Rab2 Protein Enhances Coatomer Recruitment to Pre-Golgi Intermediates

Ellen J. Tisdale§ and Michael R. Jackson

From the Department of Pharmacology, Wayne State University School of Medicine, Detroit, Michigan 48201 and the R. W. Johnson Pharmaceutical Research Institute, San Diego, California 92121

The Rab2 protein is a resident of pre-Golgi intermediates and required for vesicular transport in the early secretory pathway. We have previously shown that a peptide corresponding to the amino terminus of Rab2 (residues 2–14) arrests protein traffic prior to a rate-limiting event in VSV-G movement through pre-Golgi structures (Tisdale, E. J., and Balch, W. E. (1996) J. Biol. Chem. 271, 29372–29379). To determine the mechanism by which this peptide inhibits transport, we investigated the effect of the Rab2 peptide on the distribution of the β-COP subunit of coatomer because COPI partially localizes to pre-Golgi intermediates. We found that the peptide caused a dramatic change in the distribution of pre-Golgi intermediates containing β-COP. A quantitative binding assay was employed to measure recruitment of β-COP to membrane when incubated with the Rab2 (13-mer). Peptide-treated microsomes showed a 25–70% increase in the level of membrane-associated β-COP. The enhanced recruitment of coatomer to membrane was specific to the Rab2 (13-mer) and required guanosine 5′-3-O-(thio)triphosphate, ADP ribosylation factor, and protein kinase C-like activity. The ability to enhance β-COP membrane binding was not limited to the peptide. Similarly, the addition of recombinant Rab2 protein to the assay promoted β-COP membrane association. Our results suggest that the Rab2 peptide causes the persistent recruitment of COPI to pre-Golgi intermediates which ultimately arrests protein transport due to the inability of membranes to uncoat.

Membrane traffic in the early secretory pathway requires the Rab1 and Rab2 GTPases (1, 2). The Rab1 protein is found in the endoplasmic reticulum (ER), pre-Golgi intermediates, and early compartments of the Golgi complex (3), whereas Rab2 has been immunolocalized only to pre-Golgi intermediates (4). These transport intermediates composed of vesicles and tubular clusters (VTCs) (5) are distinct from the ER and the Golgi complex (6). VTCs are morphologically defined by the proteins ERGIC-53/gp58 (p53/gp58) (7, 8) and Rab2 (4), and are the major peripheral site for COPI recruitment (9, 10).

Pre-Golgi intermediates (VTCs) sort and recycle resident proteins from itinerant proteins destined for secretion (10–12). Although the mechanism of protein recycling is unknown, it is likely to involve the COPI coat complex (coatomer and ARF) (10, 13, 14). Coatomer is a heptameric, soluble complex composed of α, β, β′, γ, δ, ε, and ζ subunits (15). The best characterized component of coatomer is the β-COP subunit that was first identified as an 110-kDa peripheral membrane protein associated with pre-Golgi intermediates and the cis Golgi stack (16, 17). The coat complex is recruited en bloc (18) or as a subset (19, 20) to membrane after activation of the small GTPase ARF (21, 22), and phospholipase D. Phospholipase D hydrolyzes phosphatidylycholine to phosphatidic acid which in tandem with phosphatidylinositol 4,5-bisphosphate enhances coatomer membrane association (23, 24). The binding of COPI deforms the membrane which leads to bud formation and eventual release of a coated vesicle.

