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From Sorting Endosomes to Exocytosis: Association of Rab4 and Rab11 GTPases with the Fc Receptor, FcRn, during Recycling

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A longstanding question in cell biology is how is the routing of intracellular organelles within cells regulated? Although data support the involvement of Rab4 and Rab11 GTPases in the recycling pathway, the function of Rab11 in particular is uncertain. Here we have analyzed the association of these two Rab GTPases with the Fc receptor, FcRn, during intracellular trafficking. This Fc receptor is both functionally and structurally distinct from the classical Fcγ receptors and transports immunoglobulin G (IgG) within cells. FcRn is therefore a recycling receptor that sorts bound IgG from unbound IgG in sorting endosomes. In the current study we have used dual color total internal reflection fluorescence microscopy (TIRFM) and wide-field imaging of live cells to analyze the events in human endothelial cells that are involved in the trafficking of FcRn positive (FcRn$^+$) recycling compartments from sorting endosomes to exocytic sites at the plasma membrane. Our data are consistent with the following model for this pathway: FcRn leaves sorting endosomes in Rab4$^+$Rab11$^+$ or Rab11$^+$ compartments. For Rab4$^+$Rab11$^+$ compartments, Rab4 depletion occurs by segregation of the two Rab proteins into discrete domains that can separate. The Rab11$^+$FcRn$^+$ vesicle or tubule subsequently fuses with the plasma membrane in an exocytic event. In contrast to Rab11, Rab4 is not involved in exocytosis.

INTRODUCTION

Trafficking of proteins and other cellular contents in the endocytic and exocytic pathways has been extensively investigated by analyzing the routes taken by different cargo molecules. However, the molecular processes that regulate intracellular trafficking are poorly understood. Rab proteins, which are small Ras-like GTPases, are known to play regulatory functions in both endocytic and exocytic pathways (Somsel and Wandinger-Ness, 2000; Miaczynska and Zerial, 2002). These proteins can exist as membrane-bound or cytosolic proteins and are regulated by GTP-GDP exchange cycles. Rab GTPases, together with associated proteins such as soluble NSF attachment protein receptors (SNAREs) that are usually transmembrane proteins, regulate the merging of different organellar membranes (Jahn *et al.*, 2003). Although Rabs also play a role in vesicle budding (Somsel and Wandinger-Ness, 2000; Miaczynska and Zerial, 2002), less is known about these processes. In addition, knowledge as to how different Rabs are involved in an intracellular pathway such as recycling from endosomes to the plasma membrane is limited. This latter pathway is the focus of the current study.

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We have chosen to use the MHC class I–related receptor, FcRn, as a model for a recycling receptor (Ghetie and Ward, 2000). This receptor is structurally and functionally distinct from the classical Fcγ receptors (Ghetie and Ward, 2000; Ravetch and Bolland, 2001). FcRn is expressed in a diverse array of cell types and plays a pivotal role in transporting IgG within (via recycling) and across cells (via transcytosis; Medesan *et al.*, 1997; Ellinger *et al.*, 1999; Praetor *et al.*, 1999; Dickinson *et al.*, 1999; McCarthy *et al.*, 2000; Firan *et al.*, 2001; Antohe *et al.*, 2001; Claypool *et al.*, 2002; Kobayashi *et al.*, 2002; Spiekermann *et al.*, 2002). Recent studies using endothelial cells have demonstrated that after uptake of IgGs into cells by fluid phase pinocytosis, IgG molecules that bind to FcRn at the permissive pH (~6.0) for FcRn-IgG interactions in sorting endosomes are recycled away from the lysosomal route (Ober *et al.*, 2004b). These IgGs are consequently salvaged from degradation and exocytosed at the cell surface via a process in which FcRn is directly involved (Ober *et al.*, 2004a). Conversely, IgGs that do not bind to FcRn enter the lysosomal route (Ober *et al.*, 2004b). FcRn is therefore a protective receptor, and as a result this Fc receptor acts as an IgG homeostat by regulating levels of antibodies throughout the body (Ghetie *et al.*, 1996; Ghetie and Ward, 2000).

Despite models for the intracellular pathways taken by FcRn (Ghetie and Ward, 2000; Rojas and Apodaca, 2002), there is no knowledge concerning the intracellular effectors that might regulate its trafficking. Here we have investigated the relationship of two Rab proteins, Rab4 and Rab11, to FcRn trafficking in human endothelial cells. Earlier studies in different cell types have indicated that Rab4 and Rab11 are involved in at least some steps of the recycling of cargo from the early endosome back to the plasma membrane (van der Sluijs *et al.*, 1992; Daro et al., 1996; Ullrich et al., 1996; Green et al., 1997; Calhoun et al., 1998; Ren et al., 1998; Casanova et al., 1999; Duman et al., 1999; Sheff et al., 1999; McCaffrey et al., 2001). Consistent with the involvement of Rab4 in recycling, a recent study has shown that this GTPase can regulate the formation of recycling vesicles from endosomes (Pagano et al., 2004). Although Rab4 and Rab11 have been proposed to be involved in the fast and slow recycling pathways, respectively (van der Sluijs et al., 1992; Sheff et al., 1999; Sönnichsen et al., 2000), the role of Rab11 is less clear and its distribution appears to vary in different cell types (Ullrich et al., 1996; Green et al., 1997; Casanova et al., 1999; Brown et al., 2000; Sönnichsen et al., 2000; van IJzendoorn et al., 2003). Furthermore, a subset of Rabs (or their mutated variants) have been shown to regulate recycling rates (van der Sluijs et al., 1992; Ullrich et al., 1996; Ren et al., 1998; Casanova et al., 1999; Duman et al., 1999; Wilcke et al., 2000; McCaffrey et al., 2001; Khvotchev et al., 2003), but which if any, of the Rabs are directly associated with exocytic events is not known. Our goal in the current study is therefore to provide insight into the interrelationships between Rab proteins and endosomal sorting, recycling, and exocytosis, with a focus on the recycling receptor, FcRn. In turn, these studies relate to how FcRn functions as a transporter of IgGs within cells.

We have combined the use of dual color epifluorescence microscopy and total internal reflection fluorescence microscopy (TIRFM; Steyer and Almers, 2001) to investigate FcRn trafficking in live endothelial cells. This has allowed us to gain insight into how Rab4 and Rab11 might relate to the intracellular routing of FcRn and how these proteins correlate with distinct steps of the recycling pathway. In addition, the use of TIRFM has allowed us to directly visualize events at the plasma membrane such as exocytosis (Toomre et al., 2000; Schmoranzer et al., 2000; Ober et al., 2004a). We observe that although both Rab4 and Rab11 can be associated with FcRn as it leaves sorting endosomes, only Rab11 diffuses into the membrane during exocytic fusion events. We also provide data to support a mechanism by which $Rab4^{+}Rab11^{+}$ compartments can be depleted of Rab4 before membrane fusion. Taken together, our data provide new insight into the processes that are involved in endosomal to plasma membrane trafficking of recycling receptors.

MATERIALS AND METHODS

Plasmid Constructs

Expression constructs for human FcRn α-chain with a C-terminal fusion of enhanced GFP (in pEGFP-N1) and human β2-microglobulin have been described previously (Ober et al., 2004b). The human FcRn α-chain gene was recloned from pEGFP-N1 into pECFP-N1 or pEYFP-N1 (Clontech, Palo Alto, CA) as an *Eco*RI fragment using standard methods. Rab4-GFP, Rab4-YFP, Rab5-YFP, Rab11-GFP, and Rab11-YFP were generously provided by Dr. Marino Zerial (Max-Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). The constructs encode GFP or YFP fused to the N-termini of Rab proteins as in (Sönnichsen et al., 2000). Rab4-CFP was generated by recloning the Rab4 gene as a *Kpn*I-*Bam*HI fragment into pECFP-C1 (Clontech).

