Rab1a regulates sorting of early endocytic vesicles

Aparna Mukhopadhyay,1,2 Jose A. Quiroz,3 and Allan W. Wolkoff1,2,3

1Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York; 2Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, New York; 3Division of Gastroenterology and Liver Diseases, Albert Einstein College of Medicine, Bronx, New York; and 4Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona

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Mukhopadhyay A, Quiroz JA, Wolkoff AW. Rab1a regulates sorting of early endocytic vesicles. Am J Physiol Gastrointest Liver Physiol 306: G412–G424, 2014. First published January 9, 2014; doi:10.1152/ajpgi.00118.2013.—We previously reported that Rab1a is associated with asialoorosomucoid (ASOR)-containing early endocytic vesicles, where it is required for their microtubule-based motility. In Rab1a knockdown (KD) cell lines, ASOR failed to segregate from its receptor and, consequently, did not reach lysosomes for degradation, indicating a defect in early endosome sorting. Although Rab1 is required for Golgi/endoplasmic reticulum trafficking, this process was unaffected, likely due to retained expression of Rab1b in these cells. The present study shows that Rab1a has a more general role in endocytic vesicle processing that extends to EGF and transferrin (Tfn) trafficking. Compared with results in control HuH7 cells, EGF accumulated in aggregates within Rab1a KD cells, failing to reach lysosomal compartments. Tfn, a prototypical example of recycling cargo, accumulated in a Rab11-mediated slow-recycling compartment in Rab1a KD cells, in contrast to control cells, which sort Tfn into a fast-recycling Rab4 compartment. These data indicate that Rab1a is an important regulator of early endosome sorting for multiple cargo species. The effectors and accessory proteins recruited by Rab1a to early endocytic vesicles include the minus-end-directed kinesin motor KifC1, while others remain to be discovered.

Address for reprint requests and other correspondence: A. W. Wolkoff, Marion Bessin Liver Research Center, 625 Ullmann Bldg., Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461 (e-mail: allan.wolkoff@einstein.yu.edu).

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sorting; Rab1a; endocytosis; EGF; transferrin

RECEPTOR-MEDIATED ENDOCYTOSIS (RME) represents a major function of cells, including hepatocytes, in which binding of a ligand to its surface receptor initiates a cascade of processing and signaling events. The physiological consequences of this process include regulation of surface expression of proteins, sampling of the cell’s environment for growth/cell division, regulation/activation of signaling pathways, and uptake and metabolism of endogenous and xenobiotic compounds (59). Furthermore, many pathogens, such as hepatitis C virus (11), Semliki Forest virus (16), Ebola virus (2, 9, 10), and bacteria (e.g., Listeria monocytogenes, Staphylococcus aureus, and Candida albicans) (12, 43, 47, 75, 76), exploit this pathway to gain entry into host cells. A major mechanism of internalization by RME is via clathrin-coated vesicles, as seen in RME of ligands such as transferrin (Tfn) (23), EGF (19, 80), and asialoorosomucoid (ASOR) (68). Upon internalization and uncoating of clathrin-coated vesicles, cargo is transported via early endocytic vesicles (EEVs), which fuse and sort cargo into compartments in Rab11-mediated pathways may be achieved by the sharing of common effectors proteins (18, 20, 38). Vesicles containing cargo that is destined for degradation lose association with Rab5 and transition into late endosomes that are associated with Rab7 (58). Rab7 has been shown to regulate endosome-lysosome membrane fusion (13) and microtubule-based motor recruitment and motility (30, 31, 77). Association with and movement of endocytic vesicles on microtubules provide a platform for fusion/fission and sorting events (4, 5, 67). Motility on microtubules is mediated by dynein and kinesins, molecular motors that confer directionality toward the microtubule plus or minus end, depending on the motor (22). A single vesicle may be associated with multiple motors of opposing directionality (24, 25, 54, 60). The directional toward which the vesicle moves may be a function of activation/inactivation of one of these opposing motors, in part mediated by associated accessory proteins (3, 15, 40, 67).

A role for vesicle-associated proteins in recruitment of specific motors was seen in recent studies of Rab1a with respect to endocytic processing of ASOR (48). We showed that a cell line with stable knockdown (KD) of Rab1a failed to
efficiently process endocytosed ASOR. Although Rab1 has been thought to be important in Golgi/endoplasmic reticulum (ER) trafficking, the secretory pathway in these cells was unaffected, presumably due to compensation by Rab1b. Expression of Rab1b was unaffected by KD of Rab1a (48). Defective ASOR processing was attributable to the fact that EEVs prepared from Rab1a KD cells lacked the minus-end-directed kinesin motor KifC1 and had impaired motility toward the minus end of microtubules. To determine whether Rab1a serves more generally as an early endocytic regulator, we studied endocytic processing of EGF and Tfn. These proteins share the initial steps of internalization with ASOR but have different ultimate subcellular fates: EGF accompanies its receptor to lysosomes, where EGF and EGFR are degraded, while after release of iron, Tfn is recycled with its receptor to lysosomes, where EGF and EGFR are degraded, and EGF is efficiently processed by Rab1a (48). The HEK-293 cell line was maintained in RPMI medium containing L-glutamine, and sodium pyruvate in the Rab1a KD cells (48). The HEK-293 cell line was unaffected with KD of Rab1a, indicating that Rab1a is an important general regulator of early endosomal sorting.