We have reported that a peptide which corresponds to residues 2–14 of Rab2 arrests protein transport by preventing the flow of cargo through pre-Golgi intermediates (VTCs) (2). The ability of this peptide to block transport in a rapid and specific manner makes it a valuable tool to dissect events in the early secretory pathway. In this study, we took advantage of the irreversible property of the peptide to determine the mechanism by which the Rab2 (13-mer) arrests transport. Our previous observation that peptide affects transport from pre-Golgi structures prompted us to look at the distribution of transport-related proteins which co-localize to VTCs. We initially analyzed the distribution of COPI by focusing on the β-COP subunit. To our surprise, we found that the peptide had a striking effect on β-COP recruitment. Membranes incubated with peptide showed a 25–70% increase in β-COP binding compared with control. This increase in coatomer binding was specific to the Rab2 (13-mer) and required ARF, GTPγS, and protein kinase C (PKC) or a PKC-like protein. Similar results were obtained after addition of recombinant Rab2 protein to the binding assay. We propose that this enhanced COPI recruitment results in the inability of vesicles derived from pre-Golgi structures to uncoat which ultimately leads to the coupled arrest of anterograde and retrograde traffic. These data suggest that Rab2 plays a role in protein sorting and recycling from pre-Golgi intermediates.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal antiserum made to peptides corresponding to β-COP (EAGELKPEEEITVGPVQK), N-α-COP (yeast sequence KMLTKFEKSSTRRAGYC), N-β-COP (PLRLDIIKRLTARSIDYKCO, and N-ε-COP (APPAPPGAGGSGEYYCV) were generously provided by Dr. Roan Teasdale (R. W. Johnson Pharmaceutical Research Institute, La Jolla, CA). The serum was applied to cyanogen bromide-activated Sepharose 4B to which the immunizing peptide was coupled for affinity purification. The column was washed with 5 bed volumes of PBS and eluted with 0.1 M glycine, pH 2.8. The eluate was neutralized to pH 7.2, dialyzed against PBS, then concentrated. Peptides were synthesized at the R. W. Johnson Pharmaceutical Research Institute Protein Facility (La Jolla, CA). The monoclonal antibody to ARF (1D9) and ARF1 cDNA...
was a gift from Dr. Richard Kahn (Emory University, Atlanta, GA). Purified cockroach was kindly provided by Dr. M. Gerard Waters (Princeton University, Princeton, NJ). Polyclonal antiserum to Rab2 was purchased from Santa Cruz Biotechnology. Rab2 cDNA was obtained from Dr. Marino Zerial (EMBL, Heidelberg, Germany). The polyclonal antiserum to p58 was obtained from the University of Bergen, Bergen, Norway. Calphostin C was purchased from Calbiochem (San Diego, CA). Chelerythrine chloride was obtained from LC Laboratories. Digotonin was acquired from Boehringer Mannheim. HRP-conjugated antibody was purchased from Bio-Rad. Monoclonal antibody to β-COP (M3A5) and all other reagents were purchased from Sigma.

Membrane Binding Reaction—HeLa cells were washed three times with ice-cold phosphate-buffered saline (PBS). The cells were scraped off the dish with a rubber policeman into 10 mM Hepes, pH 7.2, and 250 mM mannitol, then broken with 15 passes of a 27-gauge syringe. The broken cells were pelleted at 500 × g for 10 min at 4 °C, and the supernatant was 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, 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 employed in the binding reaction as described previously (10, 14). Membranes (30 μg of total protein) were added to a reaction mixture which contained 27.5 mM Hepes, pH 7.2, 65 mM MgOAc, 5.5 mM KOAc, 1.8 mM CaCl2, 1 mM ATP, 5 mM creatine phosphate, and 0.2 units of rabbit muscle creatine kinase. Peptide or Rab2 protein were added to obtain the final concentration as indicated under “Results” and the reaction mixture incubated on ice for 30 min. Rat liver cytosol (50 μg) and 2 μM GTP-S 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 in 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol. The membrane was blocked in Tris-buffered saline (TBS) 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 (25) or a polyclonal antibody to Rab2, or a monoclonal antibody to ARP1 (1D9) (26), washed, further incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibody, developed with enhanced chemiluminescence (ECL) (Amersham), then quantitated by densitometry.

Indirect Immunofluorescence—NRK cells plated on coverslips were permeabilized with digitonin (20 μg/ml) as outlined previously (3). Coverslipped cells were fixed in ice-cold methanol for 10 min. The fixed cells were washed with PBS, then co-incubated for 30 min with a monoclonal antibody to Rab2, or a polyclonal antibody to Rab2, or with antibody to β-COP (M3A5) and a polyclonal serum to p58, or with polyclonal Rab2 serum to Rab2. Cells were washed with PBS, co-stained with Texas Red anti-rabbit antibody and fluorescein isothiocyanate anti-mouse antibody, washed, mounted, and then viewed under a Zeiss Axiovert fluorescence microscope.

