The small GTPase Rab2 is a resident of pre-Golgi intermediates and required for protein transport from the endoplasmic reticulum (ER) to the Golgi complex (Tisdale, E. J., Bourne, J. R., Khorrami-Far, R., Der, C. J., and Balch, W. E. (1992) J. Cell Biol. 119, 749–761). The Rab2 protein, like all small GTPases, contains conserved GTP-binding domains as well as hypervariable carboxy-terminal and amino-terminal domains. While the role of the carboxyl terminus in specific membrane localization is well recognized, the potential role of the variable NH2 terminus remains to be clarified. To determine whether the NH2 terminus of Rab2 was required for its activity in vivo, a trans dominant mutant of Rab2 that inhibits ER to Golgi transport was progressively truncated and analyzed for its effect on vesicular stomatitis virus glycoprotein transport in a vaccinia-based transient expression system. Deletion of the first 14 amino-terminal residues resulted in the loss of the inhibitory properties of the mutant without affecting its post-translational processing or membrane association. To assess the potential role of the NH2 terminus in Rab2 function, a peptide corresponding to the first 13 amino acids following the initiator methionine was introduced into an in vitro assay that efficiently reconstitutes transport of vesicular stomatitis virus glycoprotein from the ER to the Golgi stack. This peptide was a potent inhibitor of transport. Biochemical and morphological studies revealed that the peptide strongly interfered with assembly of pre-Golgi intermediates which mediate segregation of anterograde and retrograde transported proteins en route to the Golgi. The combined results suggest that the NH2 terminus of Rab2 is required for its function and for direct interaction with components of the transport machinery involved in the maturation of pre-Golgi intermediates.

The Rab family of small GTPases includes more than 30 members that regulate vesicular traffic between specific compartments of the endocytic and exocytic pathways of eukaryotic cells (1, 2). Rab proteins, like other members of the Ras superfamily, contain four highly conserved sequence motifs required for guanine nucleotide binding (3). In order for Rab proteins to regulate distinct transport events, non-conserved regions must confer unique function. The most divergent regions of Rab proteins are located at their amino and carboxyl termini. The function of the carboxyl terminus has been extensively characterized. Cysteine residues found at the COOH terminus of all Rab proteins are post-translationally processed by the addition of geranylgeranyl lipids, a modification essential for membrane association and function (4). Moreover, the hypervariable region composed of the terminal ∼30 amino acids dictates localization of Rab proteins to specific endocytic and exocytic compartments (5, 6).

In contrast to the well characterized role of the carboxyl terminus in small GTPase function, few studies have focused on the potential role(s) of the hypervariable amino termini found in different Rab proteins. Sequence alignments of Rab family members show that the NH2 termini are variable in length. This has led to the suggestion that these extensions, at least in some cases, participate in specific protein interactions. In support of this idea, truncation of the NH2-terminal domain of Rab5 prevents early endosome fusion (7). Consistent with this observation, peptide mimetics to this region also block the fusion of endosomes in an in vitro assay that reconstitutes this event (7). A conserved lysine found in the amino terminus of most Rab proteins has also been reported to be essential for prenylation of Rab5 by Rab geranylgeranyltransferase II (8).

Although these two studies ascribe two distinct roles for the NH2 terminus, both implicate a functional requirement for the NH2 terminus in Rab activity.

The Rab2 protein is a resident of pre-Golgi intermediates (9), which consist of clusters of small vesicles and tubules (termed vesicular-tubular clusters or VTCs) (10), and participate in the segregation of anterograde and retrograde transported protein following export from the ER (11). Although the function of Rab2 is unknown, we have previously demonstrated that it is required for protein traffic between the ER and the Golgi complex (12). A mutation in the nucleotide binding domain that restricts Rab2 to the GTP-bound form (Rab2(Q65L)) or a mutation that causes a high exchange rate (Rab2(N119I)) resulted in proteins that were potent inhibitors of ER to Golgi transport when transiently expressed in vivo.

