

Rab4 and Rab7 Define Distinct Nonoverlapping Endosomal Compartments*

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Several Rab GTPases have been localized to distinct compartments of the endocytic pathway. Rab4 is associated with early endosomes and recycling vesicles and regulates membrane recycling from early endosomes. Rab7 is localized to late endosomes and is involved in the regulation of membrane transport between late endosomes and lysosomes. Although Rab4 and Rab7 appear to regulate distinct transport events in endocytosis, it is not clear whether they perform their activities in related or entirely distinct intracellular compartments. To address this question, we generated stable cell lines expressing Rab4 tagged with a novel X31 influenza hemagglutinin (NH) epitope tag. These antibodies are characterized in this paper and were used to immunoprecipitate endocytic vesicles with cytoplasmically exposed NHRab4. Immunoprecipitated membranes contain internalized ¹²⁵I-transferrin, but are devoid of Rab7. Confocal immunofluorescence microscopy showed that the early endosomal GTPases Rab4 and Rab5 both do not codistribute with Rab7 within the same cell. These observations suggest that each of the three Rab GTPases operationally defines a distinct station of the endocytic pathway.

Receptor-mediated endocytosis is a process in which eukaryotic cells internalize proteins and solutes. The overall characteristics of this pathway, which starts with the binding of ligands to a cell-surface receptor, are relatively well understood (reviewed in Ref. 1). Ligand-receptor complexes are internalized via coated vesicles that rapidly uncoat and fuse with early endosomes. The mildly acidic pH causes dissociation of most ligands from their receptors and is required for proper sorting of ligands and receptors (2). Whereas receptors are thought to segregate into tubular extensions that bud off to form recycling vesicles that mediate transport to the plasma membrane, ligands stay behind in the lumen of the early endosomes and are often targeted for degradation in late endosomes and lysosomes. The mechanism of membrane transport from early endosomes to late endosomes and lysosomes is incompletely understood, and the prevailing theories are controversial (3). In one view, discrete transport vesicles with unique molecular properties transport their contents in a microtubule-dependent fashion between pre-existing early and late endosomes (4). A second model proposes that early endosomes gradually mature

into late endosomes due to simultaneous recycling of cargo back to the plasma membrane and fusion of vesicles containing lysosomal enzymes from the Golgi complex (5). Yet a third view holds that, in some cells, early and late endosomes are part of a single extensive tubulovesicular endocytic network (6). The lack of specific marker proteins for endocytic organelles is an important factor that precluded a clear experimental distinction between these models.

Recently, small Ras-like GTP-binding proteins of the Rab family were identified in eukaryotes, and >30 members have been cloned so far (7). Rab GTPases are associated with the cytoplasmic face of various intracellular organelles and have been shown to be important regulators of intracellular transport (reviewed in Refs. 7 and 8). Several Rab proteins have been localized to organelles of the endocytic pathway. In peripheral early endocytic compartments, Rab5 is associated with coated vesicles and early endosomes (9), whereas Rab4 is found on endocytic vesicles that recycle cargo from early endosomes (10). Thus, although Rab4 and Rab5 each localize to compartments that also contain the transferrin receptor (TfR),¹ it is now clear that Rab4 and Rab5 largely define distinct early endosomal structures.² In accordance with their localization, a phenotypic analysis of endocytosis in cells overexpressing Rab4 or Rab5 showed that Rab5 controls the fusion of incoming coated vesicles with early endosomes (9), while Rab4 is involved in membrane recycling from early endosomes to the paranuclear recycling compartment (10). At least two other Rab proteins are associated with late endocytic compartments. Rab7 (11) and Rab9 (12) are found on late endosomes, where they regulate distinct vesicular transport routes. Rab9 regulates transport between late endosomes and the *trans*-Golgi network (12) and is required for lysosome biogenesis as well (13). Rab7, on the other hand, mediates transport between early and late endocytic organelles (14, 15).

Now that the distribution of several Rab proteins has been firmly established, it has become possible to use them as compartment-specific tags, even though they transiently reside in the cytosol due to ongoing GTP hydrolysis (8). Because overexpression does not appear to cause Rab proteins to mislocalize (10, 16) to other membranes, the strict localization of a particular Rab protein allows investigations into "product-precursor" relationships of endocytic compartments, using Rab proteins as compartmental markers. We investigated here, in CHO cells stably expressing NHRab4 and the TfR, whether the endocytic vesicles that are characterized by Rab4 do exchange membranes and contents with late endocytic structures containing Rab7. Our immunoprecipitation results and confocal immunofluo-

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¹ The abbreviations used are: TfR, transferrin receptor; hTfR, human TfR; Tf, transferrin; CHO, Chinese hamster ovary; GST, glutathione S-transferase; PBS, phosphate-buffered saline.

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rescence data employing the novel NH epitope tag show that Rab4 and Rab7 are essentially associated with distinct non-overlapping endocytic compartments. In NHRab4/GRab5 (Rab5 with N-terminal vesicular stomatitis virus G protein epitope tag), we also observed that Rab5 and Rab7 are associated with distinct endocytic organelles. These results suggest that the early endocytic compartments characterized by Rab4 or Rab5 and the late endosomes associated with Rab7 are separate structures.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Transient expression in HeLa cells was carried out with the vaccinia virus T7 bacteriophage RNA polymerase system as described (17). cDNAs encoding NHRab4, MychTfR, and NHRab7 were ligated in the cytomegalovirus-driven expression plasmids pCB6 and pcDNA3. CHO cells were stably transfected with NHRab4pCB6 and MychTfRpCB6 (18) and NHRab4pCB6 and GRab5pCB6² as described and maintained in medium containing 0.6 mg/ml G418. The Rab4/NHRab7 double transfectant was generated by transfecting NHRab7pcDNA3 into a previously established Rab4 CHO cell line (10) and was maintained in medium containing 0.6 mg/ml G418 and 50 μ M methotrexate. Transfectants received 5–10 mM sodium butyrate (Sigma) 17 h prior to all experiments.