Antibodies and Reagents

Anti-EEA1 antibody was obtained from BD Biosciences (Palo Alto, CA). Alexa 568–labeled anti-mouse IgG (highly cross adsorbed) and Alexa 546– or Alexa 647–labeled transferrin were obtained from Molecular Probes (Eugene, OR).

Cells and Transfections

The human endothelial cell line HMEC-1.CDC (Pruckler et al., 1993; a dermally derived microvasculature cell line) was generously provided by Francisco Candal at the CDC (Atlanta, GA). These cells were maintained in phenol red–free HAM'S F-12K medium (Biosource International, Camarillo, CA) before use in transfections. HMEC-1 cells were transiently transfected with expression constructs (1–2 μg of FcRn and human β2-microglobulin constructs and 200 ng of Rab constructs) using Nucleofector technology (Amaxa Biosystems, Cologne, Germany) as described (Ober et al., 2004b). Immediately after transfection, cells were plated in phenol red–free HAM'S F-12K medium on coverslips (for microscopy) or wells of 24-well plates (for flow cytometry). Cells were used in experiments at 19–27 h posttransfection.

For experiments in which exocytosis of transferrin was analyzed using TIRFM, transfected cells were pulsed with 20 μg/ml Alexa 546–labeled transferrin (Molecular Probes) in phenol red-free HAM'S F-12K medium for 30 min at 37°C in a 5% CO_2 incubator, washed with prewarmed medium, and imaged as described in Ober et al. (2004b). In a subset of experiments using Rab11-GFP–transfected cells, 1 μg/ml Alexa 546–labeled transferrin was present in the medium throughout the imaging period.

Flow Cytometric Analyses

Transfected HMEC-1 cells in 24-well plates were pulsed with 10 μg/ml Alexa 647–labeled transferrin in phenol red–free HAM'S F-12K medium for 60 min at 37°C in a 5% CO_2 incubator, washed, and then chased in medium containing 1 mg/ml unlabeled holotransferrin for varying times up to 30 min. After each chase period, cells were washed with ice-cold phosphate-buffered saline (PBS) and removed from the wells by trypsinization. Cells were then washed with medium to remove trypsin and analyzed by flow cytometry on a FACScaliber (Becton Dickinson, Franklin Lakes, NJ). Data were processed using WinMDI version 2.8 (copyright of Joseph Trotter).

Immunofluorescence Studies of Fixed Cells

Transfected HMEC-1 cells were fixed using 3.4% paraformaldehyde, washed with PBS, and mounted in Prolong (Molecular Probes). For analysis of EEA1 distribution, FcRn-GFP–transfected HMEC-1 cells were fixed, permeabilized, and stained with anti-EEA1 antibody (BD Biosciences, San Jose, CA) as described previously (Ober et al., 2004b).

Live Cell Imaging

A Zeiss Axiovert 100TV inverted microscope (Zeiss, Thornwood, NY) was used for imaging with a 100× 1.65 NA Olympus objective (Olympus, Melville, NY) and a 1.6× optovar lens for additional magnification. For excitation a custom laser excitation system was used consisting of four laser lines from three lasers: 488 nm/514 nm (Laser Physics, West Jordan, UT); 543 nm (Research Electro-Optics, Boulder, CO) and 442 nm (Omnichrome/Melles Griot, Carlsbad, CA). This excitation system was used in two configurations. The 442- and 514-nm laser lines were used for the study of CFP and YFP labeled proteins, respectively. The 488- and 543-nm lines were used to image GFP and Alexa 546–labeled proteins, respectively. Images were acquired simultaneously with a dual intensified camera emission system consisting of an I-PentaMAX camera (Roper Scientific, Trenton, NJ) and a Sitcam C2400–08 camera (Hamamatsu, Bridgewater, NJ). Experiments were carried out both in dual color wide-field and total internal reflection mode.

The dual color acquisitions were carried out with a frame rate of either 10 or 5 frames per second and corresponding exposure times of 100 or 200 ms, respectively for the I-PentaMAX camera and 33 ms for the Sitcam camera. Acquired images were processed, registered, and overlaid in our custom written Matlab based software package MIATool (www4.utsouthwestern.edu/wardlab/miatool). Movies were exported in Quicktime format.

RESULTS

Both Rab4 and Rab11 Are Present in the Sorting Endosomes of HMEC-1 Cells

In earlier studies we have used HMEC-1 cells, derived from dermal microvasculature, to analyze the FcRn-mediated transport of IgGs within cells (Ober et al., 2004a, 2004b). These analyses show that IgGs that bind to FcRn in $EEA1^{+}$ early endosomes are recycled away from the lysosomal pathway (Ober et al., 2004b) and are exocytosed at the cell surface in a process involving FcRn (Ober et al., 2004a). FcRn is therefore a salvage receptor that is sorted with transferrin receptors into the recycling pathway in early (sorting) endosomes (Ober et al., 2004b). Here we have analyzed the involvement of Rab4 and Rab11 in the intracellular trafficking pathway of FcRn, with a focus on the steps from endosomal sorting to exocytosis at the plasma membrane. This has been carried out by imaging HMEC-1 cells after transfection with different Rab4, Rab11, and FcRn fluorescent protein constructs.

Immunofluorescence analyses of HMEC-1 cells cotransfected with human FcRn-CFP and either Rab4-, Rab5-, or Rab11-YFP were carried out to assess the distribution of these Rab proteins. For comparative purposes, Rab5, which recruits EEA1 to early endosomes (Simonsen et al.,

