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Synthetic peptides of the *Rab* effector domain inhibit vesicular transport through the secretory pathway

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Synthetic peptides of the putative effector domain of members of the *ras*-related *rab* gene family of small GTP-binding proteins were synthesized and found to be potent inhibitors of endoplasmic reticulum (ER) to Golgi and intra-Golgi transport *in vitro*. Inhibition of transport by one of the effector domain peptides was rapid ($t_{1/2}$ of 30 s), and irreversible. Analysis of the temporal site of peptide inhibition indicated that a late step in transport was blocked, coincident with a Ca^{2+}-dependent pre-fusion step. The results provide novel biochemical evidence for the role of members of the *rab* gene family in vesicular transport in mammalian cells, and implicate a role for a new downstream Rab effector protein (REP) regulating vesicle fusion.

Key words: GTP binding protein/*rab*/*ras*/vesicular transport

Introduction

The discovery of the *ras* superfamily of small GTP-binding proteins, which includes the *ras*, *ral*, *rho*, *rap*, YPT1, SEC4 and the *rab* gene families (Santos and Nebreda, 1989; Burgoyne, 1989), is having an important impact on diverse areas of cell biology. The *ras* gene product has been extensively characterized at the biochemical, molecular and structural levels (Barbacid, 1987; Gibbs and Marshall, 1989; DeVos, 1988; Pai *et al.*, 1989; Milburn *et al.*, 1990). Evidence for the role of small GTP-binding proteins in vesicular transport through the secretory pathway of eukaryotic cells originated from the identification of the SEC4 gene product in yeast (Salminen and Novick, 1987, 1989; Goud *et al.*, 1988; Walworth *et al.*, 1989). In the absence of functional SEC4p, carrier vesicles fail to fuse with the plasma membrane and accumulate in the cell (Goud *et al.*, 1988). Vesicular transport between earlier stages of the secretory pathway in yeast requires other small GTP-binding proteins including YPT1 (Segev *et al.*, 1988; Schmitt *et al.*, 1988; Baker *et al.*, 1990; Bacon *et al.*, 1989), SAR1 (Nakano *et al.*, 1988; Nakano and Muramatsu, 1989), and ARF (Stearms *et al.*, 1990). Incubation of a temperature-sensitive mutant of YPT1 at the restrictive temperature results in the extensive elaboration of both ER and Golgi membranes (Schmitt *et al.*, 1988) and in an inhibition of protein transport through compartments of the Golgi *in vivo* (Schmitt *et al.*, 1988; Segev *et al.*, 1988) and *in vitro* (Bacon *et al.*, 1989; Baker *et al.*, 1990). YPT1p has been localized to Golgi rich regions in both yeast and mammalian cells (Segev *et al.*, 1988). In addition, the non-hydrolyzable analog of GTP, GTPγS, blocks ER to Golgi transport *in vitro* in mammalian cells (Beckers and Balch, 1989) and yeast (Baker *et al.*, 1988; Ruohola *et al.*, 1989) and inhibits intra-Golgi transport *in vitro* (Melancon *et al.*, 1987).

Vesicular transport between compartments of the secretory pathway in mammalian cells is likely to involve the *rab* gene family (Balch, 1989). *Rab* genes have been cloned from rat (Touchot *et al.*, 1987), mouse (Haubruck *et al.*, 1987) and human libraries (Zahraoui *et al.*, 1989) and the family now includes at least eight related members. The *rab* gene products, to be referred to as 'Rab' proteins, are 21–25 kd GTP-binding proteins with primary sequence homologies to yeast YPT1 ranging from 76% (Rab1) to 36% (Rab6) (Zahraoui *et al.*, 1989). The strong sequence homology between YPT1 and the *rab1* gene product has led to the suggestion that Rab1 is the mammalian counterpart of YPT1. This hypothesis is supported by the fact that mouse *rab1* can complement mutant yeast lacking YPT1 (Haubruck *et al.*, 1989).

The striking conservation of domain structure between YPT1, members of the *rab* gene family and *ras* strongly suggest that both the secondary and tertiary structure of these proteins are also highly conserved (DeVos *et al.*, 1988; Pai *et al.*, 1989; Tong *et al.*, 1989). These include the three highly conserved sequences involved in GTP-binding, a conserved C-terminus containing cysteine residues essential for membrane association, as has been observed for Ras (Barbacid, 1987; Casey *et al.*, 1989; Hancock *et al.*, 1989; Lowry and Willusen, 1989; Schafer *et al.*, 1989; Jackson,J.H., Cochrane,C.G., Bourne,J.R., Solski,P.A., Buss,J.E. and Der,C.J., submitted), and a region of conserved sequence among the Rab proteins (amino acid residues 32–40) corresponding to the Ras effector domain (Sigal *et al.*, 1986; Willumsen *et al.*, 1986; Barbacid, 1987; Santos and Nebreda, 1989; Gibbs and Marshall, 1989). The Ras effector domain is required for regulation of GTP hydrolysis and is essential for interaction of p21 with its putative effector protein, GAP (GTPase activating protein), which stimulates GTP hydrolysis nearly 100-fold (Trahey and McCormick, 1987; Gibbs *et al.*, 1988; Cales *et al.*, 1988; Adari *et al.*, 1988; Vogel *et al.*, 1988; Marshall *et al.*, 1988; Stone *et al.*, 1988; Michaeli *et al.*, 1989). The Ras effector domain undergoes considerable conformational change reflecting the status of bound nucleotide (GDP or GTP), indicating that it is one of the key switch regions regulating Ras function (Milburn *et al.*, 1990).

If functional domains involved in GTP-binding and regulation of hydrolysis are evolutionarily constrained, then the extensive molecular, genetic, biochemical and structural information accumulated for Ras might provide a useful model for the role of Rab protein(s) in vesicular transport in the secretory pathway of mammalian cells. In the following studies, we show that using p21 as a predictive

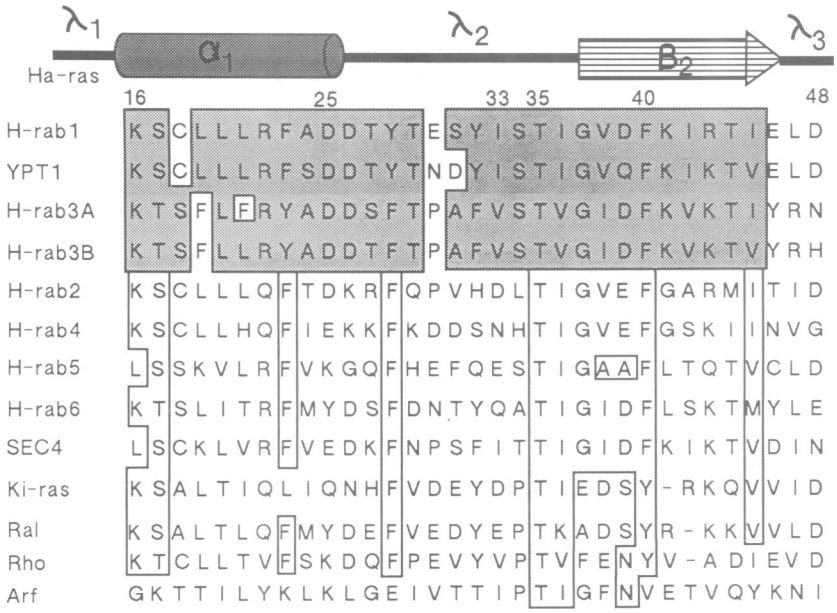

Fig. 1. Sequence alignment of the effector domain (amino acid residues 32−40) and flanking regions of Ha-*ras*, the *rab* gene family, SEC4, YPT1, *arf*, *rho* (*Aplysia*) and *ral*. λ_1, λ_2, λ_3, α_1 and β_2 are the structural motifs assigned to the Ha-*ras*−GTP complex (Pai *et al.*, 1989). The reference numbering is that of Ki-*ras*. The heavily shadowed box defines the regions of amino acid conservation between H-rab1, YPT1, H-rab3A, and H-rab3B. The lightly shadowed box define regions of amino acid conservation between all the effector domain and flanking sequences listed.

paradigm for Rab protein function, synthetic peptide analogs of putative Rab protein sequences corresponding to the Ras effector domain are found to be inhibitors of both ER to Golgi and intra-Golgi transport. These studies provide biochemical evidence for a role of the *rab* gene family in vesicular transport in the early stages of the secretory pathway of mammalian cells, and support the existence of a late-acting Rab 'effector protein', REP (rab effector protein), in the regulation of transport vesicle fusion.