Plasmid Constructions—Human Rab4 cDNA and canine Rab5 cDNA were ligated in *Eco*RI-restricted pGEX2T and pGEX1 λ T (Pharmacia, Woerden, The Netherlands), respectively, and were used for the production of fusion proteins. The polymerase chain reaction was used to create NH epitope-tagged Rab4 using the following primer set: 5'-ta-cg-gaa-ttc-cgg-atg-caa-gac-ctt-cca-gga-aat-gac-aac-agc-aca-gca-ggt-tcc-gaa-acc-tac-gat-ttt-ttg-ttt-aag-ttc-3' (sense) and 5'-t-cta-gga-att-cct-cta-aca-acc-aca-ctc-ctg-agc-g-3' (antisense).³ NHRab7 was constructed by replacing the Rab4 insert in NHRab4BSKS with Rab7 cDNA lacking the start methionine. Polymerase chain reaction products were subcloned into pBSKS, and their identity was confirmed by dideoxy sequencing of both strands (19).

Antibodies—The synthetic peptide CQDLPGNDNST, corresponding to the amino acid sequence immediately adjacent to the signal sequence of X31 influenza HA1 protein, was conjugated with bismaleimido-*N*-hydroxysuccinimide (Pierce) to keyhole limpet hemocyanin and was used for antibody production in mouse, rabbit, and guinea pig. Polyclonal antibodies against Rab4 and Rab5 were generated by immunizing rabbits with GST-Rab4 or GST-Rab5 fusion proteins. Monoclonal antibodies against the Myc epitope 9E10, the cytoplasmic tails of vesicular stomatitis virus G protein, the polymeric IgA receptor, and the human transferrin receptor were described before (20–22, 29). The affinity-purified rabbit antibody against the synthetic Rab7 peptide NALKQETEVELLYNEFPEPIKLDKNDRAKTSAES (15) was obtained from P. Chavrier.

In Vitro Transcription and Translation—MycRab4BSKS, GRab5BSKS, and NHRab7BSKS were used as template in a T7 RNA polymerase transcription system and were translated in the presence of 10 μ Ci of [³⁵S]methionine for 90 min at 30 °C in a rabbit reticulocyte system according to the manufacturer's instructions (Promega). Translation mixtures were diluted to 100 μ l with PBS and 1.0% Triton X-100 and spun for 10 min at 14,000 rpm in a microcentrifuge, and each was divided in three aliquots, from which MycRab4, GRab5, and NHRab7 were immunoprecipitated with rabbit antibodies against Rab4, Rab5, and Rab7 or with monoclonal antibodies against the epitope tags.

Immunoprecipitation and Western Blotting—Protein A-Sepharose CL-4B beads were bound either to polyclonal antibodies or, via affinity-purified goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), to hybridoma supernatants. Immunoprecipitations were carried out exactly as described, resolved by SDS-polyacrylamide gel electrophoresis, and analyzed by fluorography (23) or a PhosphorImager. Nitrocellulose and polyvinylidene difluoride transfers were blocked for at least 1 h in 0.1% Tween 20 and 5% skimmed milk powder in PBS (Blotto). Antibody incubations were performed for 60 min with affinity-purified antibodies. Detection was with horseradish peroxidase-conjugated secondary antibodies using ECL (Amersham Corp.) or ¹²⁵I-protein A (23).

Immunoisolation—NHRab4/MychTfR CHO double transfectants were incubated for 30 min in serum-free medium supplemented with 0.5% bovine serum albumin. ¹²⁵I-Tf was added to the cells at 5 μ g/ml

and internalized for 1 h at 37 °C. Cell surface-bound ¹²⁵I-Tf was subsequently removed by acid washes as described (10). The cells were resuspended in 3 mM imidazole, 250 mM sucrose, and 2 mM EDTA, pH 7.4 (homogenization buffer), and homogenized through a 24-gauge needle. The postnuclear supernatant was poured into an SW 50.1 tube and brought to 41% sucrose. Two layers of 1.5-ml 16% sucrose and 1.5-ml 10% sucrose cushions were layered over the postnuclear supernatant, and the tube was centrifuged for 1 h at 38,000 rpm. The 10–16% sucrose interface was retrieved and diluted with PBS and 0.5% bovine serum albumin to 0.25 M sucrose. Monoclonal antibodies were absorbed overnight to sheep anti-mouse labeled Immunobeads (Bio-Rad). The beads were washed with PBS and 0.5% bovine serum albumin and incubated with the retrieved membrane fractions for 3 h on a rotator in the cold room. The beads were washed four times in PBS, and a small aliquot was saved for γ -counting. The remaining beads were resuspended in Laemmli sample buffer (35).

Confocal Scanning Laser Immunofluorescence Microscopy—Double transfectants were fixed in 3% paraformaldehyde and quenched in NH₄Cl⁺/PBS, pH 7.4, for 5 min. The cells were permeabilized and blocked for 30 min in 0.1% saponin, 0.5% bovine serum albumin, and PBS (blocking buffer) and stained with the monoclonal antibody against NH (1:15) and with a rabbit antibody against Rab7. The coverslips were washed for three periods of 15 min in blocking buffer. Cells were counterstained for 30 min with 5-[[4,6-dichlorotriazin-2-yl]amino]fluorescein-labeled goat anti-rabbit antibody at 1:75 dilution (Jackson ImmunoResearch Laboratories, Inc.) or Indocarbocyanine cy3-labeled goat anti-mouse IgG at 1:200 dilution (Jackson ImmunoResearch Laboratories, Inc.). The coverslips were then mounted as described (18) and examined with a 63 \times planapo objective on a Leitz DMIRB fluorescence microscope (Leica, Voorburg, The Netherlands) interfaced with a Leica TCS4D confocal scanning laser microscope (Leica, Heidelberg, Germany) using the following filters: fluorescein isothiocyanate, λ_{ex} = 488 nm and λ_{em} = 520 nm band pass; and Texas Red or Indocarbocyanine cy3, λ_{ex} = 568 nm and λ_{em} = 590 nm long pass. Single labeling control experiments were used to ascertain the absence of fluorescence bleeding from one channel into the other.

