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The N-terminal domain of a rab protein is involved in membrane-membrane recognition and/or fusion

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Proteins of the YPT1/SEC4/rab family are well documented to be involved in the regulation of membrane transport. We have previously reported that rab5 regulates endosome-endosome recognition and/or fusion *in vitro*. Here, we show that this process depends on the rab5 N-terminal domain. Treatment of early endosomal membranes at a low trypsin concentration essentially abolished fusion and cleaved rab5 to a 1 kDa smaller polypeptide. Two-dimensional gel analysis suggested that rab5 is one of the few, if not the only, polypeptides cleaved by trypsin under these conditions. Whereas endosome fusion could be stimulated by cytosol prepared from cells overexpressing rab5 (and thus containing high amounts of the protein), this stimulation was abolished by trypsin-treatment of the cytosol. Trypsin-treated cytosol prepared from mock-transfected cells, which contains very low amounts of rab5, showed no inhibitory activity indicating that rab5 is the target of trypsin in these experiments. Purified rab5 prepared after expression in *Escherichia coli* was treated with trypsin, which cleaved the protein at the N-terminus. A synthetic peptide of rab5 N-terminal domain inhibited endosome fusion in our cell-free assay. A version of the same peptide truncated at the N-terminus or a peptide of rab3 N-terminal domain were without effects. Altogether, these observations suggest that the N-terminal domain of rab5 is involved in the process of early endosome recognition and/or fusion, presumably because it interacts with another component of the transport machinery.

Key words: endosome recognition and fusion/membrane transport/N-terminal/rab5

Introduction

The involvement of monomeric GTP-binding proteins, homologous to the protein encoded by the proto-oncogene *ras*, in the regulation of membrane transport is now well established (for reviews, see Goud and McCaffrey, 1991; Balch, 1992; Gruenberg and Clague, 1992; Pfeffer, 1992; Zerial and Stenmark, 1993). At least two families of these proteins, Sar/ARF and Ypt1/Sec4/rab, have been implicated in transport. Amongst proteins of the former family, Sar1p is required for vesicle formation in yeast (Rexach and Schekman, 1991; Oka *et al.*, 1991), and an ARF protein is a component of non-clathrin-coated vesicles in mammalian cells (Serafini *et al.*, 1991). More is known about the role of proteins of the YPT1/SEC4/rab family, which we will refer to as rab proteins. Several rab proteins have been shown to be required at specific steps of membrane transport, both in yeast (Goud *et al.*, 1988; Segev *et al.*, 1988) and in mammalian cells (Gorvel *et al.*, 1991; Plutner *et al.*, 1991; Bucci *et al.*, 1992; Lombardi *et al.*, 1993). Moreover, every member of that family that has been localized exhibits a specific subcellular distribution (Goud and McCaffrey, 1991; Gruenberg and Clague, 1992). Since rab proteins, by analogy with other GTP-binding proteins, are believed to undergo a conformational change upon GTP hydrolysis and thereby to act as a molecular 'switch', it has been proposed that they mediate membrane targeting (Bourne, 1988; Bourne *et al.*, 1990). This proposal is consistent with studies of SEC4 (Goud *et al.*, 1988) and YPT1 in yeast (Rexach and Schekman, 1991; Oka *et al.*, 1991; Segev, 1991). However, the precise function of rab proteins in membrane transport remains unclear.

Information on the structural organization of rab proteins has been obtained by comparision with the structure of ras (deVos *et al.*, 1988; Pai *et al.*, 1989; Tong *et al.*, 1989), and by mutagenesis. In their hypervariable C-terminal region (Valencia *et al.*, 1991), rab proteins contain a signal which is both necessary and sufficient for their association with the correct intracellular membrane (Chavrier *et al.*, 1991). Membrane association itself requires the prenylation of one or more C-terminal cysteine residues present in a motif functionally analogous to the ras CAAX box (Evans *et al.*, 1991). Other identified regions include the highly conserved GTP-binding motifs and the so-called 'effector' domain (Valencia *et al.*, 1991). By analogy with ras, the effector domain is believed to interact with a GAP protein, thereby stimulating the low endogenous GTPase activity of rab proteins. Effector domain peptides have, in fact, been shown to inhibit transport in the biosynthetic pathway (Plutner *et al.*, 1990). Proteins have been identified that can escort rab proteins in the cytosol (GDI), stimulate GTP hydrolysis or facilitate GDP/GTP exchange (Huang *et al.*, 1990; Sasaki *et al.*, 1990; West *et al.*, 1990; Burstein *et al.*, 1991; Tan *et al.*, 1991; Burstein and Macara, 1992). Other proteins, which include putative components of the transport machinery, are expected to interact with rab proteins. In fact, a protein interacting with rab3a/smg-25a has been identified (Shirataki *et al.*, 1992). However, it is not clear which regions of rab proteins would be involved in these interactions and except for the GTP-binding domain, the effector domain and the C-terminus, nothing is known about the role played by other regions of rab proteins.

Using an established cell-free assay, we have previously shown that rab5 is required for the fusion of early endosomes

in vitro (Gorvel *et al.*, 1991). In the present paper, we report that the N-terminal domain of rab5 is necessary for rab5 function in endosome–endosome recognition and/or fusion, presumably because this domain interacts with another component of the transport machinery.