**MATERIALS AND METHODS**

Cells, reagents, and antibodies. The human hepatoma cell line Huh7 and derivative Rab1a KD cell lines (48) were maintained in RPMI medium containing l-glutamine (Mediatech, Manassas, VA). As described previously, Rab1a was not detected by immunoblotting in the Rab1a KD cells (48). The HEK-293 cell line was maintained in DMEM containing 4.5 g/l glucose, l-glutamine, and sodium pyruvate (Mediatech). All media were supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin-streptomycin (Mediatech). Tet System-approved serum (Clontech, Mountain View, CA) was used for the Rab1a KD cells, which were further supplemented with 1.5 μg/ml blasticidin (Invitrogen, Carlsbad, CA) and 2.0 μg/ml puromycin (Clontech) to maintain the stable KD. HEK-293 cells overexpressing superfolder green fluorescent protein (sfGFP) or Rab1a-sfGFP were selected and maintained in medium containing 500 μg/ml G418 (Mediatech). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. All chemicals were of analytical grade or higher.

The following antibodies were used: rabbit anti-Rab1a (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-Rab4 (BD Transduction Laboratories, San Jose, CA), mouse anti-EGFR (EMD Millipore, Billerica, MA), mouse anti-FLAG M2, rabbit anti-Rab4, and mouse anti-β-actin (Sigma, St. Louis, MO), mouse anti-human KifC1 (Abd Serotec, Oxford, UK), Cy5-conjugated goat anti-rabbit IgG and Cy3-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA), mouse anti-Rab11 (Zymed/Invitrogen, Camarillo, CA), mouse anti-lysosomal-associated membrane protein 1 (LAMP-1; H4A3), and rabbit anti-GFP (Abcam, Cambridge, MA), rabbit anti-asiaglycoprotein receptor (ASGPR) (48, 62, 69), and anti-GFP-agarose beads (Medical and Biological Laboratories, Nagoya, Japan).

Preparation of Alexa 488-ASOR. ASOR was prepared as described previously (70) by hydrolysis of human α1-orosomucoid (Sigma) in 0.1 N sulfuric acid at 75°C for 1 h. The solution was neutralized with 1 N NaOH and dialyzed against water, and protein concentration was determined. Fluorescent ASOR was prepared by conjugation with Alexa 488-carboxylic acid succinimidyl ester (Molecular Probes, Eugene, OR) following the manufacturer’s instructions.

Endocytic uptake of fluorescent ligands. Rab1a KD and parental Huh7 cells were cultured on 35-mm uncoated plates (MatTek, Ashland, MA) and incubated for 1 h in serum-free, dye-free RPMI medium (Mediatech). The cells were chilled on ice and incubated with 20 μg/ml Tfn conjugated to Alexa 488 (Molecular Probes, Eugene, OR) or 5 μg/ml biotinylated GFP complexed to Alexa Fluor 555-streptavidin (Molecular Probes) for 1 h. Unbound ligand was washed off, and the plates were shifted to 37°C to initiate endocytosis. At indicated times, the cells were removed, fixed in 4% paraformaldehyde, and stained for Rab11 or LAMP1 using specific antibodies. For fluorescent ASOR uptake studies, cells were grown on 35-mm uncoated MatTek plates, chilled, and incubated with 10 μg/ml Alexa 488-ASOR in binding buffer (in mM: 135 NaCl, 0.81 MgSO4, 1.2 MgCl2, 27.8 glucose, 2.5 CaCl2, and 25 HEPES, pH 7.2) for 1 h on ice. Excess unbound ASOR was removed by washing, and the plates were treated similarly to those containing EGFR or Tfn.

Preparation of endocytic vesicles. Endocytic vesicles were prepared from Huh7 and Rab1a KD cells as previously described (48). Cells were incubated in serum-free medium for 1 h and then incubated on ice with 1.5 μg/ml Alexa 488-ASOR in buffer (in mM: 135 NaCl, 0.81 MgSO4, 1.2 MgCl2, 27.8 glucose, 2.5 CaCl2, and 25 HEPES, pH 7.2), 0.5 μg/ml Alexa 555-EGF, or 1 μg/ml Alexa 488-Tfn in dye-free RPMI medium for 1 h. Unbound ligand was removed by washing, and the cells were incubated at 37°C for the indicated times to initiate endocytosis. They were then washed in cold MEPS buffer (5 mM MgCl2, 5 mM EGTA, 35 mM Pipes-K2, and 0.25 M sucrose, pH 7.4) and prepared for centrifugation at 500 g. Cells were lysed in MEPS buffer containing protease inhibitor cocktail (Sigma) and protease inhibitor tablets (Roche, Mannheim, Germany) by passage through a 25-gauge 5/8-inch needle. A postnuclear supernatant was prepared after centrifugation at 2,000 g for 10 min. After adjustment to 1.4 M sucrose, vesicles were layered at the bottom of a 1.4-1.2-0.25 M discontinuous sucrose density gradient and subjected to centrifugation at 100,000 g for 2 h. Purified vesicles were harvested from the 1.2-0.25 M sucrose interface and stored in small aliquots at −80°C.