RESULTS

The available antibodies against Rab4 are of too low affinity to reliably detect the endogenous protein in nontransfected tissue culture cells. To circumvent this problem, we chose to N-terminally epitope tag Rab4. Several of the previously described epitope tags (20, 24), however, did not work satisfactorily in our hands. We therefore first generated antibodies against the QDLPGNDNST amino acid sequence from X31 influenza hemagglutinin. This hydrophilic decapeptide is situated immediately downstream of the signal sequence of influenza hemagglutinin (25) and is exposed on a surface loop of X31 influenza HA1 as suggested by the structure of the crystallized hemagglutinin bromelain fragment (26). We then placed this new epitope tag at the N terminus of Rab4 and generated stable CHO cell lines expressing NHRab4. To define the epitopes recognized by the novel NH antibodies, we performed a deletion analysis of constructs consisting of Rab4 with 5'-extensions of oligonucleotides encoding truncated versions of the NH epitope tag. Upon transient expression of these constructs in HeLa cells, the polyclonal rabbit antibody against NH recognized the entire 10-amino acid epitope tag and the truncated peptide sequences QDLP and QDLPGN (data not shown). The monoclonal NH antibody, however, immunoprecipitated only the full-length epitope tag (data not shown). To have maximum flexibility in applications of the NH antibodies, we used only the entire 10-amino acid epitope extension.

NHRab4/MychTfR CHO Double Transfectants—Previous attempts to elucidate the distribution of endogenous Rab4 and Rab7 by morphological methods have not been very successful, and Rab4 and Rab7 have not been colocalized within the same cell. This is an important biological question since the comparative localization of Rab4 and Rab7 may provide novel information on the biogenesis of early and late endocytic compartments, but also because the two GTPases have distinct roles in the regulation of the endocytic pathway. We first assessed the

³ Polymerase chain reaction primers used to generate the truncated NH epitope tag on Rab4 are available on request.

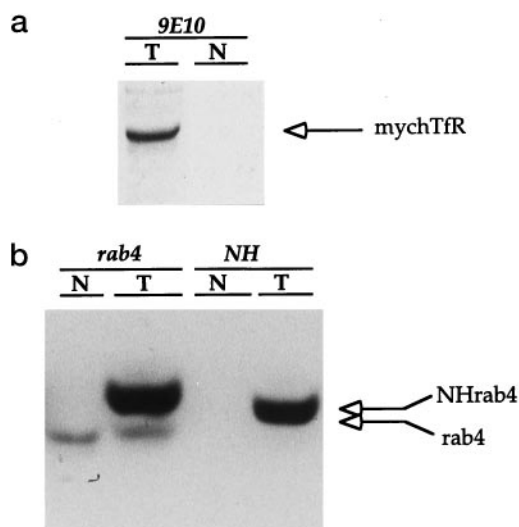


FIG. 1. Stable NHRab4/MychTfR CHO double transfectants. CHO cells expressing NHRab4 and MycTfR (*T*) or nontransfected control CHO cells (*N*) were lysed in reducing Laemmli buffer. Samples (10–15 μ g of protein) were resolved on 12.5% SDS-polyacrylamide minigels, and proteins were detected on Western blots using rabbit anti-Rab4 antibody (1:4000), rabbit anti-NH antibody (1:1000), or mouse monoclonal antibody 9E10 (1:5) against the c-Myc epitope tag on the TfR. Bands were visualized after incubation with horseradish peroxidase-labeled secondary antibodies (1:5000) and ECL.

expression levels of MychTfR and NHRab4 in the double transfectants on Western blots of detergent lysates. As shown in Fig. 1 (*upper panel*), the 9E10 antibody specifically stained a 85-kDa band of MychTfR not present in control cells. Because NHRab4 has a slightly higher molecular mass than Rab4, the Rab4 antibody revealed a doublet of 24 and 25 kDa, of which the slower migrating was identified as epitope-tagged Rab4 using the affinity-purified NH antibody (Fig. 1, *lower panel*). From densitometric scans of the two Rab4 bands, we estimated that ~5–10 times more NHRab4 was present than endogenous Rab4. Confocal immunofluorescence microscopy showed that 60–70% of the cells coexpressed NHRab4 and MychTfR, whereas the remaining cells expressed either NHRab4 or MychTfR (data not shown).

Immunoisolation of NHRab4 Compartments Containing Internalized 125 I-Tf—We previously showed that Rab4 is associated with early endocytic compartments containing the TfR and a class of vesicles that transport membrane and proteins from sorting endosomes to a recycling compartment that is located close to the centrioles (10, 18). Rab4 is not present on the plasma membrane, suggesting that its activity is restricted to the regulation of vesicular transport between early endosomes and the recycling compartment, but not to the later stages of the TfR recycling pathway. The presence of a unique recycling compartment in several cell lines was demonstrated some years ago (27, 28). Apart from the presence of cellubrevin (18) in recycling vesicles, nothing is known about the resident protein content of these vesicles or the recycling compartment itself. Isolation of this compartment and the vesicles that transport cargo to it from the early sorting endosome has not been achieved yet. As a fraction of Rab4 is localized on the recycling vesicles, we next sought to develop a purification method based on the physical properties of the marker protein that would allow us to start to characterize recycling vesicles that have Rab4 associated with their cytosolic membrane. We used the epitope tag antibody for immunoisolation of NHRab4-containing compartments.