Results

Effector domain peptides inhibit transport in vitro

The strong sequence homology between Rab and Ras proteins suggest that domains essential for Ras function may also be essential for Rab function. In particular, the domain of Rab proteins analogous to the Ras effector domain was thought likely to play a key role in transport. Amino acid residues encompassing the Ras effector domain (residues 32−40) (Figure 1) are found in the surface exposed λ_2-loop of p21 and are flanked by the α_1-helix and the β_2-sheet structural motifs (Figure 1) (Pai *et al.*, 1989; DeVos *et al.*, 1988; Tong *et al.*, 1989). Synthetic peptides encoding amino acid residues found in the Ras effector domain have recently been shown to inhibit Ras−GAP interaction potently (Schaber *et al.*, 1989). Similarly, it was reasoned that synthetic peptides prepared from the homologous Rab effector domain sequences might serve as inhibitors for interaction between Rab and a Rab-specific effector protein(s) to be referred to as REP (*rab* effector protein). Since addition of an effector domain synthetic peptide to an *in vitro* transport reaction may be anticipated to compete for the putative effector protein (REP) triggering GTP hydrolysis, effector domain peptides may inhibit transport in a fashion analogous to that observed by addition of the non-hydrolyzable analog of GTP, GTPγS (Beckers and Balch, 1989).

In addition to the effector domain, two other regions were thought to be of potential interest. The first region includes a highly conserved surface domain found in Rab proteins, analogous to the domain recognized by the neutralizing antibody Y13-259 in Ras (amino acid residues 63−73) (Sigal *et al.*, 1986). This peptide would serve as a useful control in that this domain is not essential for Ras function (Sigal *et al.*, 1986). The second region includes the highly divergent sequences found at the C-terminus of Rab proteins which contain terminal cysteine residues. The C-terminal tail region of Ras (Hancock *et al.*, 1989; Casey *et al.*, 1989), SEC4 (Walworth *et al.*, 1989) and YPT1 (Molenaar *et al.*, 1988) contain Cys residues critical for membrane-association and which, in the case of Ras, have been shown to acquire post-translational modifications by fatty acylation or prenylation (Casey *et al.*, 1989; Hancock *et al.*, 1989; Lowry and Willumsen, 1989; Schafer *et al.*, 1989; Jackson,J.H., Cochrane,C.G., Bourne,J.R., Solski,P.A., Buss,J.E. and Der,C.J., submitted). Addition of synthetic peptides encoding sequences found at the C-terminal tail of Rab proteins may be anticipated to inhibit transport *in vitro* by competition for the enzyme(s) likely to be involved in similar post-translational modifications.

In order to test the effect of synthetic peptides on the transport of protein through the secretory pathway, two different assays were used to monitor the movement of protein between sequential compartments. Vesicular trafficking of protein between the endoplasmic reticulum (ER) and the Golgi, or between Golgi compartments in mammalian cells, can be measured by following the compartment specific carbohydrate modifications associated with the transport of vesicular stomatitis virus (VSV) G protein in viral infected cells (Balch *et al.*, 1984a; Beckers *et al.*, 1987; Beckers and Balch, 1989). Efficient transport *in vitro* between the ER and the Golgi can be reconstituted readily using semi-intact cells, a population of cells in which the plasma membrane has been perforated to release >90% of the soluble cytoplasmic components, but which retain

Table I. Inhibition of ER to Golgi and intra-Golgi transport by synthetic peptides

Peptide	Sequence	ER to Golgi	Intra-Golgi
		Observed inhibition at 100 μM peptide	
Effector Domain			
rab3(30–45)	P A F V S T V G I D F K V T I	+/–	+/–
rab3(33–48)	V S T V G I D F K V K T I Y R N	+/–	+
rab3(36–51)	V G I D F K V K T I Y R N D K R	–	–
rab3AL(33–48)	V S **A L** G I D F K V K T I Y R N	++	++
rab3AL(33–37)	V S **A L** G	–	–
rab3F(33–48)	V S T **F** G I D F K V K T I Y R N	++	++
rab3K(33–48)	V S T F G I D **K** K V K T I Y R N	+/–	+
ras(33–48)	D P T I E D S Y R K Q V V I D	–	–
rasF(33–48)	D P T **F** E D S Y R K Q V V I D	–	–
ral(33–48)	E P T K A D S Y R K K V V L D	–	–
rho(33–48)	V P T V F E N Y V A D I E V D	–	–
Y13-259 epitope			
rab1/ypt1(63–74)	R F R T I T S S Y Y R G	–	–
rab3(63–74)	R Y R T I T T A Y Y R G	–	–
Carboxyl-terminus			
rab1(188–200)	Q S T P V K Q S G G G C C	–	nd
rab2(196–208)	G N Q G G Q Q A G G G C C	–	nd
rab3(188–200)	T D Q Q A P P H G D C A C	–	nd
rab4(195–207)	R R T Z A P S A Q E C G C	–	nd

Synthetic peptides (100 μM final concentration) were added to the transport assays as described in Materials and methods. All peptides were added directly to the assay buffer from 1 mg/ml stock solutions (H_2O). Legend: (–) no inhibition; (+/–) <25% inhibition; (+) >50% inhibition; (++) complete inhibition; (nd) not determined. Sequence alignment according to Touchot *et al.*, 1987 and Zahraoui *et al.*, 1989.

secretory compartments such as the ER and the Golgi as intact, functional organelles (Beckers *et al.*, 1987). Intra-Golgi transport can be differentiated from ER to Golgi transport using an assay in which VSV-G protein is transported from the *cis* to the *medial* Golgi compartments using highly enriched Golgi membranes (Balch *et al.*, 1984a). Both ER to Golgi and intra-Golgi transport assays require the addition of cytosol (the supernatant from high speed centrifugation of crude cell homogenates), and energy in the form of ATP, and both are strongly inhibited in the presence of GTPγS (Balch *et al.*, 1984b; Melancon *et al.*, 1987; Beckers and Balch, 1989).

