Co-operative regulation of endocytosis by three RAB5 isoforms

Cecilia Bucci[a], Anne Lütcke[b], Olivia Steele-Mortimer[b], Vesa M. Olkkonen[c], Paul Dupree[d], Mario Chiariello[a], Carmelo B. Bruni[a], Kai Simons[b], Marino Zerial[b,*]

[a]*Dipartimento di Biologia e Patologia Cellulare e Moleculare 'L. Califano' e Centro di Endocrinologia ed Oncologia Sperimentale del Consiglio Nazionale delle Ricerche, Via S. Pansini 5, 80131 Napoli, Italy*
[b]*European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstrasse 1, D-69012 Heidelberg, Germany*
[c]*National Public Health Institute, Department of Biochemistry, Mannerheimintie 166, SF-00300, Helsinki, Finland*
[d]*Department of Plant Sciences, Cambridge University, Downing Cambridge, CB-23EA, UK*

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Abstract Rab proteins are small GTPases involved in the regulation of membrane traffic. Rab5a has been shown to regulate transport in the early endocytic pathway. Here we report the isolation of cDNA clones encoding two highly related isoforms, Rab5b and Rab5c. The two proteins share with Rab5a all the structural features required for regulation of endocytosis. Rab5b and Rab5c colocalize with the both transferrin receptor and Rab5a, stimulate the homotypic fusion between early endosomes in vitro and increase the rate of endocytosis when overexpressed in vivo. These data demonstrate that three Rab5 isoforms cooperate in the regulation of endocytosis in eukaryotic cells.

Key words: Rab protein; Rab5 isoform; Endocytosis; Eukaryotic cell

1. Introduction

A common feature of the Ras superfamily of small GTPases is the existence of subgroups of structurally related isoforms sharing high sequence identity (80–90%) [1]. Isoforms are widely found among members of the different subfamilies. For example, three highly related Ras proteins, H-Ras, K-Ras and N-Ras, constitute a subgroup in the Ras subfamily. Other isoforms are Rap1A and Rap1B, Rap2A and Rap2B, and Ral A and Ral B. Examples of isoforms in the Rho subfamily are Rho1A and Rho1C, and Rac1 and Rac2. The ARF subfamily also contains several highly related proteins [2,3] and, recently, two isoforms of Sar1 (Sar1A and Sar1B) have been identified in mammalian cells [4].

Subgroups of highly homologous proteins exist also in the Rab protein subfamily. In the case of Rab1 two isoforms, Rab1a and Rab1b, have been discovered [5]. These two proteins have been shown to colocalize at the level of the intermediate compartment and seem to have a similar role in controlling ER to Golgi transport. However, proteins that share high sequence homology do not necessarily share the same functional role. For example, the Rab3 subgroup accounts for at least four isoforms (Rab3a, Rab3b, Rab3c and Rab3d) sharing high sequence identity (77–85%). However, while Rab3a appears to be only expressed in neurons and neuroendocrine cells and, together with Rab3c, regulates neurotransmitter release [7–11], Rab3b seems to be specifically expressed in polarized epithelial cells [12] and appears to have a divergent function [13]. The Rab3d protein is mainly expressed in adipocytes and might be involved in glucose transporter trafficking in response to insulin stimulation [14]. Finally, a possible fifth member of this subgroup has been discovered in the cytoplasmic site of zymogen granules of pancreatic acinar cells [6]. Therefore, this subgroup of closely related proteins seems to be quite heterogeneous, its members having a cell type specific localization and possibly different functions. For Rab4 two isoforms have been discovered: Rab4a is present on the early endosome and regulate recycling but the localization and the function of Rab4b are not yet known [15–17].

Rab5 is a protein localized to the plasma membrane, clathrin coated vesicles and early endosomes and is ubiquitously expressed [18,19]. The protein is essential for in vitro homotypic fusion of early endosomes and is able to increase the rate of endocytosis in vivo when overexpressed [19,20]. It has recently being renamed Rab5a since the discovery of two partial cDNA clones highly related that were then called Rab5b and Rab5c [21,22]. This indicated the presence of a subgroup of at least three Rab5 protein in mammalian cells. Interestingly in yeast, three highly related Rab5 proteins have been isolated and named Ypt51p, Ypt52p, Ypt53p [23]. These proteins share between 52% and 54% identity with canine Rab5a and seem to have similar or at least overlapping functions in the endocytic pathway, suggesting a similarity with the situation in mammalian cells. Do mammalian Rab5a, Rab5b and Rab5c perform different functions, similar to the members of the Rab3 subgroup, or do they share the same role? Given that neither the intracellular localization, nor the tissue distribution nor the function of the Rab5b and Rab5c isoforms has been investigated yet, we decided to begin the functional characterization of these proteins. In this study we report the isolation of full-length cDNA clones for Rab5b and Rab5c. Furthermore, we investigated the localization and fucntion of these proteins in BHK cells using a combination of in vitro and in vivo assays. Our results indicate that three Rab5 proteins regulate the dynamics of early endosomes fusion and the rate of endocytosis in eukaryotic cells.

2. Materials and methods

2.1. Cloning of mouse Rab5b

Based on the partial sequence previously identified [21], a 5′ RACE protocol was performed essentially as described [21]. The universal primers KXEB and KXEBpoly(C) were used in combination with the nested gene-specific primers R1 (5′-TCGAGGATCCATCTAAACACCGGA-3′) and R2 (5′-ACCTCGAGGATCCGGATTGGGTGAGGAAGC-3′). For both PCRs, an annealing temperature of 60°C was used. The amplification products of the second PCR were subcloned

*Corresponding author. Fax: (49) (6221) 387306.