To label the endocytic compartments with a tracer, we accumulated 125 I-Tf until steady state. Surface-bound 125 I-Tf was

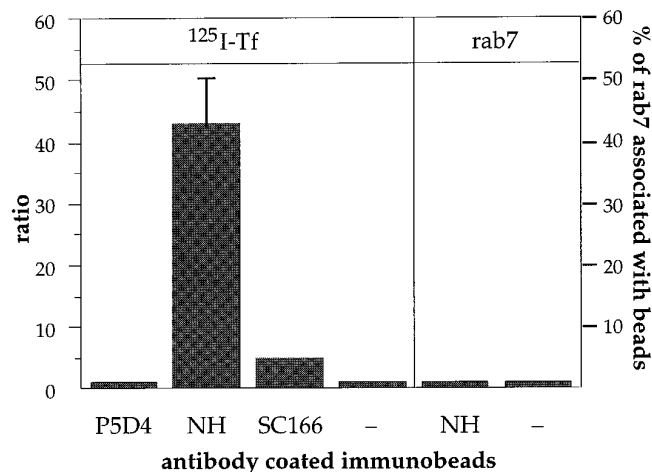


FIG. 2. Immunoisolation of NHRab4-containing organelles. 125 I-Tf was internalized for 60 min in NHRab4/MychTfR CHO cells. After removal of surface-bound 125 I-Tf, cells were fractionated. Low density membranes were isolated on a discontinuous sucrose gradient and used as input for immunoisolation using monoclonal antibodies against NH (NH), vesicular stomatitis virus G protein (P5D4), the polymeric IgA receptor (SC166), or control sheep anti-mouse Immunobeads (-). 125 I-Tf was determined in the nonbound fraction and in membranes bound to the Immunobeads in a γ -counter and plotted as bound to anti-NH antibody-coated beads over bound to P5D4-coated beads. Rab7 associated with anti-NH antibody-coated Immunobeads (NH) or control sheep anti-mouse Immunobeads (-) was analyzed by Western blotting of the beads and high speed pellets of the nonbound fraction using an affinity-purified Rab7 peptide antibody and ECL or 125 I-protein A detection.

stripped from the plasma membrane with alternate acid and neutral washes. This procedure removed >99% of the surface-bound transferrin. A postnuclear supernatant was fractionated by sucrose gradient centrifugation to produce membrane fractions enriched in Golgi and endosome membranes (29). This step was also introduced to remove the small but significant amount of NHRab4 (~15% of the total cellular pool of Rab4 protein) that might interfere with immunoisolation due to competition for antibody binding. The sucrose interface was used as input for immunoisolation with the monoclonal NH antibody. Up to 15% of internalized 125 I-Tf could be isolated on the anti-NH antibody-coated Immunobeads. It is not surprising that a limited fraction of 125 I-Tf-containing vesicles could be immunoisolated on the Immunobeads. A substantial large fraction of 125 I-Tf is present in organelles that typically do not contain Rab4, such as coated vesicles, the paranuclear recycling compartment, and transport vesicles that fuse with the plasma membrane.

To control the specificity of the immunoisolation procedure, we prepared Immunobeads with the monoclonal antibody SC166, which recognizes the cytosolic tail of the polymeric IgA receptor, or the monoclonal antibody P5D4, against the cytoplasmic tail of vesicular stomatitis virus G glycoprotein. These two proteins are normally not present in CHO cells. Using the specific NH beads, ~50 times more 125 I-Tf was immunoisolated than with the P5D4 or SC166 beads or with Immunobeads coated with only the bridging sheep anti-mouse antibody (Fig. 2). Because the beads allowed us to immunoisolate a significant fraction of early endocytic compartments, we next investigated whether Rab7 was present on the immunoisolated organelles. Unlike 125 I-Tf, however, most if not all of Rab7 was recovered in the nonbound membrane fraction of incubations containing NH-coated or control Immunobeads (Fig. 2), suggesting that Rab7 did not distribute onto early endosomes or endocytic recycling vesicles.

Confocal Immunofluorescence Microscopy of Rab7 and

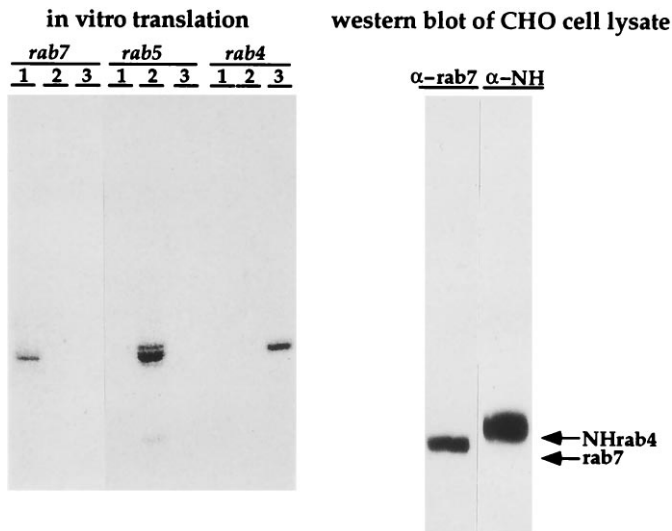


FIG. 3. Characterization of the Rab7 antibody. MycRab4BSKS, GRab5BSKS, and NHRab7BSKS were translated *in vitro* in a programmed rabbit reticulocyte lysate system at 30 °C. After 90 min, translation mixtures were divided into three equal aliquots and immunoprecipitated with antibodies against Rab7 (lanes 1), Rab5 (lanes 2), and Rab4 (lanes 3). Note that the Rab7 antibody recognized Rab7, but not Rab5 or Rab4. We also analyzed the specificity of the affinity-purified Rab7 antibody on Western blots of detergent lysate from NHRab4/MycTfR cells. In these lysates, endogenous Rab7 was the only band detected with this antibody.