To investigate the biochemical requirement for Rab2 in protein transport through the early secretory pathway, we have now focused on a region of the amino terminus (amino acids 1–14) to assess its potential role in Rab2 function. In vivo, we find that the amino terminus is critical to sustain the inhibitory phenotype of the Rab2(N119I) mutant. Moreover, a peptide synthesized to the first 13 amino acids of Rab2 following the initiating methionine was found to be a potent and specific inhibitor of protein transport using an assay that reconstitutes ER to Golgi traffic in vitro. Combined biochemical and morphological studies used to map the site of peptide inhibition revealed that the Rab2 peptide specifically arrests transport at this region of the amino terminus. The potential role of the NH2 terminus in Rab2 function has been assessed by various methods, including transient expression systems and peptide mimetics to this region. The combined results suggest that the NH2 terminus is required for Rab2 function and for direct interaction with components of the transport machinery involved in the maturation of pre-Golgi intermediates.
an early stage of the secretory pathway, preventing the normal flow of cargo through VTCs. These results demonstrate that the NH2 terminus of Rab2 is required for its activity and for interaction with as yet unidentified components which function in the assembly and maturation of VTCs.

EXPERIMENTAL PROCEDURES

Materials—A monoclonal antibody specific for the carbonyl terminus of VSV-G (P5D4) was generously provided by T. Kreis (13). Antiserum to p58 was a gift of J. SARaste (14). The polyclonal serum to α-1,2-mannosidase II was kindly supplied by M. Farquhar (University of California, San Diego, CA). Endoglycosidase H (endo H) was obtained from New England Biolabs Inc. (Beverly, MA). A monoclonal antibody specific for Rab2 (pET-Rab) was prepared as described (9) and used for the transient expression of the Rab2 mutant proteins in HeLa cells described below. A monoclonal antibody (1C5B) specific for Rab2 was prepared from a hybridoma cell line (Scripps Research Institute Protein/DNA Core Facility).

Cell Culture and Generation of Mutant Constructs—HeLa cells were cultured in minimal essential medium (Life Technologies, Inc.) and normal rat kidney (NRK) and Chinese hamster ovary (CHO) 15B cells were cultured in minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and penicillin-streptomycin. Rab2 expression constructs were generated from human Rab2 cDNA sequences (16) provided by M. Zerial (EMBL, Heidelberg, Germany). These sequences were ligated into the NdeI and BamHI sites of the pET3a vector (Novagen, Madison, WI) for expression from the T7 promoter (17). NH2-terminal deletions of Rab2 and Rab2(N119I) were generated by PCR using 5′ oligonucleotide primers, which included an NdeI site and sequence complementary to Rab2 that introduced a start codon at the respective deletion site: 5′-GCAACATTTGCGGACACAGTTG-3′; 5′-GCAAGCATATGGGTGTTGGTAAATCATGC-3′; primer 5′-GCAAACATATGGGCGACACAGGTG-3′; and 5′-GCAAACATATGGGCGACACAGGTG-3′; all mutated Rab sequences were verified by DNA sequence analysis.

Transfection Procedure—The transient expression of the Rab2 mutant proteins was as described previously (12). Briefly, HeLa cells (5 × 104/35-mm dish) were infected with a vaccinia T7 RNA polymerase (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and penicillin-streptomycin. Rab2 expression constructs were generated from human Rab2 cDNA sequences (16) provided by M. Zerial (EMBL, Heidelberg, Germany). These sequences were ligated into the NdeI and BamHI sites of the pET3a vector (Novagen, Madison, WI) for expression from the T7 promoter (17). NH2-terminal deletions of Rab2 and Rab2(N119I) were generated by PCR using 5′ oligonucleotide primers, which included an NdeI site and sequence complementary to Rab2 that introduced a start codon at the respective deletion site: 5′-GCAACATTTGCGGACACAGTTG-3′; 5′-GCAAGCATATGGGTGTTGGTAAATCATGC-3′; primer 5′-GCAAACATATGGGCGACACAGGTG-3′; and 5′-GCAAACATATGGGCGACACAGGTG-3′; all mutated Rab sequences were verified by DNA sequence analysis.