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into pGEM1 (Promega Corp.) and analyzed by double-stranded DNA sequencing using the T7 Sequencing kit (Pharmacia Fine Chemicals).

Since the sequence of the clones containing the 5′ end of murine Rab5b were found to be identical to the one of human Rab5b [22], the sequence could be extended to the 3′ end of the coding region by two sequential PCRs using sets of nested primers derived from the 5′ of the open reading frame obtained by RACE (S1: 5′-CTCCTACTTTGATAACCTG-3′; S2: 5′-CCGAGAATTCATATGACTAGCAGAAGTACAGCC-3′) and from the 3′-untranslated region of the human Rab5b (R3: 5′-CTTAGCTCTTGTGCTAGCTCC-3′; R4: 5′-ACGTGGATCCTTGTTTGCTGCTAGCCACC-3′; [22]. The first PCR was carried out under standard conditions (20 ml volume, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM of dATP, dGTP, dCTP, dTTP, 20 pmol of S1 and R3 and one unit of Amplitaq (Perkin Elmer Cetus) using 0.4 μl of first strand cDNA [21] as template. The reaction was overlaid with 20 μl of mineral oil (Sigma Immunochemicals), transferred to a programmable thermal cycler (Perkin Elmer Cetus) and incubated for 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min with a final extension at 72°C for 2 min. The PCR products were run on a 1% Seaplaque agarose (FMC Corp. Bioproducts) gel and excised. The agarose was melted and 5 μl were used as the template for the second PCR which was carried out in a 100 μl volume using 100 pmol of S2 and R4. The amplification products of the second PCR were subcloned into pGEM1. The amino acid sequence of mouse Rab5b was found to be 100% identical to human Rab5b [22].

2.2. Cloning of canine Rab5c

A lambda UNI-ZAP XR (Stratagene) MDCK II cell cDNA library [18] was probed with a PCR fragment spanning the mouse Rab5c sequence between the first and second conserved GTP-binding site motifs [21]. Out of the 300,000 plaques probed, four turned out positive. After plaque purification and in vivo excision according to the manufacturer's procedure (Stratagene), the cDNA inserts in pBluescript SK(−) were sequenced using the Pharmacia T7 polymerase kit and the supplier's protocol for double-stranded DNA templates. Two of the positive clones showed Rab5c sequence, one being apparently full-length. The complete sequence of this insert was determined by the 'primer walking' strategy. Duplicate lifts were performed using nitrocellulase sheets (Schleicher and Schuell). The probe fragment was labelled with [α-^{32}P]dCTP by random hexanucleotide priming as described [24]. The filters were hybridized overnight at 42°C in 50% formamide 5 × Denhardt's solution, 5 SSC, 1% SDS.

2.3. RNA Preparation and Northern blot analysis

RNA preparation and Northern blot hybridization were performed as described [25]. The primer pairs used for the preparation of the radiolabelled PCR probes were 5′-AGCTGAATTCAGGAAACAAGGCTG-3′/5′-CTGAGGATCCTTACTGACTCCTGGTTGGCTGCG3′ (Rab5a) and 5′-CAGCTTTGATCCCTGCCAAGGC-3′/Sp6 (Rab5b). In the case of Rab5a, the filters were washed at low stringency (2 × SSC, 0.5% SDS, 60°C).

2.4. Antibodies

Polyclonal rabbit antisera directed against Rab5a, Rab5b or Rab5c were raised using synthetic peptides (PKNEPQNPGANSARGR, KQNLGGAAGRSRGVDLHEQS and KQNAAGAPSRNRGVDLQENS, respectively) corresponding to the C-terminal hypervariable region. The peptides were coupled to keyhole limpet hemocyanin and purified as previously described [18]. The mouse hybridoma cell line 9E10 producing a monoclonal antibody against the c-myc epitope [26] was a gift from Dr. Stephen Fuller. The antibody was produced and purified as described [27]. The monoclonal antibody against the human transferrin receptor was obtained from Boehringer Mannheim (Germany).

2.5. Plasmid construction

All the cDNAs were cloned with appropriate sites into the pGEM1 vector under the T7 promoter so that they could be used in the vT7 recombinant vaccinia virus expression system. Rab5b and Rab5c cDNAs were also cloned in the pGEM-*myc* plasmid [28] that carries 13 amino acids containing the *myc* epitope recognized by the 9E10 monoclonal antibodies [26]. The Rab5a Ile^{133} mutant was constructed as preciously described [20]. The Rab5b Leu^{79}, Rab5b Ile^{133}, Rab5c Leu^{80}, Rab5c Ile^{134} mutants were made by the PCR mediated mutagenesis [29] using in the first amplification the mutated oligonucleotides 5′-GTGGTATCGCTCCAGCCCAGCAGTGTC-3′, 5′-CAGCTTTGATCCCTGCCAAGGC-3′, 5′-GTGATACCGCTCCAGTCCAGCTGTGT-3′ and 5′-CGTCATTGCACTCGCGGGGATCAAGGCAGACCTGGCCAGCA-3′, respectively. We used as outer primers oligonucleotides corresponding to the SP6 and T7 promoters present in the vector. The resulting mutated bands were isolated by agarose gel electrophoresis, purified and cloned into pGEM1 under the control of the T7 promoter. The bands were then sequenced completely to ensure that the wanted mutations were present and that no additional ones were introduced by the *Taq* polymerase.