Results and discussion

Early endosomes exhibit a striking tendency to undergo lateral (homotypic) fusion with each other *in vitro* (Davey *et al.*, 1985; Gruenberg and Howell, 1986, 1987, 1989; Braell, 1987; Diaz *et al.*, 1988; Woodman and Warren, 1988). This process is highly specific (Gruenberg *et al.*, 1989; Bomsel *et al.*, 1990; Aniento *et al.*, in press) and is regulated by NSF (Diaz *et al.*, 1989), heterotrimeric G-proteins (Colombo *et al.*, 1992), rab5 (Gorvel *et al.*, 1991), phosphorylation–dephosphorylation events (Tuomikoski *et al.*, 1989; Thomas *et al.*, 1992; Woodman *et al.*, 1992) and, possibly, annexin II (Emans *et al.*, 1992). The fusion process can be inhibited after trypsin treatment of the membranes (Diaz *et al.*, 1988; Woodman and Warren, 1988), and a high molecular weight trypsin-sensitive protein was shown to be required for the fusion of macrophage endosomes (Colombo *et al.*, 1991). Our initial goal was to use the fusion assay we have established (Gruenberg and Howell, 1986, 1987; Gruenberg *et al.*, 1989) to identify components of the early endosomal recognition/fusion machinery that may be sensitive to trypsin. Briefly, fusion is reconstituted in the assay by mixing two early endosomal fractions, one containing internalized avidin and the other internalized biotinylated horseradish peroxidase (bHRP). The avidin–bHRP complex formed upon fusion is then extracted in detergent and immunoprecipitated with antibodies against avidin, and the enzymatic activity of HRP quantified.

Trypsin sensitivity of early endosome fusion in vitro

To titrate the amount of trypsin required for inhition of fusion, early endosomal fractions (avidin- and bHRP-labeled) were pre-incubated separately, for 30 min at 4°C, in the presence of TPCK-treated trypsin. The reaction was arrested by the addition of excess soybean trypsin inhibitor. Control experiments were treated identically, except that trypsin and the inhibitor were added simultaneously. Treated fractions were then used in the fusion assay. As shown in Figure 1, early endosome fusion was essentially abolished by trypsin at a low concentration (~0.5 μg/ml), corresponding to a ratio of ~1:1000 (w/w) trypsin to the total amount of endosomal membrane protein present. Decreased fusion activity could not be explained by leakage of the fusion markers resulting from damage to endosomal membranes, since endosomes remained ≥85% latent at the end of the reaction. When only the bHRP-labeled fraction was treated with trypsin (instead of both the avidin- and the bHRP-labeled fractions), fusion was also inhibited at the same trypsin concentration (Figure 1).

Analysis of trypsin-sensitive polypeptides

In an attempt to identify the trypsin-sensitive polypeptide(s) responsible for inhibition of the fusion process, we analyzed the polypeptide composition of early endosomal membranes by two-dimensional gel electrophoresis. Cells were metabolically labeled for 16 h with [^{35}S]methionine, and then early endosomal fractions were prepared using a flotation gradient followed by immunoisolation (see

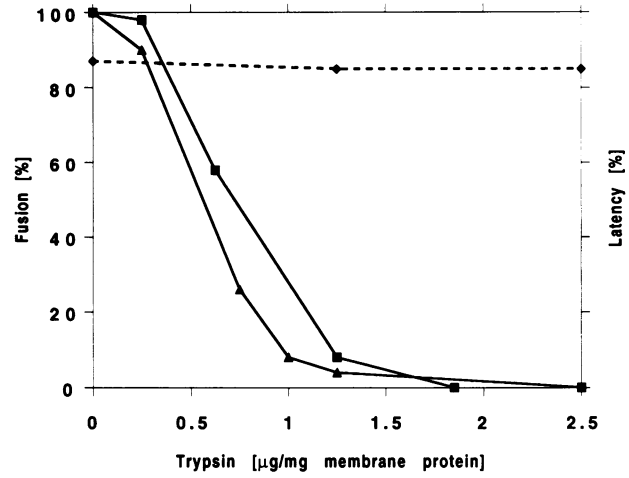

Fig. 1. Trypsin-sensitivity of endosome fusion *in vitro*. Both fractions (avidin and bHRP-labeled) were separately incubated for 30 min on ice with TPCK-treated trypsin (solid squares). Alternatively, only the bHRP-labeled fractions were treated with trypsin (solid triangles). Trypsin activity was arrested by adding soybean inhibitor (0.1 mg inhibitor/mg membrane proteins in the assay) and the fractions were used in the cell-free fusion assay. All values are expressed as a percentage of the control fusion (membranes incubated simultaneously with the inhibitor and 2.5 μg trypsin/mg membrane protein). Under all conditions, the fluid phase marker used to measure fusion (bHRP) retained its latency (~85% of the total present in the fraction) during the course of the experiment (solid diamonds). The amounts of TPCK-treated trypsin are expressed in μg per mg membrane protein present in the corresponding fractions.

Gruenberg and Gorvel, 1992, and references therein). Early endosomes were prepared with a 75-fold enrichment over the homogenate, and the *in vitro* fusion activity of these fractions was very high, corresponding to ~60% mixing of the markers following fusion in the assay (Thomas *et al.*, 1992; Emans *et al.*, 1993).

