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Two distinct effectors of the small GTPase Rab5 cooperate in endocytic membrane fusion

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Using the yeast two-hybrid system, we have identified a novel 62 kDa coiled-coil protein that specifically interacts with the GTP-bound form of Rab5, a small GTPase that regulates membrane traffic in the early endocytic pathway. This protein shares 42% sequence identity with Rabaptin-5, a previously identified effector of Rab5, and we therefore named it Rabaptin-5β. Like Rabaptin-5, Rabaptin-5β displays heptad repeats characteristic of coiled-coil proteins and is recruited on the endosomal membrane by Rab5 in a GTP-dependent manner. However, Rabaptin-5β has features that distinguish it from Rabaptin-5. The relative expression levels of the two proteins varies in different cell types. Rabaptin-5β does not heterodimerize with Rabaptin-5, and forms a distinct complex with Rabex-5, the GDP/GTP exchange factor for Rab5. Immunodepletion of the Rabaptin-5β complex from cytosol only partially inhibits early endosome fusion *in vitro*, whereas the additional depletion of the Rabaptin-5 complex has a stronger inhibitory effect. Fusion activity can mostly be recovered by addition of the Rabaptin-5 complex alone, but maximal fusion efficiency requires the presence of both Rabaptin-5 and Rabaptin-5β complexes. Our results suggest that Rab5 binds to at least two distinct effectors which cooperate for optimal endocytic membrane docking and fusion.
Keywords: effector/endosome/membrane traffic/Rab5

Introduction

A complex molecular machinery regulates vesicle-mediated transport of proteins and lipids between organelles (Rothman, 1994; Pfeffer, 1996). A cascade of membrane-bound and soluble factors sequentially drives the packaging of cargo and the recruitment of coat proteins into a coated bud, the fission of the coated bud into a coated vesicle, the targeting of the vesicle to its appropriate acceptor compartment and, ultimately, the docking and fusion of the vesicle with its target membrane. In addition, various organelles in the cell can undergo homotypic fusion reactions. All these steps are controlled by an array of different regulatory proteins including GTPases. For instance, the process of coat assembly is regulated by small GTPases of the ARF (ADP ribosylation factor) family (Stamnes and Rothman, 1993; Teal *et al.*, 1994). Arf proteins control the recruitment of soluble coat proteins to the membrane but, in addition, they can also modulate the lipid composition via activation of phospholipase D (PLD) (Brown *et al.*, 1993). Heterotrimeric G proteins are also implicated in vesicle formation as well as membrane fusion (Barr *et al.*, 1992; Colombo *et al.*, 1992). Dynamins self assemble into rings and trigger the budding coated vesicles to pinch off in a reaction that requires GTP hydrolysis (Hinshaw and Schmid, 1995; Takei *et al.*, 1995). Members of the Rho family of small GTPases are also implicated in the budding of transport vesicles and in the intracellular movement of organelles (Schmalzing *et al.*, 1995; Lamaze *et al.*, 1996; Murphy *et al.*, 1996), presumably via their action on the actin cytoskeleton (Tapon and Hall, 1997). Finally, small GTPases of the Rab family play an important role in vesicle docking and fusion (Nuoffer *et al.*, 1994; Pfeffer, 1994; Novick and Zerial, 1997), although recent reports suggest that they directly or indirectly also affect vesicle budding (Nuoffer and Balch, 1994; Riederer *et al.*, 1994; Benli *et al.*, 1996).

The large number of Rab proteins identified in mammalian cells, their localization to distinct intracellular compartments and their functional properties have suggested that each of these proteins functions as a specific regulator of intracellular transport (Pfeffer, 1994; Novick and Zerial, 1997). Like other members of the Ras superfamily, Rab proteins cyclically transmit signals to downstream effectors in a guanine-nucleotide-dependent manner. In the cytosol, Rab proteins are maintained in the GDP-bound form complexed to Rab GDI (GDP dissociation inhibitor). Upon membrane association, whereas Rab GDI dissociates and is released in the cytosol, the inactive Rab protein is converted into the GTP-bound active form by GDP/GTP exchange factors (GEFs) (Soldati *et al.*, 1994; Ullrich *et al.*, 1994). The switch between the GDP- and GTP-bound state is essential because it determines the ability to regulate the vesicle transport machinery (Rybin *et al.*, 1996).

A prerequisite for the understanding of how Rab proteins function in membrane transport is the identification of their effectors. It has been proposed that specificity in vesicle fusion is conferred by the correct pairing of vesicular soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (v-SNAREs) on vesicle membranes and cognate t-SNAREs on target membranes (Söllner *et al.*, 1993; Rothman, 1994; Rothman and Warren, 1994). Rab proteins have been demonstrated to be essential for SNAREs pairing to occur (Lian *et al.*, 1994; Søgaard *et al.*, 1994). However, these observations do not necessarily imply that v- and t-SNAREs may be direct effectors of Rab proteins (Brennwald *et al.*, 1994). Recently, Ypt1p has been shown to directly interact with the tSNARE Sed5p in yeast, although the nucleotide specificity of this

interaction has not been determined (Lupashin and Waters, 1997). Thus far, a number of different cytosolic factors have been identified in mammalian cells that, as predicted for effector molecules, interact with Rab proteins with specificity for the triphosphate conformation (Shirataki *et al.*, 1993; C.Li *et al.*, 1994; Chung *et al.*, 1995; Ren *et al.*, 1996; Stahl *et al.*, 1996; Diaz *et al.*, 1997; Wang *et al.*, 1997).

Using the yeast two-hybrid system, we have previously identified a 100 kDa protein, Rabaptin-5, which acts as an effector for the small GTPase Rab5. Rab5 regulates the transport from the plasma membrane to early endosomes as well as the homotypic fusion between early endosomes (Gorvel *et al.*, 1991; Bucci *et al.*, 1992; G.Li *et al.*, 1994). Rabaptin-5 is mainly a cytosolic protein with a small membrane pool. It displays regions with high probability for coiled-coil structure and does not share significant sequence homology with other putative Rab effectors. *In vitro* and *in vivo* studies have shown that Rabaptin-5 is recruited by Rab5 on the membrane in a GTP-dependent manner. Moreover, Rabaptin-5 is essential for the Rab5-dependent endosome fusion (Stenmark *et al.*, 1995a). Altogether, these results imply that the GTP cycle of Rab5 signals to the endocytic membrane docking and fusion machinery via Rabaptin-5.

Which other factors function in the Rab5-dependent control of endocytic membrane fusion is unclear. To address this question, we have searched for new Rab5-interacting molecules using the yeast two-hybrid system. Here we report the identification of a novel effector of Rab5. This protein shares sequence similarity with Rabaptin-5 and is required for maximal efficiency of homotypic endosome docking and fusion *in vitro*. Our studies reveal that two distinct Rab5 effectors act in concert in the process of endosome fusion.

Results

Identification of a novel Rab5-interacting protein by two-hybrid screening in yeast

To search for proteins interacting with the GTP-bound form of Rab5, we have previously screened a HeLa expression cDNA library and identified Rabaptin-5 (Stenmark *et al.*, 1995a). To search for novel Rab5-interacting proteins, we performed a new screening using a mouse brain expression cDNA library since Rab5 is abundant in brain (Bucci *et al.*, 1994). Reporter yeast cells were first transformed with a plasmid encoding a fusion between Rab5Q79L, a mutant Rab5 defective in GTP hydrolysis (Stenmark *et al.*, 1994), and the bacterial protein LexA, which recognizes specific DNA sequences upstream of the two reporter genes HIS3 and LacZ (Vojtek *et al.*, 1993). The strain was subsequently transformed with a plasmid mouse brain cDNA library encoding proteins as C-terminal fusions with the transcriptional activation domain of Gal4 (Bartel *et al.*, 1993). Library plasmids rescued from positive clones (His3 βGal+) were transformed into reporter yeast containing various control baits. Screening of 3×10^4 transformants yielded 28 independent positive clones. Two of these clones were found to interact with Rab5Q79L but not with Rab5S43N (a mutant with preferential affinity for GDP) (Stenmark *et al.*, 1995a). The interaction specificity of one of these clones, clone 2.5, containing a 1.7 kb insert, was examined in more detail in a liquid β-galactosidase assay (Table I). This clone caused a strong activation of the *LacZ* reporter gene in the presence of LexA fusions of Rab5Q79L and Rab5Q79LΔCCSN (deleted in the C-terminal isoprenylation motif), but not in the presence of fusions with Rab5S34N, Rab3AQ81L, Rab4AQ67L, Rab6Q72L (Martinez *et al.*, 1994), Rab7Q67L, Rab11Q70L (Ullrich *et al.*, 1996) or the respective mutants with preferential affinity for GDP. Furthermore, no reporter gene activation was detected with LexA fusions of RasG12V (Vojtek *et al.*, 1993). These results suggest that clone 2.5 encodes a protein that specifically interacts with the GTP-bound, active form of Rab5.