Protein Expression and Maturation Assays—NRK or CHO clone 15B cells were transfected with plasmids that encode wild-type or mutated Rab2 proteins, as described. The transient expression of the Rab2 mutant proteins in HeLa cells described below. A monoclonal antibody (1C5B) specific for Rab2 was prepared from a hybridoma cell line (Scripps Research Institute Protein/DNA Core Facility). Rab2 Is Essential for Maturation of Pre-Golgi Intermediates

RESULTS

Analysis of Transport in Vitro—NRK or CHO clone 15B cells were infected for 4 h with the temperature-sensitive VSV strain ts045, then biosynthetically labeled with 100 μCi of Tran35S-label for 10 min at the restrictive temperature (39.5 °C) to accumulate VSV-G mutant in the ER. The cells were then perfused by washing and scraping and employed in an in vivo ER to cis/medial Golgi transport assay as described (20, 21). Briefly, transport reactions were performed in a final volume of 40 μl in a buffer containing 25 mM Hepes-KOH (pH 7.2) 75 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA, 1.8 mM CaCl2, 1 mM UDP-N-acetylglucosamine (100 μM UDP-galactose and 100 μM CMP-sialic acid were added where indicated under "Results"), an ATP-regenerating 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 protein in 25 mM Hepes-KOH (pH 7.2) 250 mM NaF, 10 mM EDTA) containing the following semi-intact cells (5 × 107 cells/ml) in 50 mM Hepes-KOH, 90 mM KOAc (pH 7.2). To the assay was added the respective peptide as described under "Results." The reactions were preincubated on ice for 30 min, and subsequently incubated for 90 min at 32 °C and transferred to ice to terminate transport; the membranes were then pelleted, solubilized in buffer, and digested with endo H or endoglycosidase D (endo D). The samples were analyzed by SDS-PAGE and the fraction of VSV-G protein processed to the endo H-resistant or endo D-sensitive forms quantitated by the PhosphorImager.

Indirect Immunofluorescence—NRK cells plated on coverslips were infected with ts045 at 39.5 °C for 2–3 h, then shifted to ice and permeabilized with digitonin (20 μg/ml) as outlined previously (22). Coverslips containing the permeabilized cells were inverted and placed in tissue culture wells containing the transport mixture described above, preincubated on ice for 15 min with or without peptide, and then incubated for 80 min at 15 °C or 20 min at 32 °C. To terminate transport, the cells were fixed in 3% formaldehyde, PBS for 10 min on ice. Fixation was quenched with 10 mM ammonium chloride in PBS for 10 min, and the cells were then blocked in PBS with 5% normal goat serum for 10 min at room temperature. To detect intracellular VSV-G, cells were re-permeabilized with 0.05% saponin in PBS/normal goat serum for 10 min, washed with PBS, then incubated for 30 min with a monoclonal antibody specific for the VSV-G protein cytoplastic tail (P5D4) (13). Where indicated, cells were co-stained with antibody to α-1,2-mannosidase II, a marker for the cis/medial Golgi compartments (23) or to p58, a marker for pre-Golgi intermediates (24). Cells were then washed with PBS, costained with 30 min with Texas Red goat anti-mouse and/or fluorescein isothiocyanate goat anti-rabbit antibody, mounted, and viewed under a Zeiss Axiosvert Fluorescence microscope.

Triton X-114 Partitioning—HeLa cells were vaccinia infected/ transfected with the Rab mutants as described above, then lysed and subjected to partitioning with pre-cycled Triton X-114 (Sigma) as described (24). The aqueous and detergent-rich phases were precipitated with seven volumes of acetone for 1 h at −20 °C. After centrifugation, the resulting pellets were resuspended in sample buffer (19), separated by SDS-PAGE, and transferred to nitrocellulose (Schleicher & Schuell) in 25 mM Tris (pH 8.3), 192 mM glycine, 20% methanol. The membrane was blocked in TBS containing 5% nonfat dry milk and 0.5% Tween 20, incubated with a monoclonal antibody to Rab2 (ICSB5) washed, further incubated with a horseshadish peroxidase-conjugated antibody (Pierce), and then developed with enhanced chemiluminescence (ECL) (Amer sham). Quantitation of Rab2 protein was performed by densitometry (Molecular Dynamics, Sunnyvale, CA).

RESULTS

The NH2 Termi of Rab2 Is Required for Function in Vivo—To study the effects of mutant forms of Rab2 on vesicular traffic in vivo, we employed a transient expression system (12, 18) in which cells were co-transfected with plasmids that encode wild-type or mutant Rab, and a plasmid that encodes the cargo molecule VSV-G (12). ER to Golgi transport is measured by following the processing of VSV-G to endo H-resistant forms during transport through compartments of the Golgi stack (12, 21, 22).