Table I lists the synthetic peptides prepared for these studies and summarizes their effect on transport of VSV-G protein *in vitro*. Synthetic peptides directed against the highly conserved surface domain (amino acid residues 63–74) common to all members of the *rab* gene family, or the divergent C-terminal residues of Rab 1A, 2, 3, or 4 proteins, showed no inhibition when included in either assay at concentrations of up to 100 μM. In the latter case, these results suggest that competition for fatty acylation or prenylation either is not occurring, or is an inefficient approach to inhibiting transport. In contrast, peptides synthesized to include amino acid residues found in the putative effector domain of the Rab protein family, but not in those of Ras, Ral or Rho, inhibited transport. As shown in Table I, no inhibition of transport was observed in the presence of a 100 μM concentration of peptides spanning amino acid residues 33–48 of the Ras, Ral or Rho proteins, small GTP-binding proteins unlikely to be involved in vesicular transport. These peptides were readily soluble in the assay buffer, a prerequisite for the analysis of their effect on transport *in vitro*. In contrast, peptides synthesized to most of the Rab effector domains were largely insoluble in water with the exception of the Rab3A peptides. No inhibition was observed by the addition of Rab1, YPT1, Rab2 and Rab4 peptides spanning amino acid residues 33–48 up to a final theoretical concentration of 100 μM (data not shown). Since these peptides are insoluble in the assay buffer upon dilution from concentrated DMSO stock solutions, their effect on transport cannot be meaningfully evaluated. In contrast, the water soluble Rab3A peptide [rab3(33–48) (see Figure 1)] showed partial inhibition of both ER to Golgi transport (25%) and intra-Golgi transport (50%) at a final concentration of 100 μM in the assay (Table I). A synthetic peptide encompassing residues 30–45 of Rab3A [rab3(30–45)] yielded similar results (Table I). No inhibition was observed for a peptide spanning amino acid residues 36–51 [rab3(36–51) (Table I)]. Although previous biochemical and genetic studies would suggest that Rab1 is likely to be the small GTP-binding protein involved in ER to Golgi transport, it is important to point out that both Rab1 and Rab3 proteins are highly conserved in sequence across the putative effector domain (see Figure 1).

To examine the sequence requirements for inhibition of transport by the rab3(33–48) synthetic peptide more thoroughly, we synthesized peptide analogs with altered sequences based on recent site-directed mutagenesis studies which have identified key residues in the effector domain of Ras that are essential for Ras–GAP interaction (Adari *et al.*, 1988; Cales *et al.*, 1988; Stone *et al.*, 1988). These residues include Thr(35) and Val(36), which, when substituted with Ala(35) and Leu(36), respectively, inhibit the ability of GAP to stimulate Ras-GTPase activity *in vitro*

and block biological transformation *in vivo* (Adari *et al.*, 1988). Similarly, substitution of Lys for Tyr(40) of Ras prevents GAP stimulated GTPase activity and transformation (Cales *et al.*, 1988). When a synthetic Rab peptide was synthesized in which the Tyr at position 40 was substituted with Lys to form the rab3K peptide, a similar level of inhibition was observed to that obtained with the synthetic peptide encoding the native sequence (Table I). Surprisingly, complete inhibition of both ER to Golgi and intra-Golgi transport was observed in the presence of a rab3AL analog in which amino acid residues Thr(35) and Val(36) were substituted with Ala(35) and Leu(36), respectively (Table I). Inhibition was lost by truncation to form the peptide containing the Ala(35) and Leu(36) substitutions, but spanning only amino acid residues 33–37 (Table I), suggesting either that amino acid(s) found in the C-terminal domain of the peptide were critical for inhibitory activity of the peptide, or that a certain minimum length was essential for stabilization of an inhibitory solution conformation. To examine the effect of non-conservative substitutions in this region, Val(36) was substituted with Phe(36) to generate the rab3F peptide. Again, complete inhibition was observed at a concentration of 100 μM, suggesting that synthetic peptides containing substitutions in this restricted region generate strong inhibitors of a putative Rab–REP interaction leading to a block in vesicular transport. Similar substitutions in the Ras effector domain peptide [rasF(33–48) (Table I)] failed to elicit inhibition of transport. These results suggest that mutations in the Rab3 effector domain sequence provide a solution conformation to the rab3(33–48) peptide which are more efficient in inhibiting transport *in vitro*.

Since at least two separate transport steps, ER to Golgi and intra-Golgi transport were inhibited by the synthetic rab3 peptides [rab3, rab3K, rab3AL and rab3F (Table I)], it was important to determine whether other membrane fusion events were also sensitive to these reagents. *In vitro* fusion of endocytotic vesicles has recently been shown to be inhibited by GTPγS (Mayorga *et al.*, 1989). When the rab3 peptides were tested for their ability to inhibit endosome–endosome fusion (Braell *et al.*, 1987), no inhibition of fusion was observed at concentrations of up to 100 μM for the rab3, rab3AL and rab3F synthetic peptides (T.Redelmeier and S.Schmid, unpublished results).

The combined results of these preliminary studies argue that the rab3 synthetic peptides may be general reagents for inhibition of a common step involving a member of the *rab* gene family in vesicular transport of protein between compartments of the exocytotic pathway.

Rab3AL is a potent inhibitor of transport

The four rab3 peptides found to inhibit transport were analyzed in more detail to determine their relative potency in inhibiting either ER to Golgi, or intra-Golgi transport. As shown in Figure 2 (panel A), while rab3 and rab3K were both relatively weak inhibitors of ER to Golgi transport, both rab3F and rab3AL inhibited transport by 50% at peptide concentrations of 50 μM and 25 μM, respectively. Transport was nearly 5-fold more sensitive to the rab3AL analog than to the synthetic peptide encoding the native Rab3 effector domain sequence [rab3(33–48)] (Figure 2, panel A). A similar inhibition profile was observed for intra-Golgi transport (Figure 2, panel B) with 50% inhibition of transport

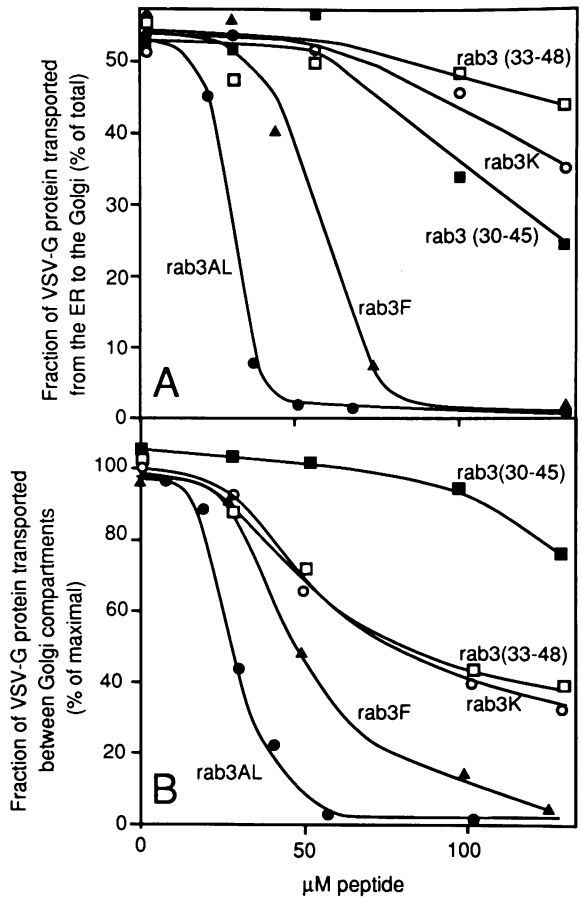

Fig. 2. Inhibition of transport by the rab3AL synthetic peptide. Synthetic peptides (see Table I) were added to ER to Golgi (**Panel A**) or intra-Golgi (**Panel B**) transport assays as described in Materials and methods at the final concentrations indicated prior to the initiation of transport. ER to Golgi transport was terminated after incubation for 90 min at 30°C by transfer to ice. Intra-Golgi transport was terminated after incubation for 60 min at 37°C.

by rab3F and rab3AL also found to require peptide concentrations of 50 μM and 25 μM, respectively.