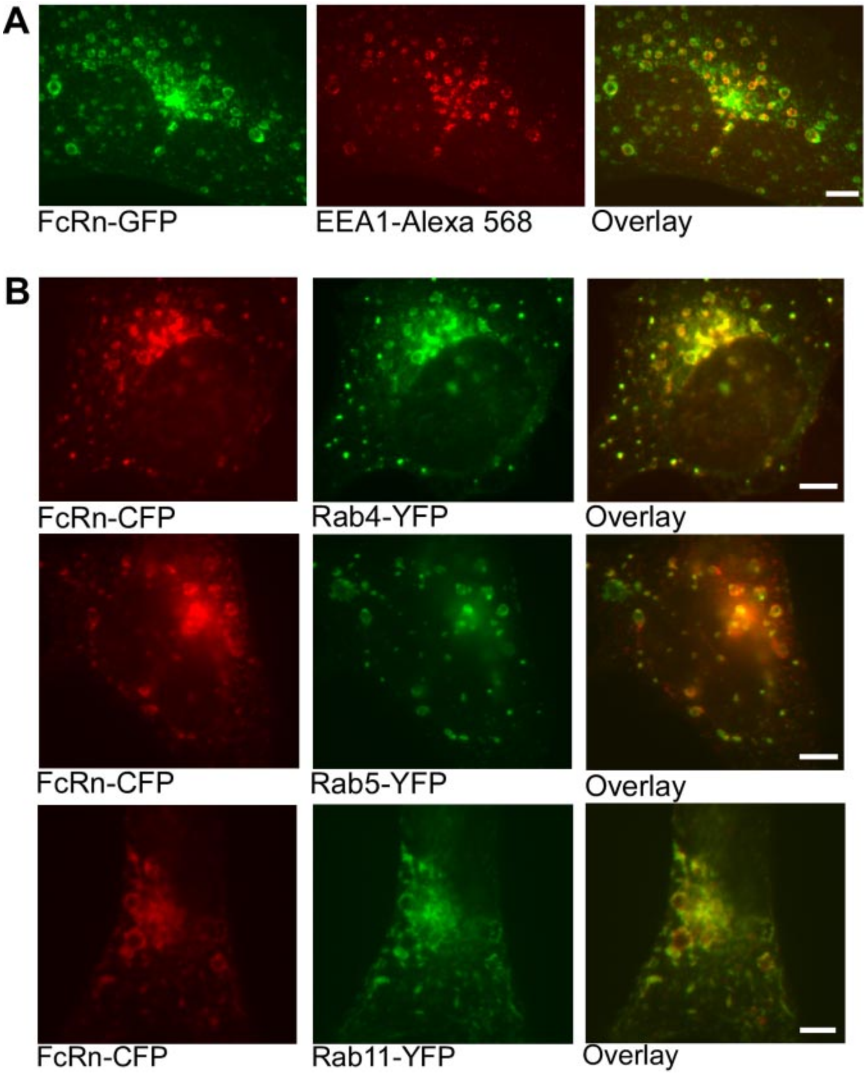

Figure 1. Analysis of the distribution of FcRn and Rab GTPases in transfected HMEC-1 cells. (A) Cells were transfected with FcRn-GFP, fixed ~20 h later, and stained with anti-EEA1 antibody. Bound anti-EEA1 was detected using Alexa 568–labeled anti-mouse Fc conjugate. (B) Cells were cotransfected with FcRn-CFP and Rab4-YFP, Rab5-YFP, or Rab11-YFP. Nineteen to 27 h after transfection, cells were fixed, mounted, and imaged. Bar, 5 μm.

1998; Christoforidis *et al.*, 1999) was included in these analyses. In previous studies we have shown that HMEC-1 cells have relatively large (1–2 μm diameter), EEA1$^+$ FcRn$^+$ endosomes that, based on transferrin and dextran distribution, can be classified as sorting endosomes (Ober *et al.*, 2004b). These endosomes are present in untransfected HMEC-1 cells and endothelial cell lines of bronchial origin (unpublished data), suggesting that they are a general feature of endothelial cells. The size of the endosomes, together with the distribution of FcRn and EEA1 on their membrane (Figure 1A), allows them to be distinguished in live cells from other organelles involved in intracellular transport such as recycling endosomes. Both Rab4 and Rab5 are localized in these FcRn$^+$ endosomes, in addition to smaller endosomal structures (Figure 1B). Significantly, Rab11 can be detected on FcRn$^+$ sorting endosomes, in addition to smaller vesicles and tubules that do not all contain FcRn at detectable levels (Figure 1B).

To ensure that overexpression of Rab4 or Rab11 does not affect intracellular trafficking (recycling) pathways, HMEC-1 cells were transfected with GFP-tagged Rab4 or Rab11, and transferrin recycling rates were assessed using flow cytometry to demonstrate that increased expression of these proteins does not perturb the recycling pathway. For cells expressing Rab proteins in the range used in the current study, no significant differences were seen between transfected and untransfected cells in transferrin recycling rates (Figure 2).

Rab4 and Rab11 Can Be Colocalized with FcRn during Trafficking into and out of Sorting Endosomes

We next analyzed the distribution and temporal behavior of FcRn and Rab proteins in live endothelial cells. HMEC-1 cells were cotransfected with various combinations of two of the following: FcRn (α chain), Rab4 or Rab11 tagged with YFP or CFP. In all cases, a construct encoding human β2-microglobulin was also cotransfected to ensure that β2-microglobulin expression was not limiting (Claypool *et al.*, 2002; Praetor and Hunziker, 2002). For these studies, a combination of epifluorescent and TIRF illumination was used to visualize intracellular and plasma membrane proximal events, respectively.

Using epifluorescent illumination, several observations were consistently made for the intracellular trafficking of Rab4 or Rab11 with respect to FcRn: first, Rab4$^+$FcRn$^+$ positive compartments can be seen to detach from sorting endosomes (Figure 3A and Supplementary Movie 1). In the example shown, a Rab4$^+$FcRn$^+$ vesicle also fuses with the sorting endosome indicating that both fusion and fission of

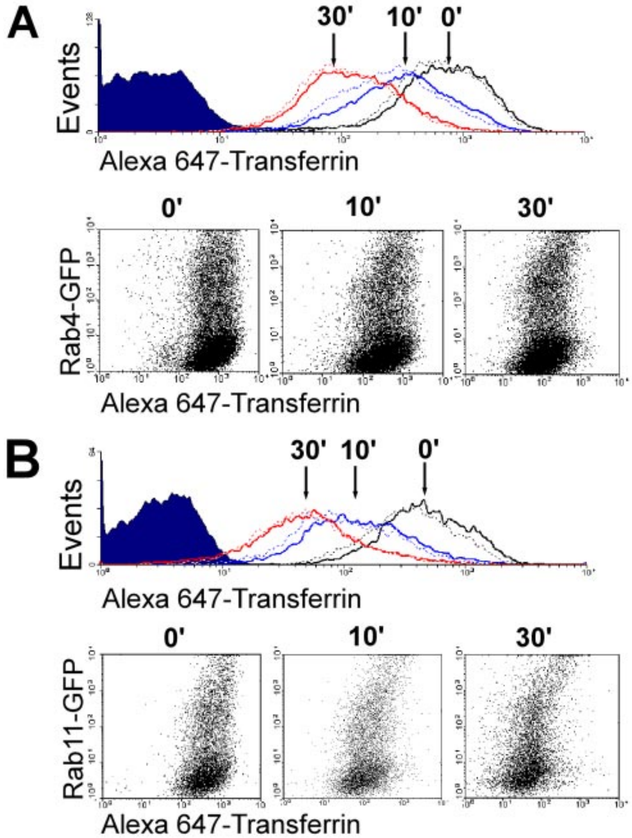

Figure 2. Transferrin recycling in HMEC-1 cells. HMEC-1 cells were either untransfected or transfected with Rab4-GFP or Rab11-GFP. Nineteen to 27 h after transfection, cells were pulsed with 10 μg/ml Alexa 647–labeled transferrin for 60 min and then harvested by trypsinization (0′ sample) or chased in medium containing 1 mg/ml holotransferrrin for 10 or 30 min. After each chase time, cells were harvested, and cell associated transferrin levels were quantitated by flow cytometry. (A) Transferrin levels in Rab4-GFP–transfected cells (solid lines) and untransfected cells (dotted lines) shown as histogram plots (top panel). The filled histogram represents autofluorescence levels in cells that were not treated with Alexa 647–labeled transferrin. The bottom panel shows dot plots at each chase time, indicating levels of Rab4-GFP expression and transferrin associated with the cells. (B) The analyses are the same as in A, except that cells were transfected with Rab11-GFP.

these compartments with sorting endosomes are possible. Fission and fusion of Rab4$^+$FcRn$^+$ compartments were observed at similar frequencies (11 fission and 8 fusion events; 19 events analyzed). Second, and similar to the observations for Rab4, Rab11$^+$FcRn$^+$ vesicles and tubules can detach from the sorting endosomes (Figure 3B and Supplementary Movie 2). Fusion of Rab11$^+$FcRn$^+$ vesicles or tubules with sorting endosomes was seen at a frequency that is slightly greater than the number of events in which Rab11 is observed leaving the sorting endosome (15 fusion events vs. 10 fission events; 25 events analyzed; Figure 3C and Supplementary Movie 3). Thus, for both Rab4 and Rab11, bidirectional trafficking into and out of sorting endosomes occurs.