**Immunoblot analysis.** Immunoblot analysis was performed as we have described previously (7). Total protein (60 μg) was subjected to SDS-PAGE under reducing conditions (350 mM β-mercaptoethanol; Sigma) and transferred to a polyvinylidene difluoride (PVDF) membrane (Perkin Elmer, Boston, MA). The PVDF membrane was blocked with Tris-buffered saline (50 mM Tris-HCl and 150 mM NaCl, pH 7.6) containing 0.1% Tween 20 and 0.1% nonfat dry milk prior to incubation with primary antibody diluted appropriately in Tris-buffered saline, 0.1% Tween 20, and 2% nonfat dry milk. Immunoblot analysis was then performed using appropriate horseradish peroxidase-conjugated secondary antibody. Immunofluorescence studies on vesicles and cells. Immunofluorescence studies on vesicles were performed in an optical chamber as described previously (50, 52, 53). Vesicles were blocked in buffer containing 35 mM Pipes-K2, 5 mM MgCl2, 1 mM EGTA, 0.5 mM EDTA, 2 mg/ml BSA, 4 mM DTT, and 2 mg/ml vitamin C with 5 mg/ml casein, pH 7.4, and then incubated with specific antibodies at a dilution of 1:50 for 6 min. After extensive washing, the vesicles were incubated with secondary antibody for 6 min prior to final washes in assay buffer (35 mM Pipes-K2, 5 mM MgCl2, 1 mM EGTA, 0.5 mM EDTA, 2 mg/ml BSA, 4 mM DTT, and 2 mg/ml vitamin C, pH 7.4). Vesicles were imaged immediately. Cells were fixed in 4% paraformaldehyde for immunofluorescent staining and permeabilized by a quick wash in 0.01% saponin diluted in PBS. Cells were blocked in 10% fetal bovine serum in PBS for 30 min and then incubated with primary antibody diluted appropriately in blocking solution for 1 h at room temperature. Unbound antibody was removed by washing with PBS, and the cells were incubated with secondary antibody appropriately diluted in blocking solution for 1 h. Subsequent to incubation, unbound secondary antibody was removed with PBS, and the cells were imaged within 1–2 days.

**EGF degradation assay.** Control Huh7 and Rab1a KD cells were grown on 6-cm culture plates. Cells were incubated in serum-free RPMI medium for 30 min prior to incubation with 100 ng/ml EGF (Sigma) diluted in serum-free RPMI medium on ice for 30 min. A control plate was incubated in serum-free RPMI medium in the absence of EGF. At the end of the incubation, the unbound ligand was removed by washing, warm serum-free RPMI medium was added,

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and the cells were incubated at 37°C for the indicated times. To stop endocytic processing, the cells were chilled and lysates were prepared in buffer containing 50 mM Tris, 150 mM NaCl, 0.5% NP-40, and 5 mM MgCl₂, pH 7.6, with protease inhibitors. The lysates were subjected to immunoblotting to assay for the EGFR.

Preparation of 293 cells expressing Rab1a-sGFP. Human Rab1a cloned into pGEX4T-1 was obtained as a kind gift from Dr. William E. Balch (Scripps Research Institute, La Jolla, CA). Rab1a was amplified from this construct by PCR using the forward primer 5′-GCCACTCGAGCAATGTCCAGCATGAATCC and the reverse primer 5′-CCAGGCGCATGATTGAAAACC. PCR product was introduced into Xhol and SacII sites at the 5′ and 3′ ends of Rab1a, respectively, which were used for insertion into sfGFP-C1 (obtained as a kind gift from Dr. Erik Snapp, Albert Einstein College of Medicine, Bronx, NY). Plasmid DNA from clones was sequenced to verify that Rab1a had been inserted in the correct orientation. At 1 day prior to transfection, 293 cells at a low passage were seeded at 30% confluence in 6-cm culture dishes. On the day of transfection, the cells were incubated in serum-free medium for 30 min prior to addition of transfection complexes. Two micrograms of DNA were used for the preparation of transfection complexes with Lipofectamine Plus reagent (Invitrogen) following the manufacturer’s instructions. At 48 h posttransfection, the cells were split and seeded into selection medium containing 500 μg/ml G418. After 2 wk, the cells were assayed for Rab1a and sfGFP expression via immunoblotting and fluorescence microscopy.