To insure that rab3AL was not inhibiting transport as a consequence of non-specific disruption of the integrity of cellular membranes, rab3AL was incubated in the presence of intact cells transporting VSV-G protein from the ER to the Golgi. No inhibition of transport *in vivo* was observed in the presence of 150 μM peptide (data not shown). The presence of an intact plasma membrane precludes access of the peptide to both cytoplasmic factors and organelles normally highly accessible in semi-intact cells, suggesting that it is unlikely that the peptide can either penetrate or disrupt the membranes involved in the transport of VSV-G protein *in vitro*. In addition, the distribution of polar and non-polar residues of rab3AL on a helical wheel projection provides no evidence for the presence of an amphipathic structure which would confer detergent-like properties on the peptide.

Transport is rapidly inactivated by rab3AL

We have previously shown that the non-hydrolyzable analog of GTP, GTPγS, irreversibly inhibits ER to Golgi transport with a $t_{1/2}$ of 2–3 min during incubation of semi-intact cells

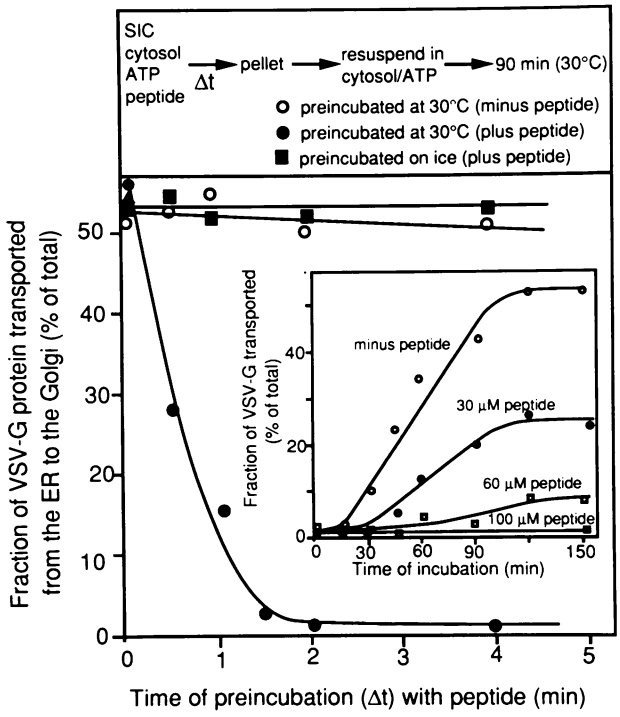

Fig. 3. Rab3AL rapidly inactivates VSV-G protein transport from the ER to the Golgi. ER to Golgi transport assays containing semi-intact cells (SIC), cytosol, ATP and (±) rab3AL (60 μM) were preincubated on ice in the presence of peptide (closed squares), or at 30°C in the absence (open circles) or presence (closed circles) of peptide. After the indicated time of preincubation (Δt), cells were rapidly pelleted (5 s in an Eppendorf microfuge) and resuspended in an assay buffer (without peptide) containing fresh cytosol and ATP, and incubated for a total of 90 min at 30°C. (**Insert**) ER to Golgi transport assays as described in the Materials and methods contained the indicated concentration of rab3AL prior to initiation of transport. Assays were incubated at 30°C and transport terminated at the indicated time by transfer to ice.

at 30°C (Beckers and Balch, 1989). To determine the time-course of inactivation by peptide, semi-intact cells were preincubated with cytosol and ATP for increasing time in the presence of excess peptide (60 μM) prior to pelleting and resuspension of cells in the absence of peptide. Under these conditions, a rapid and irreversible inactivation of transport was observed. Transport between the ER and the Golgi was inhibited with a $t_{1/2}$ of 30 sec (Figure 3). In contrast, when cytosol (in the absence of semi-intact cells) was pretreated with peptide and the peptide removed by gel filtration, no transport inhibition was observed (data not shown). A similar lack of inhibition was observed when both cells and cytosol were incubated for increasing time on ice in the presence of peptide prior to pelleting and incubation in the absence of peptide (Figure 3, closed squares). Since incubation of semi-intact cells on ice in the presence of 0.01% detergent inactivates transport (data not shown), these results also rule out the possibility of a non-specific detergent effect of the peptide on membrane integrity. These results also suggest that an essential transport component(s) which recognizes the Rab protein effector domain may be rapidly and non-competitively inhibited by peptide.

To determine whether inactivation by peptide results in a reduction of the extent of transport, the rate of transport, or both, semi-intact cells were incubated in the presence of peptide for an extended 150 min incubation. As shown in Figure 3 (insert), increasing concentrations of peptide in the transport reaction reduced both the observed rate and the extent of transport, suggesting that the concentration of REP is a potential regulatory step in ER to Golgi transport.

Peptides are generally sensitive to a wide range of exo- and endo-proteases. Although inhibition was found to be very rapid *in vitro*, it was important to determine the stability of the peptide in an incubation cocktail containing semi-intact cells and cytosol to accurately assess the potency of the peptide. Since the rab3AL peptide contains several trypsin cleavage sites (see Table I), we first tested whether peptide pretreated with trypsin would retain its ability to inhibit transport. Pretreatment of peptide for 1 min on ice with trypsin (1 μg trypsin/μg peptide) prior to addition to the assay completely inactivated the peptide (data not shown). However, in contrast the trypsin sensitivity, rab3AL was found to be stable in the presence of semi-intact cells and cytosol for at least 10 min (data not shown). These results, coupled with the ability of rab3AL to rapidly inactivate transport (Figure 3) indicate that the concentration of rab3AL added to the assay is an accurate measure of the sensitivity of vesicular transport to the peptide.

Rab3AL inhibits a late, Ca^{2+}-dependent step in transport

GTPγS and the calcium chelator EGTA inhibit different steps in the transport of protein between the ER and the Golgi. GTPγS inhibits an early step in transport (Beckers and Balch, 1989); EGTA inhibits a late step immediately prior to fusion of a carrier vesicle to the *cis* Golgi compartment (Beckers and Balch, 1989; Beckers,C.J.M., Plutner,H. and Balch, W.E., submitted). To define the temporal site of inhibition by peptide, transport of VSV-G protein was initiated by incubation at 30°C. Subsequently, peptide was added after different times of incubation to individual transport reactions. These reactions were subsequently incubated for a total time of 90 min to allow any VSV-G protein which had been transported beyond the peptide-sensitive step at the time of peptide addition to progress to the *cis* Golgi compartment for processing by α-1,2-mannosidase I. As shown in Figure 4 (closed circles), addition of peptide at both early or late incubation times resulted in an immediate inhibition of transport. In contrast, in a parallel experiment in which transport was interrupted by the addition GTPγS, inhibition was lost after the first 15–20 min of incubation (Figure 4, open squares). Since the kinetics of GTPγS inhibition reflect inhibition of an early step involved in the binding of GTP or a GTP–protein complex to a transport intermediate, the kinetics of inhibition by peptide suggests that the protein recognizing the Rab effector domain is temporally uncoupled from the acquisition of GTP (or a GTP–protein complex), and occurs at a late step preceding fusion to the *cis* Golgi compartment.