Rab4—Having shown that the early endosomal marker ^{125}I -Tf could be immunisolated in NHRab4-containing membranes, whereas Rab7 was retained in the nonbound membrane fraction, we next examined the intracellular distribution of Rab4 and Rab7 proteins using confocal immunofluorescence microscopy. We first assessed the specificity of the Rab7 antibody with respect to Rab4 and Rab5. As shown in Fig. 3, the antibody against Rab7 detects a single band of 25 kDa after immunoprecipitation of *in vitro* translated NHRab7 in a rabbit reticulocyte system. Neither Rab4 nor Rab5 was recognized by the affinity-purified Rab7 antibody, and Rab7 could not be immunoprecipitated with Rab4 or Rab5 antibodies (Fig. 3). To check whether the Rab7 antibody cross-reacted with other intracellular proteins, detergent lysate from NHRab4/MycTfR CHO cells was analyzed by Western blotting (Fig. 3). Fig. 3 shows a single band corresponding to the molecular mass of Rab7 (24 kDa), indicating that the affinity-purified Rab7 antibody was specific for Rab7.

NHRab4/MycTfR CHO double transfectants were fixed and labeled with monoclonal antibodies against NHRab4 and the affinity-purified Rab7 peptide antibody. Rab4 was present in discrete peripheral cytoplasmic spots and crescent-shaped structures in the perinuclear area (Fig. 4b). Rab7, however, was associated with large doughnut-shaped membranes that were concentrated in the perinuclear area (Fig. 4a), but also present in the peripheral cytoplasm. Rab7 staining appeared to be completely distinct from the Rab4-containing structures (Fig. 4b) as evidenced in Fig. 4c, which shows the merged images. The absence of Rab7 in early endocytic organelles was also investigated using a second marker of these compartments. For this purpose, we labeled the NHRab4/MycTfR CHO cells with antibodies against hTfR (Fig. 4e) and compared its distribution with Rab7 (Fig. 4d). Again, the labeling pattern of Rab7 was distinct from compartments of the early endocytic pathway, confirming the immunoisolation experiments (Fig. 2) and the nonoverlapping distribution with Rab4.

As the hypervariable region of Rab proteins contains amino acids that are required for their association with a specific

organelle (30), it is conceivable that this targeting mechanism might become compromised after overexpression of a Rab protein. We therefore established a CHO double transfectant in which NHRab7 was expressed together with non-epitope-tagged Rab4 and investigated the distribution of Rab4 and Rab7 by confocal immunofluorescence microscopy. As shown in Fig. 4g, the distribution of NHRab7 as detected with the monoclonal NH antibody was very similar if not identical to that of endogenous Rab7, labeled with the affinity-purified Rab7 antibody (*cf.* Fig. 4a). Upon overexpression of NHRab7, the localization of Rab4 (Fig. 4h) was complementary to that of NHRab7 as seen in the merged image (Fig. 4i). Thus, we find the same distinct distributions of Rab4 and Rab7 whether or not Rab7 is overexpressed, suggesting that, at these expression levels, the targeting machinery for Rab7 and Rab4 is not saturated. While this manuscript was submitted, Meresse *et al.* (15) reported very similar distribution of endogenous and overexpressed Rab7 in HeLa cells.

Confocal Immunofluorescence Microscopy of Rab7 and Rab5—To investigate the relationship between the early endocytic organelles that can be characterized by the associated small GTPase Rab5 and the endosomal compartments containing Rab7, we again performed double label confocal immunofluorescence microscopy. We used a CHO cell line that expresses NHRab4 and GRab5, in which Rab5 is N-terminally epitope-tagged with part of the cytoplasmic tail of vesicular stomatitis virus G protein. This double transfectant contained ~10 times more NHRab4 and GRab5 than the non-epitope-tagged endogenous CHO proteins.² Results of the double labeling experiments with antibodies against the vesicular stomatitis virus G epitope tag and Rab7 are shown in Fig. 5. Most of the red GRab5 staining was in the peripheral cytoplasm and around the nucleus (Fig. 5b), whereas endogenous CHO Rab7 was concentrated in distinct looking punctate structures around the nucleus. It is clear, however, after merging the two images, that the GRab5 (Fig. 5b) and Rab7 staining patterns were complementary and that there was essentially no colocalization as evidenced by the absence of the yellow areas within the same optical section or the same membrane compartment (Fig. 5c). Because the Rab7 antibody gives some cytosolic staining, the merged confocal images in Figs. 4c and Fig. 5c occasionally have a light orange hue. In agreement with the localization experiments in the NHRab4/MycTfR cells, we did not find overlapping distributions between NHRab4 and Rab7 in the NHRab4/GRab5 cells. Thus, we have shown that the early endocytic compartments that are operationally defined by Rab5 or Rab4 do not overlap with Rab7 compartments, suggesting that each of these endosomal Rab proteins define distinct classes of endocytic organelles.