2.6. Cell culture and transfection

BHK cells were grown at 37°C in GMEM supplemented with 10% tryptose phosphate broth, 5% foetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 10 μg/ml streptomycin in a 5% CO_2 incubator.

The cells were split on 24-well plates the day before the experiment so that they could be 80% confluent before transfection. Infection by the vT7 recombinant vaccinia virus and subsequent lipofection using DOTAP (Boehringer Mannheim) were performed as previously described [19]. Briefly the cells were infected with 5–10 pfu/cell of vaccinia recombinant T7 virus for 30 min at room temperature with intermittent shaking in GMEM and subsequently transfected with 2.0 μg of the appropriate plasmid using DOTAP following the manufacturer instructions. The transfection was always performed in the presence of 10 mM hydroxyurea, blocking DNA replication to prevent the virus to enter in the late phase. Four to five hours after transfection the cells were processed either for immunofluorescence or for internalization assays. In the case of transfection with hTR we further incubated the cells for 90 min at 37°C with cycloheximide (10 μg/ml). The transfection was prolonged to 7 h when the cells were needed for the preparation of cytosol used in the fusion assay.

2.7. Confocal immunofluorescence microscopy

Immunofluorescence was performed essentially as described [30]. Before fixation with 3% paraformaldehyde cells on 10 mm diameter round coverslip were permeabilized with 0.1% saponin in 80 mM PIPES (pH 6.8), 5 mM EGTA and 1 mM $MgCl_2$ for 3′. After fixation the cells were washed with PBS and then a solution of 50 mM NH_4Cl in PBS was applied to quench. The first antibodies when used on overexpressed cells were diluted so that the endogenous protein was not detectable. Secondary antibodies, FITC- and TRITC-labelled donkey anti-mouse and anti-rabbit affinity purified were obtained from DiaNova (Germany). In the case of double immunofluorescence the two different antibodies were added simultaneously to the cells. The coverslips were viewed with the EMBL confocal microscope and the optical section examined were 0.4 μm thick.

2.8. In vitro fusion of early endosomes

The assay was performed essentially as described [20,31]. Briefly BHK cells were allowed to internalize avidin or biotinylated horseradish peroxidase (bHRP). After internalization cells were washed extensively and homogenized; subsequently early endosomal fractions were prepared using a flotation gradient [20]. The two populations of early endosomes were mixed in the presence of ATP, rat liver cytosol at a concentration of 4 mg/ml, and cytosol from BHK cells overexpressing Rab5a, Rab5b, Rab5c, Rab5a Ile^{133}, Rab5b Ile^{133} or Rab5c Ile^{134}. After 45 min at 37°C the avidin-bHRP complexes were immunoprecipitated with an anti-avidin antibody in the presence of detergents and the amount of fusion quantified measuring the HRP enzymatic activity using σ-dianisidine as previously described [32].

2.9. Estimation of transferrin endocytosis

Human transferrin was labelled to a specific activity of 10^7 cpm/mg using Iodogen [33]. Internalization of ^{125}I-transferrin was performed essentially as previously described [27]. After 4 h of transfection cells were washed with cold PBS containing 0.1% BSA and were allowed to bind ^{125}I-transferrin for 90 min on ice. The cells were then washed extensively on ice with PBS/BSA and then incubated for different times at 37°C. After the incubation the medium was collected. Cells were then treated with 3 mg/ml of pronase (Sigma) for 30 min on ice and subsequently collected and spun at 2,500 rpm. The medium preciously collected, the supernatant and the cells represented the amount of transfer-

rin recycled, membrane-bound and internalized, respectively. The measurements of ^{125}I-transferrin were performed using an NE 1612-γ-counter (Nuclear Enterprises).

3. Results

3.1. Molecular cloning of Rab5b and Rab5c cDNAs

We previously described the isolation of partial clones for Rab5b and Rab5c from mouse kidney using a PCR-based cloning approach [21]. Using a combination of cDNA library screening and RACE protocols we have obtained cDNAs encoding the full-length proteins and determined their nucleotide sequence. Fig. 1A shows the alignment of the deduced amino acid sequences of canine Rab5a and Rab5c, and murine Rab5b. Rab5b and Rab5c are 88% and 91% identical to the Rab5a protein, respectively. The highest sequence identity is in the central region spanning the four conserved elements forming the GTP-binding site, whereas the highest sequence divergence is displayed by the extreme N- and C-terminal domains. In view of the established role of the C-terminus in targeting Rab proteins to their membranes [34,35], it is noteworthy that a RxRGVDLxE sequence and the Cysteine-motif (-CCSN) responsible for the geranylgeranylation of Rab5a [36] are shared by the three isoforms.

We next examined the pattern of expression of Rab5a and Rab5b in comparison with that of Rab5c [25] by Northern blot hybridization on adult mouse tissues. Transcripts of 2.8 kb, 3.6 kb and 1.8 kb were detected using the Rab5a, Rab5b (Fig. 1B) and Rab5c probes (not shown), respectively. The three messenger RNAs were found to be ubiquitously expressed. Only in the intestine the level of Rab5b mRNA appeared to be considerably lower than that from the other tissues examined.