Previous studies demonstrated that a mutation in the GTP-binding domain of Rab2 (Rab2(N119I)) resulted in a protein that potently inhibited transport of VSV-G between the ER and the Golgi complex (12). If the NH2 terminus contains critical residues for Rab2 function, deletion of these residues would eliminate the dominant inhibitory phenotype of the mutant. Overexpression of wild-type Rab2 caused a moderate decrease (25%) in the processing of VSV-G to endo H-resistant forms (Fig. 1A, lane b). A similar partial decrease in protein transport through late Golgi compartments has been observed in cells that overexpress wild-type Rab5 (25). In contrast, cells that express amino-terminal truncated wild-type Rab2 proteins missing 11 (Fig. 1A, lane c) and 14 (Fig. 1A, lane d) residues transported VSV-G to the trans-Golgi as efficiently as the controls (Fig. 1A, lane a). Analogous NH2-terminal deletions of the Rab2(N119I) mutant, which inhibits ER to Golgi transport (Fig. 1B) (12), led to a progressive loss in the ability of this
FIG. 1. Deletion of the amino terminus of a Rab2 mutant leads to loss of function in vitro. Amino-terminal deletion mutants of wild-type Rab2 and Rab2(N119I) were generated, then co-transfected with a plasmid encoding VSV-G into HeLa cells infected with vaccinia T7 RNA polymerase recombinant virus as described under “Experimental Procedures.” Panel A, a, control (VSV-G only); b, Rab2 wild-type; c, Rab2(Δ11); d, Rab2(Δ14); e, Rab2(ΔCC); f, Rab2(Δ14-ΔCC). Panel B, a, control (VSV-G only); b, Rab2(N119I); c, Rab2(N119I-Δ11); d, Rab2(N119I-Δ14); e, Rab2(N119I-ΔCC); f, Rab2(N119I-Δ14-ΔCC). The fraction of VSV-G processed to endo H-resistant forms (% of total) was measured for each construct. The results are typical of three independent experiments.

A critical lysine residue found in the NH2 terminus of Rab5 and other Rab proteins (see Table II) has been reported to be mutant to arrest vesicular traffic (Fig. 1B, lanes c and d). These results suggest that the deleted amino acids are necessary for both wild-type Rab2 and Rab2(N119I) function.

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A critical lysine residue found in the NH2 terminus of Rab5 and other Rab proteins (see Table II) has been reported to be mutant to arrest vesicular traffic (Fig. 1B, lanes c and d). These results suggest that the deleted amino acids are necessary for both wild-type Rab2 and Rab2(N119I) function. Comparative analysis in the presence of cytosol and ATP (21, 22) showed that Rab2 mutants were completely lost when either His6- or hemagglutinin-tagged proteins were used to reconstitute transport of VSV-G from the ER to the Golgi stack (20, 21). This assay utilizes tsO45 VSV-G, a protein that has a thermoreversible defect in export from the ER. Cells were infected for 4 h at the restrictive temperature (39.5 °C) to retain VSV-G exclusively in the ER (22), then rapidly transferred to ice and perforated to generate semi-intact cells. Export of VSV-G from the ER was initiated by incubation of perforated cells at the permissive temperature (32 °C) in the presence of cytosol and ATP (21, 22).

To facilitate purification of recombinant Rab2 for use in vitro, wild-type and mutant forms were epitope-tagged at their NH2 termini. Surprisingly, and in contrast to epitope-tagged Rab1 proteins, which can be modified with His6 or Myc, or hemagglutinin tags at their NH2 terminus and retain full activity (26, 27), the inhibitory activity of Rab2 mutants was completely lost when either His6- or hemagglutinin-tagged proteins were transfected in vitro (data not shown). While these results suggest that the NH2-terminal domain of Rab2 is required for function, the inability to epitope-tag active forms of Rab2 made it difficult to obtain sufficient amounts of wild-type or mutant proteins from recombinant sources to study function in vitro. We therefore examined whether peptidomimetics to the NH2 terminus of Rab2 or other Rab proteins might interfere with the transport of VSV-G between the ER and the Golgi stack in vitro.