Immunoprecipitation of KifC1-FLAG and Rab1a-sGFP. On the day of transfection, 293 cells expressing Rab1a-sGFP or sfGFP alone were seeded to achieve 50% confluence. Mouse KifC1 expressed as a kind gift from Dr. E. Balch (Scripps Research Institute, La Jolla, CA) was cloned into pGEX4T-1 was obtained as a kind gift from Dr. William Balch (Scripps Research Institute, La Jolla, CA). Rab1a was amplified from this construct by PCR using the forward primer 5′-GCCACTCGAGCAATGTCCAGCATGAATCC and the reverse primer 5′-CCAGGCGCATGATTGAAAACC. PCR product was introduced into Xhol and SacII sites at the 5′ and 3′ ends of Rab1a, respectively, which were used for insertion into sfGFP-C1 (obtained as a kind gift from Dr. Erik Snapp, Albert Einstein College of Medicine, Bronx, NY). Plasmid DNA from clones was sequenced to verify that Rab1a had been inserted in the correct orientation. At 1 day prior to transfection, 293 cells at a low passage were seeded at 30% confluence in 6-cm culture dishes. On the day of transfection, the cells were incubated in serum-free medium for 30 min prior to addition of transfection complexes. Two micrograms of DNA were used for the preparation of transfection complexes with Lipofectamine Plus reagent (Invitrogen) following the manufacturer’s instructions. At 48 h posttransfection, the cells were lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% NP-40, and 5 mM MgCl₂, pH 7.4) containing protease inhibitors. sfGFP was immunoprecipitated from the lysates by incubation with anti-GFP-agarose beads (Medical and Biological Laboratories) overnight at 4°C on a rotator. On the following day, the beads were washed multiple times in lysis buffer and then eluted with 2% SDS-containing Laemmli sample buffer at 75°C for 10 min. The eluate was immunoblotted for FLAG to test for the presence of KifC1.

Image acquisition and processing. Images were acquired with a ×60/1.4 numerical aperture Olympus objective on an Olympus IX71 inverted microscope containing automated excitation and emission filter wheels maintained at 37°C. Data were collected through a CoolSNAP HQ cooled charge-coupled device camera (Photometrics, Roper Scientific, Tucson, AZ) regulated by MetaMorph software (Molecular Devices, Sunnyvale, CA). Fluorescent images were analyzed using ImageJ 1.39u (National Institutes of Health public domain; http://rsb.info.nih.gov/ij/) and Adobe Photoshop CS2 version 9.0.2 (Adobe Systems, San Jose, CA) software. Colocalization of vesicle-associated fluorescent proteins was quantified using the ImageJ Co-localization macro written by Dr. John W. Murray to run in ImageJ 1.39u. This macro is based on previous methods (50) and is described by Mukhopadhyay et al. (48). The macro functions by segmenting images of vesicles into discrete spots and quantifying fluorescence at each spot in images of alternate fluorescence channels of the same field. It employs the SpotEnhancing filter written by Daniel Sage (Biomedical Imaging Group, Lausanne, Switzerland) and the Analyze Particles function of ImageJ 1.39u. Threshold intensity is chosen automatically on the basis of image intensity and standard deviation. z-Series images were acquired on a confocal microscope utilizing a multichannel white light source with 4',6-diamidino-2-phenylindole, FITC, rhodamine, and Cy5 filter settings on a CARVII spinning-disk imager (Crisel Instruments, Rome, Italy) containing a back-illuminated electron-multiplying charge-coupled device camera (iXon 897, Andor Technologies, Bellows Falls, VT) and a ×60/1.4 numerical aperture oil immersion lens. Images were pseudocolored, merged, and processed using ImageJ.

Statistical analysis. Statistical analysis was performed using χ² or Student’s t-test, as appropriate, and Microsoft Excel 2000.

Results

Segregation of ASOR from its receptor is reduced in Rab1a KD cells. Previously we observed slower endocytic processing and degradation of ASOR by live-cell imaging by cells with a stable KD of Rab1a than control cells, resulting in intracellular accumulation of this ligand (48). In the present study we investigated this finding in more detail using parental Huh7 and Rab1a KD cells grown on MatTek plates and incubated with Alexa 488-ASOR in the cold. At the end of incubation, the plates were washed free of unbound Alexa 488-ASOR and transferred to 37°C to initiate endocytosis. At specific times, the plates were removed, chilled, and fixed in 4% paraformaldehyde. An early step in ASOR endocytosis is segregation of receptor from ligand into separate vesicles (5). To examine this process, we used specific antibody to stain cells for ASGPR and imaged them by confocal microscopy. Figure 1, A and B, shows representative z-series images of Huh7 and Rab1a KD cells that were fixed 10 and 60 min after endocytic uptake of ASOR. By 10 min after initiation of endocytosis, there was little colocalization between ASGPR (red) and ASOR (green) in control cells, indicating segregation into separate vesicles of receptor from ligand. At this time, the plane of focus toward the top of the cell (Fig. 1A) and toward the base of the cell (Fig. 1B) for the majority of ASGPR was different from that for ASOR. In contrast, ASGPR and ASOR remained colocalized in common vesicles in Rab1a KD cells, even after 60 min of internalization (arrows in Fig. 1B). These findings suggest impaired segregation of ASOR from its receptor in the absence of Rab1a.