Purification of Recombinant Rab2 Protein and in Vitro Prenylation—Rab214 was generated by polymerase chain reaction using a 5′-oligo-nucleotide primer which introduced a start codon at the deletion site. The cDNA for Rab2 wild type and Rab214 were cloned into pET3A (Novagen, Milwaukee, WI) and introduced into BL21 (DE3) pLysS (Novagen). A 1-liter culture was grown to OD600 of 0.4–0.5 and induced with 0.4 mM isopropyl-β-thiogalactopyranoside at 37 °C for 3 h. The cells were centrifuged at 5000 × g for 10 min at 4 °C, and the cell pellet resuspended in 50 mM Tris, pH 7.4, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidase, 1 mM EDTA, and 1% Triton X-100, then homogenized by 20 passes with a Dounce tissue grinder. Lysosome (400 μg/ml), DNase I (40 μg/ml), and 25 mM MgCl2 were added to the homogenate and allowed to digest for 30 min on ice at 4 °C, then centrifuged at 22,000 × g for 30 min at 4 °C. The supernatant was applied to a 70-ml column containing Q Sepharose Fast Flow (Pharmacia) equilibrated with Buffer A (50 mM Tris, pH 7.4, 10 mM MgCl2, and 1.0 mM EDTA). washed with 2 bed volumes of Buffer A, then eluted with a linear NaCl gradient (0–400 mM) in Buffer A. Three-ml fractions were collected and an aliquot of each separated by SDS-PAGE and immunoblotted with a Rab2 polyclonal antibody. Rab2-enriched fractions were pooled, concentrated, and applied to a 200-ml column containing Sephacryl S-100 (Pharmacia) and eluted with Buffer A. Fractions containing Rab2 or Rab214 proteins were identified by SDS-PAGE and immunoblotting, then pooled, concentrated, and prenylated in an in vitro reaction. Briefly, the isoprenylation reaction was performed in a total volume of 50 μl that contained 5 μg of recombinant Rab2 or Rab214 protein, 10 μg of phosphatidylcholine (Sigma), 25 μl of rat liver cytosol, 10 mM MgCl2, 1 mM ATP, 5 mM creatine phosphate, and 0.2 units of rabbit muscle creatine kinase. The reaction was incubated for 1 h at 37 °C and then desalted through a 1-ml column of Sephade G-25 (Pharmacia) and concentrated. The protein concentration was determined by Micro BCA Protein Assay Reagent Kit (Pierce).

Purification of Recombinant ARF and Preparation of ARF-depleted Cytosol—Recombinant myristoylated ARF was prepared from the BL21 bacterial strain that had been co-transformed with a plasmid encoding N-myristoryltransferase and wild type ARF1 (27). The transformed cells were induced with 1.0 mM isopropyl-β-thiogalactopyranoside for 3 h at 25 °C in the presence of 50 μM myristate. The cells were lysed and the expressed ARF1 purified on a DEAE-Sephacel and AcA54 Ultrogel columns as described by Weiss et al. (28). For ARF depletion, rat liver cytosol was fractionation on a Superose 6 column equilibrated in 25 mM Hepes, pH 7.2, 125 mM KOAc, 2.5 mM MgOAc, and 1 mM dithiothreitol (28). ARF-enriched and ARF-depleted fractions were identified by Western blotting. Those fractions lacking ARF were pooled, and concentrated to the original volume.

RESULTS
N-terminal Peptide to Rab2 Alters β-COP Distribution—We analyzed the distribution of β-COP in permeabilized NRK cells incubated with or without peptide for 80 min at 15 °C by indirect immunofluorescence. Cells were incubated at this reduced temperature to accumulate and enhance visualization of pre-Golgi intermediates (VTCs). In control cells, anti-β-COP antibody labeled the juxtanuclear Golgi complex and vesicular structures scattered throughout the cytoplasm and in proximity to the Golgi complex (Fig. 1A). This distribution of β-COP is in agreement with that found in other cell types (6, 9, 10). In contrast, cells incubated in the presence of the Rab2 peptide displayed prominent β-COP-labeled structures that ringed the nucleus in a collar-like manner (Fig. 1B). These cells did not appear to contain peripherally-located β-COP-labeled vesicles. The β-COP containing ring-like structure partially overlapped with VTCs located near the cis Golgi region that stained with the antibody to p58 (29) (Fig. 1D) and with antibody to Rab2 (Fig. 1H). Both of these polypeptides are considered marker proteins for VTCs (4, 7, 8). As we observed with antibody to β-COP, p58 (Fig. 1D) and Rab2 (Fig. 1H)-labeled vesicles were not found in peripheral locations. In control cells, p58 (Fig. 1C) and Rab2 (Fig. 1G) were localized in vesicular elements that were concentrated in the Golgi region and dispersed throughout the cytoplasm. This staining pattern is similar to that observed with antibody to β-COP. This striking change in VTC distribution prompted us to further explore the relationship of Rab2 to COPI recruitment.