The immunoisolated fractions were treated with TPCK-trypsin as above, at the lowest concentration required to inhibit fusion [1:1000 (w/w) trypsin:endosomal protein], and the reaction was arrested with soybean trypsin inhibitor. The fractions were then analyzed using high resolution two-dimensional gels (Thomas *et al.*, 1992; Emans *et al.*, 1993) and autoradiography. Comparision of autoradiograms from several experiments showed that the polypeptide patterns of the trypsin-treated samples were essentially identical to those of the untreated controls (even after longer exposure times). However, the intensity of two low molecular weight polypeptides was changed by the trypsin treatment. In Figure 2, the enlarged lower half of a typical autoradiogram shows that the intensity of one labeled polypeptide decreased after treatment, whereas the intensity of a faster migrating (~1 kDa smaller), slightly more acidic polypeptide, which remained membrane-associated, increased.

Rab5 associated with early endosomal membranes is trypsin-sensitive

The only polypeptide sensitive to trypsin (Figure 2) under conditions inhibiting fusion (Figure 1) exhibited a molecular weight similar to that of the low molecular weight GTP-binding protein rab5, which is known to be required for early endosome fusion (Gorvel *et al.*, 1991). Therefore, we investigated the trypsin sensitivity of rab5 associated with early endosomal membranes.

Early endosomal fractions were prepared using the

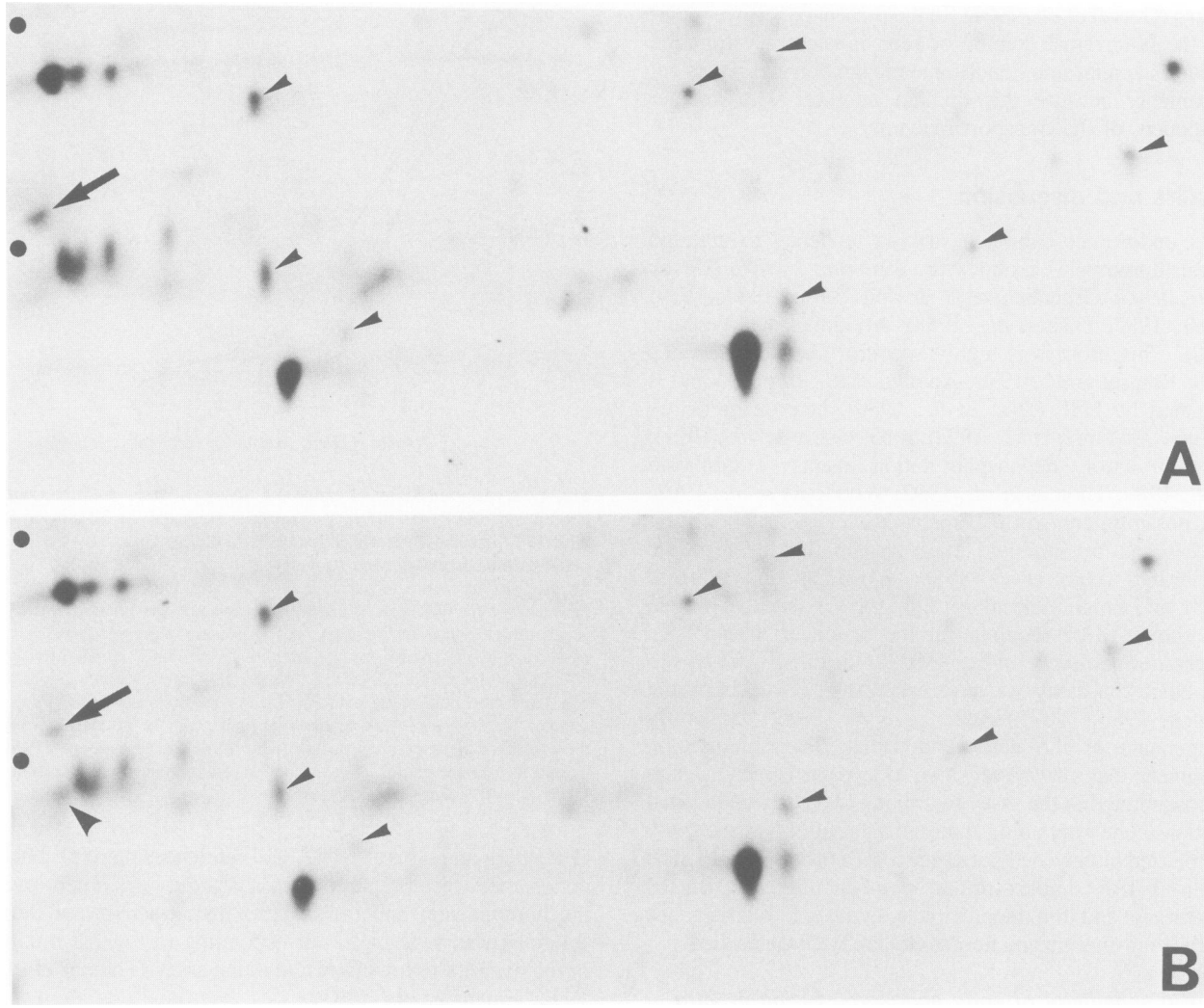

Fig. 2. Two-dimensional gels of trypsin-treated endosomal fractions. Immunoisolated early endosomal fractions were prepared from cells metabolically labeled with [^{35}S]methionone and treated with trypsin under conditions which inhibit fusion [corresponding to 1:1000 (w/w) trypsin:membrane protein] as in Figure 1. (**A**) Control: membranes were incubated simultaneously with trypsin and the inhibitor; (**B**) trypsin and inhibitor were added sequentially. The samples were analyzed by two-dimensional gel electrophoresis followed by autoradiography. Isoelectric focusing was from left (alkaline pH) to right (acidic pH) and the second dimension from top (high molecular weight) to bottom (low molecular weight). Only the lower halves of the gels are shown (molecular weight markers of 30 and 46 kDa are indicated). The arrow indicates the only polypeptide that exhibited a detectable decrease in intensity after the treatment (presumably rab5), and the large arrowhead the only spot that exhibited a detectable increase in intensity (presumably a cleaved form of rab5). Small arrowheads indicate examples of polypeptides not affected by the treatment.