These data indicate that mammalian cells express at least three isoforms of Rab5 which share high amino acid sequence identity and display a similar tissue expression and distribution. Previous studies have demonstrated that distinct structural domains specify the membrane localization and activity of RAb5a [34,37] and other Rab proteins [35,38]. The C-terminal domain functions in targeting the protein to the early endosomes but it is not sufficient to confer Rab5a function [34,37]. The N-terminus and the regions predicted to undergo nucleotide-dependent conformational changes (the effector domain or switch1, α2/L5 or switch2 and α3/L7) are additionally required for the regulatory role of Rab5a in endocytic transport [31,37].

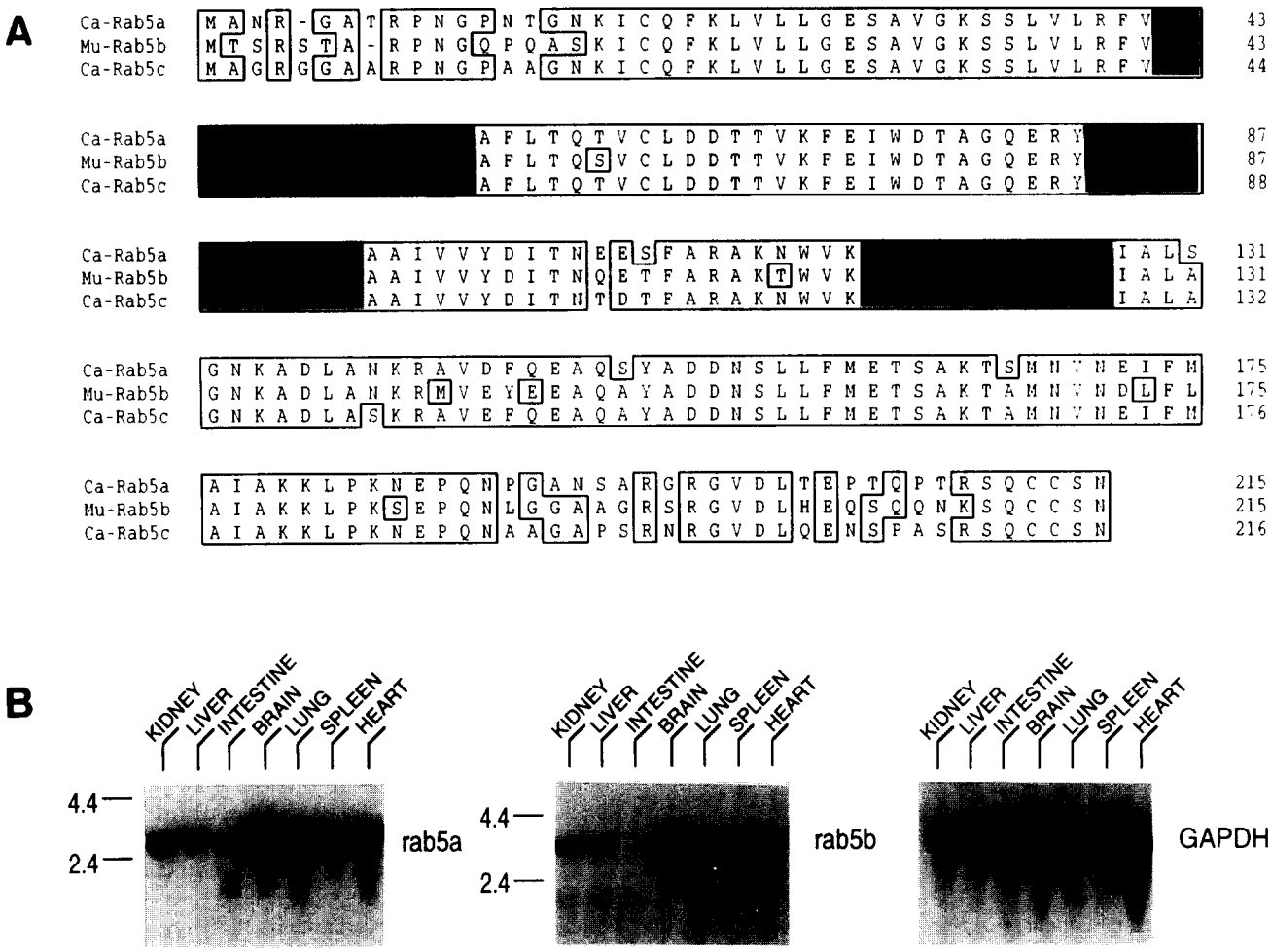

Fig. 1. Amino acid sequence and mRNA expression of Rab5a, Rab5b and Rab5c. (A) Amino acid sequence comparison between canine Rab5a, murine Rab5b and canine Rab5c. All conserved residues are boxed. The effector loop2, α2/L5 and α3/L7 regions are shaded. The nucleotide sequences of Rab5b and Rab5c are available from the EMBL/GenBank/DDB under the Accession Numbers X84239 and Z27110, respectively. (B) Northern blot analysis of Rab5a and Rab5b on adult mouse tissues. The mRNA for Rab5a and Rab5b are 2.8 kb and 3.6 kb long, respectively. The amount of RNA loaded on each lane was checked by hybridization with a GAPDH probe.