Rab11, but not Rab4, Is Associated with Exocytic Fusion Events

Dual color TIRFM on a rapid time scale was next used to visualize events in proximity to the cell membrane. We have previously shown that this approach can be used to observe the FcRn-mediated exocytosis of IgG ligand (Ober *et al.*, 2004a). In this earlier study we demonstrated that some exocytic events involve complete fusion of FcRn$^+$IgG$^+$ vesicles with the plasma membrane, whereas for others only partial fusion occurs. The exocytic events involving full fusion were the focus of the current study. These full fusion events are characterized by a "puff" of FcRn-fluorescent protein fluorescence as the exocytic compartment fuses with the plasma membrane.

Dual color TIRFM of cells cotransfected with FcRn-CFP or YFP and individual YFP or CFP labeled Rab proteins allowed us to determine which Rab proteins might fuse with the membrane during exocytosis of FcRn. Multiple fusion events of Rab11$^+$FcRn$^+$ positive compartments could be observed and a representative example is shown in Figure 4A and Supplementary Movie 4. This was visualized as rapid, simultaneous diffusion of FcRn-CFP and Rab11-YFP into the plasma membrane. The intensity plots for both FcRn and Rab11 at the exocytic site show a rapid rise followed by a decrease to background levels (Figure 4A). Thus, Rab11 appears to remain associated with the exocytic vesicle during fusion with the plasma membrane.

In marked contrast to the observations for Rab11, the levels of Rab4-CFP associated with FcRn-YFP during exocytic events were consistently at the level of background signal (Figure 4B and Supplementary Movie 5). Similar data were also observed for cells transfected with Rab4-YFP and FcRn-CFP (unpublished data), indicating that the difference in behavior between Rab4 and Rab11 is not due to variations of the fluorescent proteins used in the fusion constructs. As would be expected from the function of Rab5 (Somsel and Wandinger-Ness, 2000; Miaczynska and Zerial, 2002), in Rab5-YFP/FcRn-CFP–transfected cells, Rab5 diffusion into the plasma membrane during FcRn exocytosis could also not be detected (unpublished data).

We investigated whether the exocytic events that we observe are on the recycling or biosynthetic pathway. Dual color TIRFM was used to analyze the exocytosis of recycled transferrin (Alexa 546 labeled) in HMEC-1 cells transfected with either FcRn-GFP or Rab11-GFP. In these experiments, FcRn or Rab11 diffusion into the plasma membrane was frequently accompanied by transferrin release (89% of 37 events for FcRn, 76% of 42 events for Rab11; Figure 5). The data therefore indicate that for the majority of exocytic events analyzed, Rab11 is associated with exocytic vesicles or tubules on the recycling pathway.

Taken together, Rab11 appears to remain associated with FcRn$^+$ vesicles and tubules during exocytic fusion with the plasma membrane. In contrast, although Rab4 is involved in the early stages of recycling from the sorting endosome, this Rab protein is apparently dissociated from exocytic vesicles/tubules before exocytosis. Thus, the two Rabs can be clearly distinguished by their different behavior during the late stages of the recycling pathway.

Rab4 and Rab11 Can Form Discrete Domains That Separate

The observation that only Rab11, but not Rab4, diffuses into the plasma membrane during FcRn exocytosis prompted us to investigate further the distribution of Rabs inside HMEC-1 cells, with the goal of better understanding the site at which stage bifurcation of Rab4 versus Rab11 into nonexocytic versus exocytic pathways occurs. We also analyzed the relative frequencies at which Rab4$^+$Rab11$^+$ versus Rab11$^+$ (no detectable Rab4) compartments leave sorting endosomes, because the latter compartments do not neces-

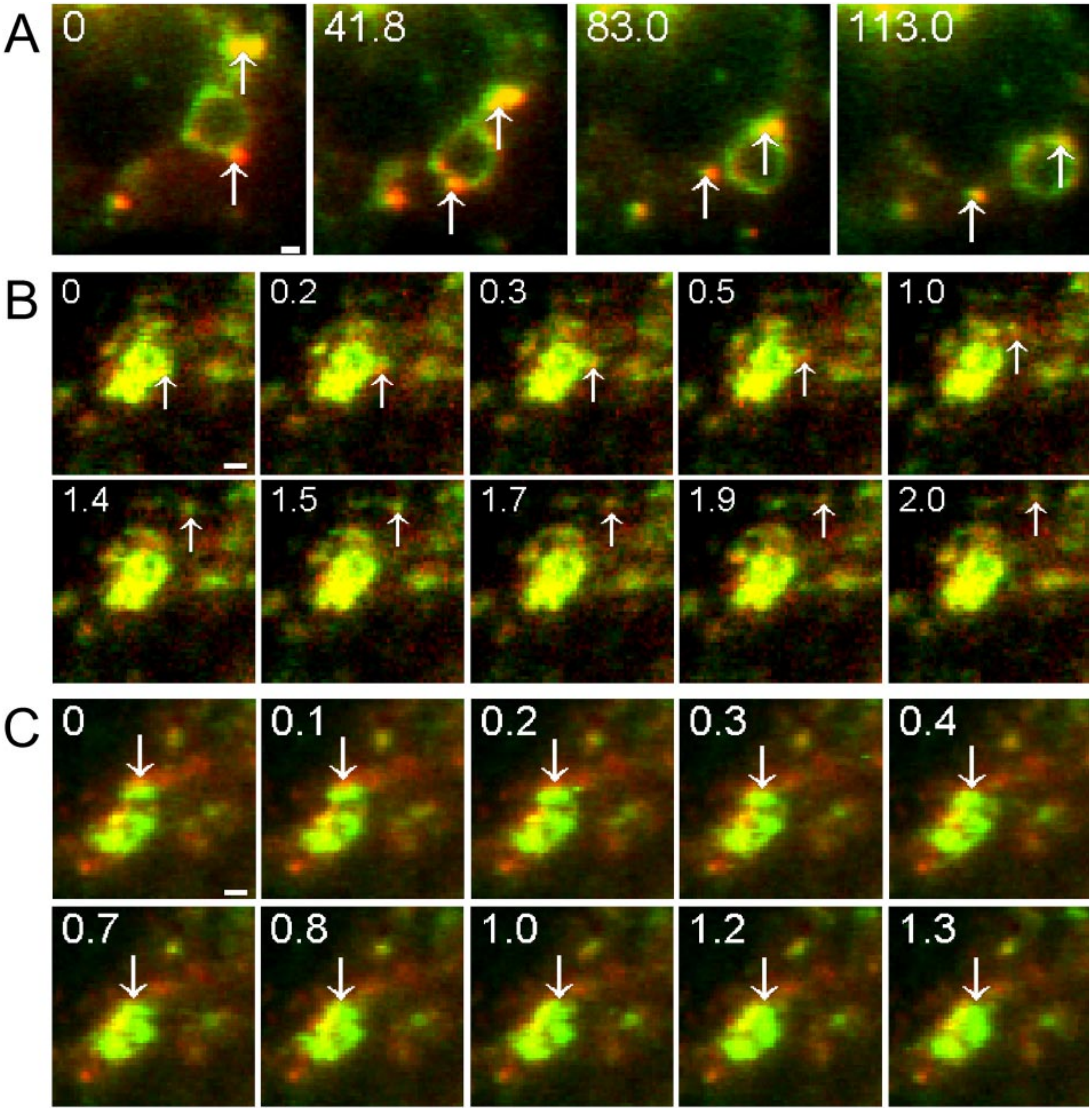

Figure 3. Dual color epifluorescence analyses of Rab4, Rab11, and FcRn in live, transfected HMEC-1 cells. Cells were cotransfected with FcRn-CFP and Rab4-YFP (A) or FcRn-CFP and Rab11-YFP (B and C) and imaged as described in the *Materials and Methods.* The images show single sorting endosomes within the cells. Individual frames are shown, with the first frame arbitrarily set to time zero. Times in seconds corresponding to each frame are indicated. (B and C) The same sorting endosome that is imaged at different times. Arrows indicate Rab^+FcRn^+ compartments leaving (A and B) and merging (A and C) with sorting endosomes. CFP and YFP are pseudocolored green and red, respectively. Bar, 1 μm. Figures 3, A, B, and C correspond to Supplementary Movies 1, 2, and 3, respectively.