flotation gradient, treated with trypsin as above [1:1000 (w/w) trypsin:endosomal protein], recovered by centrifugation and analyzed by Western blotting using an antibody raised against a C-terminal epitope of rab5 (see Chavrier *et al.*, 1990a). As shown in Figure 3A, the membrane-associated form of rab5 was cleaved by trypsin. The cleavage product, which was still recognized by the antibody after Western blotting (Figure 3) or immunoprecipitation (not shown), was ~1 kDa smaller, corresponding to a shift in mobility similar to that observed in two-dimensional gels (Figure 2). In addition, rab5 (Figure 3A) and the metabolically labeled trypsin-sensitive polypeptide (indicated by an arrow in Figure 2) exhibited a very similar behavior; in both cases, although a minor amount of cleaved protein was already detected in the control (presumably reflecting cleavage occurring during preparation of the membranes), only 50% of the total amount of protein was cleaved by trypsin and the cleavage product remained membrane-associated (Figure 2B and Figure 3A, lane 2). These experiments show that rab5 can be cleaved by trypsin and suggest that the target of trypsin in our assay is the rab5 protein.

The rab5 protein may be the target of trypsin

As rab5 was a good candidate for the trypsin-sensitive polypeptide, we determined its mobility in our two-dimensional gels. To establish its position unambigously, rab5 was overexpressed using the T7 RNA polymerase recombinant vaccinia virus system (Chavrier *et al.*, 1990a; Gorvel *et al.*, 1991) and early endosomal membranes were prepared using the flotation gradient. These fractions were then treated with trypsin as above [1:1000 (w/w) trypsin:endosomal protein], recovered by centrifugation and analyzed by two-dimensional gel electrophoresis. The polypeptides were then transferred to nitrocellulose and overlaid with [α-^{32}P]GTP (Lapetina and Reep, 1987; Bucci *et al.*, 1992; Huber *et al.*, 1993), in order to reveal the position of small GTP-binding proteins.

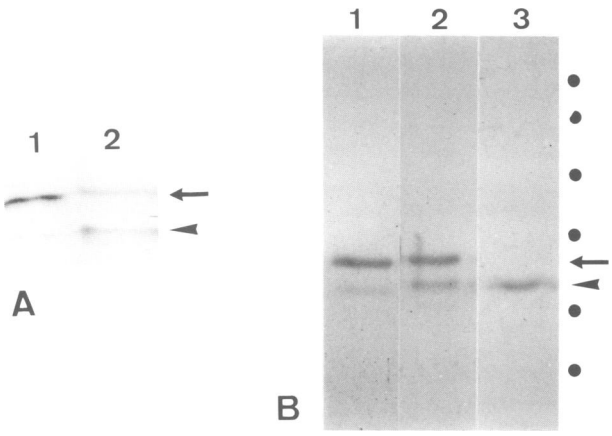

Fig. 3. Western blotting of endogenous rab5 and Coomassie-staining of rab5 produced in *E.coli*. (**A**) An immunoisolated endosomal fraction was treated without (lane 1) or with trypsin (lane 2) as in Figures 1 and 2, and then analyzed by electrophoresis in 12.5% acrylamide gels and Western blotting using the anti-rab5 antibody. (**B**) The rab5 protein produced in *E.coli* was purified and then treated as in Figures 1–3, using 0 (lane 1), 0.13 (lane 2) or 1.3 (lane 3) μg trypsin/mg rab5 protein. The molecular weight markers are indicated (14, 30, 46, 69, 92 and 200 kDa). In both panels, arrows indicate the position of intact rab5 and arrowheads the cleaved form of rab5. The cleaved form of rab5 exhibited the same apparent mobility (~1 kDa smaller than rab5) in panels A and B.

Figure 4 shows the enlarged lower half of a typical blot, comparable to the autoradiogram shown in Figure 2. In the absence of trypsin, several GTP-binding proteins could be detected in the fraction. As expected after overexpression, the rab5 spot, which migrates at the same position in the absence of overexpression (not shown), is most heavily labeled with [α-^{32}P]GTP. A comparision between autoradiograms showed that the mobility of overexpressed rab5 was identical to that of the trypsin-sensitive polypeptide observed after metabolic labeling in Figure 2. The other GTP-binding proteins present in the fractions have not yet been identified, and their pattern differs significantly from those obtained with other subcellular fractions (Huber *et al.*, 1993). They presumably include rab proteins involved in other steps of membrane transport connected to early endosomes (van der Sluijs *et al.*, 1991). After trypsin-treatment, overexpressed rab5 was cleaved to a slightly more acidic and ~1 kDa smaller polypeptide, while other GTP-binding proteins present in the fraction were not affected. The overexpressed rab5 appeared more sensitive to trypsin than endogenous rab5 (Figure 3A), although both are clearly membrane-associated, suggesting that the latter is partially protected, possibly via interactions with other proteins. The trypsin-treated form of rab5, which retained the capacity to bind GTP on blots, migrated at the same position as the metabolically labeled product of trypsin cleavage identified