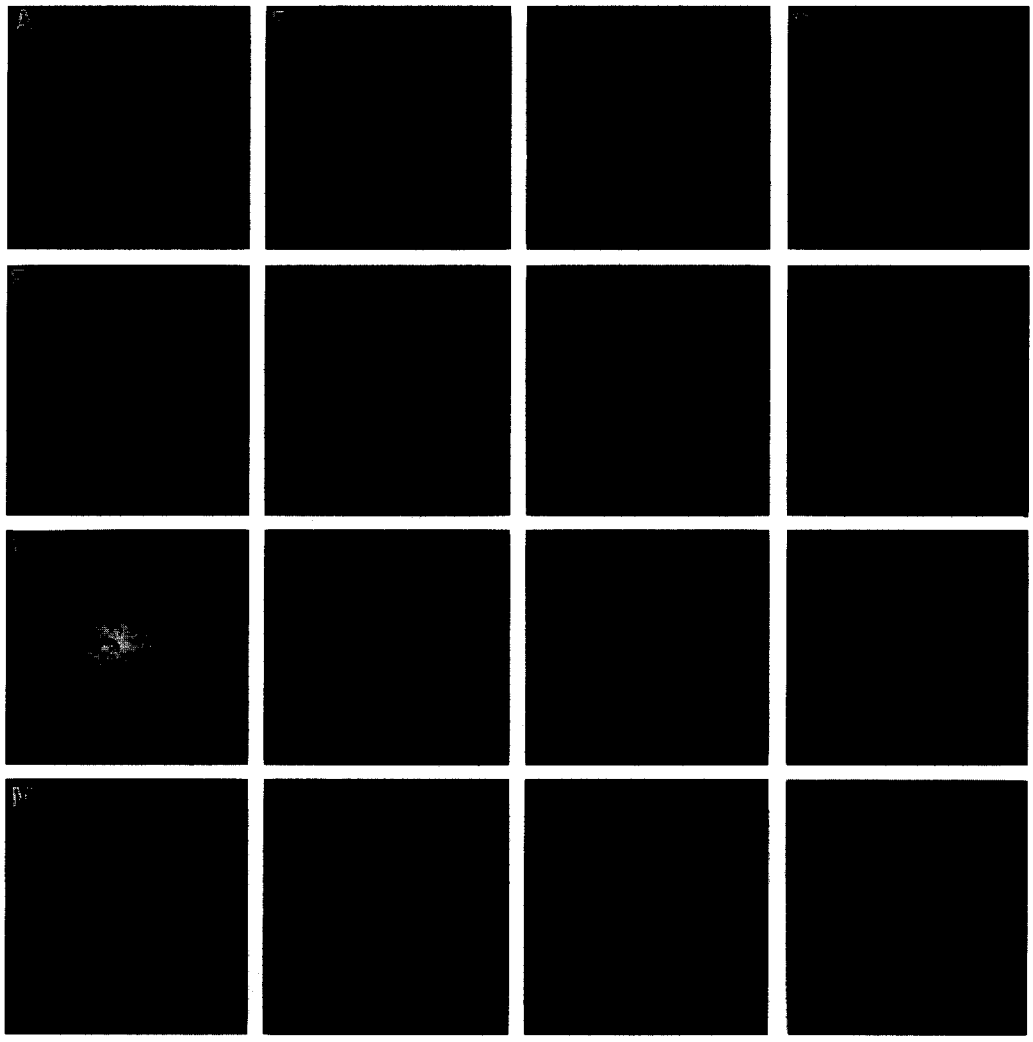

Fig. 2. Localization of Rab5a, Rab5b and Rab5c by double immunofluorescence microscopy. BHK cells grown on coverslip were infected with the vT7 vaccinia recombinant virus and either transfected with the human transferrin receptor (hTR) or with Rab5 constructs. Double immunofluorescence and confocal immunofluorescence analysis was performed as described in section 2. All these images are one optical section of 0.4 μm. Bar 10 μm. (A–H) Infected and transfected cells expressing hTR were stained using anti-hTR antibody (A,C,E,G) and affinity purified anti-Rab5b (B,D) or anti-Rab5c antibodies (F,H). (I–L) BHK cells transfected with myc-Rab5b (I,J) or myc-Rab5c (K,L) plasmids were stained with 9E10 monoclonal anti-myc antibody (I,K) and with affinity purified anti-Rab5a polyclonal antiserum (J,L). (M–P) BHK cells were transfected with Rab5b Leu^{79} (M,N) or Rab5c Leu^{80} (O,P) plasmids and stained with affinity purified anti-Rab5b or anti-Rab5c antibodies, respectively.

Given the high sequence conservation in these structural elements, the three Rab5 isoforms can be predicted to share the same localization and functional role. We therefore tested this hypothesis experimentally by determining the intracellular localization and functional role of Rab5b and Rab5c in Baby Hamster Kidney (BHK) cells.

3.2. Localization of Rab5b and Rab5c

To determine the intracellular localization of Rab5b and Rab5c proteins, polyclonal rabbit antisera were raised against peptides corresponding to the C-terminal region, as this displays the lowest sequence conservation among the three isoforms. The antibodies were affinity-purified as described in section 2 and tested against the various isoforms by immunoblotting and immunofluorescence staining on BHK cells overexpressing either Rab5a, or Rab5b or Rab5c using the T7 RNA polymerase-recombinant vaccinia virus system [19,39]. The antibodies resulted to be specific for either isoform. As for anti-Rab5a antibodies [18], anti-Rab5b and -Rab5c antibody recognized a single band by Western blot analysis. Both the intensity of the immunoblot signal and that of the immunofluorescence labelling were enhanced upon overexpression of the protein against which the antibody was raised but not of the other isoforms (data not shown). The affinity purified antibodies were therefore used to investigate the intracellular localization of the individual Rab5 isoforms. The Rab5a protein has been previously localized to the plasma membrane, clathrin coated vesicles and early endosomes and its immunofluorescence staining pattern overlaps with that of transferrin receptor (TR) [18,19]. We first examined the localization of Rab5b and Rab5c proteins in BHK cells expressing the human hTR) using the vaccinia virus-based system by double immunofluorescence confocal microscopy. Most of the endocytic structures labeled by the anti-hTR monoclonal antibody were also stained posi-