To define the temporal role of REP in vesicular transport more explicitly, we examined the effect of peptide on a late transport intermediate in which VSV-G protein accumulates during prolonged incubation in the presence of EGTA (Beckers and Balch, 1989). Transport from this intermediate requires the addition of μM concentrations of Ca^{2+} to the assay, but is insensitive to the addition of GTPγS, suggesting that an earlier step involving binding of GTP (or a GTP–protein complex) is complete. However, it is

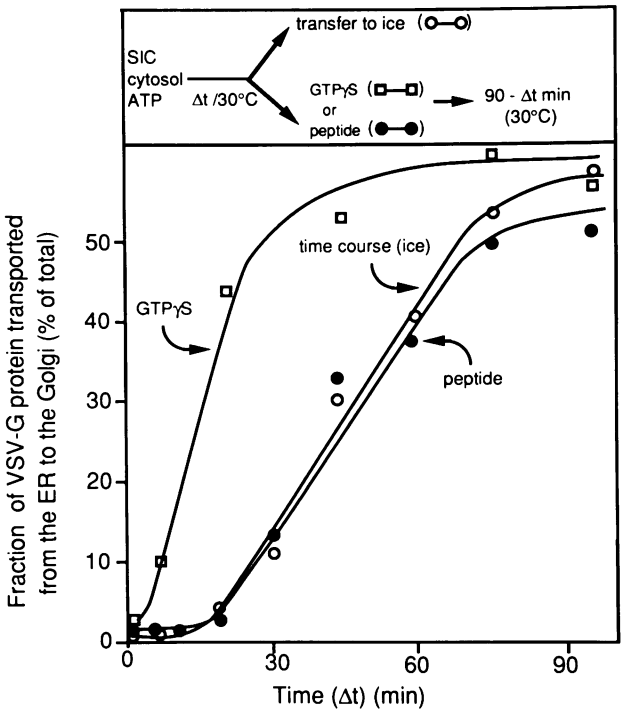

Fig. 4. Peptide inhibits a late step in ER to Golgi transport. ER to Golgi transport assays containing semi-intact cells (SIC), cytosol and ATP were incubated for the indicated time (Δt) at 30°C prior to transfer to ice (open circles), addition of rab3AL (60 μM, closed circles), or addition of GTPγS (3 μM) (open squares). Assays supplemented with peptide or GTPγS at Δt were incubated for a total of 90 min at 30°C prior to termination of transport by transfer to ice.

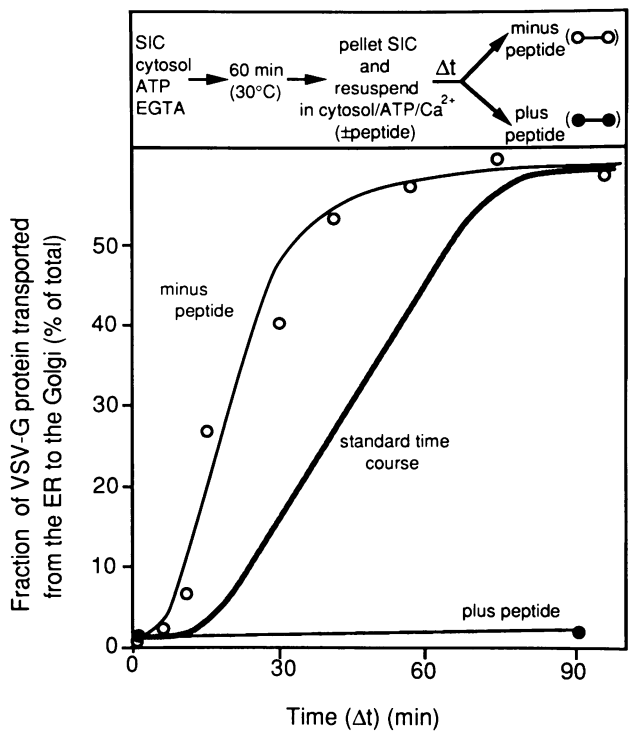

Fig. 5. Peptide inhibits transport from the CA^{2+}-dependent step. ER to Golgi transport assays containing semi-intact cells (SIC), cytosol and ATP were preincubated for 60 min at 30°C in the presence of 5 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA). Subsequently, semi-intact cells were pelleted and incubated for the indicated time (Δt) in the presence of fresh cytosol, Ca^{2+} and ATP and in the absence (open circles) or presence (closed circles) of rab3AL. The time course begins at the time of reincubation in the absence of EGTA. Heavy solid line: a typical time course for transport of VSV-G protein from the ER to Golgi.

unknown whether GTP hydrolysis occurs during the time period in which VSV-G protein accumulates in the EGTA intermediate, or occurs after release from the EGTA block by the addition of Ca^{2+} to the transport reaction. As shown in Figure 5 (open circles), transport of VSV-G protein from the EGTA intermediate to the *cis* Golgi compartment occurs without a lag period and with a nearly 2−3 fold increase in rate compared to export from the ER (Figure 5, solid line), indicative of the accumulation of VSV-G protein in a late, downstream intermediate. Addition of peptide to the transport reaction prior to release from the EGTA block potently inhibited transport (Figure 5, closed circles). It is apparent that transport from this late step requires the activity of a downstream component which recognizes the Rab effector domain and may be involved in triggering subsequent fusion events.

Discussion

Specificity of inhibition of rab3 synthetic peptides

The strong sequence homology between Ras and members of the *rab* gene family has provided a useful paradigm to identify potential functional domains within Rab proteins which may be important for their role in vesicular transport. The current results directly demonstrate that peptides encoding a region of amino acid sequence defined as the putative effector domain of Rab proteins potently inhibit transport between the ER and the Golgi, and between compartments of the Golgi. These peptides appear to inhibit transport selectively within the exocytotic pathway; no inhibition was observed in the case of endosome−endosome fusion, an event associated with transport of protein through the endocytotic pathway. Since synthetic peptides encoding the highly conserved domain spanning amino acid residues 63−74 [equivalent to the functionally non-essential hydrophilic surface domain recognized by the Y13-259 antibody on Ras (Sigal *et al.*, 1986)], and those encoding a portion of the C-terminal tail failed to inhibit transport, it is clear that the addition of synthetic peptides in concentrations of up to 100 μM does not result in a general non-specific inhibition of transport *in vitro*. The inability of the peptide to inhibit transport in intact cells coupled with its inability to inhibit transport during preincubation on ice rules out the trivial interpretation that inhibition is simply due to a non-specific detergent effect on membranes. Finally, the ability to make conservative or non-conservative substitutions of specific amino acid residues which potentiate the inhibitory capacity of the peptide, presumably through alteration of the solution conformation of the peptide, argues against a non-specific charge effect.

Although we have not completed an exhaustive survey of synthetic peptides encompassing putative effector domain sequences for all GTP-binding proteins, peptides encoding amino acid residues 33−48 of the Ras, Ral, and Rho effector domains failed to inhibit transport. In particular, rasF(33−48) was totally inactive, in contrast to the strong inhibition observed with the equivalent rab3F(33−48) peptide. We conclude from these results that the inhibition