Rab2 (13-mer) Increases Membrane-associated β-COP—A quantitative binding assay was used to measure β-COP recruitment to membranes in the presence of the Rab2 peptide (10, 21). For this assay, microsomes were prepared from whole permeabilized cells and washed with 1 mM KCl to remove pre-bound COPI. These membranes were preincubated in buffer for 30 min on ice with or without peptide, and then supplemented with GTP-S and rat liver cytosol and incubated at 37 °C for 10 min. The peptide concentrations employed in the binding assay were equivalent to those which inhibit protein traffic in an in vitro transport assay (2). To terminate the reaction, membranes were collected by centrifugation, separated by SDS-PAGE, transferred to nitrocellulose, then probed with an affinity purified antibody to β-COP. We observed in peptide-treated membranes a marked increase in β-COP recruitment that occurred in a dose dependent manner and required GTP-S (Fig. 2A).
2). The amount of membrane-associated β-COP increased ~50% when incubated with 25 μM peptide. In our previous studies (2), this peptide concentration reduced ER to Golgi transport by 50% in an in vitro transport assay. To learn if recruitment was specific to the Rab2 (13-mer), peptides were synthesized to the amino terminus of other Rab proteins and evaluated in the binding assay (see Table I). Stimulation of β-COP membrane binding was specific to the Rab2 (13-mer). As shown in Fig. 3, a randomized form of Rab2 (13-mer) had no effect on β-COP binding even at a concentration that results in maximum recruitment by the wild-type peptide. Likewise, a peptide that corresponds to the first 7 amino acids of Rab2 had no influence on β-COP membrane binding. The amount of membrane-associated β-COP was not affected by control peptides made to the analogous N-terminal domains of Rab5 and Rab3A. We also noted that a peptide to the amino terminus of Rab1B, a protein essential for ER to Golgi transport failed to recruit coatomer above the control level. These results show that the enhanced recruitment of β-COP to membrane was specific to the Rab2 (13-mer).

The availability of affinity-purified antibodies that recognize the α, β', and ε subunits of coatomer allowed us to test for co-recruitment after peptide treatment. In all cases, we observed a linear increase in membrane binding of these subunits comparable to β-COP (data not shown). It appears that the peptide stimulates, at the very least, recruitment of a subcomplex if not all coatomer subunits.

The Rab2 Peptide Increases the Rate of β-COP Recruitment—We performed a time course in which membrane was incubated with or without peptide to determine whether the Rab2 (13-mer) affects the rate of coatomer recruitment to membrane. As shown in Fig. 4A, the Rab2 peptide accelerated coatomer binding that resulted in a \( t_{1/2} \) of ~7.5 min for the control. No increase in β-COP recruitment was observed after ~10 min of incubation for both control and peptide-treated membranes. The apparent saturability was not due to the rate of COPI binding, since longer incubations times did not result in an increased level of membrane-bound β-COP (data not shown). To further learn if the mem-

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**Fig. 1.** Rab2 (13-mer) causes redistribution of β-COP-labeled structures. NRK cells grown on coverslips were permeabilized with digitonin, and then incubated in a complete transport mixture in the absence (A, C, E, and G) or presence (B, D, F, and H) of 50 μM Rab2 peptide for 80 min at 15 °C. The distribution of β-COP (A and B) and p58 (C and D), or β-COP (E and F) and Rab2 (G and H) was determined by indirect immunofluorescence as described under “Experimental Procedures.” In control cells, antibody to β-COP labeled the Golgi region and peripherally located pre-Golgi intermediates or VTCs. These structures overlap with vesicles that stain with antibody to p58 (panel C) and with antibody to Rab2 (panel G). Peptide-treated cells displayed punctate β-COP-labeled vesicles that ringed the nucleus. This ring-like structure co-distributed with vesicles that stained with antibody to p58 (panel D) and with antibody to Rab2 (panel H).