sitate the need for Rab4 depletion before exocytosis. Cells were therefore cotransfected with Rab4-CFP and Rab11-YFP and imaged using epifluorescent microscopy. Although $Rab11^+$ vesicles/tubules can be seen to leave sorting endosomes without detectable levels of Rab4 (70%; 10 events analyzed), 30% of "leaving" $Rab11^+$ compartments contained levels of Rab4 that could be readily detected. In one additional case, a leaving $Rab4^+$ compartment could be seen that did not contain detectable levels of Rab11, suggesting that such events involving only Rab4 are relatively rare.

An example of a $Rab4^+Rab11^+$ compartment leaving a sorting endosome is shown (Figure 6A and Supplementary Movie 6). In the example shown, the $Rab4^+Rab11^+$ vesicle splits into $Rab4^+$ and $Rab11^+$ compartments shortly after fission from the sorting endosome. In the cytosol, we also observed that $Rab4^+Rab11^+$ vesicles/tubules can form discrete domains which then detach from each other (Figure 6, B and C and Supplementary Movie 7). This suggests a mechanism by which $Rab4^+Rab11^+$ tubules can segregate before exocytosis to generate separate $Rab4^+$ and $Rab11^+$ compartments.

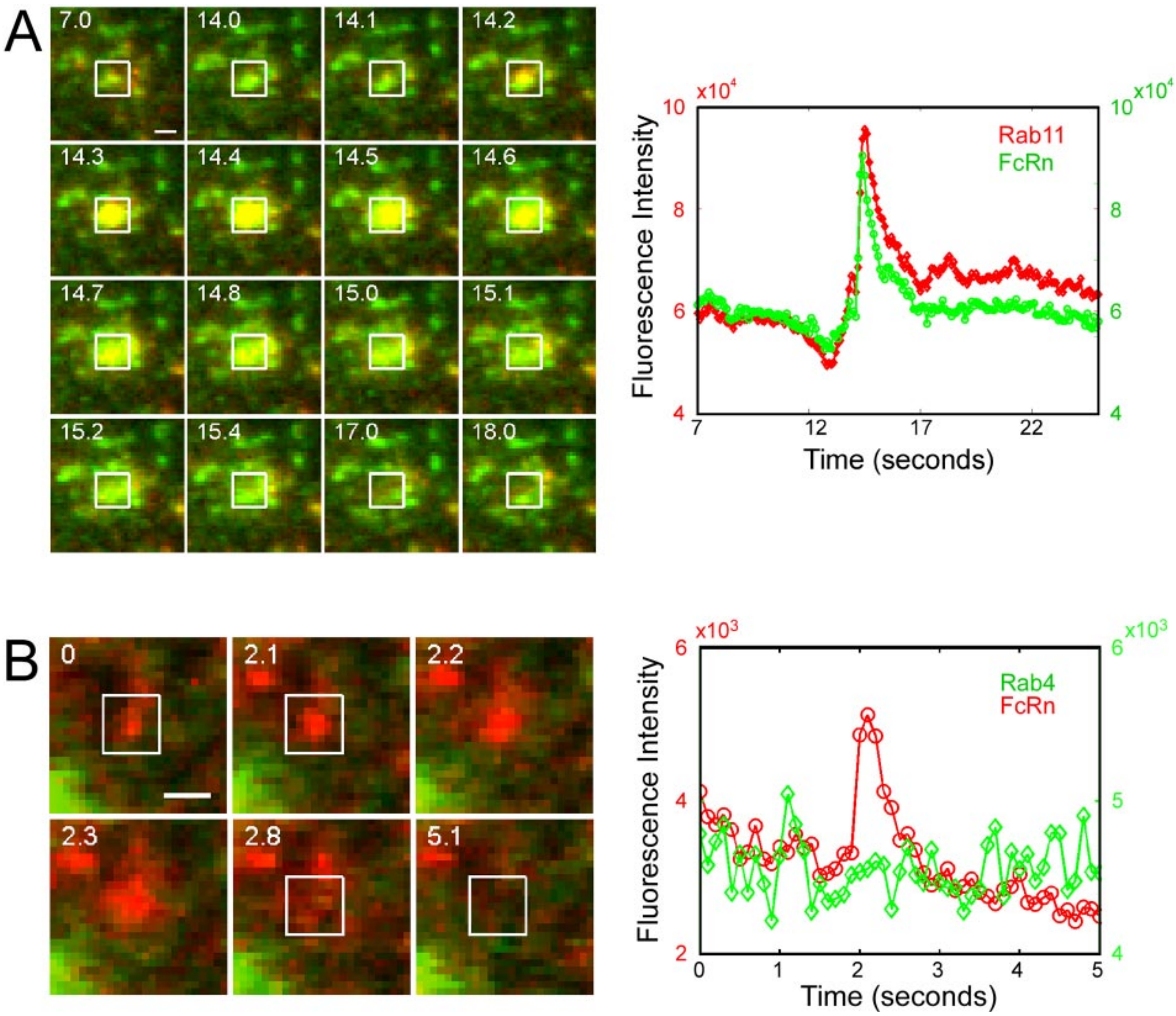

Figure 4. Dual color TIRFM analyses of exocytic events in transfected HMEC-1 cells. Cells were cotransfected with FcRn-CFP and Rab11-YFP (A) or FcRn-YFP and Rab4-CFP (B) and imaged as described in *Materials and Methods.* Times in seconds corresponding to each frame are indicated. Boxes indicate the exocytic site on the cell surface. Plots show fluorescence intensities corresponding to FcRn and Rab proteins as a function of time. CFP and YFP are pseudocolored green and red, respectively. Bar, 1 μm. Figures 4, A and B, correspond to Supplementary Movies 4 and 5, respectively.

Bifurcation of Rab4 and FcRn Pathways

As Rab11 and FcRn both fuse with the plasma membrane during exocytosis, we also analyzed whether behavior similar to that observed for segregation of Rab4 and Rab11 could be seen for Rab4 and FcRn. Cells were cotransfected with Rab4-YFP and FcRn-CFP and imaged using epifluorescent illumination. Figure 7A (Supplementary Movie 8) shows that compartmentalization of Rab4 and FcRn within tubules/vesicles can occur. The Rab4$^+$ and FcRn$^+$ domains can then separate into Rab4$^+$ and FcRn$^+$ vesicles. In this same series of images, a vesicle with distinct Rab4$^+$ and FcRn$^+$ compartments that do not separate during the course of the imaging can also be seen. In some cases, fission can occur but loss of FcRn from the Rab4$^+$ compartment is incomplete (Figure 7B). This suggests that segregation of compartments can be mediated by iterative steps, in addition to the single-step separations that are observed in Figures 6 and 7A. Taken together, our data indicate that Rab4 can be depleted from Rab4$^+$Rab11$^+$FcRn$^+$ compartments by segregation of Rab4 into separate domains followed by fission.