Table I. Interaction between clone 2.5 and various LexA fusions, assayed as LacZ reporter gene activity

LexA fusion	Activation domain fusion	β-galactosidase activity (U)
Rab5Q79L	–	0.1 ± 0.1
–	2.5	0.1 ± 0.0
Rab5	2.5	3.46 ± 0.21
Rab5S34N	2.5	0.18 ± 0.01
Rab5I53M	2.5	7.73 ± 0.33
Rab5Q79L	2.5	266 ± 2
Rab5ΔCCSN	2.5	13.6 ± 2.6
Rab5Q79LΔCCSN	2.5	425 ± 3
Rab3A	2.5	0.09 ± 0.02
Rab3AAT36N	2.5	0.09 ± 0.06
Rab3AQ81L	2.5	0.05 ± 0.03
Rab3C	2.5	0.15 ± 0.04
Rab3CQ81L	2.5	0.06 ± 0.01
Rab4A	2.5	0.09 ± 0.01
Rab4AS22N	2.5	0.09 ± 0.01
Rab4AQ67L	2.5	0.11 ± 0.01
Rab4B	2.5	0.06 ± 0.01
Rab4BQ67L	2.5	0.13 ± 0.09
Rab6	2.5	0.09 ± 0.04
Rab6T27N	2.5	0.07 ± 0.04
Rab6Q72L	2.5	0.07 ± 0.00
RabQ67L	2.5	0.06 ± 0.01
Rab11Q70L	2.5	0.10 ± 0.05
Rab17	2.5	0.06 ± 0.01
Rab22	2.5	0.06 ± 0.01
RasG12V	2.5	0.1 ± 0.01

L40 reporter yeast cells transformed with 2.5 plasmid, plasmids encoding LexA fusions of the proteins indicated, or both, were grown to OD_{600} values of ~1.0 in synthetic medium lacking tryptophan, leucine or both. β-galactosidase activity was then measured, using *o*-nitrophenyl-β-D-galactoside (Sigma) as a substrate (Guarente *et al.*, 1983). β-galactosidase activities (in relative units) are presented as mean values ± SEM, obtained with three independent transformants.

Clone 2.5 encodes a hydrophilic protein with similarity to Rabaptin-5

A sequence database search showed that the cDNA sequence of clone 2.5 completely matches the cDNA of a protein called FRA for Fos-related antigen (DDBJ/EMBL/GenBank accession number #U34932). This open reading frame has been identified in a search for proteins immunologically related to c-Fos. However, this protein does not share significant sequence homology with c-Fos or related proteins, but rather shares sequence homology with Rabaptin-5. Clone 2.5 corresponds to the C-terminal part (residues 154–554) of the open reading frame, which has a calculated M_r of 61.97 kDa and a predicted pI of

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  1 .....................................MAAAPAALA  9
                                        .|   .|
  1 MAQPGPASQPDVSLQQRVAELEKINAEFLRAQQQLEQEFNQKRAKFKELY 50
          .         .         .         .         .
 10 LDPQPQEEQKDA...SESSELSRLRAELAGALAEMETMKAVAEVSESTKA 56
    |..:.: ...:|   .. .:|::||.:| :| ||||.:||:|.|||.||.
 51 LAKEEDLKRQNAVLQAAQDDLGHLRTQLWEAQAEMENIKAIATVSENTKQ 100
          .         .         .         .         .
 57 EAVAAVQRQCQEEVASLQAILKDSISSYETQIAA.LKQERQQQQQDSEEK 105
    ||:..|.|| .|||||||::|:.:..|| |:   |.||| | .| .|
101 EAIDEVKRQWREEVASLQAVMKETVRDYEHQFHLRLEQERTQWAQYREYA 150
          .         .         .         .         .
106 ERELGHLKQLLARAHPLDSLEKQMEKAHEDSEKLREIVLPMEQETAELKV 155
    |||::.|:. |. ::. :.||.:|.||:||.|||||.:|:|||.|||.||
151 EREIADLRRRLSEGQEEENLENEMKKAQEDAEKLRSVVMPMEKEIAALKD 200
          .         .         .         .         .
156 KLLRAEELIQEIQ..................................... 168
    ||  ||: |.|::
201 KLITEAEDKIKELEASKVKELNHYLEAEKSCRTDLEMYVAVLNTQKSVLQE 250
          .         .         .         .         .
169 ..........................RRPRQPAS..LHGSTELL...... 184
                               ::.:|.|.  : :|  ||
251 DAEKLRKELHEVCHLLEQERQQHNQLKHIWQKANDQFLESQRLLMRDMQR 300
  .         .         .         .         .
185 PLSRNPSPPLEPSEE..........PSGDAG...PAAEAFAHNCDD.... 217
    :|..:.|.:  ..||          .| |||   |.:::|... :|
351 ALTQEESSAQLSNEEEHLDSTRGSVHSLDAGLLLPSGDPFSKSDNDMFKD 400
          .         .         .         .         .
218 .............SASISSFSLG.................GAAGSVS. 234
                 |:|: | .||                .:|:|||
401 GLRRAQSTDSLGTSGSLQSKALGYNYKAKSAGNLDESDFGPLVGADSVSE 450
          .         .         .         .         .
235 ......................LRGPQGLSPEQEETASLVSTGTLVPEGI 262
                          |: .::.|||||||||:|..| . |:
451 NFDTASLGSLQMPSGFMLTKDQERAIKAMTPEQEETASLLSSVTQGMESA 500
          .         .         .         .         .
263 YLPPPGYQLVPDSQWEQLQVEG........................... 284
    |:.|.||.||.:.:|: || |.
501 YVSPSGYRLVSETEWNLLQKEVHNAGNKLGRRCDMCSNYEKQLQGIQIQE 550
          .         .         .         .         .
285 ..............RQLQKELESVRRERDELQEGLSRSNEDCAKQMQVLL 320
                  || ..:||...::::||:: :..|.||:..|: .|:
551 AEIRDQVKKLQLMLRQANDQLEKIMKDKQELEDFIKQSSEDSSHQISALV 600
          .         .         .         .         .
321 AQVQNSEQLLRTLQGTVSQAQERVQRQMAELATSHKCLSQEVKRLNEENR 370
     ..|.|| || .||..:|||.  || ||| |  |:. :|:|: ||..:|
601 LRAQASEILLEELQQGLSQAKRDVQEQMAVLMQSREQVSEELVRLQKDND 650
          .         .         .         .         .
371 GLRAEQLPSSALQGSEQQEDQDEALPSSIQELHQLVRHTRQQARARQQAQ 420
    :|.:.:    .|:.| ||.: |  ||...:.|::|| . |::  . . |.
651 SLQGKH....SLHVSLQQAE.DFILPDTTEALRELVLKYREDIINVRTAA 695
          .         .         .         .         .
421 EHEAERLRIEIVKLREALDEETAAKASLEGQLRVQREETDVLEASLCSLR 470
    :| .|:|: ||: |:|.::.| . |..||: |.:: |:..  ||::||:
696 DHVEEKLKAEILFLKEQIQAEQCLKENLEETLQLEIENCKEEIASISSLK 745
          .         .         .         .         .
471 IETERVQQEH.................................HKAQLTD 487
     | ||:. |.                                || :.:
746 AELERIKVEKGQLESTLREKSQQLESLQEIKISLEEQLKKETAAKATVEQ 795
          .         .         .         .         .
488 LLSEQRAKALRLQAELETSEQVQRDFVRLSQALQVRLEQIRQADTLEQVR 537
    |: |::.|| |||.||:.|||||||||:|||.||||.||.|||||.||.:|
796 LMFEEKNKAQRLQTELDVSEQVQRDFVKLSQTLQVQLERIRQADSLERIR 845
    .
538 SILDEAPLRDIKDIKDS 554
    .||::..| ||.::.:.
846 AILNDTKLTDINQLPET 862
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Fig. 1. Alignment of the amino acid sequences of Rabaptin-5β and Rabaptin-5. The alignment was performed with the GAP program of the GCG package. The predicted coiled-coil domains are underlined (amino acids 22–49; 276–376; 403–508 with $P > 0.6$) using the coil program with a window of 21 (Lupas, 1996). The upper sequence corresponds to Rabaptin-5β, the lower one to Rabaptin-5.