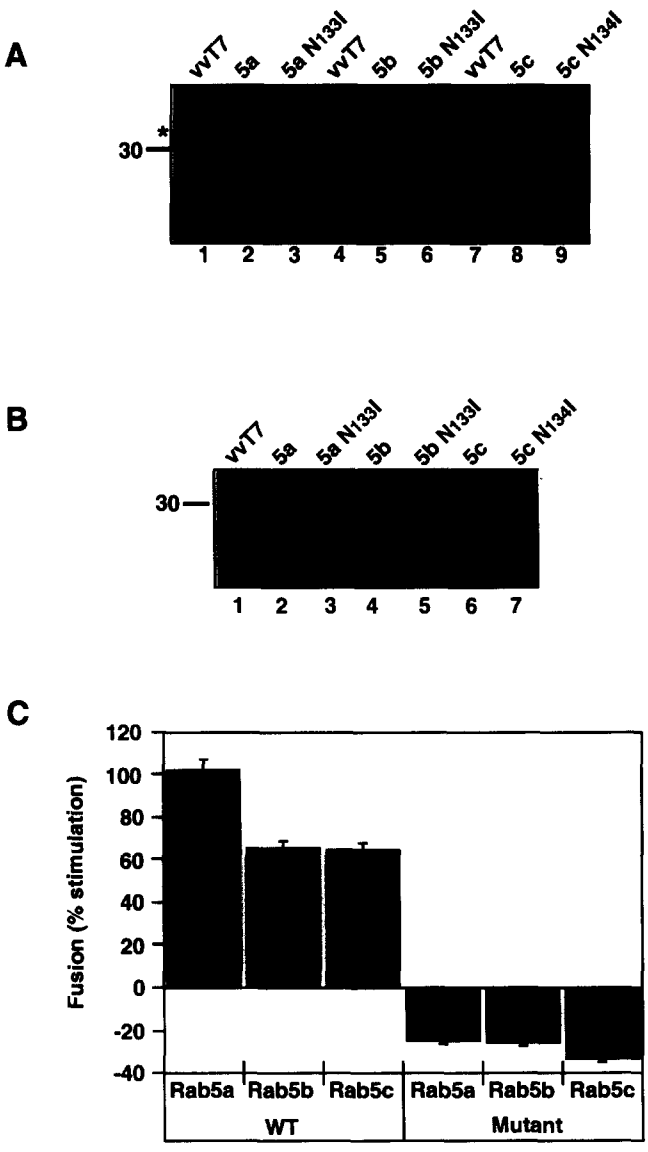

Fig. 3. Cytosols containing overexpressed Rab5b or Rab5c are able to stimulate early endosome fusion in vitro. Cytosol was prepared from BHK cells infected with vT7 and transfected with plasmids encoding Rab5a, Rab5b, Rab5c, Rab5a Ile^{133}, Rab5b Ile^{133} or Rab5c Ile^{134} for 7 h. (A) Western blot analysis on cytosol prepared from transfected cells was performed using affinity purified anti-Rab5a, anti-Rab5b and anti-Rab5c antisera. The additional band present in lane 1–3 and indicated with an asterisk is due to cross-reactivity of the anti-Rab5a antisera with a vaccinia virus protein. The Rab5b Ile^{133} protein migrates slower compared to w.t. (B) GTP overlay on the different cytosol containing overexpressed Rab5s w.t. and mutant proteins. (C) Cytosol overexpressing the different Rab5 proteins and their corresponding mutant was used in the in vitro fusion assay between early endosomes. The effect on fusion is expressed as percentage of stimulation or inhibition of the fusion activity obtained with cytosol from control BHK cells infected with the vT7 vaccinia recombinant virus.

tive with both the anti-Rab5b and anti-Rab5c affinity purified antibodies (Fig. 2A-H). To directly compare the localization of the Rab5 isoforms, we tagged the Rab5b and Rab5c proteins at their N-termini with the c-*myc* epitope and we transiently expressed them in BHK cells using the vaccinia T7 RNA polymerase system. The staining pattern of the anti-c-*myc* monoclonal antibody 9E10 was then compared with that of the affinity-purified anti-Rab5a polyclonal antibody. Extensive colocalization between myc-Rab5b and Rab5a (Fig. 2I–J), and between myc-Rab5c and Rab5a (Fig. 2K–L) was observed. These morphological results indicate that the three Rab5 isoforms colocalize within hTR-positive endocytic compartments.