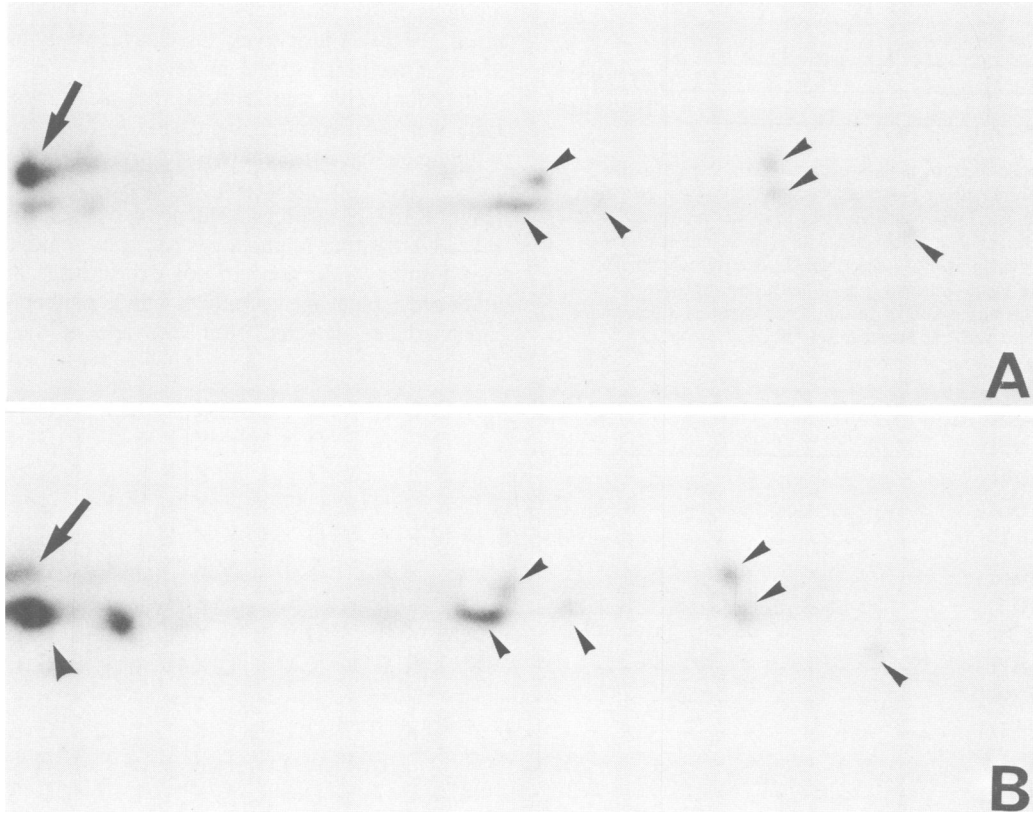

Fig. 4. GTP overlay after rab5 overexpression. Rab5 was overexpressed using the vaccinia/T7 RNA polymerase system and early endosomal fractions were prepared using the flotation gradient (Gorvel *et al.*, 1991). The fractions were then treated as in Figures 1–3. Two-dimensional gels are as in Figure 2. (**A**) Trypsin and inhibitor were added simultaneously; (**B**) trypsin and inhibitor were added sequentially. After treatment the fractions were analyzed on two-dimensional gels, transferred to nitrocellulose and overlaid with [α-^{32}P]GTP to reveal small GTP-binding proteins. The arrow indicates the position of intact overexpressed rab5 and the large arrowhead the cleavage product after trypsin treatment. As shown in panel B, the latter form still binds GTP. In this as in other experiments, we have observed that a fraction of the overexpressed rab5 protein migrates at a slightly more acidic position than the major spot indicated by the arrow (panel A). A similar spot is observed for the cleaved form of rab5 in panel B. Small arrowheads show the position of other small GTP-binding proteins present in the fraction, which were unaffected by trypsin.

in Figure 2. Altogether, these experiments demonstrate that rab5 is a target of trypsin in our assay.

Cytosolic rab5 is inactivated by trypsin

As a next step, we took advantage of our previous finding that cytosol prepared from cells overexpressing rab5 (and thus containing high amounts of the protein) stimulates endosome fusion *in vitro* (Gorvel *et al.*, 1991). In Figure 5, we show that this stimulation of fusion was abolished after pre-treatment of the cytosol with trypsin under the same conditions as used for the membranes [1:1000 (w/w) trypsin:cytosol protein]. As its membrane-associated form (Figures 3 and 4), rab5 was then cleaved but still recognized by our C-terminal antibodies (not shown). Addition of soybean trypsin inhibitor during the pre-treatment step blocked the effects of trypsin on fusion (Figure 5), and stimulation by cytosolic rab5 was then as effective as previously observed (Gorvel *et al.*, 1991). The inhibitory effect of trypsin on fusion activity depended on the rab5 protein alone, since trypsin treatment of cytosol prepared from mock-transfected cells, which contains very low amounts of rab5 (Gorvel *et al.*, 1991; Steele-Mortimer *et al.*, 1993), did not inhibit fusion.