DISCUSSION

In the current study we have investigated the association of Rab4 and Rab11 with the compartments involved in the recycling of the Fc receptor, FcRn, in human endothelial cells. In earlier analyses we showed that FcRn salvages IgGs from lysosomal degradation in the sorting endosomes in a process that overlaps with the transferrin pathway (Ober *et al.*, 2004b), and FcRn-bound IgG is subsequently exocytosed (Ober *et al.*, 2004a). Here we provide data to support distinct functions for Rab4 and Rab11 in the recycling pathway. Significantly, we show that in endothelial cells both Rabs can be associated with the recycling pathway at the level of sorting steps in endosomes, but only Rab11 fuses with the plasma membrane during exocytic events involving FcRn. Our current studies therefore yield new insight concerning

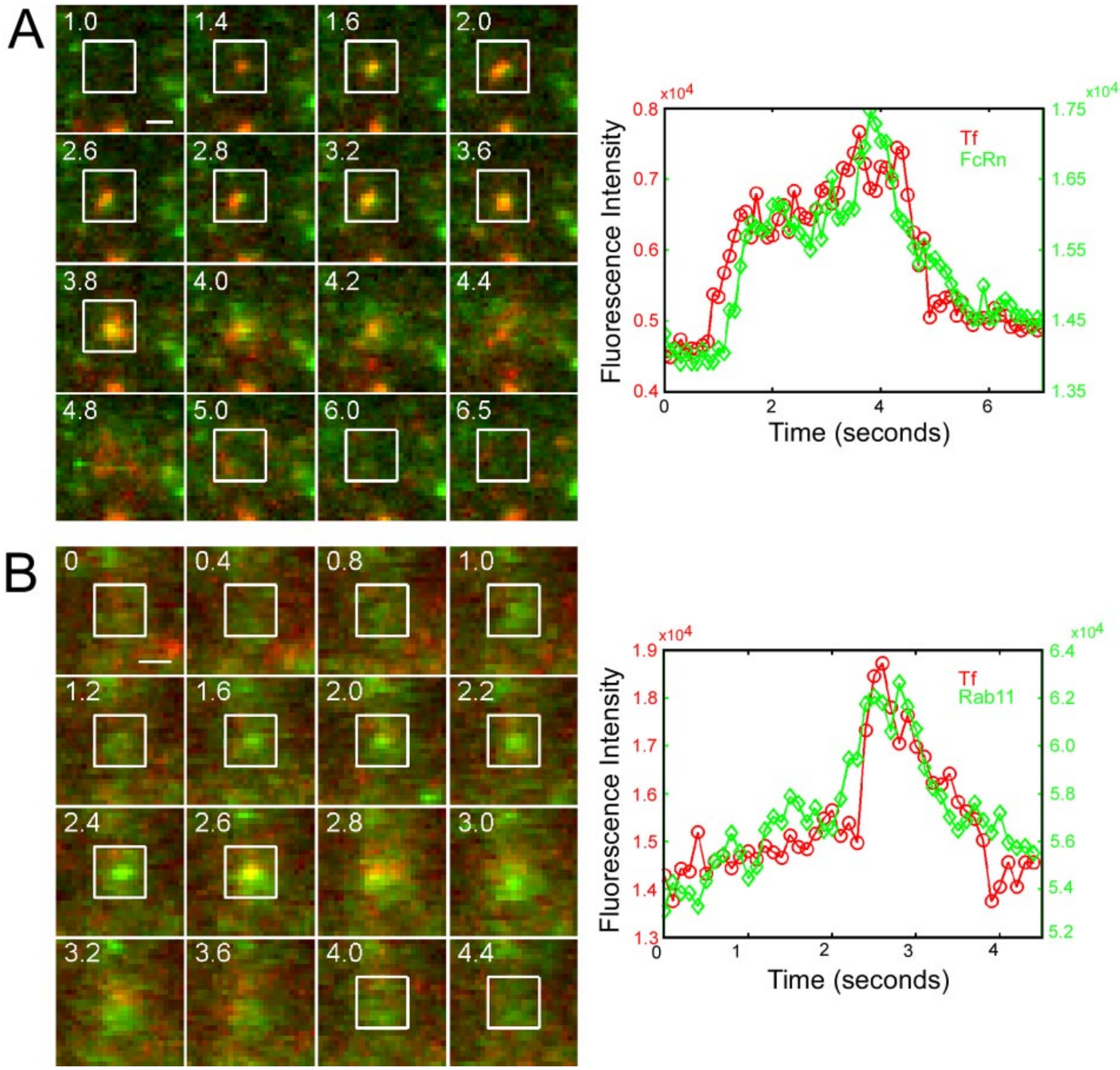

Figure 5. Dual color TIRFM analyses of exocytic events in transfected HMEC-1 cells. Cells were cotransfected with FcRn-GFP (A) or Rab11-GFP (B). Cells were either pulse-chased (A) or continuously incubated (B) with Alexa 546–labeled transferrin and imaged as described in *Materials and Methods.* Times in seconds corresponding to each frame are indicated. Boxes indicate the exocytic site on the cell surface. Plots show fluorescence intensities corresponding to FcRn, Rab11, and transferrin as a function of time. GFP and Alexa 546–labeled transferrin are shown in green and red, respectively. Bar, 1 μm.

the processes that are involved in the trafficking of a recycling receptor from endosomes to exocytic sites at the plasma membrane.

The analysis of Rab protein distribution in transfected endothelial cells indicates that both Rab4 and Rab11 are associated with FcRn$^+$EEA1$^+$ endosomes. In earlier studies we have identified these compartments as sorting endosomes (Ober *et al.*, 2004b). In analyses in human epidermal cells, Rab4 was reported to be associated with compartments leaving sorting endosomes on the recycling pathway (Sönnichsen *et al.*, 2000). These recycling endosomes led into either a rapid recycling pathway, or into Rab4$^+$Rab11$^+$ perinuclear recycling endosomes that participate in slower recycling (Sönnichsen *et al.*, 2000). Consistent with this model, in vitro analyses of Rab4 function indicate that this GTPase can regulate the formation of recycling vesicles from endosomes (Pagano *et al.*, 2004), although the role of Rab11 was not analyzed in these studies. Here we most frequently observe either Rab11$^+$ or Rab4$^+$Rab11$^+$ compartments leaving sorting endosomes. The apparent discrepancy in the association of Rab11 with fission events from sorting endosomes could be due to the different cell types being analyzed and/or differences in the imaging conditions. For example, the use of intensified cameras in the current study results in a higher sensitivity of detection than that typically achieved using confocal imaging. Taken together, our data provide evidence to support a possible role for Rab11, in addition to Rab4, in endosomal sorting. However, our observations do not exclude the possibility that additional Rab11 accumulates in the Rab4$^+$Rab11$^+$ vesicles/tubules that leave the sorting endosomes, which would be consistent with a previously proposed model (Sönnichsen *et al.*, 2000).