observed by the addition of the Rab3A effector domain peptides to the assay reflects competitive inhibition of the activity of a member(s) of the *rab* gene family in transport. Since the studies described here focus on a synthetic peptide encompassing the putative effector domain of the Rab3A protein, can we conclude that Rab3A is the key small GTP-binding protein regulating transport between two different (ER to Golgi and intra-Golgi) stages of the secretory pathway? We think that this is an unlikely interpretation for several reasons. First, the Rab3A effector domain peptides were water soluble, a property essential for analysis of their function in our assay. The equivalent Rab1A effector domain peptide and its close homolog, the yeast YPT1 peptide, were insoluble under the current assay conditions, rendering interpretation of their effect on transport difficult. Both Rab2 and Rab4 effector domain peptides were similarly insoluble in the assay buffer (unpublished observations). Second, alignment of the Rab3A effector domain and its flanking sequences with other members of the Rab family, SEC4, YPT1, Ras, Arf and Ral, reveals a rather striking conservation of this region exclusive to members of the Rab family and YPT1 (Figure 1). This sequence conservation is >95% for Rab1, Rab3 and YPT1 effector domains (Figure 1, heavily shaded region). An increasing number of non-conservative substitutions are found between this group and the Rab2, 4, 5 and 6 effector domains as well as the more divergent SEC4, Ras, Ral and Arf proteins (Figure 1). We interpret this strong sequence homology between Rab1, Rab3 and YPT1 effector domains to indicate that the rab3AL peptide is a general reagent which is likely to inhibit interaction of both Rab1 or Rab3 with their effector protein(s). The peptide may be too small to include additional information which directs specificity to effector binding. This interpretation suggests that Rab1 is the likely candidate for a functional role in ER to Golgi transport, consistent with its high homology to YPT1 (Haubruck *et al.*, 1987; Touchot *et al.*, 1987). We think it unlikely that the Rab3A protein is a logical candidate for regulation of ER to Golgi transport in that its expression is restricted to brain tissue and is principally associated with synaptic vesicles (Touchot *et al.*, 1987; Zahraoui *et al.*, 1989; Mollard *et al.*, 1990). Whether the putative effector protein (REP) is identical for all the members of the Rab family, or is a member of a family of divergent proteins each specific for an individual Rab protein, remains to be determined. Unique GAP- like GTPase stimulating proteins have been detected for K-*rev* (Kitayama *et al.*, 1989) and Ras (Trahey and McCormick, 1987; Gibbs *et al.*, 1988). Both proteins contain identical effector domains (amino acid residues 32–40), but differ in their flanking sequences. Since Ras and K*rev* are responsible only to their respective GAPs (G.Bokoch and C.Der, personal communication), these results have led to the suggestion that flanking regions contain essential information for the specificity of interaction, and the GAP-like proteins are likely to constitute a diverse family of proteins specific for each of the numerous small GTP-binding proteins.

What is the mechanism by which the peptide inhibits transport *in vitro*? Synthetic peptides spanning the effector region of Ras compete efficiently with the binding of Ras to GAP in an *in vitro* competition assay (Schaber *et al.*, 1989). Ras synthetic peptides encompassing residues 17–44 and 17–37 (Figure 1) potently compete for Ras–GAP binding with IC_{50}s of 2.4 and 0.4 μM, respectively

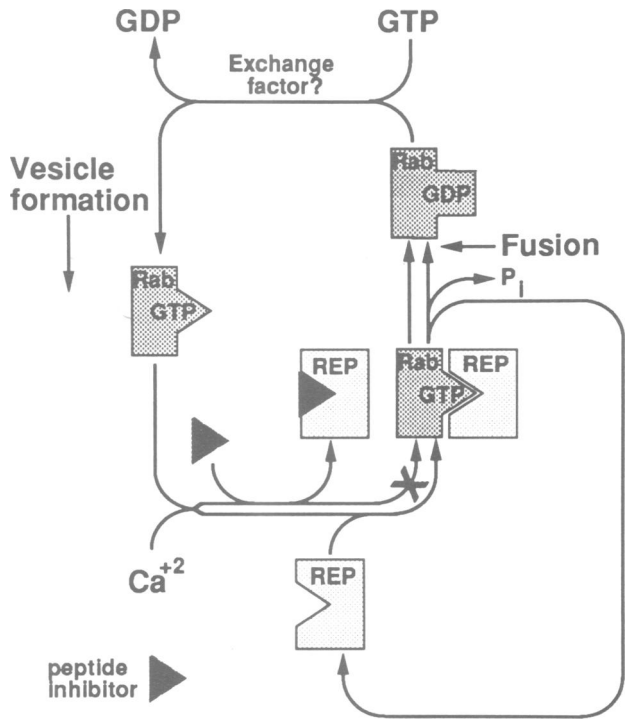

Fig. 6. A rab/REP (rab effector protein) cycle is required for transport of protein between the ER and the Golgi compartments. The site of transport inhibition by the peptide is indicated with an X.

(Schaber *et al.*, 1989) when compared to the value observed for Ha-Ras (110 μM) (Vogel *et al.*, 1988). In a similar study, synthetic peptides to the C-terminal sequence of the α-subunit of G_t, essential for G_t–transducin interaction (Hamm *et al.*, 1987; Deretic and Hamm, 1987), were shown to compete efficiently and specifically for the binding of G_t to light-activated rhodopsin (Hamm *et al.*, 1988). We would anticipate that the rab3AL peptide inhibits in an analogous fashion through competition for the binding between Rab and REP. It was surprising that the Ala(35) and Leu(36) substitutions generated a peptide (rab3AL) with stronger inhibitory properties than the native sequence considering that similar substitutions in Ras result in a reduction in GTPase stimulation by GAP (Vogel *et al.*, 1988; Schaber *et al.*, 1989). One possible explanation for this result is that the solution conformation of the mutant peptide is more adaptable for binding to REP then the native effector domain sequence. Alternatively, subtle differences may exist between the Ras and Rab protein effector domains which cannot be evaluated at the present time in the absence of molecular or structural studies of Rab.

A model for Rab/REP function in vesicular transport

What is the role of Rab and REP in directing vesicular trafficking? In the simplest view (Figure 6), our results favor an interpretation that a Rab(GTP complex) protein acquired early in transport may play a key role in regulating a late step in transport. In this case, a membrane-associated Rab protein, possibly recruited during vesicle formation (Figure 6) would interact at a late step in transport with an acceptor-associated REP protein. In the presence of the Rab(GTP)–REP ternary complex, GTP hydrolysis would occur, leading to Ca^{2+}-dependent vesicle fusion. Addition

of peptide would inactivate REP rendering the fusion reaction defective (Figure 6). In support of this model, we found that inhibition was rapid and irreversible only when both semi-intact cells and cytosol, but not cytosol alone, were incubated at 30°C in the presence of peptide. In addition, inhibition by peptide is largely preceded by the transport step sensitive to GTPγS (Beckers and Balch, 1989). If Rab−GTP is unable to metabolize GTP and GDP in the absence of REP, then transport is blocked. However, in the case of peptide, the block is at a time-point in transport which presumably reflects the requirement for GTP hydroylsis, not at the time-point in which GTP−GTPγS or a Rab−GTP−GTPγS complex is recruited by the forming vesicle. Since the step at which the peptide blocks transport was found to be late, coincident with the Ca^{2+}-dependent step, it is apparent that during ER to Golgi transport fusion of a vesicular carrier to the *cis* Golgi compartment requires both Rab effector function (REP) and Ca^{2+} (Figure 6). These are likely to be independent events since Ca^{2+} will not rescue a GTPγS inhibited transport reaction (Beckers and Balch, 1989). These results also suggest that the effector function detected in the transport assay is a downstream event, and that the rate of transport is not limited by the activity of REP under normal incubation conditions.

It remains to be determined whether the putative REP is a factor which directly stimulates GTP hydrolysis as suggested by Ras/GAP analogy. In yeast, RAS can interact with both adenylate cyclase (De-Feo-Jones *et al.*, 1983; Kataoka *et al.*, 1985; Barbacid, 1987; Gibbs, 1988; Gibbs *et al.*, 1989; Schaber *et al.*, 1989) and IRA proteins, the latter being the putative yeast homolog to mammalian GAP (Tanaka *et al.*, 1990). These results raise the possibility that the Rab−GTP complex and other small GTP-binding proteins may interact with effector proteins which have roles other than to directly stimulate GTP hydrolysis. In this model, the Rab−REP complex would be anticipated to require additional proteins to trigger hydrolysis. Current experiments directed at purifying and characterizing the factor(s) responsive to peptide will clarify these issues.