4.6 (Figure 1). The protein sequence is hydrophilic, with no potential signal sequence or transmembrane domains. The N- and C-terminal regions are predicted to be mainly α-helical and contain heptad repeats characteristic of coiled-coil domains (Lupas, 1996). According to the

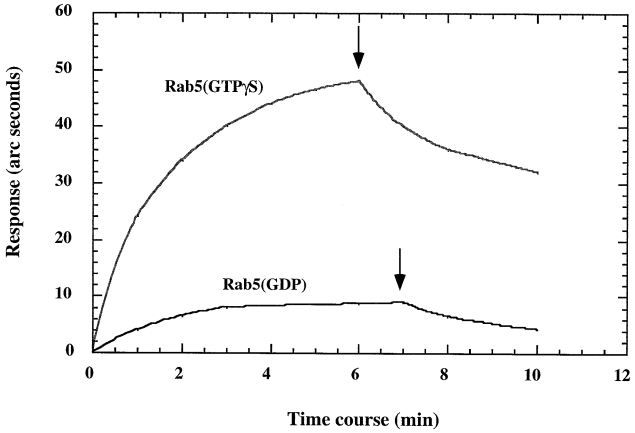

Fig. 2. Sensograms of Rab5 binding to Rabaptin-5β. The aminosilane surface of the Biosensor cuvette was derived with Rabaptin-5β. Rab5–GTPγS or Rab5–GDP were bound at a concentration of 0.2 μM. The arrows indicate the wash out of Rab5 with binding buffer.

pairwise program (Berger *et al.*, 1995), two coiled-coil domains in the C-terminus are linked via a hinge to one coiled-coil domain in the N-terminal part. Protein 2.5/FRA shares 42% identity and 62% similarity with Rabaptin-5. This similariy is highest in the coiled-coil domains but protein 2.5/FRA nevertheless lacks one coiled-coil domain in the N-terminal part (Figure 1).

The interaction between Rab5 and protein 2.5/FRA was confirmed biochemically. Binding of bacterially expressed recombinant protein 2.5/FRA to recombinant Rab5 was measured in a Biosensor (Davies and Knight, 1993). For this purpose, protein 2.5/FRA was coupled to aminosilane coated biosensor cuvettes and incubated with GTPγS- or GDP-loaded Rab5. Consistent with the data from the two-hybrid system, the results shown in Figure 2 indicate that protein 2.5/FRA interacted with the GTP-bound form of Rab5, whereas low binding was observed with Rab5–GDP and no significant interaction was detected with the control, Rab1. Due to its specific interaction pattern and its sequence similarity with Rabaptin-5, we therefore name the protein Rabaptin-5β.

Cellular distribution of Rabaptin-5β

To characterize Rabaptin-5β functionally, we raised antisera in rabbits against the full-length protein expressed in *Escherichia coli*. The antisera were affinity-purified and first characterized by immunoblot analysis. In agreement with the predicted molecular mass of Rabaptin-5β, the affinity purified antibody recognized a 64 kDa protein. While the same band was also detected by the anti-Rabaptin-5, indicating that the two proteins are both structurally and immunologically related, the anti-Rabaptin-5β antibody did not appear to cross-recognize Rabaptin-5. The specificity of the anti-Rabaptin-5β antibody allows us to discriminate between the two proteins. Analysis of different cell lines revealed that Rabaptin-5β is expressed in several cell lines (Figure 3) and organs (Table II) examined. We used quantitative Western blot analysis, using recombinant Rabaptin-5 and Rabaptin-5β as standards, to estimate the relative abundance of the two proteins in the cytosols of rat liver, rat brain, bovine brain and HeLa cells. Interestingly, Rabaptin-5β was found to be less abundant than Rabaptin-5 in HeLa, rat brain and

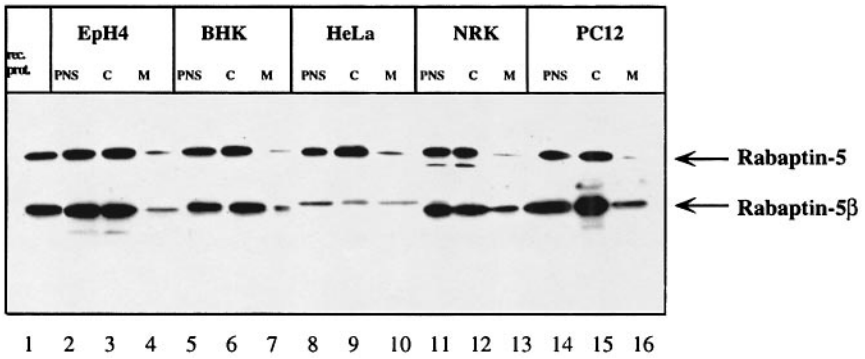

Fig. 3. Cellular distribution of Rabaptin-5β and of Rabaptin-5. EpH4, BHK, HeLa, NRK and PC12 cells were fractionated into high-speed pellet (M) and supernatant (C). Proportional amounts of supernatant (20 μg, lanes 4, 7, 10, 13 and 16) and pellet (12 μg, lanes 3, 6, 9, 12 and 15) were analyzed by SDS–PAGE followed by immunoblotting using affinity-purified anti-Rabaptin-5 and anti-Rabaptin-5β antibodies. Postnuclear supernatants (32 μg, lanes 2, 5, 8, 11 and 14) and recombinant His_6-Rabaptin-5 and Rabaptin-5β (25 ng, lane 1) were analyzed as controls.

Table II. Quantitative analysis of Rabaptin-5β and Rabaptin-5 in cytosols

Cytosol	Rabaptin-5β (pmol/mg)	Rabaptin-5 (pmol/mg)	Molar ratio Rabaptin-5β:Rabaptin-5
Rat liver	11.7	12	1:1
Rat brain	4.6	60	1:13
Bovine brain	0.78	17	1:22
HeLa cell	0.62	9	1:14

Quantitative analysis of Rabaptin-5β and Rabaptin-5 in rat liver, rat brain, bovine brain and HeLa cell cytosols was performed by immunoblot analysis of different concentrations of each cytosol referred to standard curves of recombinant Rabaptin-5β and Rabaptin-5. Purified antibodies anti-Rabaptin-5 and anti-Rabaptin-5β were used for this Western blot.

bovine brain cytosol with molar ratios of 1:14, 1:13 and 1:22, respectively (Table II). In contrast, an equal molar ratio between Rabaptin-5β and Rabaptin-5 could be observed in rat liver cytosol with 11.7 pmol/mg and 12 pmol/mg of each, respectively (Table II). This variable abundance was also evident in the various cell lines examined (Figure 3). This suggests that, although it is expressed ubiquitously, the level of expression of Rabaptin-5β differs depending on the cell type and tissue.