The peptides generated are summarized in Table II. As shown in Fig. 2 (open triangles), incubation of semi-intact cells with the peptide that corresponds to the amino-terminal 13 amino acids of Rab2 following the initiating methionine strongly inhibited the processing of VSV-G to endo H-resistant forms in a dose-dependent manner. Transport was reduced by 50% at a peptide concentration of 25 μM (Fig. 2). This concentration is 5-fold lower than that reported to be required for the equivalent Rab5 NH2-terminal peptide to inhibit endosome

| Rab protein | Detergent phase | Aqueous phase |
|-------------|----------------|---------------|
| Rab2wt endogenous | 78% | 22% |
| Rab2wt (expressed) | 53% | 47% |
| Rab2(Δ11) | 47% | 53% |
| Rab2(Δ14) | 52% | 48% |
| Rab2(ΔCC) | 5% | 95% |
| Rab2(N119I) | 42% | 58% |
| Rab2(N119I-Δ11) | 41% | 59% |
| Rab2(N119I-Δ14) | 48% | 52% |
| Rab2(N119I-ΔCC) | 5% | 95% |
| Rab2(N119I-Δ14-ΔCC) | 5% | 95% |

*a* Triton X-114 extraction was performed as described under “Materials and Methods.”
**Rab2 Is Essential for Maturation of Pre-Golgi Intermediates**

**Table II**

| Peptides synthesized for in vitro assay |
|----------------------------------------|
| Rab protein | N-terminal alignment | Peptide |
|------------|---------------------|---------|
| Rab1B      | --RPEYDYLKLLLIGD--  | NPEYDYLKLLLIGD |
| Rab2       | --MAYAYLFK--        | AAYAYLFK |
| 7-mer      | --MAYAYLFK--        | AAYAYLFK|
| 13-mer     | --MAYAYLFK--        | AAYAYLFK|
| Random     | --MAYAYLFK--        | AAYAYLFK|
| Rab3A^b    | --MASATDSRYGQKESSDQNFDYMFKNKMI--  | ASATDSRYGQK |
| Rab5^b     | --MNRRATRGPGNPTNGKICQKL--  | ANRGATRPNGPNTNGK |

^a The N-terminal regions of Rab proteins are aligned as described (9).
^b Underline indicates peptide used in present studies.
^c The lysine residue critical for prenylation of Rab5 is shown in bold.
^d The N-terminal peptide inhibits endosome fusion (7).

**FIG. 2. A peptide that corresponds to the NH2 terminus of the Rab2 protein inhibits ER to Golgi transport.** Semi-intact NRK cells were preincubated with peptides that correspond to the NH2-terminal sequence of either Rab1B (closed circles), Rab2 (7-mer) (closed triangles), Rab2 (13-mer) (open triangles), randomized Rab2 (13-mer) (open squares), Rab3A (open circles), and Rab5 (closed squares) for 30 min on ice in a transport mixture as described under “Experimental Procedures.” The cells were then transferred to 32 °C and incubated for a total of 90 min. The fraction of VSV-G protein processed to the endo H-resistant forms (% of total) was determined as described under “Experimental Procedures.”

The intracellular localization of VSV-G from the ER to pre-Golgi forms is composed of clusters of ER-derived carrier vesicles and short tubular elements (referred to as VTCs) (10, 11, 21, 22). Subsequent transport of VSV-G from VTCs to the cis/trans-Golgi compartments results in its processing to the first endo H-resistant R1 oligosaccharide form, which terminates in GlcNAc residues. Transport through sequential Golgi compartments to the trans Golgi network results in processing to the endo H-resistant Rf form, which contains galactose and sialic acid (21). Each of these forms has unique mobility in SDS-PAGE, allowing us to quantitate ER to Golgi and intra-Golgi transport separately (21).

To determine the rate at which transport of VSV-G to endo H-resistant Golgi forms was inactivated by the Rab2 (13-mer) peptide, semi-intact NRK cells were incubated at 32 °C in the absence (Fig. 3A, closed circles) or presence (Fig. 3A, open circles) of an inhibitory concentration of peptide (75 μM). At the time indicated (Δt in Fig. 3A) cells were pelleted, resuspended in transport mixture that lacked the peptide, and subsequently incubated for a total time of 90 min at 32 °C. Peptide was found to rapidly inhibit transport and prevent the processing of VSV-G to total endo H-resistant forms with a t½ of ~45 s (Fig. 3A, open circles). This inhibition was irreversible, which suggests that the peptide binds and inactivates a critical transport factor within minutes of incubation in vitro.