3.3. Morphological alterations of the early endosomes upon overexpression of w.t. and mutant Rab5 isoforms

Rab5a is a rate-limiting regulatory GTPase for the early endocytic pathway in mammalian cells. Overexpression of wild-type Rab5a results in the appearance of enlarged early endosomes [19]. Larger vesicles than normal were also observed in cells overexpressing the Rab5b and Rab5c proteins (Fig. 2I–L) compared to control cells (Fig. 2A–H), suggesting that, similar to Rab5a, the two Rab5 isoforms regulate the dynamics of the early endosome compartment. We wanted to confirm this point by investigating whether the expression of dominant interfering mutants causes alterations in the morphology of endocytic organelles, as previously shown for Rab5a. Expression of the GTPase-deficient mutant Rab5a Leu^{79} has a more prominent effect than wild type Rab5a in expanding the size of the early endosomes [27]. In contrast, expression of the Rab5a Ile^{133} and Rab5a Asn^{34} mutants leads to the accumulation of very small endocytic structure at the periphery of the cell [19,27]. We therefore introduced similar mutations in the Rab5b and Rab5c proteins, transiently expressed these molecules in BHK cells using the vaccinia expression system and analysed the endocytic profiles in the transfected cells by immunofluorescence confocal microscopy using the affinity purified anti-Rab5b and anti-Rab5c antibodies. Expression of the Rab5b Ile^{133} and Rab5c Ile^{134} mutants caused the appearance of smaller endocytic vesicles than those present in control cells (not shown). In contrast, cells expressing the Rab5b Leu^{79} (Fig. 2M,N) and Rab5c Leu^{80} (Fig. 2O,P) mutants displayed very large endocytic structures similar to those obtained upon expression of the Rab5a Leu^{79} mutant protein [27]. Thus, expression of wild type and mutant Rab5a, Rab5b and Rab5c proteins induce similar morphological alterations of the early endosome compartment.

3.4. Rab5b and Rab5c regulate the fusion of early endosomes in vitro

Rab5a controls the homotypic fusion between early endosomes in vitro [20]. To further characterize the functional properties of Rab5b and Rab5c, we prepared cytosols from BHK cells overexpressing the Rab5a, Rab5b, Rab5c, Rab5a Ile^{133}, Rab5b Ile^{133} or Rab5c Ile^{134} proteins and tested them in the fusion assay. Western blot analysis using affinity-purified anti-Rab5a, -Rab5b, and -Rab5c antibodies confirmed that the various proteins were expressed at comparable levels in the cytosol from transfected BHK cells (Fig. 3A). As expected from previous studies [19], binding of [α^{32}P]GTP by ligand blotting was detected for the wild type but not for the mutant proteins (Fig. 3B). Fig. 3C shows the results of the fusion assay. While cytosols containing the overexpressed wild type Rab5a, Rab5b and Rab5c stimulated the early endosomes fusion by up to 100%, the mutant proteins decreased the efficiency of fusion. Rab5a appeared to exert the highest stimulatory effect whereas Rab5c Ile^{134} was the strongest inhibitor. These differences however, may reflect small variations in the concentration of cytosolic isoprenylated proteins.

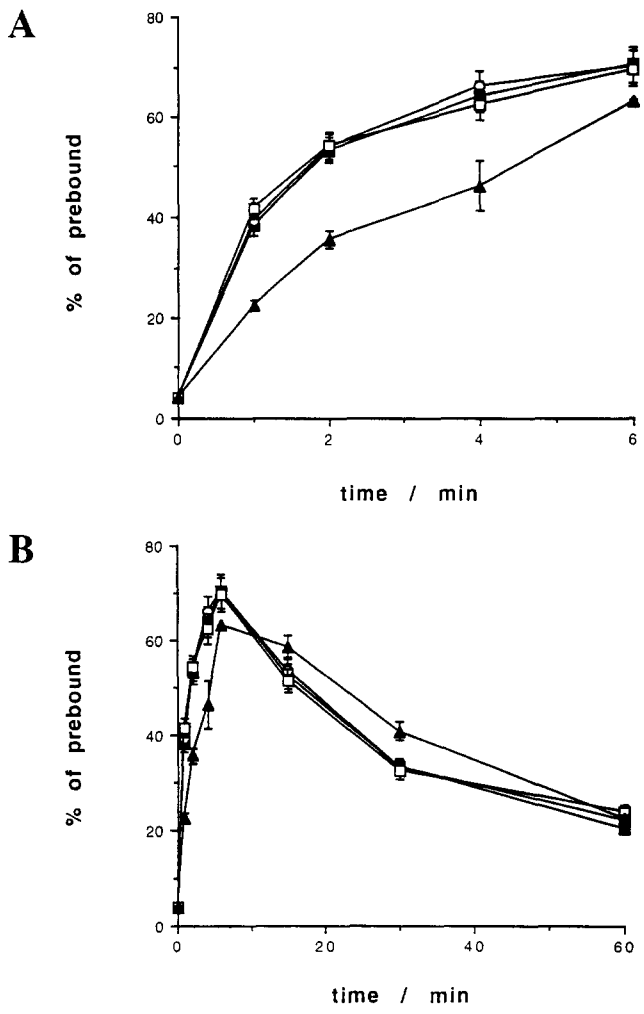

Fig. 4. Rab5b and Rab5c regulate, like Rab5a, the kinetics of transferrin endocytosis in vivo. BHK cells were transfected for 4 h with the human transferrin receptor (hTR) plasmid alone (closed triangle) or together with one of the following plasmids: Rab5a (closed square), Rab5b (open circle), Rab5c (open square). The cells were then allowed to bind ^{125}I-hTf for 2 h at 0°C. After binding the cells were washed extensively and incubated at 37°C for different periods of time to allow internalization. Internal transferrin was calculated as the percentage of total transferrin bound that became pronase resistent after internalization. Separate plots of the first 6 min of internalization (A) and of the entire experiment (B) are shown to visualize better the kinetics of internalization and recycling of ^{125}I-hTf. Each time point represents the mean of triplicate samples and the bar is the stand ard error of the mean when larger then the size of the symbol.