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**Fig. 2.** Rab2 peptide stimulates β-COP membrane recruitment. Microsomes were prepared from HeLa cell homogenates as described under “Experimental Procedures,” then preincubated with or without increasing concentrations of the Rab2 peptide for 30 min on ice. Cytosol and GTPγS were added to one set of membranes (closed bar), whereas the other set received only cytosol (striped bar) and then all membranes incubated for 10 min at 37 °C to promote COPI binding. Microsomes were collected by centrifugation, separated by SDS-PAGE, and immunoblotted with affinity-purified antibody to β-COP. Rab2 peptide treatment resulted in a marked increase in membrane-associated β-COP that required GTPγS.
branes were saturated with β-COP or if cytosol was limiting, microsomes were preincubated with or without the Rab2 peptide and then GTPγS and increasing amounts of cytosol added. Fig. 4B shows that control and peptide-treated membranes were saturated with β-COP when incubated with 0.1 mg of cytosol (total protein). Incubation of membranes with higher cytosol concentrations did not result in additional β-COP recruitment. Peptide-treated membranes showed an ~30% increase in membrane-bound β-COP compared with control membrane. These combined results suggest that the Rab2 (13-mer) not only affects the extent of β-COP recruitment but also causes a rapid recruitment of COPI to membrane.

**Rab2 Protein Increases Membrane-associated β-COP**—The Rab2 peptide inhibits protein traffic in an irreversible manner (2) which may result from the inability of the Rab2 protein to bind to pre-Golgi elements. We pursued this question by first establishing a dose-response curve for the Rab2 protein. The addition of 200 ng of Rab2 protein saturated the membranes (Fig. 5). The microsomal binding assay was then performed with increasing peptide concentrations in the presence of 200 ng of recombinant Rab2 protein. The amount of membrane-bound Rab2 protein did not change when co-incubated with 10–75 μM peptide (Fig. 5, inset). These results suggest that the Rab2 (13-mer) does not compete with Rab2 protein binding to membrane.

We reasoned that if there was no competition between the peptide and the Rab2 protein for binding, then the Rab2 (13-mer) might mimic the function of the intact protein and therefore potentiate Rab2 activity. To address this possibility, the microsomal binding assay was performed by incubating membranes with three concentrations of recombinant Rab2 protein with GTPγS and cytosol for 15 min at 37 °C. As we observed

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**Table I**

Peptides tested in membrane binding assay

| Rab protein | Peptide          |
|-------------|------------------|
| Rab1B       | NPEYDYLFKLLLIGD  |
| Rab2 (7-mer)| AYAYLFK         |
| Rab2 (13-mer)| AYAYLFKHHID     |
| Rab2 (random)| ARKGYIDLYTFI   |
| Rab3A       | ASATDSRYGK      |
| Rab5        | ANRGATRPNPNTGNK |
with the Rab2 (13-mer), Rab2 protein membrane binding resulted in a dose-dependent increase in membrane-associated β-COP (Fig. 6A). This result was further investigated in the morphological assay which showed that the Rab2 protein changed the intracellular distribution of β-COP. In these cells we observed large β-COP containing structures located in the perinuclear region (Fig. 7C). These structures appeared to overlap with juxtanuclear structures that labeled with antibody to p58 (Fig. 7D) and with antibody to Rab2 (Fig. 7D, inset). This accumulation of COPI containing intracellular structures in response to the Rab2 protein is consistent with the enhanced β-COP recruitment observed in the microsomal binding assay.