In our present model (Figure 6), the Rab protein involved in ER to Golgi transport in mammalian cells may be viewed simply as regulating information flow during transport, preventing key steps triggering vesicle fusion until appropriate membrane−receptor associations have occurred and so imparting specificity to the transport reaction. A similar interpretation of the role of GTP-binding proteins in transport has been suggested on the basis of genetic interactions involved in the delivery of vesicles to the plasma membrane in yeast (Bourne, 1988; Walworth *et al.*, 1989). In support of this, both YPT1 (Kaiser and Schekman, 1990) and SEC4 (Salminen and Novick, 1987) proteins are associated with vesicle membranes and function at a late targeting of fusion step in transport. Since the current assay conditions only allow us to detect the latest step in transport sensitive to peptide which measures Rab effector domain function, our data do not rule out the possibility that vesicular transport is regulated by a sequential series of GTP-dependent steps, a likely possibility considering that at least two other small GTP-binding proteins, SAR1 (Nakano and Muramatsu, 1989) and ARF (Stearms *et al.*, 1990) may also be essential for ER to Golgi transport. If multiple GTP hydrolysis events are required, the same or different effector protein(s) may interact with one or more small GTP-binding proteins to regulate earlier steps of transport involved in vesicle formation and targeting. In this model, the cycle proposed in Figure 6 would be reiterated several times during a single round of transport, a hypothesis we are currently testing.

Since the Ras paradigm enabled us to design synthetic peptides which are specific inhibitors of vesicular transport along the exocytotic pathway, similar strategies may be useful for studying the role of other small GTP-binding proteins likely to be involved in the regulation of diverse processes in the cell.

Materials and methods

Materials

Semi-intact cells used for analysis of ER to Golgi transport were prepared from clone 15B cells infected with tsO45 strain of vesicular stomatitis virus (VSV) using the swelling method as described previously (Beckers *et al.*, 1987). *Trans* ^{35}S-label ([^{35}S]methionine and [^{35}S]cysteine, >1000 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Irvine, CA). Donor and acceptor membrane fractions for analysis of intra-Golgi transport were prepared from VSV-infected and uninfected 15B cells, respectively, as described previously (Balch *et al.*, 1984a). GTPγS was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Endoglycosidase D (endo D) was obtained from Boehringer Mannheim Biochemicals or prepared from the culture supernatant of *Diplococcus pneumoniae* as described previously (Beckers *et al.*, 1987).

Peptide synthesis

The following peptides were synthesized by Multiple Peptide Systems (La Jolla, CA) (nomenclature according to Table I): rab3(30−48), rab3(36−51), rab3AL(33−48), rab3K(33−48), rab1(33−48), YPT1(33−48), rab1F(33−48), ras(33−48), rasF(33−48), ral(33−48), and rho(33−48). We thank R.Houghten (Multiple Peptide Systems, CA) for providing us with the following peptides: rab1/ypt1(63−75), rab3(63−75), rab1(C-terminus), rab2(C-terminus), rab3(C-terminus), rab4(C-terminus). Rab3AL(33−37), rab3F(33−48), and rab3(33−48) were synthesized by solid phase synthesis on an ABI automated peptide synthesizer (Model 430) (Merrifield, 1963). Deprotection and removal of the peptide from the resin support was effected by treatment with HF (Sakaibara *et al.*, 1967). All peptides (C-terminal amide derivatives) were purified using preparative HPLC on a reverse-phase C_{18} silica column (Vydac 10 mm × 250 mm, 218TP (Vydac, CA) using a 0−65% gradient of acetonitrile containing 0.05% TFA (Rivier *et al.*, 1984). Homogeneity was demonstrated by analytical HPLC, and identity was confirmed by amino acid analysis and sequence analysis.

Incubation conditions and analysis of transport

The ER to Golgi transport assays using semi-intact cells were performed as described previously (Beckers *et al.*, 1987; Beckers and Balch, 1989). Briefly, transport incubations contained in a final total volume of 40 μl (final concentration); 25 mM HEPES-KOH, pH 7.2, 90 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA, 1.8 mM Ca^{2+}, 1 mM ATP, 5 mM creatine phosphate, 0.2 IU of rabbit muscle creatine phosphokinase, 25 μg cytosol and 5 μl (25−30 μg) protein; 5 × 10^5 to 1 × 10^6 semi-intact cells). Transport was initiated by transfer to 30°C. Assays were supplemented with additional reagents as indicated in the Results. After termination of transport by transfer to ice, the membranes were pelleted by a brief (30 s) centrifugation in an Eppendorf microfuge at top speed. The pellet was subsequently solubilized in an endo D digestion buffer and digested with endo D as described previously (Beckers and Balch, 1989). Digestion was terminated by adding a 5× concentrated gel sample buffer (Beckers and Balch, 1989) and boiling for 5 min. The samples were analyzed by SDS−PAGE using 7.5% acrylamide gels (Laemmli, 1970) and autoradiographed; the fraction of VSV-G protein processed to the endo D sensitive form was determined by densitometry (Beckers and Balch, 1989). Intra-Golgi transport was determined as described previously (Balch *et al.*, 1984a)

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References

Adari,H., Lowy,D.R., Willumsen,B.M., Der,C.J. and McCormick,F. (1988) *Science*, **240**, 518–521.

Bacon,R.A., Salminen,A., Ruohola,H., Novick,P. and Ferro-Novick,S. (1989) *J. Cell Biol.*, **109**, 1015–1022.

Baker,D., Hicke,L., Rexach,M., Schleyer,M. and Schekman,R. (1988) *Cell*, **54**, 335–344.

Baker,D., Wuestehube,L., Schekman,R., Botstein,D. and Segev,N. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 355–359.

Balch,W.E. (1989) *J. Biol. Chem.*, **264**, 16965–16969.

Balch,W.E., Dunphy,W.G., Braell,W.A. and Rothman,J.E. (1984) *Cell*, **39**, 405–416.

Balch,W.E., Glick,B.S. and Rothman,J.E. (1984) *Cell*, **39**, 525–536.

Barbacid,M. (1987) *Annu. Rev. Biochem.*, **56**, 779–827.

Beckers,C.J.M. and Balch,W.E. (1989) *J. Cell Biol.*, **108**, 1245–1256.

Beckers,C.J.M., Keller,D.S. and Balch,W.E. (1987) *Cell*, **50**, 523–534.

Beckers,C.J.M., Block,M.R., Glick,B.S., Rothman,J.E. and Balch,W.E. (1989) *Nature*, **339**, 397–398.

Bourne,H.R. (1989) *Cell*, **53**, 669–671.

Braell,W.A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1137–1141.

Burgoyne,R.D. (1989) *Trends Biochem. Sci.*, **14**, 394–396.

Cales,C., Hancock,J.F., Marshall,C.J. and Hall,A. (1988) *Nature*, **332**, 548–551.

Casey,P.J., Solski,P.A., Der,C.J. and Buss,J.E. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8323–8327.

De-Feo-Jones,D., Scolnick,E.M., Koller,R. and Dhar,R. (1983) *Nature*, **306**, 707–709.

Deretic,D. and Hamm,H. (1987) *J. Biol. Chem.*, **262**, 10839–10847.