DISCUSSION

Although the pathway of receptor-mediated endocytosis is relatively well understood, it is only recently that the first proteins were identified that may be involved in the regulation of the various budding, targeting, and fusion events between compartments of this pathway. The discovery of the ARF and Rab families of small GTPases and the localization of at least five of these proteins (11, 12, 29, 31) to early and late endocytic compartments have been significant steps forward in the understanding of how the endocytic pathway is structurally and functionally organized. The assignments of individual Rab proteins to specific endocytic compartments was based on double labeling morphological methods or subcellular fractionation experiments using endocytosed proteins as markers. These probes therefore at best can be considered as kinetic markers. This less strict definition of a marker protein potentially creates ambiguity in the localization of endocytic Rab proteins as

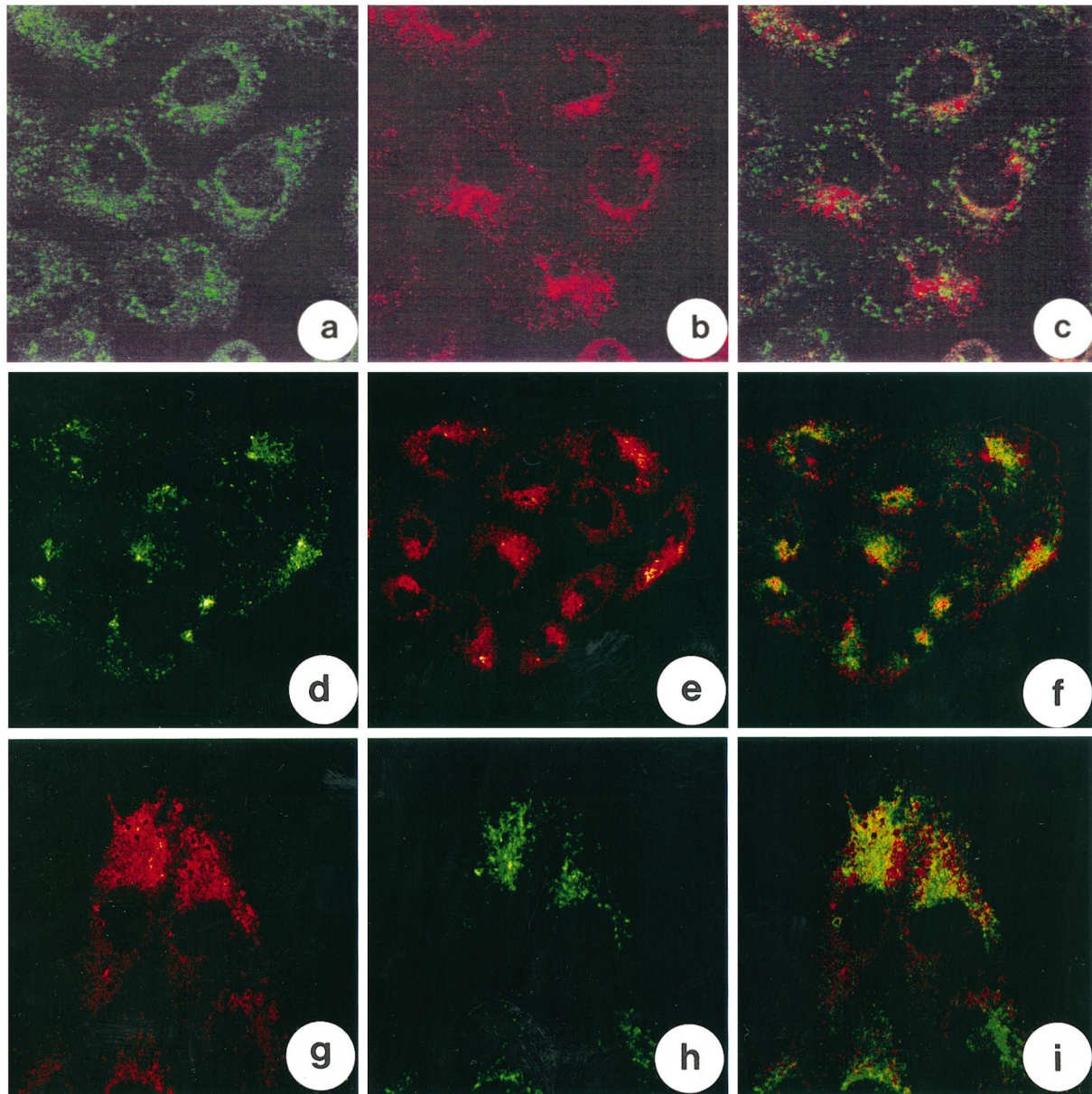


FIG. 4. Confocal immunofluorescence microscopy of Rab4 and Rab7. The relative distributions of Rab4 and Rab7 were visualized in NHRab4/MyhTfR and Rab4/NHRab7 CHO cells by scanning laser confocal microscopy. *a*, distribution of endogenous Rab7 as detected with the rabbit anti-Rab7 peptide polyclonal antibody and visualized using 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein-conjugated anti-rabbit IgG (green); *b*, distribution of NHRab4 as detected with the anti-NH monoclonal antibody and visualized with Texas Red-conjugated anti-mouse IgG (red); *c*, merging of the images in *a* and *b* (the yellow color indicates areas of overlap between Rab4 and Rab7 distribution); *d*, distribution of Rab7 as detected with the Rab7 antibody (green); *e*, localization of the hTfR as detected with the monoclonal antibody OKT9 (red); *f*, merging of the images in *d* and *e*; *g*, localization of expressed NHRab7 (red) as detected with the NH antibody; *h*, distribution of Rab4 as detected with the affinity-purified anti-Rab4 antibody (green); *i*, merging of the images in *g* and *h*.