From our earlier work we know that overexpression of Rab2 wild-type caused a decrease (25%) in the processing of VSV-G to endo H-resistant forms (2). Deletion of the first 14 amino-terminal residues from Rab2 wild-type did not affect prenylation, but restored transport to control levels. To determine if a similar truncation of Rab2 wild-type could modify the ability of the protein to recruit β-COP, we generated recombinant Rab2 Δ14 protein and introduced the mutant protein into the binding assay. Fig. 6B shows that membranes treated with Rab2 Δ14 did not recruit β-COP to the level obtained by incubation with the wild-type protein. Furthermore, the truncated protein did not alter β-COP distribution when introduced into the morphological assay (Fig. 7E). These cells displayed a similar distribution of VTCs labeled with p58 (Fig. 7F) and Rab2 (Fig. 7F, inset) compared with control (Fig. 7, A and B). These combined results support our contention that the peptide functions as a bona fide domain of the Rab2 protein and more importantly, demonstrates that the amino-terminal residues of Rab2 are necessary to promote COPI binding.

**Fig. 6. Recombinant Rab2 protein promotes β-COP membrane association.** Recombinant Rab2 wild-type protein was purified and isoprenylated in vitro, as described under "Experimental Procedures." HeLa cell microsomes were incubated with 0, 50, 100, or 200 ng of recombinant Rab2 protein (panel A) or recombinant Rab2Δ14 protein (panel B) in assay buffer supplemented with GTPγS and cytosol for 15 min at 37 °C. Membranes were pelleted, separated by SDS-PAGE, transferred to nitrocellulose, and the blot probed with a monoclonal antibody to β-COP and a polyclonal antibody to Rab2. After incubation with HRP-conjugated secondary antibodies, the blot was developed with ECL and the amount of recruited Rab2 and β-COP was quantitated by densitometry. A co-linear increase in Rab2 and β-COP was noted with increasing peptide concentration. The amino-terminal truncated form of Rab2 had no effect on β-COP recruitment.

**Fig. 5. Rab2 amino-terminal peptide does not compete with Rab2 protein membrane binding.** HeLa cell microsomes were incubated with increasing concentrations of purified recombinant Rab2 protein in an assay buffer supplemented with GTPγS for 15 min at 37 °C. The membranes were pelleted, separated by SDS-PAGE, transferred to nitrocellulose, and the blot probed with a polyclonal antibody to Rab2. After incubation with an HRP-conjugated secondary antibody, the blot was developed with ECL and the amount of recruited Rab2 quantitated by densitometry. Membranes are saturated with 200 ng of Rab2 protein. Inset, HeLa cell membranes were preincubated with 200 ng of recombinant Rab2 protein in the presence or absence of increasing Rab2 peptide concentrations for 30 min on ice. GTPγS was then added and the reactions shifted to 37 °C for 15 min. Microsomes were collected by centrifugation, separated by SDS-PAGE, and immunoblotted with polyclonal antibody to Rab2, and quantitated by densitometry. The amount of membrane-bound Rab2 did not change with increasing peptide concentration which suggests that the Rab2 (13-mer) does not interfere with Rab2 protein binding to membrane.
to the cytosolic concentrations of these components employed in the assay. Fig. 8B shows control membranes bound a small amount of coatomer. The addition of exogenous ARF stimulated β-COP binding. Similarly, coatomer recruitment was significantly enhanced when ARF was added to microsomes treated with the Rab2 (13-mer) or the Rab2 protein. Both Rab2 peptide- and Rab2 protein-incubated membranes recruited more β-COP than control. As further proof for an ARF requirement in the assay, we depleted ARF from cytosol by gel filtration on a Superose 6 FPLC column (28). The binding reaction was then performed with the ARF-deficient, coatomer-containing cytosol in the presence or absence of the Rab2 (13-mer) or Rab2 protein (Fig. 8C). In these reactions, the amount of membrane-associated β-COP was negligible. However, the addition of purified recombinant ARF to the assay restored β-COP binding. As we observed with the purified components, the level of membrane-bound COPI is greater in reactions containing Rab2 peptide and Rab2 protein. These membranes also recruit more β-COP than microsomes incubated with purified components suggesting that an additional cytosolic factor may promote membrane binding. Interestingly, the amount of bound ARF is comparable between control and treated membranes (Fig. 8C, anti-ARF). Most likely ARF acts catalytically to facilitate coatomer recruitment. These combined results suggest that the Rab2 peptide and protein do not “by-pass” ARF but require ARF-dependent assembly of β-COP containing coat complex onto membrane.