Fractionation of EpH4, BHK, HeLa, NRK and PC12 cells showed that Rabaptin-5β is mainly cytosolic, whereas a minor fraction is membrane bound. Similar results were obtained for Rabaptin-5 (Figure 3). However, Rabaptin-5β seems more enriched than Rabaptin-5 on the membrane of HeLa cells (Figure 3, lane 10).

Rabaptin-5β is recruited by Rab5Q79L on endosomal membranes in vivo

The results from the yeast two-hybrid system suggest that Rabaptin-5β interacts specifically with the GTP form of Rab5. To determine whether, similarly to Rabaptin-5, Rabaptin-5β could interact with and be recruited by Rab5 on endosomes *in vivo*, we co-expressed the myc epitope-tagged Rabaptin-5β with Rab5 mutants in BHK cells and studied the localization of the two proteins by confocal immunofluorescence microscopy. Cells were permeabilized with saponin prior to fixation in order to wash out the excess of exogenous cytosolic protein. Cells overexpressing Rab5Q79L displayed expanded early endosomes due to the stimulation of endocytosis and increase of early endosomes fusion (Figure 4A and B). Using anti-Rab5 and anti-myc, we observed extensive colocalization between Rab5Q79L and Rabaptin-5β (Figure 4A and B). In contrast, we observed no recruitment of Rabaptin-5β on membranes expressing Rab11Q70L, which is localized to the recycling endosomes and the trans-Golgi network (TGN) (Ullrich *et al*., 1996) (Figure 4C and D). By staining cells fixed prior to permeabilization, we verified that Rabaptin-5β was detected in the cytoplasm of cells co-transfected with Rab11Q70L, indicating that the lack of membrane recruitment is not due to lack of expression of the protein. Cells overexpressing Rab5S34N displayed fragmented early endosomes (Stenmark *et al*., 1994), which were also poorly labeled by the anti-Rabaptin-5β antibody (data not shown). These results confirm the specific interaction with the GTP-bound form of Rab5 observed in the two-hybrid system and indicate that, like Rabaptin-5, Rabaptin-5β is recruited by Rab5GTP on endosomal membranes *in vivo*.

Rabaptin-5β does not interact with Rabaptin-5 but binds Rabex-5

The repetitive pattern of hydrophobic and hydrophilic residues in the heptad repeats allows the homo- or hetero-oligomeric association of helices into a coiled-coil structure (Lupas, 1996). Rabaptin-5 contains predicted coiled-coil repeats and undergoes dimerization both *in vitro* and *in vivo* (Vitale *et al*., 1998). Since Rabaptin-5β has high probability for coiled-coil domains, we investigated whether the two proteins could form heterodimers. For this purpose, immunoprecipitation experiments from bovine brain cytosol were performed using anti-Rabaptin-5 antibodies. After immunoprecipitation with anti-Rabaptin-5β-specific serum, the precipitate and the supernatant were probed with anti-Rabaptin-5 serum. Rabaptin-5 was not detected in the precipitate (Figure 5A, panel beads, lane IR-5β) but was easily detectable either in the corresponding supernatant (panel sup, lane IR-5β) or when the immunoprecipitation was performed with anti-Rabaptin-5 serum (panel beads, lane IR-5). A control in which preimmune serum replaced anti-Rabaptin-5β serum gave no Rabaptin-5β signal (panel beads, lane PI). Conversely, immunoprecipitations performed with anti-Rabaptin-5 serum showed Rabaptin-5 and Rabaptin-5β when the blot was probed with anti-Rabaptin-5 serum, but only Rabaptin-5β when the blot was probed with anti-Rabaptin-5β serum (panel

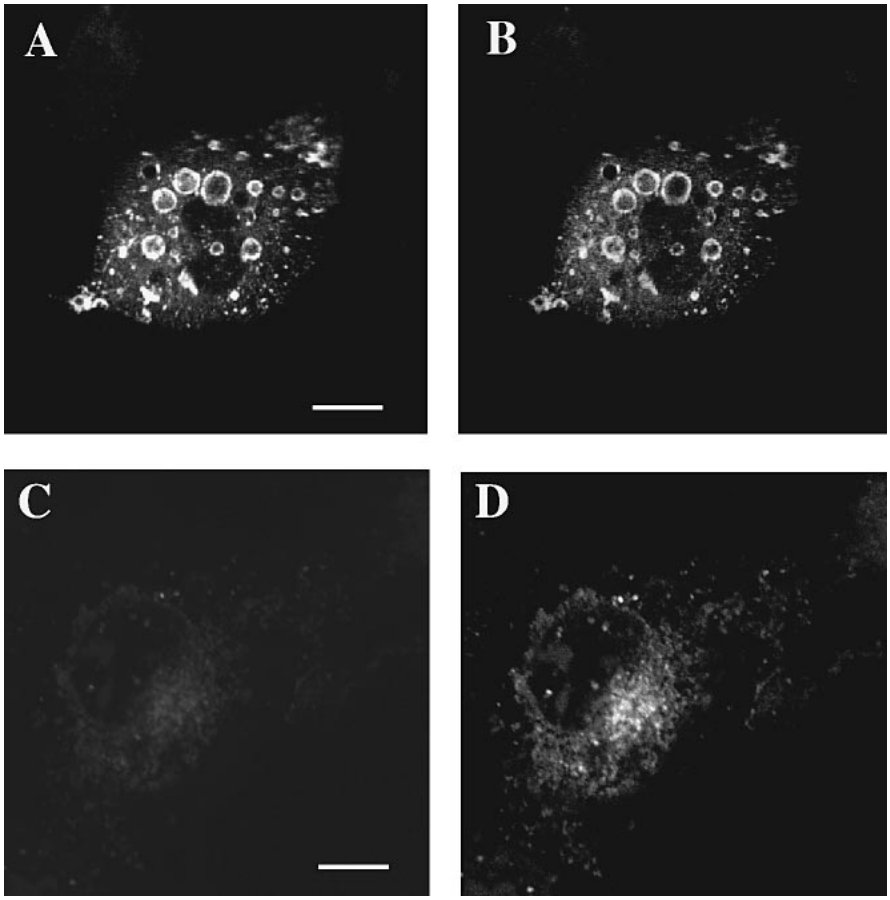

Fig. 4. Endosome targeting of Myc-tagged Rabaptin-5β in cells co-expressing Rab5Q79L. BHK cells were transfected with Myc-Rabaptin-5β and cotransfected with either Rab5Q79L (A and B) or Rab11Q70L (C and D). The cells were permeabilized with saponin prior to fixation and stained with anti-Myc monoclonal (**A** and **C**) and anti-Rab5 (**B**) or anti-Rab11 (**D**) affinity-purified polyclonal antibodies. Coverslips were viewed with a Zeiss confocal microscope. Scale bar, 10 μm.

beads, lane IR-5). This is due to the cross-reaction of the Rabaptin-5 serum, as shown previously. These immunoprecipitation experiments indicate that Rabaptin-5 and Rabaptin-5β do not form hetero-oligomers in cytosol.

It has been shown recently that Rabaptin-5 interacts specifically with a 60 kDa protein called Rabex-5, which is an exchange factor for Rab5 (Horiuchi *et al.*, 1997). In order to know whether Rabaptin-5β could also interact with Rabex-5, we performed co-immunoprecipitation experiments. After immunoprecipitation with anti-Rabaptin-5β-specific serum, the precipitate was probed with anti-Rabex-5 serum, and Rabex-5 was detected (Figure 5B, lane IR-5β). Rabex-5 was also detected in the precipitates resulting from immunoprecipitations with anti-Rabaptin-5 serum (lane IR-5) and with anti-Rabex-5 serum itself (lane IRabex-5). A negative control, in which preimmune serum was used, gave no Rabex-5 signal (lane PI). Altogether, these immunoprecipitation experiments indicate that Rabaptin-5 and Rabaptin-5β interact separately with Rabex-5, forming two independent complexes.