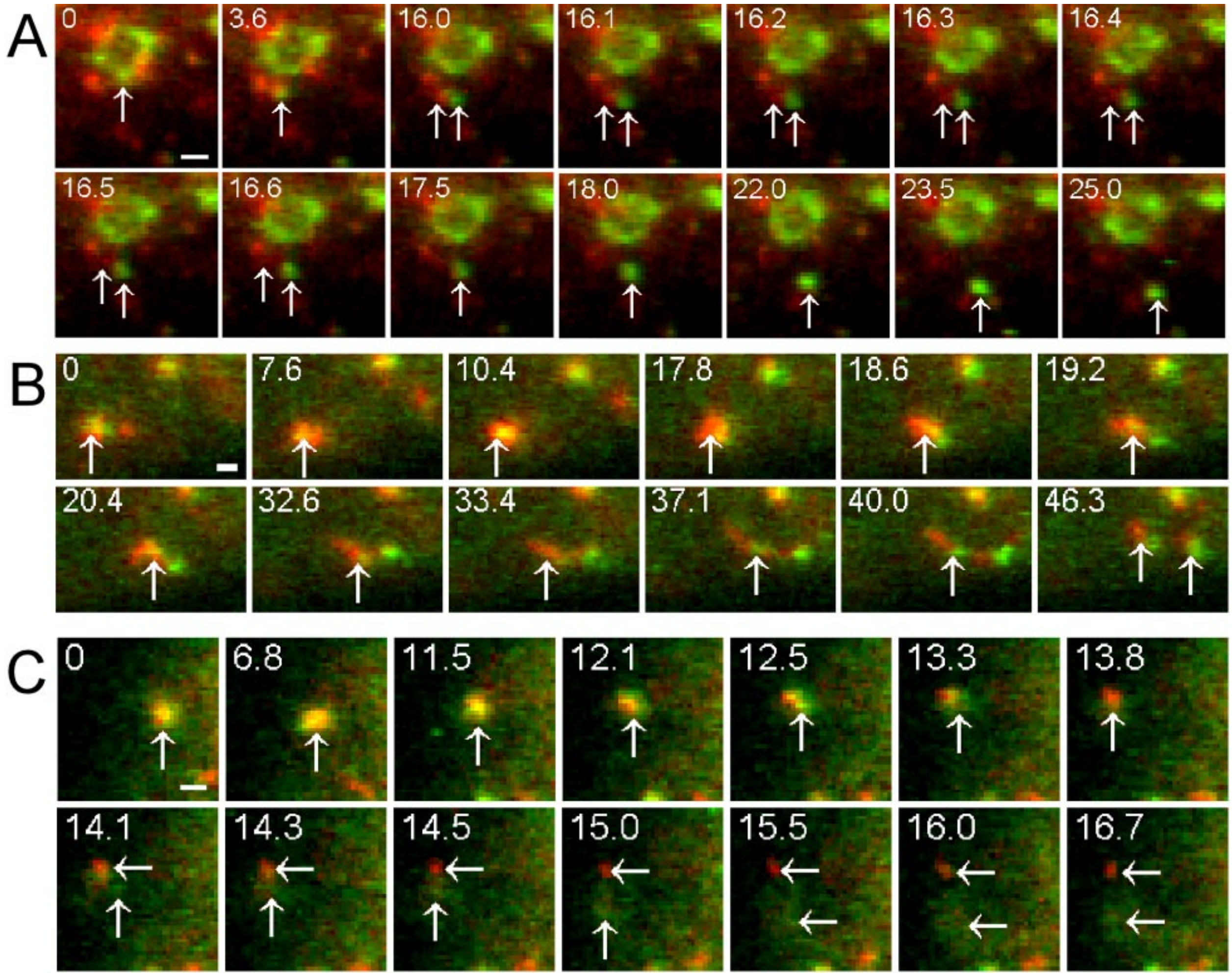

Figure 6. Analysis of behavior of Rab4 and Rab11 in transfected HMEC-1 cells. Cells were transfected with Rab4-CFP and Rab11-YFP and imaged as described in *Materials and Methods.* (A) A Rab4$^+$Rab11$^+$ vesicle leaving a sorting endosome and then separating into individual Rab4$^+$ and Rab11$^+$ compartments (indicated by arrows). (B) A Rab4$^+$Rab11$^+$ tubule (arrow), showing domain organization of the two Rab proteins. The tubule separates at the Rab4-Rab11 junction. (C) A Rab4$^+$Rab11$^+$ tubule that separates into Rab4$^+$ and Rab11$^+$ compartments (indicated by arrows). CFP and YFP are pseudocolored green and red, respectively. Bar, 1 μm. Figures 6, A and C, correspond to Supplementary Movies 6 and 7, respectively.

Significantly, although both Rab4 and Rab11 can be present in the same tubules and vesicles that separate from sorting endosomes, only Rab11 is seen to diffuse into the plasma membrane during exocytosis. The question therefore arises as to how Rab11 segregates from Rab4 to generate an exocytic compartment? Several models could be envisaged. First, gradual depletion of Rab4 could occur via a steady, continuous process. Second, Rab4 and Rab11 could segregate into domains that subsequently separate. Our data favor the latter possibility, but do not exclude the additional involvement of the former. We observe clear domains of Rab4 and Rab11 in tubules and vesicles that can segregate to generate Rab4$^+$ and Rab11$^+$ compartments. In addition, Rab4$^+$FcRn$^+$ compartments can lose their Rab4$^+$ content by a similar mechanism. In some cases, however, loss of FcRn from a Rab4$^+$ compartment is incomplete, suggesting that Rab4 depletion can occur via iterative steps. Thus, our studies are consistent with a model in which Rab4$^+$Rab11$^+$FcRn$^+$ compartments leave the sorting endosome and then bifurcate into Rab4$^+$ and Rab11$^+$FcRn$^+$ organelles. The fate of the Rab4$^+$ compartments is currently unknown, because their movement out of the focal plane limits the duration for which they can be tracked. Our observations also raise the question as to how fission of Rab4$^+$ from Rab11$^+$ compartments is mediated. It is conceivable that divalent Rab effectors, analogous to those described for Rab4 and Rab5 (De Renzis *et al.,* 2002), exist for Rab4 and Rab11 and are involved in the separation process.

Endothelial cells grow in vivo as polarized monolayers with apical and basolateral surfaces. For the current analyses, transiently transfected HMEC-1 cells have been used as subconfluent monolayers, which by analogy with epithelial cells (Brown *et al.,* 2000) might have some polarized character. In polarized (and nonpolarized) Madin-Darby canine kidney (MDCK) cells, Rab11 has been proposed to be a marker for apical recycling endosomes, but not for recycling endosomes that are involved in transferrin trafficking (Casanova *et al.,* 1999; Brown *et al.,* 2000; Wang *et al.,* 2000). This contrasts with data for nonpolarized cells where Rab11 is associated with the pericentriolar recycling compartment that plays a role in transferrin recycling (Ullrich *et al.,* 1996; Green *et al.,* 1997; Ren *et al.,* 1998; Sheff *et al.,* 1999). For