3.5. Rab5b and Rab5c regulate endocytosis in vivo

We next examined whether the Rab5b and Rab5c proteins also had the ability to increase the kinetics of endocytosis in vivo. For these experiments we transiently expressed in BHK cells hTR alone or in combination with one of the three wild type or mutant proteins. Consistent with previous data [19], overexpression of Rab5a increased the rate of transferrin endocytosis, and this effect was shared by wild type Rab5b and Rab5c (Fig. 4A). In contrast, expression of the Rab5a Ile^{133}, Rab5b Ile^{133} and Rab5c Ile^{134} mutant proteins caused a ~40% inhibition in the rate of transferrin internalization (data not shown). Recycling to the cell surface (Fig. 4B) was slightly accelerated by the wild type proteins.

In conclusion, these data demonstrate that the three Rab5 isoforms share a regulatory function on transport in the early endocytic pathway.

4. Discussion

We have cloned the cDNAs encoding full-length Rab5b and Rab5c proteins. Together with Rab5a they form a subgroup of closely related isoforms. Similar subgroups exist also for other Rab proteins. Given that the Rab3 subgroup seems to be heterogeneous, its members displaying cell-type specific expression and function, it became important to investigate the function of the Rab5 subgroup. To rigorously address the role of the three Rab5 proteins in mammalian cells we used a combination of approaches. We showed that the mRNAs for the three genes are expressed in all organs examined and that, by confocal immunofluorescence microscopy, the three isoforms colocalize at the level of plasma membrane and early endosomes. Moreover, Rab5b and Rab5c stimulated early endosome fusion in vitro and transferrin endocytosis in vivo. Thus, the ability of Rab5a to regulate transport in the early endocytic pathway is also shared by the Rab5b and Rab5c isoforms. Since Rab5b and Rab5c proteins appear to be ubiquitously expressed, like Rab5a, we conclude that three Rab5 proteins regulate endocytosis in eukaryotic cells.

The Rab5a, Rab5b and Rab5c proteins colocalize to a TR-positive endosomal compartment. One implication of our study is that these three GTPases must share a common determinant for targeting to the early endosomes. Previous experiments have shown that the targeting signal for Rab proteins is present in the C-terminal hypervariable domain [34]. The C-terminus of Rab7 is sufficient to target Rab5a to the late endosomes [34] and the C-terminus of Rab5a can target Rab6 to the plasma membrane and early endosomes [37]. Similarly, the hypervariable C-terminal domains of Ypt1p and Sec4p in yeast were shown to be responsible for the targeting of the two proteins [35]. Comparison of the primary sequence of the three Rab5 isoforms revealed a conserved RxRGVDLxE motif that may be important for the interaction with the specific membrane targeting machinery.

The C-terminal hypervariable domain is required for organelle targeting but is not sufficient to confer functional specificity. The N-terminus and the regions involved in the conformational change occurring upon GTP-hydrolysis, α2/L5 and α3/L7 as well as the effector loop, have been found to be specific structural determinants for the activity of Rab proteins [35,37,38]. When these regions were transplanted on Rab6 they transmitted Rab5a function to the chimeric protein [37]. Interestingly, while the N-terminal domain is more divergent, the three Rab5 proteins are highly conserved in the α2/L5, α3/L7 regions and effector domain. This suggests that the three proteins are likely to have common regulatory factors. Three Rab5-like proteins, named Ypt51p, Ypt52p and Ypt53p, with a conserved α2/L5 region have been identified in yeast and found to have slightly different but overlapping functions in the endocytic pathway [23]. The strong conservation between the isoforms and the presence of subgroups throughout evolution suggest an important role of the three proteins in the regulation of endocytic trafficking.

The presence of three Rab5 isoforms may reflect an evolutionary need to ensure Rab5 function even in the presence of

harmful mutation. Alternatively, each isoform could fulfill a distinct role and be responsible for a fine regulation of the early endocytic pathway. However, the differences between them could be too subtle to be detected even with the spectrum of techniques we employed. For example, although the three Rab5 proteins are localized to the same compartment as determined by confocal immunofluorescence microscopy, they could still be restricted to specific microdomains of the early endosomes. Electron microscopy studies will be required to address this question, provided that antibodies with high sensitivity of detection become available. Rab5 isoforms may also have different localization and function in polarized cells. Rab5a is associated with, and regulates endocytosis from, both the apical and the basolateral domain in epithelial cells [40]. Similarly, in neurons Rab5a has a role in both axonal and dendritic endocytosis [41]. It is not known whether Rab5b and Rab5c are restricted to either endocytic domain.

It will also be important to establish whether the activity of the three Rab5 proteins is differentially regulated. In the case of the Rab4 subgroup, Rab4a and Rab4b may have a similar role in endocytosis [17] (Bucci and Zerial, unpublished data). However, whereas Rab4a is phosphorylated in the C-terminal domain by the cdc2 kinase during mitosis and upon phosphorylation becomes cytosolic [42], Rab4b does not contain this phosphorylation site. Among the three Rab5 isoforms, only Rab5c contains a putative phosphorylation site for cdc2 kinase (S196) in its C-terminus. It remains to be demonstrated whether this site is indeed phosphorylated during mitosis and whether this post-translational modification plays a role in selectively regulating membrane association of Rab5c.

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