Rabaptin-5 and Rabaptin-5β cooperate in homotypic early endosome fusion in vitro

We next tested the function of Rabaptin-5 in a cell-free assay of homotypic fusion between early endosomes from HeLa cells. This assay is cytosol and ATP dependent and is based on the detection of an immunocomplex formed between biotinylated transferrin, internalized in one population of early endosomes, and sheep anti-human transferrin antibody, internalized in another population of early endosomes (Woodman and Warren, 1988; Horiuchi *et al*., 1997). We estimated the contributions of Rabaptin-5β and Rabaptin-5 in the fusion reaction, taking into account the relative amounts of Rabaptin-5 and Rabaptin-5β in different cytosols (Table II). Rabaptin-5 was more abundant than Rabaptin-5β (molar ratio 14:1 and 13:1 in HeLa and rat brain cytosol, respectively). To investigate the requirement for Rabaptin-5β in the fusion reaction, the protein was depleted from HeLa (Figure 6A) and rat brain cytosol (data not shown). Immunoblot analysis demonstrated that ~90% of Rabaptin-5β was immunodepleted from the cytosol under the conditions used (Figure 6A, lane 2). In parallel, immunodepletions of both cytosols were performed with anti-Rabaptin-5 serum. As the anti-Rabaptin-5 serum cross-recognizes Rabaptin-5β (Figure 6A, lane 4), the cytosol could be depleted for both Rabaptin-5 proteins. As shown in Figure 6B, mock depletion of the cytosol with preimmune serum did not affect fusion. However, depletion of HeLa cytosol with anti-Rabaptin-5β serum inhibited fusion moderately but significantly at every time point analyzed in this kinetic study. Depletion of both Rabaptin-5 and Rabaptin-5β strongly inhibited homotypic early endosome fusion at each time point tested, with a maximal inhibition (85%) after 45

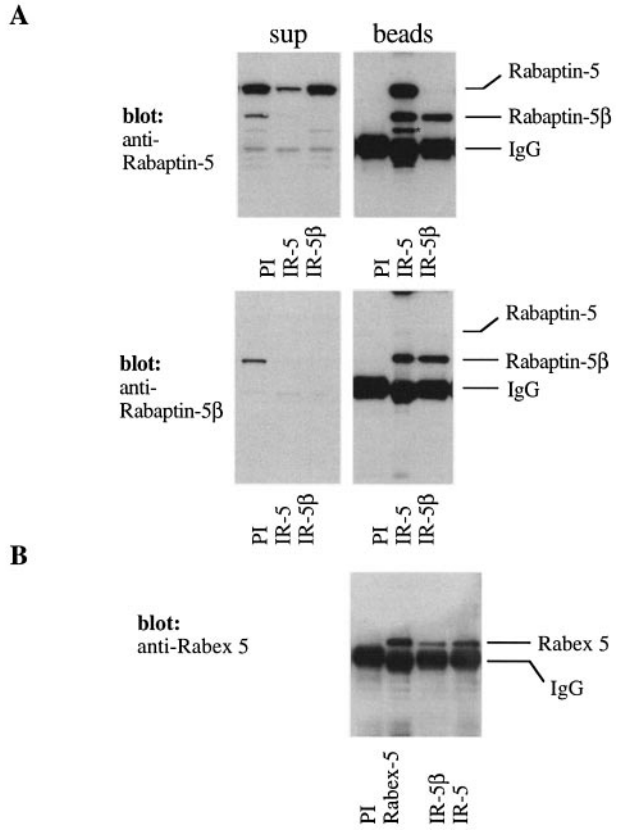

Fig. 5. Rabaptin-5β does not form heterodimers with Rabaptin-5 but does with Rabex-5. (**A**) Aliquots of 100 μg of bovine brain cytosol were immunoprecipitated with either anti-Rabaptin-5 (IR-5) or anti-Rabaptin-5β (IR-5β) serum and the precipitates (right panels) as well as the supernatants (left panels) were probed for Rabaptin-5 (upper panels) or Rabaptin-5β (lower panels). As a control, samples were also immunoprecipitated with preimmune serum (PI) and the precipitates as well as the supernatants were analyzed as described above. * represents a degradation product. (**B**) Aliquots of 100 μg of bovine brain cytosol were immunoprecipitated with either anti-Rabex-5 (IRabex-5), anti-Rabaptin-5β (IR-5β) or Rabaptin-5 (IR-5) and the precipitates were probed for Rabex-5. A control using preimmune serum (PI) was performed as described in (A).

min. Similar results were obtained using rat brain cytosol (data not shown). The addition of recombinant Rabaptin-5β alone inhibited the fusion reaction in a concentration-dependent manner (Figure 6C), suggesting that the presence of Rabex-5 is necessary for the activity of the Rabaptin-5β complex, as shown previously for Rabaptin-5 (Horiuchi *et al*., 1997).

The contribution of Rabaptin-5β may be underestimated in the fusion assay described above due to its low expression in HeLa cytosol (22 nM Rabaptin-5 versus 1.5 nM Rabaptin-5β). Unfortunately, rat liver cytosol alone, which contains equal amounts of Rabaptin-5 and Rabaptin-5β, could not support early endosome fusion in this assay. To investigate the requirement for the two Rabaptin-5 complexes in early endosome fusion further, we modified the ratio of Rabaptin-5 to Rabaptin-5β in cytosol. First, HeLa cytosol was supplemented with rat liver cytosol in order to increase the content in soluble Rabaptin-5β complex. The fusion reaction was carried out at high cytosol concentration (9 mg/ml) and the molar ratio of Rabaptin-5 to Rabaptin-5β was adjusted at 1.3:1. Using this heterologous system, we found that removal of Rabaptin-5β alone inhibited early endosome fusion by only 22% (Figure 6D lane 5), whereas depletion of both Rabaptin-5 complexes resulted in a more substantial inhibition (77%) of the fusion activity. Thus, even in the presence of quantitatively similar amounts of Rabaptin-5 and Rabaptin-5β complexes, the degree of inhibition resulting from Rabaptin-5β immunodepletion was similar to that observed in the presence of HeLa cytosol alone (Figure 6B). Secondly, we examined the ability of cytosol fractions containing either the Rabaptin-5 complex alone (Ni100) (Horiuchi *et al.*, 1997) or both Rabaptin-5 and Rabaptin-5β complexes (Ni10) to rescue the inhibition of endosome fusion caused by immunodepletion of the two Rabaptin-5 complexes. In the presence of high concentrations of cytosol, addition of the Rabaptin-5 complex alone restored the fusion activity but only to a limited extent (~70% fusion; Figure 6D, lanes 7 and 8), whereas in the presence of lower cytosol concentrations this fraction was shown previously to stimulate fusion (Horiuchi *et al.*, 1997). The lack of stimulatory activity on fusion observed here is probably due to the saturating amounts of cytosol used in this assay (data not shown). The fusion activity was more efficiently recovered when the fraction containing both Rabaptin-5 and Rabaptin-5β complexes was added, even if the concentration of the Rabaptin-5 complexes in the reaction (3 or 6 nM Rabaptin-5, 1.5 or 3 nM Rabaptin-5β; Figure 6D, lanes 9 and 10, respectively) was lower than that of the Rabaptin-5 complex provided by the Ni100 fraction (20 or 40 nM Rabaptin-5; Figure 6D, lanes 7 and 8, respectively). Immunodepletion of the Ni10 fraction of either Rabaptin-5β alone or of both Rabaptin-5 proteins demonstrated that the rescue of fusion activity depends on both Rabaptin complexes. Depletion of Rabaptin-5β significantly inhibited the rescue of fusion (Figure 6D, compare lane 11 with 12), whereas depletion of both Rabaptin-5 proteins completely abolished it (Figure 6D, lane 13). Altogether, these results suggest that the two Rabaptin-5 complexes contribute to a different extent to the fusion activity but that both Rabaptin-5 complexes are necessary to confer maximal efficiency of early endosome fusion *in vitro*.

Discussion

We have identified a novel protein, Rabaptin-5β, which interacts specifically with the active form of Rab5 and behaves as a Rab5 effector. Rabaptin-5β is recruited to early endosomes by Rab5 in a GTP-dependent manner and depletion of the protein from cytosol partially, but significantly, inhibits the fusion of early endosomes *in vitro*. Our results suggest that at least two distinct Rab5 effectors function in endosome fusion.