The time period in transport sensitive to the Rab2 peptide was determined by the addition of peptide after increasing time of incubation at 32 °C. One set of cells served as controls and were transferred to ice at the time of peptide addition to determine the extent of processing of VSV-G to the transient endo H-resistant Rf form and the terminal Rf form (Fig. 3B, closed and open circles, respectively). A second set of cells supplemented with peptide at the indicated time (Δt) were incubated for a total of 150 min (Fig. 3B, circles) to chase any VSV-G that had migrated beyond the peptide-sensitive step to downstream compartments of the Golgi stack. Processing of VSV-G to the R1 form in controls transferred to ice occurred with an ~10-min lag period (Fig. 3B, closed squares) with 50% processing to the R1 form by 20 min of incubation. In contrast, transport through the peptide-sensitive step (Fig. 3B, closed circles) preceded processing of VSV-G to the R1 form in the control (Fig. 3B, closed squares) by ~10 min. In this case, 50% of the VSV-G was transported past the peptide-sensitive step by the 10-min time point based on processing of VSV-G to the R1 form (Fig. 3B, open circles). At no time did the peptide lead to the accumulation of VSV-G in the R1 form (Fig. 3B, closed circles), as VSV-G was efficiently chased to the fully processed Rf form diagnostic of delivery to trans Golgi compartments (Fig. 3, open circles). These results demonstrate that the peptide specifically interferes at an early step in protein transport which precedes delivery of VSV-G to the Golgi stack.

**Rab2 Peptide Inhibits ER to Golgi Transport at a Step Coincident with Inhibition by GTPyS—The intracellular localiza-**
the partial accumulation of VSV-G in ER-derivied vesicles, which co-localize with VTCs (26, 28, 31). Semi-intact NRK cells were incubated at 32 °C in a transport mixture for increasing time prior to the addition of GTPγS (Fig. 5, closed triangles) or peptide (Fig. 5, closed squares), and subsequently incubated for a total of 90 min. Transport of VSV-G through the peptide-sensitive step was nearly coincident with that of the GTPγS-sensitive step. In both cases, the block in transport preceded the processing of VSV-G to the R₁ form by ~5–10 min and >80% of the VSV-G was transported through this step by 15 min (Fig. 5). Since the t₁/₂ of inhibition by GTPγS (2–3 min) (28) is slightly slower than that of peptide (Fig. 3; ~45 s), it is evident that the peptide interferes with events temporally associated with the uncoating of ER-derived vesicles, possibly involving in the delivery of cargo to pre-Golgi VTCs.

**Peptide Acts Prior to a Rate-limiting Step in the Maturation of 15 °C VTCs**—Inhibition of intact or permeabilized cells at reduced temperature (15 °C) leads to the accumulation of VSV-G protein in VTCs as determined morphologically (22, 32, 33). To determine if peptide inhibited transport from these 15 °C-VTCs, semi-intact cells were first preincubated at 15 °C for 80 min (Fig. 6, open symbols). When these cells were subsequently transferred to 32 °C (Fig. 6, open circles), no lag was observed in the processing of VSV-G to the endo D-sensitive form (Fig. 6, closed squares). In contrast, export from the ER has a 15–20-min lag period preceding processing of VSV-G (Fig. 6, open circles). These results illustrate that VSV-G accumulated in VTCs during the preincubation period. Addition of peptide immediately prior to release from the 15 °C block pre-
of the Rab2 peptide on transport relative to the site of action of calphostin C, semi-intact NRK cells were incubated at 32 °C and at the indicated time (Δt), supplemented with either 120 nM calphostin C (Fig. 7, closed circles) or 75 μM Rab2 (13-mer) peptide (Fig. 7, open circles), then incubated for a total of 90 min. The temporal site of inhibition by calphostin C (Fig. 7, closed circles) in protein traffic markedly preceded the block by the Rab2 peptide (Fig. 7, open circles). These results are consistent with the possibility that the transport component sensitive to the Rab2 peptide is downstream of events directing the recruitment of VSV-G to ER-derived vesicular carriers.