Calphostin C Inhibits Rab2 Peptide and Rab2 Protein Recruitment of β-COP—It has been reported that ARF/coat assembly increases in response to protein kinase C (PKC) (30). We know from our previous studies that the specific PKC inhibitor calphostin C, arrests protein traffic prior to the block by the Rab2 peptide (2) and therefore PKC or a PKC-like protein functions upstream of Rab2. To determine whether PKC influenced Rab2 peptide and Rab2 protein activity, coatomer binding was assessed in the presence of calphostin C.
DISCUSSION

We previously reported that a peptide corresponding to the amino terminus of Rab2 was a potent and irreversible inhibitor of ER to Golgi transport. The Rab2 peptide did not interfere with vesicle budding from the ER nor did it prevent intra-Golgi trafficking (2). These results suggested that the Rab2 (13-mer) specifically arrested transport from VTCs. Since Rab2 immunolocalizes to pre-Golgi intermediates and the Rab2 peptide interferes with traffic from these intermediates, we wished to learn if the peptide influenced other transported-related proteins which also target to these structures. Our thought was that this information might shed light on the mechanism by which the Rab2 peptide inhibits protein trafficking. To address this question, we analyzed the distribution of COPI by focusing on the β-COP subunit. We observed a dramatic change in β-COP distribution after peptide treatment. Vesicular structures containing β-COP accumulated near the nucleus and in proximity to the Golgi complex. Similar results have been reported for anoxic pancreatic acinar cells (34). In those studies, ATP-depletion did not alter the Golgi stack but greatly reduced the number of ER to Golgi transport vesicles that were replaced by cytoplasmic, fibrillar aggregates containing β-COP. These aggregates represent cytoplasmic pools of coatamer that self-associate after disruption of ER to Golgi traffic. Although like the anoxic condition the organization of the Golgi complex and the endoplasmic reticulum did not change after Rab2 peptide treatment, we observed a different β-COP phenotype. Our studies show structures that overlapped when labeled with antibody to β-COP, antibody to p58, and antibody to Rab2, indicating that the Rab2 peptide did not cause β-COP membrane dissociation to the cytosol. On the contrary, we believe that VTCs are perpetually coated with COPI which interferes with subsequent trafficking through the Golgi complex. This result is similar to the effect produced by GTPγS when added to the intra-Golgi transport assay (35). In that case, coated vesicles and coated membranes form which cannot uncoat and inhibit subsequent fusion events necessary for intercisternal transport. Since VTCs in peptide-treated cells are “stalled,” components are not retrieved from these structures that are necessary for cargo transport from the ER. Our previous obser-
ivation that the Rab2 (13-mer) caused ts045 VSV-G to accumulate in punctate structures which were smaller and less abundant than VTCS that accumulate at 15 °C, supports this interpretation (2). We predict that prolonged incubation with the Rab2 peptide would ultimately result in complete inhibition of transport from the ER as well as recycling from pre-Golgi and Golgi compartments.

The results of our microsomal binding assay show that the Rab2 (13-mer) increased the rate and extent of COP1 membrane association and that this enhanced recruitment is specific to the Rab2 peptide. We did not observe a similar level of β-COP binding after incubation with a scrambled peptide or with amino-terminal peptides made to other Rab proteins. Interestingly, the Rab1B peptide and Rab2 peptide share 8 amino acids, yet the Rab1B peptide did not interrupt protein traffic (2) or affect COP1 recruitment. The ability of the Rab2 protein to promote β-COP membrane association and the lack of recruitment by the Rab2Δ14 protein supports our contention that the peptide functions as a bona fide Rab2 domain.

Combined biochemical and morphological data suggests that the peptide acts at a post-ER budding step but before VSV-G exit from pre-Golgi structures. These structures segregate anastrophe from retrograde-transported proteins, an event that requires the participation of ARF and coatomer (10). Our results indicate that ARF is necessary for Rab2 peptide and Rab2 protein-stimulated COP1 binding. We suggest that ARF functions catalytically to facilitate Rab2 peptide and Rab2 protein enhancement of COP1 binding. This requirement for ARF is consistent with the numerous publications showing ARF involvement in COP1 recruitment (10, 21, 22). In particular, addition of an activated form of ARF (ARF1 Q71L) causes VSV-G to accumulate in pre-Golgi structures that contain β-COP and p53/p58 (10). Furthermore, ARF1 can mediate the in vitro recruitment of the COP1 coat to subcellular fractions enriched in cis Golgi/intermediate compartment membranes (26).

