which efficiently blocks ER to Golgi transport, does not affect Rab2-stimulated vesicle formation. Although GAPDH is required for transport in the early secretory pathway, membrane-associated GAPDH is not essential for vesicle formation, indicating that GAPDH is not a critical component of the budding machinery. Instead, we believe that phospho-GAPDH is part of a protein complex that assembles on a VTC subcompartment in response to Rab2. This complex would serve as an export site to coordinate events required for retrograde-vesicle budding and microtubule nucleation. The observation that phospho-GAPDH is not required for vesicle release presents the possibility that there is an additional PKCα/λ substrate(s) located on the VTC that is not detected because phosphorylation results in membrane dissociation. This interpretation would be consistent with the observation that numerous transport-related proteins undergo cycles of phosphorylation-dephosphorylation, which influences the protein cellular location and modulates their activity (36–38).

From these combined results, we propose that Rab2 binding to the VTC subdomain initiates a cascade of events that results in the recruitment of PKCα/λ, GAPDH, and other unidentified effectors from the cytosol. Rab2 in complex with its effectors defines the site of retrograde-vesicle budding from VTCs. PKCα activation is a critical event that results in the phosphorylation of GAPDH and signals the downstream recruitment of COPI, which ultimately leads to the release of vesicles enriched in recycling components. Because VTCs are the secretory pathways counterpart to endosomes, the suggestion that Rab2 binds to and promotes membrane recycling from a VTC subcompartment is consistent with the finding that Rab5, Rab4, and Rab11 associate with endosome microdomains and regulate sequential transport events (39).

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