**Rab2 Peptide Reduces the Formation of VSV-G-containing VTCs**—In order to define morphologically the site of accumulation of VSV-G in response to the Rab2 (13-mer) peptide, NRK cells were infected with tsO45-VSV-G for 3 h at the nonpermissive temperature to restrict VSV-G to the ER. The cells were then permeabilized (22), incubated in the presence or absence of peptide for 20 min at 32 °C, and the distribution of VSV-G determined by indirect immunofluorescence (20). Control cells efficiently transported VSV-G to a perinuclear location that contained the compact Golgi complex (Fig. 8A). Unlike the control, peptide-treated cells accumulated VSV-G in small, punctate structures, which were scattered through the peripheral cytoplasm (Fig. 8B). These structures were much smaller, less abundant, and more peripherally located compared with VTCs, which accumulate at reduced temperature (Fig. 8C). Incubation of cells at reduced temperature in the presence of the peptide also led to substantial reduction in the appearance of VSV-G in VTCs (data not shown). Although we were concerned that the peptide might directly affect the integrity of the Golgi complex, as has been suggested for other studies employing the amphipathic peptide mastoparan (36), we found no change in the structure of the Golgi as assessed by the distribution of the cis/medial Golgi marker α-1,2-mannosidase II following exposure to peptide (Fig. 8, compare panel A, inset (minus peptide)) to panel B, inset (plus peptide)). These morphological results are consistent with our biochemical data and further demonstrate that the change in the size and abundance of VTCs observed in the presence of peptide may reflect the inability of ER-derived vesicles to deliver VSV-G to pre-Golgi intermediates.
that an epitope tag at the NH₂ terminus neutralizes Rab2 transport is in agreement with results reported for Rab5 in interaction with specific components involved in ER to Golgi required for prenylation of Rab2.

This demonstrates that the affects observed with the Rab2 NH₂ terminus were found to play a critical role in endosome fusion (7). The necessity of the NH₂ terminus for Rab2 function is also compatible with the observation that a Rab5/6 chimera, which was generated by substitution of the two switch regions (α2/L5 and α3/L7) and the Rab5 carboxyl terminus for the equivalent domains in Rab6, did not generate a functional Rab5 protein. A biologically active protein capable of promoting endosome fusion was generated only after the Rab5 NH₂ terminus was added to the chimera (6).

DISCUSSION

The NH₂ Terminus of Rab2 Is Required for Its Function—We have evaluated the role of the Rab2 NH₂ terminus in ER to Golgi transport and have provided new evidence that the amino termini of small GTPases can impart specific function. Our results demonstrate that the Rab2 (13-mer) peptide was a potent inhibitor of transport through the early secretory pathway. Addition of a randomized Rab2 (13-mer) peptide, a truncated (7-mer) peptide, or similar peptide mimetics of the Rab3A and Rab5 amino-terminal domains had no effect on transport. This demonstrates that the effects observed with the Rab2 (13-mer) peptide are specific. A requirement for the amino terminus of Rab2 for normal function is supported by the fact that progressive deletions of the amino-terminal residues of the wild-type Rab2 or Rab2(N119I) mutant resulted in loss of activity in vivo. These results are consistent with our observation that an epitope tag at the NH₂ terminus neutralizes Rab2 function. Although it has been reported that a conserved lysine in the amino terminus of Rab5 is essential for post-translational processing by Rab geranylgeranyltransferase II in vitro (8), we found that truncations which removed this lysine residue did not alter the distribution of Rab2 or Rab2(N119I) between membranes and cytosol. In contrast, deletion of the carboxyl-terminal cysteines of Rab2(N119I) and its amino truncated (Δ14) form resulted in proteins that partitioned to the aqueous phase. It is apparent that the NH₂ terminus is not required for prenylation of Rab2.

The requirement for the Rab2 amino terminus to promote its interaction with specific components involved in ER to Golgi transport is in agreement with results reported for Rab5 in which its NH₂ terminus was found to play a critical role in endosome fusion (7). The necessity of the NH₂ terminus for Rab2 function is also compatible with the observation that a Rab5/6 chimera, which was generated by substitution of the two switch regions (α2/L5 and α3/L7) and the Rab5 carboxyl terminus for the equivalent domains in Rab6, did not generate a functional Rab5 protein. A biologically active protein capable of promoting endosome fusion was generated only after the Rab5 NH₂ terminus was added to the chimera (6).

Rab2 Is Required for the Maturation of Pre-Golgi Intermediates—The ability of the peptide to act rapidly and irreversibly to arrest transport allowed us to map its site of action in the secretory pathway. It is apparent that the peptide was specific for a step in ER to Golgi transport, as no effect could be detected during transport of VSV-G between the cis, medial, or trans Golgi compartments. These findings are consistent with the fact that Rab2 is localized to VTCs (9, 37).