Because of the difficulties and inaccuracies in quantitative analysis of z-series colocalization studies, we quantitatively analyzed colocalization of ASOR and ASGPR in vesicles in a postnuclear supernatant prepared from cells that had taken up ASOR for 20 or 60 min. Representative micrographs are shown in Fig. 1C, and these results are summarized for multiple experiments in Fig. 1D. In postnuclear supernatants prepared from control Huh7 cells, 51% of ASOR-containing vesicles colocalized with receptor (ASGPR) following 20 min of internalization (n = 689), and 48% colocalized with ASGPR at 60 min (n = 470). In contrast, in vesicles prepared from Rab1a KD cells, 75% of ASOR-containing vesicles were also associated with ASGPR following 20 min of internalization (n = 530, P < 0.001), signifying reduced vesicle sorting. By 60 min, ASOR and ASGPR were colocalized in only 57% of these vesicles (n = 375), signifying that the rate of sorting of ligand from receptor into separate vesicles was slowed in Rab1a KD vesicles compared with control. We previously showed that degradation of ASOR is substantially reduced in Rab1a KD compared with control Huh7 cells (48). Thus, by 60 min in control Huh7 cells, much less ASOR remains, and it is likely that the higher colocalization in Huh7 cells can be mostly attributed to the population of vesicles that failed to internalize or internalized slowly. It should be noted that, unlike other experiments in this report that used vesicles purified on a sucrose density gradient from postnuclear supernatant, the vesicles used in these colocalization studies had not...
Fig. 1. Reduced segregation of asialoorosomucoid (ASOR) from its receptor asialoglycoprotein receptor (ASGPR) in Rab1a knockdown (KD) cells. Control Huh7 and Rab1a KD cells were incubated with Alexa 488-ASOR (green) on ice and then transferred to 37°C for 10 or 60 min to internalize Alexa 488-ASOR. A and B: representative z-plane confocal images of fixed Huh7 (control) and Rab1a KD cells immunostained for ASGPR (red). a–l: 1- to 1.5-μm confocal cuts from the top to the bottom of the cells. Arrows indicate areas of colocalization. Scale bars, 10 μm. C: endocytic vesicles purified from cells 20 and 60 min after internalization of Alexa 488-ASOR (green) were stained for ASGPR (red) using specific antibody. Arrows indicate areas of colocalization. Scale bars, 5 μm. D: percent colocalization of ASOR and ASGPR in isolated vesicles in multiple experiments performed as described in C. Numbers in parentheses indicate total number of vesicles counted. *P < 0.001.
been subjected to sucrose density gradient separation and likely contained plasma membrane and residual receptor-bound ASOR. This cell surface ASOR was ignored in the whole cell images but could add to the total percentage of ASOR-ASGPR colocalization, accounting for a higher-than-expected percentage of colocalization in control cells.

Efficient endocytosis of ASOR results in delivery to lysosomes (7) for degradation. To study this event, cells were fixed following endocytosis of fluorescent ASOR for 20 or 60 min. The lysosomal marker LAMP-1 (4) was then immunolocalized in these cells, and colocalization with ASOR was examined. Representative single-plane confocal images in which the plane of maximum green (ASOR) was chosen are shown in Fig. 2A. Representative z-series sections from the top to the bottom of cells that had internalized ASOR for 60 min are shown in Fig. 2B. As indicated by areas of colocalization (arrows), in control Huh7 cells, ASOR (green) colocalized to lysosomes (red) by 60 min of endocytosis. In contrast, there was little colocalization between LAMP-1 and ASOR in Rab1a KD cells. In fact, as seen in Fig. 2B, most of the LAMP-1 in Rab1a KD cells was present in a plane of focus (more at the top of the cell) different from that in which ASOR was observed (more at the bottom of the cell) at all times tested. These results indicate that reduced segregation of receptor from ligand in the absence of Rab1a correlates with decreased trafficking of ASOR to lysosomes. Thus Rab1a is required for efficient endocytic processing of ASOR at the early endocytic sorting step.

Rab1a is required for efficient endocytic trafficking of EGF and Tfn. ASOR trafficking through cells follows the typical clathrin-mediated endocytic pathway (68). To test whether Rab1a is a global mediator of endocytic sorting of ligands entering via clathrin-coated pits, we studied endocytic trafficking of two other ligands, EGF and Tfn. These two ligands were chosen, as all three, ASOR, EGF, and Tfn, have common early steps of endocytosis, namely, formation of the clathrin-coated pit, internalization of the EEV, loss of the clathrin coat, and fusion and sorting at the early endocytic compartment. However, after sorting at the early endocytic compartment, the subsequent stages of processing diverge. While ASOR segregates from its receptor and is targeted to lysosomes for degradation, EGF, along with its receptor, is sorted into the lysosomal degradation pathway (8, 19, 79, 82). In contrast, Tfn, along with its receptor, recycles back to the cell surface (78, 80, 81).