kinetic markers are not true resident marker proteins in the sense that they specifically designate a unique compartment. Instead, they are transiently localized to a given compartment, and some time later, they can be found in the next endocytic organelle of their intracellular itinerary. The problem with this approach is clearly exemplified by Rab5 and Rab4. These two Rab proteins were previously both localized to what was called early endosomes (9, 10). Rab5 was found on the plasma membrane and coated vesicles and colocalized with the TfR. Rab4, on the other hand, was found on early endocytic structures because it cofractionated with internalized Tf and partially colocalized with the TfR. A phenotypic characterization of transfected fibroblasts overexpressing each of these two proteins or mutants thereof revealed that Rab4 and Rab5 produced distinct, essentially complementary endocytosis pheno-

types (9, 10). The question then arose whether this was due to intrinsic differences between two distinct proteins that are associated with the same organelle or whether they might be associated with a subset of the compartments of the TfR cycle. Functional assays and morphological analysis on cell lines expressing moderate levels of NHRab4 and GRab5² showed that Rab4 and Rab5 define a set of distinct but slightly overlapping endocytic compartments that also contain cellubrevin. Thus, although Rab5 and Rab4 each colocalized with the TfR, they colocalize to only a very limited extent with each other and appear to act at distinct sites in the early endosomal network.

In this work, we extended this approach to investigate whether Rab7 is specifically associated with late endosomes or whether Rab7 is also distributed over early endocytic compartments that are defined by the presence of either Rab4 or Rab5.

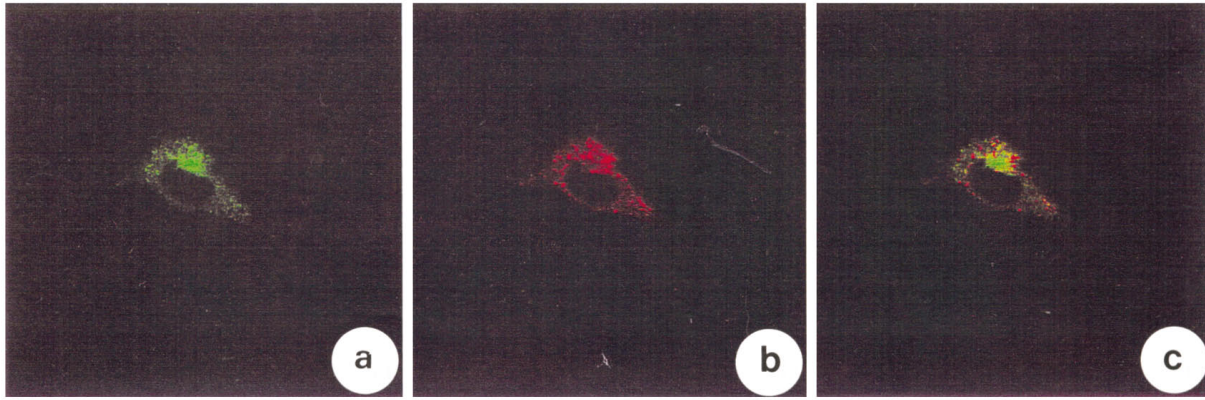


FIG. 5. Confocal immunofluorescence microscopy of GGrab5 and Rab7. The relative distributions of Rab5 and Rab7 were visualized in CHO cells stably transfected with NHRab4 and GGrab5 by scanning laser confocal microscopy. *a*, distribution of endogenous Rab7 as detected with the rabbit anti-Rab7 peptide polyclonal antibody and visualized using 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein-conjugated anti-rabbit IgG (green); *b*, distribution of GGrab5 as detected with the monoclonal P5D4 antibody and visualized with Texas Red-conjugated anti-mouse IgG (red); *c*, merging of the images in *a* and *b* (the yellow color indicates areas of overlap between Rab5 and Rab7 distribution).

For this purpose, we performed morphological and biochemical experiments in stable CHO double transfectants expressing NHRab4/MychTfR or NHRab4/GGrab5. We found, by confocal immunofluorescence microscopy and immunoisolation using the new NH antibody, that Rab4 and Rab7 each define completely nonoverlapping endocytic organelles. Although the NHRab4/GGrab5 cells did not have the same good morphology as the other CHO double transfectant, we still found >90% of GGrab5 in a distinct membrane compartment than Rab7.

Our observation that Rab4 and Rab7 define distinct compartments in biochemical and morphological assays also provides an explanation for a previous finding made by Desjardins *et al.* (32) on the biochemical characterization of phagosomes in J774 macrophages. In a pulse-chase approach followed by cell fractionation, they observed a time dependent increase in the association of Rab7 with phagocytic compartments with a concomitant decrease in the amount of Rab5 present on the isolated phagosomes. At relatively early time points, phagosomes predominantly contained Rab5 (as well as some Rab7), probably reflecting their close resemblance to the plasma membrane protein composition from which phagosomes initially derive. At none of the investigated time points, however, was Rab4 detected on the phagosomes. Our results, however, suggest that Rab4 and Rab7 define distinct compartments of the endocytic pathway. These observations indicate that Rab7 cannot associate with the compartment on which cytoplasmic Rab4 docks. We already showed that Rab4-containing vesicles are involved in the transfer of internalized Tf from early endosomes to the paranuclear recycling compartment. This would suggest that membrane destined to be recycled to the plasma membrane very quickly segregates from the route to late endosomes, causing it to be essentially unavailable to fusion with phagosomes. Alternatively, phagosomes may lack the ability to fuse with recycling vesicles and therefore are unable to acquire Rab4.

Previously, it was shown that Rab7 distribution overlaps only to a very small extent with a Q79L Rab5 mutant (33). Because this Q79L Rab5 mutant does not hydrolyze GTP to a significant extent, it is not a substrate for GDP dissociation inhibitor. Therefore, it may remain in association with the endocytic target compartment that the Rab5-containing coated vesicles fuse with. Thus, although Q79L Rab5 and Rab7 appeared to colocalize to a very small extent, this may in fact be an overestimation due to the inability of Q79L Rab5 to be dissociated from the more downstream endocytic target compartment. Biochemical cell fractionation experiments (16) also

document the distribution of Rab5 and Rab7 to distinct regions of discontinuous sucrose gradients. We extended these observations for Rab7 and Rab5 using a morphological steady-state approach. Confocal immunofluorescence microscopy showed that the distributions of transfected GGrab5 and endogenous Rab7 are essentially nonoverlapping.