Combined morphological and biochemical data suggest that Rab2 may be involved in the assembly or maturation of pre-Golgi intermediates at a step preceding Ca²⁺-dependent fusion of vesicles to the cis Golgi compartment. Rab2 function at an earlier step is in agreement with the close temporal relationship between the site of peptide inhibition and that observed for GTPγS, a reagent that allows partial vesicle budding from the ER, but prevents further uncoating and delivery of cargo to tubular elements of VTCs (11, 30). Although transit of VSV-G from VTCs accumulated at 15 °C to the cis Golgi was peptide-sensitive, a brief (5-min) incubation at 32 °C rendered transport insensitive to peptide. This leads us to conclude that the Rab2 function sensitive to peptide precedes the reduced temperature defect. Kinetic studies also demonstrated that the peptide temporarily inhibited transport at a step following the site of action of calphostin C, which is known to prevent vesicle budding and export of VSV-G from the ER (34). While this result is consistent with the interpretation that peptide does not interfere with budding, it does eliminate the possibility as the release of individual vesicles from the ER cannot be detected in semi-intact cells (11). Indirect immunofluorescence showed that VTCs that accumulated VSV-G in the presence of peptide were reduced in size and abundance compared to VTCs that accumulate at 15 °C. We conclude that at least one prominent biochemical and phenotypic effect of the peptide, like that of GTPγS, is to block the delivery of cargo present in ER-derived vesicles to recycling compartments of VTCs. Based on previous studies, which implicate members of the Rab family in docking and fusion events at different steps of the exocytic and endocytic pathway (1, 38), we propose that a Rab2-specific component(s) may therefore play a critical role in the assembly of VTCs.

The need for both Rab2 and a second Rab protein, Rab1, in ER to Golgi transport is presently unclear. The evolutionary divergence (~40% identity) (9) between Rab1 and Rab2 would suggest that these proteins are likely to have unique functions. We have shown previously that Rab1 is essential for ER to Golgi transport in vitro (12, 27) and in vivo in mammalian cells (21, 26, 39). The Rab1 homologue in yeast, Ypt1p, is also essential for ER to Golgi transport (27, 38), although a homologue for Rab2 has yet to be detected in yeast. In contrast to the highly localized distribution of Rab2 to pre-Golgi intermediates, Rab1 can be detected in the ER, pre-Golgi intermediates, and early compartments of the Golgi stack (40, 41). Depletion of Rab1 blocks the appearance of VSV-G in VTCs in vitro (40, 42). Moreover, a Rab1A mutant restricted to the GDP-bound form (Rab1A(S25N)) (which blocks recruitment of wild-type Rab) inhibits export of VSV-G from the ER in vivo and in vitro (12, 27), while a mutant with a high nucleotide exchange rate

**Fig. 1. Rab2 (13-mer) reduces the rate of VSV-G accumulation in pre-Golgi VTCs.** NRK cells grown on coverslips were infected with tsO45 VSV for 3 h at 39.5 °C to retain tsO45 VSV-G in the endoplasmic reticulum. The cells were rapidly shifted to ice, permeabilized with digitonin (A and B) (22), and then incubated in a complete transport mixture in the absence (A) or presence (B) of 75 μM Rab2 peptide at 32 °C for 40 min. The distribution of VSV-G was determined by indirect immunofluorescence as described under “Experimental Procedures.” In panel C, infected NRK cells were incubated for 80 min at 15 °C to accumulate VSV-G in pre-Golgi intermediates, and then examined using indirect immunofluorescence as described above. Small arrows denote VTCs.
Rab2 Is Essential for Maturation of Pre-Golgi Intermediates

This study investigates the role of Rab2 in the transport of cargo from the ER to Golgi compartments. A dual requirement of Rab1 and Rab2 in transport is observed: Rab2 is required for transport of cargo to VTCs and Rab1 for the subsequent fusion of VTCs to the Golgi stack. Consistent with the latter interpretation, VSV-G accumulates in enlarged VTCs at the cis face of the Golgi stack when semi-intact cells are incubated either in the presence of peptides specific for the effector domain of Rab1 or with the Rab1(N124I) mutant. Further experiments are in progress to address these two possibilities and determine the specific roles of Rab2 and Rab1 in ER to Golgi transport.

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