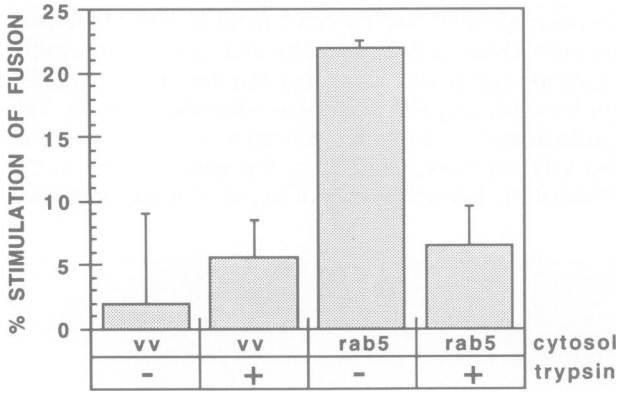

Fig. 5. The cytosolic form of rab5 is inactivated by trypsin. BHK cytosol prepared from cells overexpressing rab5 (rab5) or mock-transfected cells infected with vaccinia virus (vv) was pre-treated with trypsin (+), which was then inactivated with trypsin inhibitor, and then added to the fusion assay as described. In control experiments (−), cytosols were incubated with trypsin and trypsin inhibitor simultaneously. The stimulation of fusion is expressed as a percentage of the value obtained with rat liver cytosol only.

The N-terminus of rab5 is involved in endosome fusion

Cleavage of rab5 must have occurred near the C- or the N-terminus of the protein, since the cleavage product migrated only sightly faster in gels (~1 kDa). In addition, the cleaved form of rab5 remained membrane-associated, being recovered on endosomal membranes after flotation (Figures 3 and 4). Rab5, like other rab proteins, is associated with membranes via geranyl geranylation of a C-terminal cysteine (Kinsella and Maltese, 1991). Therefore, if trypsin cleaved the protein at a C-terminal site between the epitope of the anti-rab5 antibody (Figure 3A) and the geranyl geranylated cysteine, the cleavage product would have to bind to another membrane component in order to remain membrane-associated. This component would have to be relatively abundant, when compared with endogenous rab5, since the cleavage product remained membrane-associated even after ~5-fold overexpression of rab5 (Figure 4). Until now, we have not detected any such component after immunoprecipitation from endosomal membranes using anti-rab5 antibodies (with or without trypsin treatment), except for low amounts of the cytosolic escort protein GDI (Steele-Mortimer *et al.*, 1993). Alternatively the cleavage may have occurred at the N-terminus of the protein.

In order to characterize further the site of trypsin cleavage, rab5 was purified after expression in *Escherichia coli*. As with the endogenous and overexpressed rab5, a shift of ~1 kDa could be observed following trypsin treatment (Figure 3B). N-terminal sequencing of the cleavage product revealed that four amino acids had been removed by trypsin (see Figure 6). In none of our experiments did we detect additional cleavage products, and neither were other intermediates detected after cleavage of the endogenous

rab3a	M A S A T D S R Y G Q K E S S D Q N F D Y M F K I L I I G N
rab3b	M A S V T D G K H G V K D A S D Q N F D Y M F K L L I I G N
rab4	- - - - - - - - - - - - - - - M S E T Y D F L F K F L V I G N
rab6	- - - - - - - - - M S T G G D F G N P L R K F K L V F L G E
rab1	- - - - - - - - - - - M S S M N P E Y D Y L F K L L L I G D
rab2	- - - - - - - - - - - - - - - - M A Y A Y L F K Y I I I G D
rab5	- - M A N R G A T R P N G P N T G N K I C Q F K L V L L G E
rab8	- - - - - - - - - - - - - - - M A K T Y D Y L F K L L L I G D
rab7	- - - - - - - - - - - - - - - M T S R K K V L L K V I I L G D
rab9	- -
rab10	- - - - - - - - - - - - - - M A K K T Y D L L F K L L L I G D
rab11	- - - - - - - - - - - M G T R D D E Y D Y L F K V V L I G D
rab4b	- - - - - - - - - - - - - - - M A E T Y D F L F K F L V I G S
YPT1	- - - - - - - - - - - - - - - M N S E Y D Y L F K L L L I G N
YPT3	- - - - - - - - - - - - M C Q E D E Y D Y L F K T V L I G D
SEC4	- - M S G L R T V S A S S G N G K S Y D S I M K I L L I G D
H-ras	- M T E Y K L V V V G A

Fig. 6. N-terminal sequence of rab proteins. The N-terminal domains of different rab proteins were aligned according to Chavrier *et al.* (1990b). The sequences of the rab3b and rab5 N-terminal domain peptides are underlined. A small arrow indicates the trypsin cleavage site of *E.coli* rab5, as determined by N-terminal sequencing of the band recovered from the gel shown in Figure 3, lane 3.

protein present on early endosomal membranes (Figures 2–4), suggesting that cleavage had occurred at a single site.