Rabaptin-5β shares sequence homology with Rabaptin-5, a previously identified Rab5 effector. The two proteins appear to differ, however, in some structural and functional properties. First, similar to Rabaptin-5, amino acid sequence analysis of Rabaptin-5β reveals the presence of a repetitive pattern of hydrophobic and hydrophilic residues predicted to form coiled-coil structures. However, the organization of the heptad repeats of Rabaptin-5β differs from that of Rabaptin-5 in that it lacks one predicted coiled-coil domain in the N-terminal region. Secondly, the relative level of expression of Rabaptin-5 and Rabaptin-

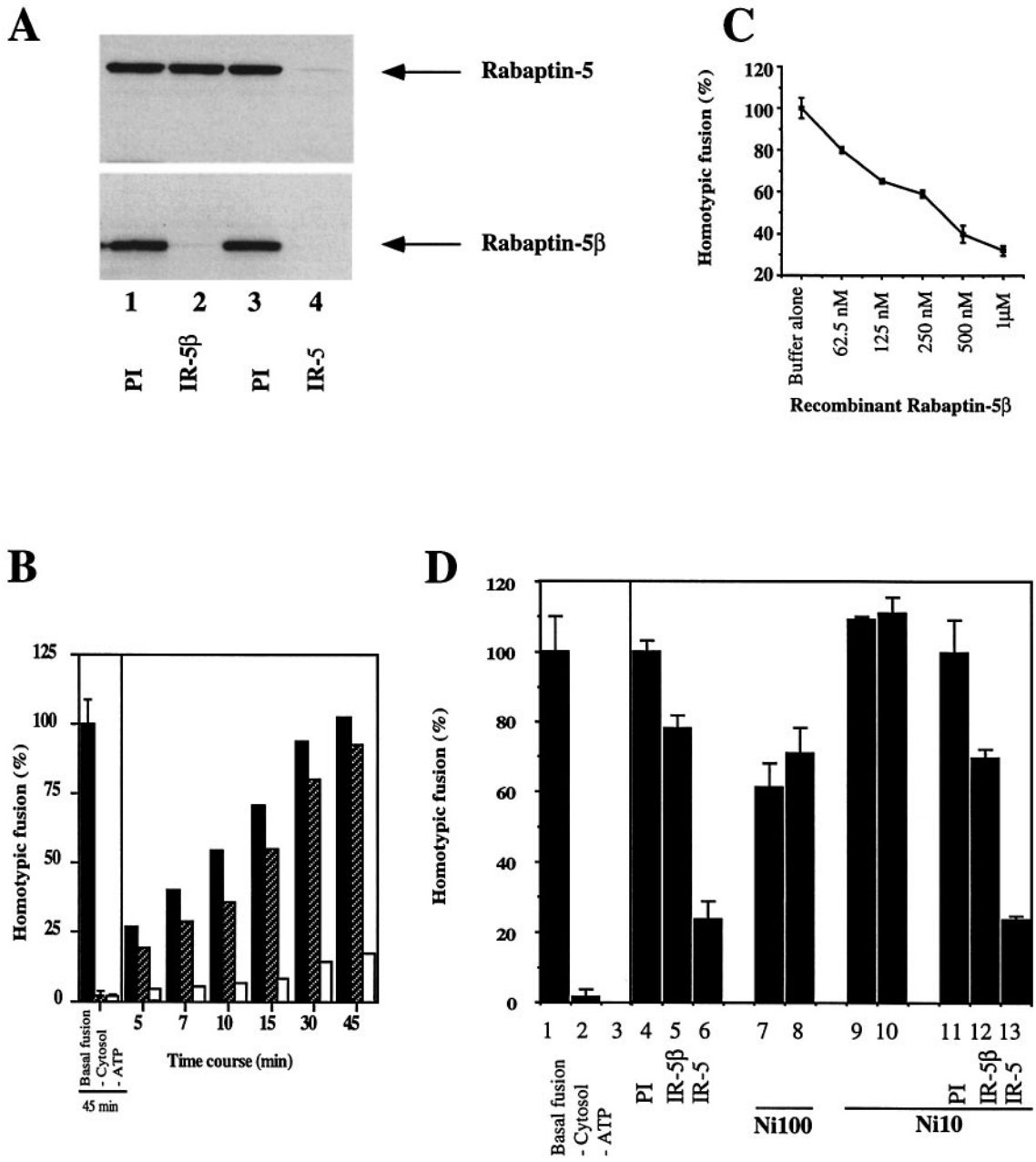

Fig. 6. Rabaptin-5β is required in homotypic EE–EE fusion in a cell-free system. (**A**) Aliquots of 560 μg of HeLa cytosol were incubated with 8 μl of either preimmune or anti-Rabaptin-5β serum (lanes 1 and 2), preimmune or anti-Rabaptin-5 serum (lanes 3 and 4) immobilized onto 20 μl of Protein A agarose beads (see Materials and methods). Beads were then pelleted and the supernatants subjected to SDS–PAGE. Immunoblots were stained using anti-Rabaptin-5 (upper part) and with anti-Rabaptin-5β (lower part) antibodies. (**B**) Time course of early endosome fusion in the presence of the various HeLa cytosol preparations described in (A). The data are expressed as percentages of the basal fusion reaction and are representative of three independent experiments. Black bars correspond to reactions performed in the presence of cytosol treated with preimmune serum, hatched bars to cytosol treated with Rabaptin-5β serum and white bars to cytosol treated with Rabaptin-5 serum. (**C**) Recombinant Rabaptin-5β inhibits *in vitro* homotypic endosome fusion. Increasing concentrations of Rabaptin-5β were added in the *in vitro* homotypic endosome fusion assay carried out in the presence of 7 mg/ml HeLa cytosol. (**D**) The two complexes Rabaptin-5–Rabex-5 and Rabaptin-5β–Rabex-5 are necessary for *in vitro* homotypic endosome fusion. The homotypic fusion of early endosomes was carried out under the following conditions: the basal reaction was performed in the presence of 3 mg/ml HeLa cytosol supplemented with 6 mg/ml rat liver cytosol (lane 1), without cytosol (lane 2), without ATP regenerating system (lane 3), or in the presence of cytosol immunodepleted with preimmune (PI, lane 4), anti-Rabaptin-5β (IR-5β, lane 5) or anti-Rabaptin-5 serum (IR-5, lane 6). For the rescue experiments, the fusion reaction in the presence of immunodepleted cytosol for both Rabaptin-5 complexes (lane 6) was supplemented with either 2 or 4 μl of Ni100 fraction (lanes 7 and 8, respectively), 2 or 4 μl of Ni10 fraction (lanes 9 and 10, respectively), or 4 μl of Ni10 fraction immunodepleted with preimmune, anti-Rabaptin-5β or anti-Rabaptin-5 serum (lanes 11, 12 and 13, respectively) as indicated.

5β varies depending on the cell type. Thirdly, Rabaptin-5β appears to be more enriched on membranes of HeLa cells compared with Rabaptin-5. Fourthly, depletion of Rabaptin-5β has a small but significant inhibitory effect on endosome fusion, whereas the additional depletion of Rabaptin-5 causes a much stronger block of fusion activity. Moreover, despite the general ability of coiled-coil proteins to form hetero-oligomers, Rabaptin-5 and Rabaptin-5β

from cytosol do not form heterodimers to a detectable extent, although they are both able to interact specifically with Rabex-5, an exchange factor for Rab5 (Horiuchi *et al.*, 1997). This argues that the two proteins are independently recruited by Rab5–GTP on the endosomal membrane.