Using Alexa 555-EGF, we conducted experiments similar to those with ASOR, imaging its endocytic uptake in control and Rab1a KD cells. Figure 3A represents single-plane confocal
Fig. 3. Endocytic processing of EGF is delayed in the absence of Rab1a. A: representative single-plane confocal images of control Huh7 and Rab1a KD cells incubated with Alexa 555-EGF (red) on ice, shifted to 37°C for 0, 5, and 30 min, and fixed. Arrows indicate large aggregates of EGF at 30 min of endocytosis in Rab1a KD cells. B: fixed Huh7 and Rab1a KD cells prepared as described in A were stained with the lysosomal marker LAMP-1 (green). Arrows indicate areas of colocalization with Alexa 555-EGF, primarily in control Huh7 cells. C: confocal z-plane images of Huh7 and Rab1a KD cells that had endocytosed EGF (red) for 60 min before fixation and immunostaining for LAMP-1 (green). From left to right: 1- to 1.5-μm confocal cuts from the top to the bottom of the cells. Arrows indicate areas of colocalization, primarily in control Huh7 cells. Scale bars, 10 μm. D and E: EGF was added to Huh7 and Rab1a KD cells on ice, and cells were incubated at 37°C for 0–60 min to initiate endocytosis. Lysate was prepared and assayed by immunoblotting for EGF receptor (EGFR) and actin (loading control). Control —EGF represents lysates from cells that were not incubated with EGF. Position of molecular weight markers (in kDa) is indicated by lines at left of each immunoblot.
images of EGF uptake. In control cells, surface EGF at 0 min internalized into punctate structures by 5 min, and by 30 min the EGF signal had started to diminish due to degradation. Similar to the control cells, Rab1a KD cells also internalized most of the EGF by 5 min, but after 30 min, EGF was present in large aggregates (arrows in Fig. 3A). Thus, similar to results with ASOR, endocytic processing of EGF was impaired in the absence of Rab1a.

To further investigate this finding, cells that had taken up fluorescent EGF were stained for the lysosomal marker LAMP-1 (Fig. 3B and C). As expected, EGF (red) in control cells colocalized with lysosomes (green) by 30 min (arrows). Representative confocal sections from the top to the bottom of the cells are shown in Fig. 3C for control Huh7 and Rab1a KD cells following 60 min of EGF internalization. As indicated by the arrows in Fig. 3C, EGF colocalized with LAMP-1 at all levels in the control cells. In contrast, in Rab1a KD cells there was little, if any, colocalization of EGF with LAMP-1.

As EGF remains bound to its receptor EGFR throughout its endocytic processing and degradation, reduced EGFR levels can be used as a biochemical surrogate for EGF degradation. To perform this assay, we incubated cells with unlabeled EGF on ice, washed off the excess, and shifted the cells to 37°C for various times before preparing a lysate. Total protein was quantified in each lysate, and equal amounts were separated by SDS-PAGE, transferred onto a PVDF membrane, and immunoblotted for EGFR. To assess the levels of EGFR at the onset of the experiment, a control plate was incubated in the absence of EGF (Control – EGF in Fig. 3, D and E). In control Huh7 cells (Fig. 3D), EGFR levels progressively decreased with time, indicating endocytosis and eventual degradation of EGF and its receptor. In contrast, there was no decrease in EGFR levels over time in the Rab1a KD cells (Fig. 3E). These results correlate with the microscopy studies, confirming impaired endocytic processing of EGF in cells lacking Rab1a.

Similar experiments were performed using Alexa 488-Tfn to investigate the role of Rab1a in endocytic trafficking of Tfn. Representative single-plane confocal images showing endocytic uptake of Tfn over time in control and Rab1a KD cells are presented in Fig. 4. In control Huh7 cells, Tfn was progressively taken up into punctate vesicular structures and then gradually disappeared by 60 min, as apotransferrin was recycled back to the cell surface and released. In Rab1a KD cells, Tfn was taken up into vesicular structures and by 15 min

Fig. 4. Rab1a is required for endocytic trafficking of transferrin (Tfn). Control Huh7 and Rab1a KD cells were incubated with Alexa 488-Tfn on ice and then incubated at 37°C for 0, 5, 15, 30, and 60 min to initiate endocytosis. Representative single-plane confocal images are shown. In Huh7 cells, Tfn was progressively taken up into punctate vesicular structures and then gradually disappeared by 60 min, as apotransferrin was recycled back to the cell surface and released. In Rab1a KD cells, Tfn was taken up into vesicular structures and by 15 min appeared in large juxtanuclear aggregates (arrows). Scale bar, 10 μm.