These experiments suggest that the rab5 N-terminal domain might be involved in the process of endosome recognition and/or fusion. In order to test this hypothesis, we synthesized a peptide of 17 amino acids corresponding to the N-terminal domain of rab5, and tested this peptide in our endosome fusion assay. As a control, we used a peptide corresponding to the N-terminal domain of rab3b, a small GTP-binding protein associated with synaptic vesicles (Fischer von Mollard *et al.*, 1991) and possibly involved in secretion in acinar cells (Padfield *et al.*, 1992). This peptide was selected as a control, because, when compared with most other monomeric GTP-binding proteins (Chavrier *et al.*, 1990b; Valencia *et al.*, 1991), both rab3 and rab5 contain relatively long N-terminal extensions forming an additional domain (see Figure 6). As shown in Figure 7, fusion was inhibited in our assay by relatively low concentrations of the rab5 N-terminal peptide under conditons where the rab3b N-terminal peptide or a rab2 C-terminal peptide were without effect. Moreover, a truncated version of the rab5 N-terminal peptide, lacking the four N-terminal residues removed by trypsin (see Figure 6), had no effect on endosome fusion (not shown). Several peptides corresponding to other regions of rab5 have also been shown to be ineffective (Lenhard *et al.*, 1992). Our findings agree well with recent studies of rab5/rab6 chimeras showing that the N-terminal domain of rab5 is absolutely required for stimulation of endocytosis *in vivo* (Stenmark *et al.*, 1994). Until now, it has been difficult to demonstrate that a structural domain of rab proteins [other than the GTP-binding motifs and the effector domain in some cases, see Plutner *et al.* (1990)] is directly involved in the regulation of membrane transport. In fact, canine rab5 can rescue ypt5-disrupted *Schizosaccharomyces pombe* cells, despite little apparent sequence homology in the variable regions of the protein (Armstrong *et al.*, 1993). Our data strongly indicate that the rab5 N-terminus is necessary for rab5 function in mammalian cells, presumably because it is required for interactions with other components of the machinery controlling early endosome recognition/fusion.

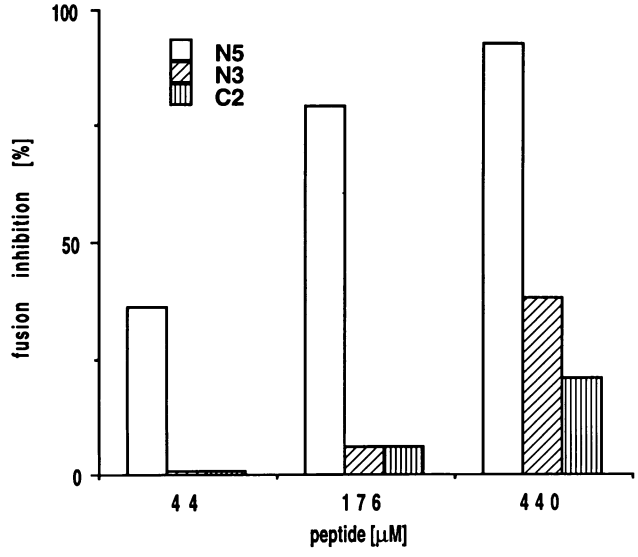

Fig. 7. Peptide inhibition of early endosome fusion. *In vitro* fusion of early endosomes was carried out as described in the legend to Figure 1. Before the assay, the mixture was preincubated on ice in the presence of either the rab5 (N5) or the rab3b (N3) N-terminal domain peptide (see Figure 6) at the indicated concentrations, and the peptides remained present throughout the assay. As an additional control, a peptide of the C-terminal domain of rab2 (C2) was used (Chavrier *et al.*, 1990a).

Materials and methods

Cells and reagents

Baby hamster kidney (BHK) cells were grown, maintained and metabolically labeled with [^{35}S]methionine for 16 h as previously described (Gruenberg *et al.*, 1989). The monoclonal antibody against a C-terminal peptide of rab5 was a kind gift of Angela Wandinger-Ness (Northwestern University, Evanson, IL) and David Vaux (Sir William Dunn School of Pathology, Oxford University). The monoclonal antibody against a C-terminal peptide of the spike glycoprotein G of vesicular stomatitis virus was a kind gift of Thomas Kreis (University of Geneva; Kreis, 1986). The peptides of canine rab2 C-terminal domain, human rab3 N-terminal domain and canine rab5 N-terminal domain were synthesized by Dominique Nalis (EMBL, Heidelberg); all were >98% pure, as measured by HPLC analysis.

Cytosol preparation

Cytosol was prepared as previously described (Gorvel *et al.*, 1991; Aniento *et al.*, 1993) at protein concentrations of 24 mg/ml for rat liver cytosol, 10–15 mg/ml for BHK cytosol and 5–8 mg/ml for mock-transfected or transfected cells. Transfection with rab5 cDNA was carried out using the vaccinia/T7 polymerase system; cells were infected with virus for 30 min and then transfected for 8 h using DOTAP (Boehringer, Mannheim) as described by Bucci *et al.* (1992).

Fusion assay

The cell-free assay we have established (Gruenberg and Howell, 1986; Gruenberg *et al.*, 1989; Tuomikoski *et al.*, 1989; Bomsel *et al.*, 1990; Gorvel *et al.*, 1991; Thomas *et al.*, 1992; Emans *et al.*, 1993; Aniento *et al.*, in press) was used to measure the fusion between early endosomes. Briefly, avidin and bHRP were internalized separately into two cell populations, by fluid phase endocytosis for 5 min at 37°C. The cells were homogenized and early endosomal fractions prepared using a flotation gradient (Gorvel *et al.*, 1991; Thomas *et al.*, 1992; Emans *et al.*, 1993). In the assay, the avidin- and bHRP-labeled endosome fractions were then combined at 4°C in the presence of 5 mg/ml BHK cytosol, ATP and salts. When indicated, peptides were added and the mixture pre-incubated for 60 min at 4°C. In some experiments, we used 25 μl (0.6 mg) rat liver cytosol (Aniento *et al.*, 1993) complemented with 25 μl (125 μg) BHK cytosol prepared from cells overexpressing rab5 or from mock-transfected, vaccinia-infected cells. In all cases, fusion was then allowed to proceed for 45 min at 37°C. At the end of the reaction, the avidin–bHRP complex formed upon fusion was immunoprecipitated with an anti-avidin antibody in the presence of detergents. The extent of fusion was quantified by measuring the enzymatic activity of the bound bHRP.