DeVos,A.M., Tong,L., Milburn,M.V., Matias,P.M., Jancarik,J., Noguchi,S., Nishimura,S., Miura,K., Ohtsuka,E. and Kim,S.-H. (1988) *Science*, **239**, 888–893.

Gibbs,J.B. and Marshall,M.S. (1989) *Microbiol. Rev.*, **53**, 171–185.

Gibbs,J.S., Schaber,M.D., Allard,W.J., Sigal,I.S. and Scolnick,E.M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5026–5030.

Goud,B., Salminen,A., Walworth,N.C. and Novick,P.J. (1988) *Cell*, **53**, 753–768.

Hamm,H.E., Deretic,D., Hofmann,K.P., Schleicher,A. and Kohl,B. (1987) *J. Biol. Chem.*, **262**, 10831–10838.

Hamm,H.E., Deretic,D., Arendt,A., Hargrave,P.A., Koenig,B. and Hofmann,K.P. (1988) *Science*, **241**, 832–835.

Hancock,J.F., Magee,A.I., Childs,J.E. and Marshall,C.J. (1989) *Cell*, **57**, 1167–1177.

Haubruck,H., Disela C., Wagner P. and Gallwitz, D. (1987) *EMBO J.*, **6**, 4049–4053.

Haubruck,H., Prange,R., Vorgias,C. and Gallwitz,D. (1989) *EMBO J.*, **8**, 1427–1432.

Kaiser,C.A. and Schekman,R. (1990) *Cell*, in press.

Kataoka,T., Powers,S., Cameron,S., Fasano,O., Goldfarb,M., Broach,J. and Wigler,M. (1985) *Cell*, **40**, 19–26.

Kitayama,H., Sugimoto,Y., Matsuzaki,T. Ikawa,Y. and Noda,M. (1989) *Cell*, **56**, 77–84.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Lowy,D.R. and Willumsen,B.M. (1989) *Nature*, **341**, 384–385.

Marshall,M.S., Hill,W.S., Ng,A.S., Vogel,U.S., Schaber,M.D., Scolnick, E.M., Dixon,R.A.F., Sigal,I.S. and Gibbs,J.B. (1989) *EMBO J.*, **8**, 1105–1110.

Melancon,P., Glick,B.S., Malhotra,V., Weidman,P.J., Serafini,T., Gleason, M.L., Orci,L. and Rothman,J.E. (1987) *Cell*, **51**, 1053–1062.

Merrifield,R.B. (1963) *J. Am. Chem. Soc.*, **85**, 2149–2154.

Michaeli,T., Field,J., Ballester,R., O'Neill,K. and Wigler,M. (1989) *EMBO J.*, **8**, 3039–3044.

Milburn,M.V., Tong,I., DeVos,A.M., Brunger,A., Yamaizumi,Z., Nishimura,S. and Kim,S.-H. (1990) *Science*, **247**, 939–945.

Molenaar,C.M.T., Prange,R. and Gallwitz,D. (1988) *EMBO J.*, **7**, 971–976.

Mollard,G.F.V., Mignery,G.A., Baumert,M., Perin,M.S., Hanson,T.J., Burger,P.M., Jahn,R. and Sudhof,T.C. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 1988–1992.

Nakano,A., Brada,D. and Schekman,R. (1988) *J. Cell Biol.*, **107**, 851–863.

Nakano,A. and Muramatsu,M. (1989) *J. Cell Biol.*, **109**, 2677–2691.

Pai,E.F., Kabsch,W., Krengel,U., Holmes,K.C., John,J. and Wittinghofer, A. (1989) *Nature*, **341**, 209–214.

Rivier,J., McClintock,R. and Anderson,H. (1984) *J. Chromatogr.*, **288**, 303–328.

Ruohola,H., Kabcenell,A.K. and Ferro-Novick,S. (1989) *J. Cell Biol.*, **107**, 1465–1476.

Sakaibara,S., Shimoniski,Y., Kishida,Y., Okada,M. and Sugihara,H. (1984) *Bull. Chem. Soc. Jap.*, **40**, 2164–2167.

Salminen,A. and Novick,P.J. (1987) *Cell*, **49**, 527–538.

Salminen,A. and Novick,P.J. (1989) *J. Cell Biol.*, **109**, 1023–1036.

Santos,E. and Nebreda,A.R. (1989) *FASEB J.*, **3**, 2151–2162.

Schaber,M.D., Garsky,V.M., Boylan,D., Hill,W.S., Scolnick,E.M., Marshall,M.S., Sigal,I.S. and Gibbs,J.B. (1989) Ras interaction with the GTPase-activating protein (GAP). *Proteins: Structure, Function, and Genetics*, **6**, 306–315.

Schafer,W.R., Kim,R., Sterne,R., Thorner,J., Kim,S.-H. and Rine,J. (1989) *Science*, **245**, 379–385.

Schmitt,H.D., Puzicha,M. and Gallwitz,D. (1988) *Cell*, **53**, 635–647.

Segev,N., Mulholland,J. and Botstein,D. (1988) *Cell*, **52**, 915–924.

Sewell,J.L. and Kahn,R.A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4620–4624.

Sigal,I.S, Gibbs,J.B., D'alonzo,J.S. and Scholnick,E.M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 4725–4729.

Stearms,T., Willingham,N.C., Botstein,D. and Kahn,R.A. (1990) *Proc. Natl. Acad. Sci. USA*, in press.

Stone,J.C., Vass,W.C., Willumsen,B.M. and Lowry,D.R. (1988) *Mol. Cell. Biol.*, **8**, 3565–3569.

Tanaka,K., Nakfuku,M., Satoh,T., Marshall,M.S., Gibbs,J.B., Matsuoto, K., Kaziro,Y. and Toh-e, A. (1990) *Cell*, **60**, 803–807.

Tong,L., de Vos,A.M., Milburn,M.V., Jancarik,J., Noguchi,S., Nishimura,S., Miura,K., Ohtsuka,E. and Kim,S.-H. (1989) *Nature*, **337**, 90–93.

Touchot,N., Chardin,P. and Tavitian,A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 8210–8214.

Trahey,M. and McCormick,F. (1987) *Science*, **238**, 542–545.

Trahey,M., Wong,G., Halenbeck,R., Rubinfeld,B., Martin,G.A., Ladner, M., Long,C.M., Crosier,W.J., Watt,K., Koths,K. and McCormick,F. (1988) *Science*, **242**, 1697–1700.

Vogel,U.S., Dixon,R.A.F., Schaber,M.D., Diehl,R.E., Marshall,M.S., Scolnick,E.M., Sigal,I.S. and Gibbs,J.B. (1988) *Nature*, **335**, 90–93.

Wagner,P., Molenaar,C.M.T., Rauh,A.J.G., Brokel,R., Schmitt,H.D. and Gallwitz,D. (1987) *EMBO J.*, **6**, 2373–2379.

Walworth,N.C., Goud,B., Kabcenell,A.K. and Novick,P.J. (1989) *EMBO J.*, **8**, 1685–1693.

Willumsen,B.M., Papageorge,A.G., Kung,H.F., Bekesi,E., Robins,T., Johnson,M., Vass,W.E. and Lowy,D.R. (1986) *Mol. Cell. Biol.*, **6**, 2646–2654.

Wilson,D.W., Wilcox,C.A., Flynn,G.C., Chen,E., Kuang,W.-J., Henzel, W.J., Block,M.R., Ullrich,A. and Rothman,J.E. (1989) *Nature*, **339**, 355–359.

Zahraoui,A., Touchot,N., Chardin,P. and Tavitian,A. (1989) *J. Biol. Chem.*, **264**, 12394–12401.

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