Their structural similarity and existence in a complex with Rabex-5 might suggest that Rabaptin-5 and Rabaptin-5β are redundant in their function. We think that three observations argue against this possibility. First, the degree of inhibition of early endosome fusion obtained upon immunodepletion of the proteins does not quantitatively correlate with the relative amount or ratio of Rabaptin-5 and Rabaptin-5β complexes present in the fusion reaction. Secondly, our studies with the purified fractions of cytosol suggest that, while fusion activity can be mostly supported in the presence of the Rabaptin-5 complex alone (Horiuchi *et al.*, 1997), both Rabaptin-5 and Rabaptin-5β are required for maximal fusion activity in our *in vitro* assay. With respect to the efficiency of fusion, we propose that the two Rab5 effectors cooperate to yield optimal docking and fusion between early endosomes. Thirdly, in view of the differences in expression level, it is conceivable that Rabaptin-5 and Rabaptin-5β may fulfil specific requirements in transport in the early endocytic pathway in different cell types. For example, the high concentration of Rabaptin-5β detected in rat liver suggests that this protein may play a critical role in the regulation of endocytosis in hepatocytes. Rab5 regulates the heterotypic fusion between endocytic vesicles and early endosomes (Horiuchi *et al.*, 1997) as well as the homotypic fusion between early endosomes (Gorvel *et al.*, 1991). Furthermore, in polarized epithelial cells, Rab5 is a rate-limiting factor controlling both apical and basolateral endocytosis (Bucci *et al.*, 1994). The multiple regulatory actions of Rab5 may explain the requirement for diverse effector molecules. It is possible that the *in vitro* fusion assay that we have used in this study (using endosomes from HeLa cells) is not optimal to fully reflect the functional role of Rabaptin-5β.

Besides the Rabaptins, other effectors of Rab proteins have been identified. While it is intriguing that all these proteins share no detectable sequence homology, they all appear to be soluble factors that can be recruited on the membrane by the active form of Rab proteins. For example, Rabphilin-3a is a peripheral membrane protein that is recruited by Rab3a (and possibly by Rab3c) in the GTP-bound form on synaptic vesicles and on chromaffin granules (Chung *et al.*, 1995; Stahl *et al.*, 1996). Another putative Rab effector, Rim, interacts with Rab3a and Rab3c in the GTP-bound form and is localized to presynaptic active zones (Wang *et al.*, 1997). Rab8ip/GC kinase has been found to interact with Rab8 in a GTP-bound form. Rab8 could activate Rab8ip by translocating the kinase from cytosol to the membrane where it would phosphorylate specific membrane bound or soluble components regulating transport from the TGN to the plasma membrane (Ren *et al.*, 1996). Recently, a novel Rab9 effector has been identified that is recruited on late endosome membranes and functions in recycling from this compartment to the TGN (Diaz *et al.*, 1997). Since genetic studies in yeast have suggested that v- and t-SNARE complex formation depends on the activity of Rab proteins (Søgaard *et al.*, 1994), it is not clear at present whether Rab proteins can also directly interact with SNAREs in a GTP-dependent manner. Ypt1p is the only Rab family member which has been shown to interact directly with the t-SNARE Sed5p in yeast (Lupashin and Waters, 1997). Perhaps the interaction between Rab proteins and SNAREs is, in most cases, only indirect and is mediated by the soluble effector proteins so far identified.

The finding that Rab5 has more than one effector is reminiscent of the multiplicity of effectors of other members of the Ras superfamily of small GTPases. A large number of cytosolic factors have been discovered to bind members of the Rho family of small GTPases (Narumiya, 1996). For example, p140mDia and p160ROCK are strong candidates for Rho effectors, mediating its action on the cytoskeleton. p140mDia induces actin polymerization by recruiting an actin binding protein, profilin, to the site of Rho action (Watanabe *et al.*, 1997). p160 ROCK, once recruited by GTP-Rho to the membrane, initiates a phosphorylation cascade leading to activation and aggregation of integrin molecules with extracellular matrix proteins, triggering the sequencial assembly of focal adhesions and stress fibers (Ishizaki *et al.*, 1997). Thus, a common feature of small GTPases is their ability to transduce their signal to multiple proteins in order to control several distinct activities in parallel leading to a coordinated biological response.

A peculiar feature of Rabaptin-5 and Rabaptin-5β as Rab effectors is that they both exist in cytosol as high molecular weight complexes. We estimated the M_r of cytosolic Rabaptin-5 by sedimentation on linear glycerol gradient and gel filtration chromatography to be ~330 kDa, and obtained evidence that Rabaptin-5 is present in a multi-protein complex (Horiuchi *et al.*, 1997). Cytosolic Rabaptin-5β is engaged in a similar complex, given that its calculated M_r is ~300 kDa (data not shown). Moreover, as with Rabaptin-5, Rabaptin-5β is complexed to the Rab5 nucleotide exchange factor Rabex-5 (Horiuchi *et al.*, 1997). It is likely, therefore, that the membrane recruitment of Rabaptin-5β is also coupled to nucleotide exchange on Rab5. The sequence motifs that mediate the interaction between Rabex-5 and Rabaptin-5 or Rabaptin-5β are not known but presumably lie in the regions conserved between the two effector molecules. High sequence conservation is observed in the C-terminal regions of Rabaptin-5 and Rabaptin-5β, where the Rab5-binding site is located (Vitale *et al.*, 1998). Interestingly, the N-terminus of Rabaptin-5 appears to contain a distinct Rab-interacting domain which is specific for the GTP-bound form of Rab4 (Vitale *et al.*, 1998). Since the N-terminal region of Rabaptin-5β shares high sequence similarity with that of Rabaptin-5, it is possible that Rabaptin-5β could also bind Rab4. We are presently testing this possibility. How these molecules interact with the rest of the docking and fusion machinery is still an open question, the solution of which will require the identification of their interacting partners on the membrane.

Materials and methods

Plasmids

pLexA-Rab5, pLexA-Rab5S34N, pLexA-Rab5I53M, pLexA-Rab5Q79L, pLexA-Rab5ΔCCSN, pLexA-Rab5Q79LΔCCSN, pLexA-

Rab11Q70L, pLexA-Rab17, pLexA-Rab7Q67L and pLexA-Rab22 were obtained by cloning Rab DNAs from the respective pGEM-1 constructs into the polylinker sites of pBTM116 (Chavrier *et al.*, 1990; Lütcke *et al.*, 1993; Olkkonen *et al.*, 1993; Stenmark *et al.*, 1994; Ullrich *et al.*, 1996).

The pLexA-Rab6 constructs were from I.Janoueix-Lerosey and B.Goud, the pLexA-RasG12V construct was from A.Vojtek, and the pLexA-Rab4 constructs were from M.McCaffrey. The pLexA-Rab3 constructs were obtained by cloning Rab3 DNAs from the respective pGEM-4Z constructs, given kindly by R.Regazzi, into the polylinker sites of pBTM116 (Vojtek *et al.*, 1993).

pGEM-Rabaptin-5β was constructed by PCR of clone FRA (AN: U34932) from a rat library, using the primers CGGAATTCTCCATATGGCAGCTGCGCCAGC and CGGGATCCTCGAGCTGAGACCTGCAGTCAAC, followed by the cloning of a 1800 bp *Nde*I–*Xho*I fragment behind the epitope for the monoclonal anti-Myc antibody 9E10 into pGEM-1 (Promega). pMAL-Rabaptin-5β was constructed by PCR using the primers CGGAATTCTCCATATGGCAGCTGCGCCAGC and CGTCTAGACTGAGACCTGCAGTCAAC, followed by the cloning of a 1800 bp *Eco*RI–*Xba*I fragment into the *Eco*RI–*Xba*I sites of pMAL-C2 (New England Biolabs).

pGEM-UEP and pHAT-UEP have been described previously (Stenmark *et al.*, 1995a).

Two-hybrid screening

The yeast reporter strain L40 (Vojtek *et al.*, 1993) (MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ) was transformed with pLexA-Rab5Q79L using a lithium acetate based method and grown on synthetic medium lacking tryptophan. This transformant was then transformed with library DNA (mouse brain cDNA library, kindly provided by J.Camonis). The transformants were grown for 8 h in synthetic medium lacking tryptophan and leucine, and plated on synthetic medium lacking histidine, leucine, tryptophan, uracil and lysine. Colonies were picked 4.5 days after plating and tested for β-galactosidase activity (Vojtek *et al.*, 1993), using a replica filter assay. Library plasmids from positive clones were rescued into *E.coli* HB101 cells plated on leucine-free medium and subsequently analyzed by transformation tests and DNA sequencing.