Trypsin-treatment of early endosomal membranes or cytosol

The cell-free assay was used to quantify the fusion activity of trypsin-treated early endosomal membranes. Each fraction (~20 μg protein in 50 μl) labeled with either avidin or bHRP was separately treated with TPCK-treated trypsin at concentrations varying between 0.1 and 1.0 μg/ml (corresponding to 0.25–2.5 ng trypsin/μg protein) for 30 min at 4°C. Trypsin activity was then stopped by adding 1 μl of homogenization buffer (3 mM imidazole pH 7.4, 250 mM sucrose) containing 5 μg of soybean trypsin inhibitor and the mixture was further incubated for 30 min at 4°C. As a control, TPCK-trypsin and the inhibitor were added simultaneously to the fractions and the mixture incubated for 60 min at 4°C. These fractions were then tested in the fusion assay (see above). The latency of trypsin-treated early endosomes containing avidin or bHRP was measured as described (Gorvel *et al.*, 1991). BHK cytosol was treated with 2.5 ng trypsin/μg cytosolic protein for 30 min on ice; the enzyme was then inhibited with 5.0 ng soybean trypsin inhibitor and the mixture was incubated for a further 15 min. In control experiments, trypsin and soybean trypsin inhibitor were added simultaneously and the samples were incubated for 45 min.

Analysis of early endosomal polypeptides

In order to analyze the polypeptide composition of early endosomes after trypsin treatment, fractions were prepared using a combination of two previously established protocols, a flotation gradient and immunoisolation. Briefly, cells were metabolically labeled for 16 h with [^{35}S]methionine and the spike glycoprotein G of vesicular stomatitis virus was then implanted

into the plasma membrane by low pH-mediated fusion of the virus envelope with the plasma membrane (White *et al.*, 1980; Gruenberg and Howell, 1985, 1986). The G-protein was internalized into early endosomes for 5 min at 37°C (Gruenberg and Howell, 1987; Gruenberg *et al.*, 1989; Thomas *et al.*, 1992; Emans *et al.*, 1993) and the cells were homogenized. Early endosomes were first separated from the plasma membrane and late endosomes by flotation on a sucrose/D_2O step gradient (Gorvel *et al.*, 1991; Emans *et al.*, 1993), and then immunoisolated (Gruenberg and Howell, 1986, 1987; Gruenberg *et al.*, 1989; Thomas *et al.*, 1992; Emans *et al.*, 1993) using a solid support coated with an antibody against the cytoplasmic domain of the G-protein (Kreis, 1986). The immunoisolated fraction was then washed in PBS to remove unbound vesicles, and treated sequentially with TPCK-trypsin and soybean trypsin inhibitor under the same conditions as described above. The fractions were analyzed by high resolution two-dimensional gel electrophoresis and autoradiography.

Transfer to nitrocellulose blots and [α-^{32}P]GTP overlay

Small GTP-binding proteins separated by two-dimensional gel electrophoresis were transferred to nitrocellulose and detected by GTP overlay. The protocol for transfer and GTP overlay (Bucci *et al.*, 1992; Huber *et al.*, 1993) was modified from the method of Lapetina and Reep (1987). Briefly, the two-dimensional gels were washed twice for 15 min each in 50 mM Tris−HCl pH 7.5 containing 20% glycerol and electrophoretically transferred to nitrocellulose paper in 10 mM $NaHCO_3$/3 mM Na_2CO_3 pH 9.8. The nitrocellulose was (i) rinsed for 30 min in binding buffer (50 mM NaH_2PO_4 pH 7.5, 10 μM $MgCl_2$, 2 mM DTT, with 4 μM ATP as competing substrate), (ii) incubated for 120 min with [α-^{32}P]GTP (1 μCi/ml, specific activity 2903 Ci/mmol, 1 Ci = 37 GBq) and (iii) rinsed for 60 min with several changes of binding buffer. The nitrocellulose was then air-dried and [α-^{32}P]GTP-binding was visualized by autoradiography (24 h, −80°C) using Kodak X-Omat AR film with an intensifying screen. To determine the molecular masses, prestained SDS−PAGE molecular weight standards (Bio-Rad) were co-electrophoresed in the second dimension and transferred to nitrocellulose.

Purification of rab5 produced in E.coli

Canine rab5 protein was expressed in *E.coli* using the pET-vector expression system (Zaharaoui *et al.*, 1989; A.Wandinger-Ness and M.Zerial, in preparation). The cells were then lysed and rab5 was purified using a Q-Sepharose column, as described by Tucker *et al.* (1986). The protein was further purified by running a 0−0.5 M NaCl gradient through an S-Sepharose column equilibrated with 25 mM HEPES pH 6.8, 10 mM $MgCl_2$, 100 mM GDP. Peak fractions were pooled and buffer replaced with 50 mM Tris pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM GDP, on a PD-10 column.

Analytical techniques

We used the high resolution two-dimensional gel electrophoresis system established by Celis and his collaborators (Celis *et al.*, 1990), as in our previous studies (Thomas *et al.*, 1992; Emans *et al.*, 1993). Protein determination was according to Bradford (1976).

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