The Rab2 (13-mer) binds to membrane. However, the peptide did not interfere with Rab2 protein membrane association. Although it appears that the peptide is not a competitive inhibitor for Rab2 protein membrane-binding sites, the Rab2 (13-mer) might influence Rab2 function by interaction with a downstream Rab2 effector. This effector could require interaction with the Rab2 amino terminus to regulate coat recruitment. The Rab2 peptide would therefore block the functional interaction of Rab2 with the downstream effector. This inhibition would lead to uncontrolled coatomer binding ultimately inhibiting vesicular traffic in the early secretory pathway. We have observed enhanced β-COP recruitment when membranes are incubated with Rab2 protein and GTP (data not shown). However, a significant increase in β-COP binding was obtained when GTPγS was included in the assay. The irreversible binding of the Rab2 protein to membrane mimics the irreversible nature of the Rab2 (13-mer). We propose that in steady state, Rab2 plays a role in coatomer recruitment to pre-Golgi intermediates (VTCS). This recruitment process requires PKC or a PKC-like protein.

Protein kinase C activity has been found in regulated (36) and constitutive exocytosis (31) and is required for receptor traffic through the endocytic path (37). All members of this family of isoenzymes have an amino-terminal regulatory domain that contains binding sites for phospholipid/diacylglycerol and calcium, and a carboxyl-terminal catalytic domain that binds ATP and has kinase activity (38). A variety of compounds are available which specifically bind to these domains and either activate or inhibit. These compounds allow one to determine if PKC exerts an effect independent of kinase activity. We evaluated the effect of calphostin C in the binding assay because PKC regulates ARF and COPI (30). Additionally, PKC inactivation by calphostin C arrests transport in the early secretory pathway before the site of Rab2 peptide inhibition (2, 39). Calphostin C binds to the PKC regulatory domain and blocks binding of diacylglycerol. In this study, calphostin C inhibited Rab2 (13-mer) and Rab2 protein stimulation of β-COP membrane binding which indicates that PKC or a PKC-like protein functions upstream of the Rab2 protein. The kinase activity of this enzyme is not required based on the result that chelerythrine chloride which binds to the catalytic domain had no influence on β-COP recruitment.

The role of PKC in mediating intracellular trafficking independent of phosphorylation has been reported for ER to Golgi transport (39) and in the production of post-Golgi vesicles (40). In both cases, a phorbol-ester binding protein was proposed to participate in the respective transport event. However, unlike the soluble phorbol ester-sensitive protein required for ER to Golgi transport, we believe that the PKC-like molecule required in Rab2 activity is membrane associated. Membranes preincubated with calphostin C, then pelleted and resuspended in cytosol and GTPγS do not bind β-COP (data not shown). PKC isoforms are differentially expressed in tissue and cell-type and each translocate to a specific intracellular site upon activation by phorbol esters (41, 42). It is possible that the PKC-like molecule required for Rab2 activated β-COP recruitment differs from the phorbol ester-binding protein that regulates exit from the ER.

The Proposed Role of Rab2 in the Secretory Pathway—The phylogenetic tree of the Ras superfamily shows that Rab2 is most closely related to Rab4 which is interpreted to mean that these proteins are similar in function (43). Therefore, it is conceivable that the role of Rab2 in exocytosis is analogous to the role of Rab4 in endocytosis. In the endocytic pathway, Rab4 regulates the early recycling step from the “sorting endosome” to the plasma membrane (44–46). Since COPI-coated vesicles are involved in retrograde transport (13), the Rab2 protein may function in protein sorting and recycling events from pre-Golgi intermediates or “sorting exosomes” (7, 10). In this case, Rab2 would regulate vesicular traffic through a subcompartment within pre-Golgi elements. Such a subcompartment might function to sort and recycle escaped ER proteins that possess retrieval sequences. The recruitment of COPI to this subcomponent may result in vesicles that contain recycling proteins. The Rab4 protein plays a similar role in endocytosis by controlling a rate-limiting step in receptor transport from early endosomes to recycling endosomes (44).

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