Fig. 5. Tfn is delayed in reaching Rab4-associated compartments in Rab1a KD cells. Huh7 and Rab1a KD cells were incubated with Alexa 488-Tfn on ice and then shifted to 37°C for 2, 5, and 30 min to initiate endocytosis. Endocytic vesicles were purified from these cells and immunostained for Rab4. Colocalization of Rab4 and Tfn in vesicles was quantified as described in MATERIALS AND METHODS. By 5 min in control cells, 50% of the Tfn-containing endocytic vesicles were associated with Rab4, and association remained for 30 min. Association with Rab4 was significantly reduced at 5 min in Tfn-containing vesicles prepared from Rab1a KD cells but increased over time, so that, by 30 min after Tfn internalization, percentage of vesicles colocalizing with Rab4 was similar for control and Rab1a KD vesicles. Numbers in parentheses indicate total number of vesicles counted. *P < 0.001.

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appeared in large juxtanuclear aggregates. Tfn remained primarily in this region until the end of the experiment (60 min). This indicates that, similar to ASOR and EGF, Rab1a is also required for efficient trafficking of Tfn and Tfn recycling is substantially reduced in the absence of this Rab protein.

**Rab1a regulates early endocytic sorting of Tfn.** Recycling of internalized Tfn back to the cell surface requires Rab4 and Rab11 (21, 57, 73). The pathways mediated by each of these Rab proteins differ. Rab4 is implicated in fast recycling of Tfn \((t_{1/2} = 5\, \text{min})\) (61, 73), whereas Rab11 is implicated in a slow-recycling route \((t_{1/2} = 15–20\, \text{min})\) (26). We investigated whether there was a differential role of these proteins in the recycling of Tfn in control and Rab1a KD cells. We purified vesicles containing Tfn that had been endocytosed by these cells for 2, 5, and 30 min and immunostained them for Rab11 and Rab4 in an optical chamber. Consistent with previous reports, by 5 min in control cells, 50% \((n = 5,940)\) of the Tfn-containing endocytic vesicles were associated with Rab4 and remained associated with Rab4 for 30 min (Fig. 5). In contrast, vesicles prepared from Rab1a KD cells following 5 min of Tfn internalization showed significantly reduced [36% \((n = 7,784)\) vs. 50% \((n = 5,940), P < 0.001) association with Rab4 that increased over time, so that, by 30 min after Tfn internalization, the percentage of vesicles colocalizing with Rab4 was similar for control and Rab1a KD vesicles.

In contrast to results with Rab4, 5 min after internalization of Tfn (Fig. 6A), Rab11 was preferentially colocalized to vesicles prepared from Rab1a KD cells (50%, \(n = 8,442)\) compared with those from control Huh7 cells (37%, \(n = 9,577, P < 0.001)\). To validate this result in vivo, cells fixed at various times after endocytic uptake of fluorescent Tfn were stained with antibody against Rab11. As seen in the representative single-plane images in Fig. 6B and z series in Fig. 7, colocalization of Rab11 (red) with Tfn-containing endocytic vesicles (green) was reduced in control cells compared with Rab1a KD cells. These colocalization images also revealed that the juxtanuclear aggregates of Tfn in Rab1a KD cells are actually in Rab11 recycling compartments. From these results, we conclude that while, in control cells, Tfn is sorted into a Rab4 compartment for fast recycling, in the absence of Rab1a, sorting into the Rab4 compartment is delayed and Tfn is missorted into a Rab11 compartment, which accounts for slower recycling and intracellular accumulation of ligand.

**Rab1a mediates sorting of early endosomes by recruiting the minus-end-directed kinesin KifC1.** Endocytosis represents a dynamic process that requires motility of vesicles on microtubules (4, 5, 67), mediated by the molecular motors dynein and kinesins. Previously we reported that, compared with results with endocytic vesicles prepared from control Huh7 cells, ASOR-containing vesicles prepared from Rab1a KD cells lack the minus-end-directed kinesin KifC1 (48). Experiments were performed to determine whether Rab1a recruits KifC1 to vesicles by interacting directly with it. Rab1a was expressed in 293 cells as a sfGFP fusion protein. A control cell line expressing sfGFP only was created. These cell lines were