Taken together, our observations document that the early endocytic markers Rab4 and Rab5 are excluded from the late endosomes containing Rab7 and lysosomal membrane glycoproteins (data not shown). Because this set of small GTPases is associated with mutually exclusive organelles, we find it difficult to reconcile our findings with the notion that Rab4- or Rab5-containing early endocytic organelles mature into late endosomes (34) containing Rab7. Instead, the data suggest that Rab7, Rab5, and Rab4 may define distinct intracellular structures.

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REFERENCES

- Kornfeld, S., and Mellman, I. (1989) *Annu. Rev. Cell Biol.* **5**, 483–525
- van Weert, A. W. M., Dunn, K. W., Geuze, H. J., Maxfield, F. R., and Stoorvogel, W. (1995) *J. Cell Biol.* **130**, 821–834
- Helenius, A., Mellman, I., Wall, D., and Hubbard, A. L. (1983) *Trends Biochem. Sci.* **8**, 45–50
- Gruenberg, J., Griffiths, G., and Howell, K. E. (1989) *J. Cell Biol.* **108**, 1301–1316
- Stoorvogel, W., Strous, G. J., Geuze, H. J., Oorschot, V., and Schwartz, A. L. (1991) *Cell* **65**, 417–427
- Hopkins, C. R., Gibson, A., Shipman, M., and Miller, K. (1990) *Nature* **346**, 335–339
- Zerial, M. (1995) in *Small GTPases* (Zerial, M., and Huber, L. A., eds), pp. 295–306, Oxford University Press, New York
- Pfeffer, S. R., Dirac-Svestrup, A. B., and Soldati, T. (1995) *J. Biol. Chem.* **270**, 17057–17059
- Bucci, C., Parton, R., Mather, I., Stunnenberg, H., Simons, K., and Zerial, M. (1992) *Cell* **70**, 715–728
- van der Sluijs, P., Hull, M., Webster, P., Goud, B., and Mellman, I. (1992) *Cell* **70**, 729–740
- Chavrier, P., Parton, R. G., Hauri, H. P., Simons, K., and Zerial, M. (1990) *Cell* **62**, 317–329
- Lombardi, D., Soldati, T., Riederer, M., Zerial, M., and Pfeffer, S. (1993) *EMBO J.* **12**, 677–682
- Riederer, M. A., Soldati, T., Shapiro, A. D., Lin, J., and Pfeffer, S. R. (1994) *J. Cell Biol.* **125**, 573–582
- Feng, Y., Press, B., and Wandinger Ness, A. (1995) *J. Cell Biol.* **131**, 1435–1452
- Meresse, S., Gorvel, J. P., and Chavrier, P. (1995) *J. Cell Sci.* **108**, 3349–3358
- Gorvel, J. P., Chavrier, P., Zerial, M., and Gruenberg, J. (1991) *Cell* **64**, 915–925
- van der Sluijs, P., Bennett, M. K., Anthony, C., Simons, K., and Kreis, T. E. (1990) *J. Cell Sci.* **95**, 545–553
- Daro, E., van der Sluijs, P., Galli, T., and Mellman, I. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 9559–9564
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.*

- 74, 5463–5467
20. Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) *Mol. Cell. Biol.* **5**, 3610–3616
21. Kreis, T. E. (1986) *EMBO J.* **5**, 931–941
22. Solari, R., Kuhn, L., and Kraehenbuhl, J.-P. (1985) *J. Biol. Chem.* **260**, 1141–1145
23. van der Sluijs, P., Hull, M., Huber, L., Male, P., Goud, B., and Mellman, I. (1992) *EMBO J.* **11**, 4379–4389
24. Field, J., Nikawa, J. I., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A., and Wigler, M. (1988) *Mol. Cell. Biol.* **8**, 2159–2165
25. Gething, M. J., Bye, J., Skehel, J., and Waterfield, M. (1980) *Nature* **287**, 301–307
26. Wilson, I. A., Skehel, J. J., and Wiley, D. C. (1981) *Nature* **289**, 367–373
27. McGraw, T. E., Greenfield, L., and Maxfield, F. R. (1987) *J. Cell Biol.* **105**, 207–214
28. Apodaca, G., Katz, L. A., and Mostov, K. E. (1994) *J. Cell Biol.* **125**, 67–77
29. van der Sluijs, P., Hull, M., Zahraoui, A., Tavitian, A., Goud, B., and Mellman, I. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 6313–6317
30. Chavrier, P., Gorvel, J. P., Stelzer, E., Simons, K., Gruenberg, J., and Zerial, M. (1991) *Nature* **353**, 769–772
31. Peters, P., Hsu, V., Ooi, C. E., Finazzi, D., Teal, S. B., Oorschot, V., Donaldson, J. G., and Klausner, R. D. (1995) *J. Cell Biol.* **128**, 1003–1017
32. Desjardins, M., Huber, L. A., Parton, R. G., and Griffiths, G. (1994) *J. Cell Biol.* **124**, 677–688
33. Stenmark, H., Parton, R., Steele-Mortimer, O., Lutcke, A., Gruenberg, J., and Zerial, M. (1994) *EMBO J.* **13**, 1287–1296
34. Dunn, K. W., and Maxfield, F. R. (1992) *J. Cell Biol.* **117**, 301–310
35. Braakman, I., Litty, H. H., Wagner, K. R., and Helenius, A. (1991) *J. Cell Biol.* **114**, 401–411

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