Cloning of Rabaptin-5β

cDNA was prepared from rat brain poly(A) + RNA (50 ng; Clontech) using an oligo(dT) primer (100 pmoles) annealed at 68°C for 5 and 10 min at room temperature, in a reaction mixture containing DTT (1 mM), dNTPs (1 mM) and AMV (10U, Life Science) for 90 min at 42°C. The sscDNA obtained was amplified by PCR using the primers CGGAATTCTCCATATGGCAGCTGCGCCAGC and CGGGATCCGGGGTACCCCGGAGGGGG on one hand, and the primers CGGAATTCGGGGTACCAGCTTGTTC and CGGGATCCTCGAGCTGAGACCTGCAGTCAAC on the other hand. The 840 bp *Nde*I–*Kpn*I and 960 bp *Kpn*I–*Bam*HI fragments of the 1800 bp product were cloned into the *Nde*I–*Bam*HI sites of the pET16b vector.

Cells and transfection

BHK-21 cells were infected for 30 min with T7 RNA polymerase recombinant vaccinia virus (vT7) and then transfected with pGEM-1 plasmids containing the cDNA of interest, by use of DOTAP (Boehringer, Mannheim), in order to obtain transient overexpression (Stenmark *et al.*, 1995b). The transfection time was 4 h.

Confocal immunofluorescence microscopy

Cells grown on 10 mm round coverslips were either permeabilized with 0.05% saponin prior to fixation or fixed directly in 3% paraformaldehyde and permeabilized with 0.1% Triton X-100 afterwards, as described. Overexpressed proteins were visualized with affinity-purified antibodies against Rab5C-terminal peptide (Chavrier *et al.*, 1990), purified mouse anti-myc mAb 9E10 (Evan *et al.*, 1985) and purified polyclonal antibody anti-Rab11 (Ullrich *et al.*, 1996). The secondary antibodies used were a rhodamine-labeled donkey anti-rabbit antibody and a FITC-labeled donkey anti-mouse antibody (both from DiaNova). Cells were mounted on Mowiol and viewed in the Zweiss confocal microscope, using the excitation wavelengths 529 and 476 nm.

Recombinant proteins

DH5α *E.coli* cells, transformed with pMAL-Rabaptin-5β, were incubated for 3 h at 37°C in the presence of 0.3 mM IPTG to induce expression of the MBP-Rabaptin-5β fusion protein. The protein was purified on amylose resin, according to the native purification protocol from the manufacturer (New England Biolabs) and cleaved with factor Xa in order to separate the MBP part from the Rabaptin-5β protein. Further purification was done by gel chromatography on a superdex 200 FPLC column (Pharmacia). By this method, Rabaptin-5β was >90% pure. The purification of His_6-Rabaptin-5 was done as described (Stenmark *et al.*, 1995).

Biosensor assays

Rabaptin-5β (0.1 mg/ml) was coupled to aminosilane coated biosensor cuvettes (Affinity, Germany) using Bis(Sulfosuccinimidyl)suberate (BS^3) as a crosslinking agent, following the manufacturer's instructions. Interactions with Rab5–GTPγS or Rab5–GDP (0.2 μM each) were monitored on an IAsys Auto+Biosensor (Affinity) in 20 mM HEPES pH 7.4, 2 mM $MgCl_2$, 100 mM NaCl, 1mM DTT. The nucleotide exchange in Rab5 was done as described (Sasaki *et al.*, 1990). After binding to the cuvette, the surface was regenerated by washing with 10 mM HCl and then with 20 mM HEPES pH 7.4, 1 M NaCl.

Cell fractionation and immunoblot analysis of Rabaptin-5β and Rabaptin-5

EpH4, BHK, HeLa, NRK and PC12 cells were grown to confluence in four Costar plates (10 cm) each, scraped into PBS and then pelleted and homogenized in 400 μl of 250 mM sucrose and 3 mM imidazole (pH 7.2) by five passages through a 22-gauge canule. Nuclei and debris were pelleted by centrifugation at 4000 r.p.m. for 5 min in an Eppendorf centrifuge. The postnuclear supernatant was centrifuged at 60 000 r.p.m. for 1 h at 4°C in a Beckmann TLA-100 rotor, and aliquots of the pellet and supernatant fractions were analyzed by SDS–PAGE followed by immunoblotting.

Immunodepletion of cytosol

The immunodepletions of HeLa, rat brain and rat liver cytosols were performed by incubating the respective cytosols with 20 μl of Protein A agarose beads (Boehringer) coated with 8 μl of preimmune, anti-Rabaptin-5 or anti-Rabaptin-5β serum at 4°C for 1 h, followed by washing of the beads with phosphate-buffered saline (PBS).

Immunoprecipitations

Twenty μl of either anti-Rabaptin-5β, anti-Rabaptin-5 or anti-Rabex-5 or preimmune serum were coupled to protein A-agarose (50 μl) for 30 min at 4°C. After three washes in PBS 1×, 100 μg of bovine brain cytosol were precipitated for 2 h at 4°C. Immunoprecipitates were washed three times with PBS 1× and resuspended in sample buffer. After boiling for 5 min at 95°C, eluates and supernatants were analyzed by SDS–PAGE, blotted onto nitrocellulose and probed with the indicated antibodies, followed by ECL detection (Amersham International).

In vitro fusion assay

The *in vitro* early endosome fusion assay was carried out as described previously (Horiuchi *et al.*, 1997). Briefly, two distinct enriched populations of early endosomes labeled with either biotinylated transferrin of a sheep anti-human transferrin antibody were prepared from HeLa cells by sucrose sedimentation. The basal fusion reaction consisted of the two endosome-enriched fractions incubated for 45 min at 37°C in the presence of either 7 mg/ml of HeLa cytosol or 3 mg/ml HeLa cytosol supplemented with 6 mg/ml rat liver cytosol, unlabeled transferrin and an ATP regeneration system (17.3 mM creatine phosphate, 87 mg/ml creatine kinase and 2.2 mM ATP). The fusion was quantified by incubation of the fusion mix with wash buffer (50 mM Tris pH 7.5, 100 mM NaCl, 2 g/l BSA and 2% Triton X-100) and streptavidin-coated magnetic beads (Dynal). After two washes in wash buffer, the samples were incubated with a rabbit anti-sheep secondary antibody coupled to a ruthenium trisbipyridine chelate (IGEN) and measured with an Origen Analyzer (IGEN) (Woodman and Warren, 1988). The data are expressed as the percentage of the basal fusion reaction.

Purification of Rabaptin-5β and Rabaptin-5 complexes

The purification of Rabaptin-5 complex was done as described in Horiuchi *et al.* (1997). For the last step, the Rabaptin-5 Mono Q peak was incubated at 4°C for 2 h with 300 μl of Ni-NTA agarose (Qiagen) equilibrated with buffer A (20 mM HEPES–KOH, pH 7.2, 5 mM $MgCl_2$, 1 mM DTT). The beads were transferred to a 10 ml chromatographic column (Bio-Rad) and washed with 15 ml of buffer A containing 10 mM imidazole (Ni10 elution fraction). At this step, the fraction was enriched in Rabaptin-5β complex. The Rabaptin-5 complex eluted with 1 ml of buffer A containing 100 mM imidazole (Ni100 elution fraction). As reported previously (Horiuchi *et al.*, 1997), this fraction is devoid of

Rabaptin-5β. Quantitative analysis of Rabaptin-5β and Rabaptin-5 in the fractions was performed by immunoblot using the corresponding recombinant proteins expressed in *E.coli* as standards. The Ni 100 fraction contained 200 nM Rabaptin-5 whereas the Ni10 fraction contained 30 nM Rabaptin-5 and 15 nM Rabaptin-5β.

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