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**Fig. 6.** Tfn more rapidly enters a Rab11-associated recycling compartment in Rab1a KD cells. Huh7 and Rab1a KD cells were incubated with Alexa 488-Tfn on ice and then shifted to 37°C for 2, 5, and 30 min to initiate endocytosis. A: endocytic vesicles were purified from these cells and immunostained for Rab11. Colocalization of Rab11 and Tfn in vesicles was quantified as described in MATERIALS AND METHODS. Numbers in parentheses indicate total number of vesicles counted. *P < 0.001. B: representative single-plane images of cells fixed and immunostained for Rab11 (red). Colocalization of Rab11 (red) with Tfn-containing endocytic vesicles (green) was reduced in control Huh7 cells compared with Rab1a KD cells. As indicated by arrows, juxtanuclear aggregates of Tfn in Rab1a KD cells, which are visible in Fig. 4, are associated with Rab11, likely in a recycling compartment. Scale bar, 20 μm.
transiently transfected with constructs encoding mouse KifC1-FLAG (54) or the empty FLAG vector (pCMV-5c, Sigma). Lysates were prepared 72 h posttransfection, and sfGFP was immunoprecipitated using anti-GFP-agarose beads. The immunoprecipitate was eluted, subjected to SDS-PAGE, and processed for immunoblotting for FLAG and GFP. KifC1 expression was similar in lysates from control and Rab1a-expressing cells (Fig. 8, top). Although there was a consistently weak background band corresponding to KifC1 following immunoprecipitation of unlinked sfGFP, KifC1 was much more abundant in the sfGFP-Rab1a immunoprecipitate (Fig. 8, top). Recovery of sfGFP content in the immunoprecipitates was not substantially different whether or not it was coupled to Rab1a (Fig. 8, bottom).

To determine whether Rab1a mediated recruitment of KifC1 to vesicles other than those involved in ASOR endocytosis, we studied association of KifC1 with EGF-containing endocytic vesicles. Endocytic vesicles purified 2 and 5 min after uptake of fluorescent EGF were immunostained for KifC1, and colocalization was quantified (Fig. 9). Little (2%) colocalization of KifC1 was observed with EGF-containing vesicles from Rab1a KD cells compared with 10% (n = 1,335) colocalization of vesicles derived from parental Huh7 cells at 2 min and 5% (n = 1,293) at 5 min (P < 0.001).

Fig. 7. Accumulation of Tf in a Rab11 compartment in Rab1a KD cells. Huh7 and Rab1a KD cells were incubated with Alexa 488-Tf (green) on ice and then shifted to 37°C for 0, 2, 5, 15, and 30 min to initiate endocytosis. Cells were fixed and immunostained for Rab11 (red), and confocal z-plane images were obtained. From left to right: 1- to 1.5-μm confocal cuts from the top to the bottom of the cells. Arrows indicate areas of colocalization of Tf and Rab11, primarily in Rab1a KD cells at 15 and 30 min of endocytosis. Scale bars, 10 μm.
DISCUSSION

We previously reported that Rab1a associated with endocytic vesicles containing the hepatocyte-specific ligand ASOR plays an essential role in their microtubule-based minus-end-directed motility (48). Stable cell lines with small interfering RNA-mediated KD of Rab1a exhibited slower processing and degradation of ASOR, correlating with reduced recruitment of the minus-end-directed kinesin KifC1 to ASOR-containing EEVs. Although Rab1a has been described as important in ER-Golgi trafficking, there was no change in morphology of the ER or Golgi system in the KD cells, likely attributable to preserved expression of Rab1b. The current study shows a requirement for Rab1a not only for endocytic trafficking of ASOR, but also for trafficking of Tfn and EGF, ligands that also enter cells via clathrin-coated pits but have different subcellular fates (19). Our data indicate that, in the absence of Rab1a, all three ligands accumulate within cells and are delayed in reaching their normal subcellular destinations. Endocytosis of ASOR, Tfn, and EGF shares the initial steps of endocytosis that include internalization, scission, uncoating, and fusion/fusion events, which lead to sorting into a recycling or a degradation route. Since lack of Rab1a impairs trafficking of all three ligands, we conclude that it has an important role in one or more of these initial processes. We suggest that the common defect is at the level of sorting. This conclusion is supported by data showing delayed segregation of ASOR from its receptor in cells lacking Rab1a, a step that normally occurs within 5 min of internalization (6) and is characteristic of early endocytic sorting. Studies of Tfn trafficking also support this conclusion, as vesicles lacking Rab1a are missorted into a Rab11-associated slow-recycling route and accumulate in Rab11-containing compartments.

Sorting of early endosomes is a highly regulated process required for trafficking of ligands to their final subcellular destinations, but the regulatory mechanisms are poorly understood. Sorting is believed to be accomplished via formation of microdomains consisting of tubular formations that sort cargo into the recycling route and flat lattices that sequester cargo for the degradation route (32). A role for Rab5 in early endosomal sorting in which it mediates homotypic fusion of vesicles has been described (42, 44–46, 63). Exit into various routes is accomplished via other protein mediators. An important role has been described for Rab4 in the exit of recycling cargo back to the plasma membrane via the fast-recycling route (t1/2 = 5
GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.M. and A.W.W. are responsible for conception and design of the research; A.M. and J.A.Q. performed the experiments; A.M. and A.W.W. analyzed the data; A.M. and A.W.W. interpreted the results of the experiments; A.M., J.A.Q., and A.W.W. prepared the figures; A.M. and A.W.W. drafted the manuscript; A.M. and A.W.W. edited and revised the manuscript; A.M., J.A.Q., and A.W.W. approved the final version of the manuscript.

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