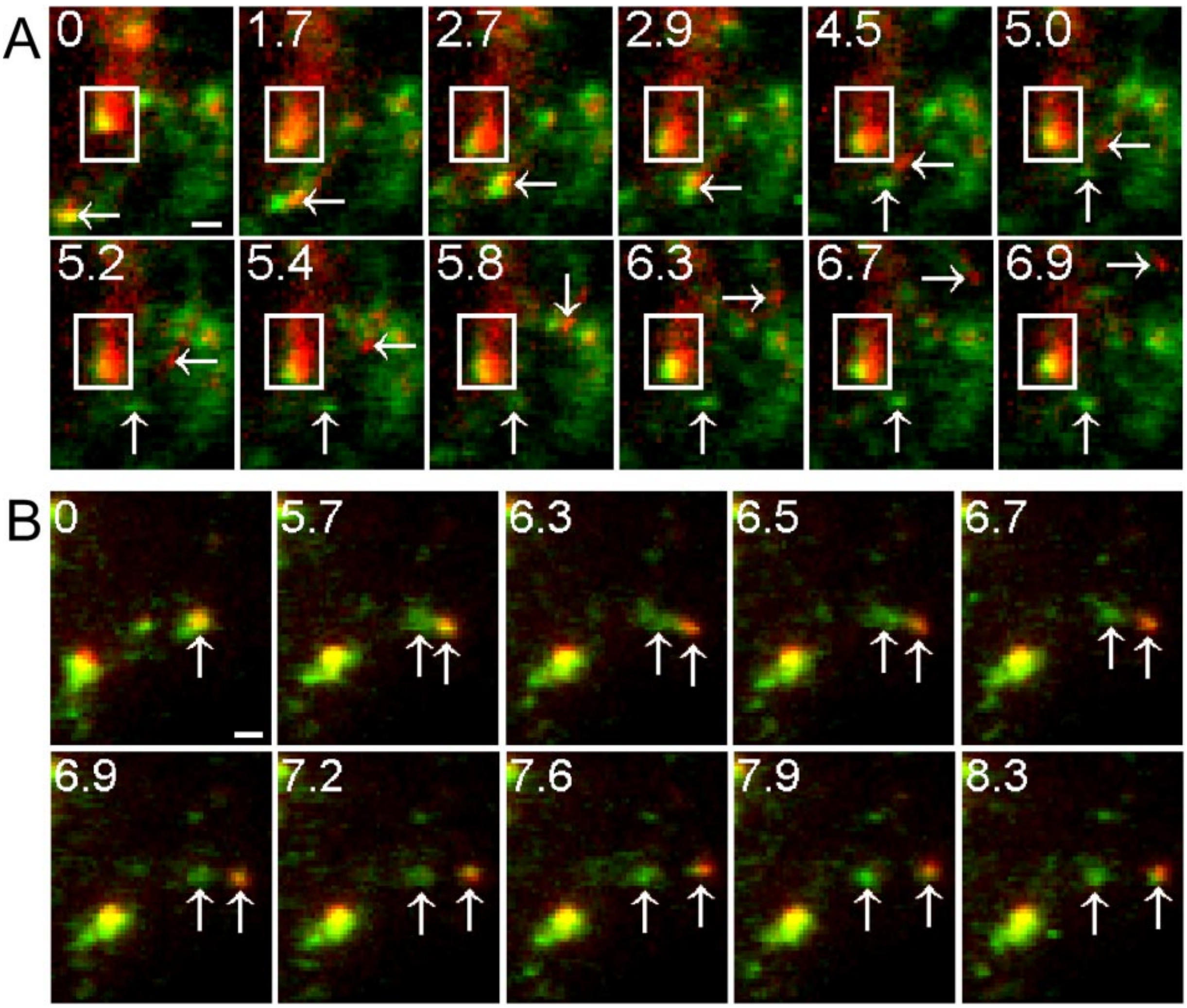

Figure 7. Analysis of compartmentalization of Rab4 and FcRn in transfected HMEC-1 cells. Cells were cotransfected with Rab4-YFP and FcRn-CFP and imaged as described in *Materials and Methods.* (A) A $Rab4^+FcRn^+$ tubule that separates into $Rab4^+$ and $FcRn^+$ compartments (indicated by arrows). The boxed region encloses a $Rab4^+FcRn^+$ compartment in which domains of Rab4 and FcRn can be seen, but separation does not occur. (B) A $Rab4^+FcRn^+$ tubule that separates into $Rab4^+$ and $FcRn^+$ compartments, with some residual FcRn remaining with the $Rab4^+$ compartment (indicated by arrows). CFP and YFP are pseudocolored green and red, respectively. Bar, 1 μm. Figure 7A corresponds to Supplementary Movie 8.

several reasons, the data in the current study indicate that Rab11 function in HMEC-1 cells correlates more closely with that in nonpolarized cells: first, our earlier studies (Ober *et al.*, 2004b) demonstrated that the recycling pathways taken by the transferrin receptor and FcRn overlap at the sorting steps in endosomes. We have also observed that transferrin exocytosis is frequently accompanied by FcRn or Rab11 diffusion into the plasma membrane. Thus, in the HMEC-1 cells used here the recycling pathways taken by the transferrin receptor and FcRn not only overlap at the sorting steps in endosomes (Ober *et al.*, 2004b), but also during exocytosis. Second, Rab11 is distributed throughout the cytosol in all HMEC-1 cells analyzed, rather than more condensed compartments reported for both polarized and nonpolarized MDCK cells (Brown *et al.*, 2000).

In several different cell types, analyses of transferrin recycling demonstrate the involvement of both fast and slow pathways (Sheff *et al.*, 1999; Hao *et al.*, 2002). The fast pathway invokes direct transit of recycling components from sorting endosomes to the plasma membrane, whereas the slow pathway involves passage from sorting endosomes to the endocytic recycling compartment (Sheff *et al.*, 1999 and reviewed in Maxfield and McGraw, 2004). From the current analyses we cannot distinguish whether our events are on the slow or fast recycling pathways. It is possible, however, that $Rab11^+$ vesicles/tubules take a more direct route to the plasma membrane than $Rab4^+Rab11^+$ vesicles/tubules that subsequently segregate into $Rab4^+$ and $Rab11^+$ compartments. However, the movement of these compartments across different focal planes imposes limitations on tracking their itineraries.

In addition to its role in recycling (Ullrich *et al.*, 1996; Green *et al.*, 1997; Calhoun *et al.*, 1998; Ren *et al.*, 1998; Casanova *et al.*, 1999; Duman *et al.*, 1999; Sheff *et al.*, 1999), Rab11 has been proposed to be involved in sorting from both endosomes to the *trans*-Golgi network (TGN; Wilcke *et al.*, 2000) or from the TGN to the plasma membrane (Chen *et al.*, 1998). Rab4 has also been reported to be associated with the Golgi (de Wit *et al.*, 2001). It is therefore possible that not all of the (exocytic) events that we observe are on the recycling pathway. However, this possibility is made unlikely by our analyses of transferrin exocytosis where we show that the majority of exocytic events seen for FcRn and Rab11 also involve transferrin release. Our data also raise the question as to how Rab11 is recaptured from the cell surface after the exocytic events. Several possible routes could be envisaged: for example, plasma membrane–bound Rab11 could be endocytosed or bind to GDP dissociation inhibitors (GDIs) that play a role in maintaining cytosolic pools of Rabs (Pfeffer and Aivazian, 2004).

In summary, our data are consistent with the following pathways for FcRn recycling from the sorting endosome: FcRn separates from the sorting endosome in association with Rab4 and Rab11 or with Rab11 only. Subsequent to separation from Rab4$^+$Rab11$^+$ compartments, Rab4 is localized to separate domains in the recycling vesicle/tubule that dissociate to leave Rab11$^+$FcRn$^+$ compartments. Rab11$^+$FcRn$^+$ compartments subsequently undergo full fusion with the plasma membrane to result in diffusion of Rab11 and FcRn out from the center of the exocytic site. These analyses therefore provide insight into the association of Rab GTPases with FcRn during the trafficking of this receptor from sorting endosomes to exocytic release at the plasma membrane.

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