ARH cooperates with AP-1B in the exocytosis of LDLR in polarized epithelial cells

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The autonomous recessive hypercholesterolemia protein (ARH) is well known for its role in clathrin-mediated endocytosis of low-density lipoprotein receptors (LDLRs). During uptake, ARH directly binds to the FxNPxY signal in the cytoplasmic tail of LDLR. Interestingly, the same FxNPxY motif is used in basolateral exocytosis of LDLR from recycling endosomes (REs), which is facilitated by the epithelial-specific clathrin adaptor AP-1B. However, AP-1B directly interacts with neither the FxNPxY motif nor the second more distally located YxxØ sorting motif of LDLR. Here, we show that ARH colocalizes and cooperates with AP-1B in REs. Knockdown of ARH in polarized epithelial cells leads to specific apical missorting of truncated LDLR, which encodes only the FxNPxY motif (LDLR-CT27). Moreover, a mutation in ARH designed to disrupt the interaction of ARH with AP-1B specifically abrogates exocytosis of LDLR-CT27. We conclude that in addition to its role in endocytosis, ARH cooperates with AP-1B in basolateral exocytosis of LDLR from REs.

Introduction

Columnar epithelial cells polarize their plasma membrane into apical and basolateral domains, each with their own distinct assortment of transmembrane proteins and lipids (Martin-Belmonte and Mostov, 2008). Polarized sorting of newly synthesized or recycling receptors takes place either at the TGN or in recycling endosomes (REs) dependent on specific sorting determinants (Mellman and Nelson, 2008; Fölsch et al., 2009). Whereas apical sorting information is often decoded in a protein’s ectodomain or transmembrane anchor, sorting to the basolateral membrane is frequently facilitated by tyrosine- or dileucine-based sorting signals encoded in the cytoplasmic tails of transmembrane proteins. These cytoplasmic tail signals are recognized by cytosolic adaptor proteins and are generally cis dominant over apical sorting information (Rodriguez-Boulan et al., 2005; Fölsch, 2008). Tyrosine-based sorting signals conform to either FxNPxY or YxxØ consensus sequences.

Low-density lipoprotein receptor (LDLR) encodes two different types of basolateral sorting signals in its cytoplasmic tail (see Fig. 5 A). The proximal signal is an FxNPxY motif that is co-linear with the endocytic motif, ensuring incorporation of LDLR into clathrin-coated pits, followed by a cluster of negative charges (Matter and Mellman, 1994). The distal signal is a noncanonical YxxØ motif and comprises the amino acids GYSY again followed by a cluster of negative charges (see Fig. 5 A; Matter and Mellman, 1994; Koivisto et al., 2001). Here, we use two well-established LDLR mutant proteins in which either the proximal or the distal signal is inactivated (Matter et al., 1992). LDLR-CT27 was truncated after amino acid 27 of LDLR’s cytoplasmic tail and contains only the proximal FxNPxY motif (see Fig. 5 A). LDLR(Y18A) contains a tyrosine-to-alanine mutation at position 18 that disrupts the FxNPxY motif while maintaining the distant sorting determinant (see Fig. 5 A).

YxxØ sorting motifs are recognized by the medium subunits of heterotetrameric clathrin adaptor protein (AP) complexes, AP-1 through AP-4 (Bonifacino and Traub, 2003). They are each composed of two large subunits (γ, α, δ, or ε), and (β1–β4), one medium subunit (μ1–μ4), and one small subunit (σ1–σ4; Boehm and Bonifacino, 2001; Brodsky et al., 2001). The medium subunits recognize YxxØ motifs (Owen and Evans, 1998), and the carboxy domains of the large subunits interact with accessory proteins and clathrin (Edeling et al., 2006). AP-2 plays a role in clathrin-mediated endocytosis. AP-2 large subunits interact with AP-1B, and AP-1B directly binds to the FxNPxY motif in the cytoplasmic tail of LDLR, but it does not directly interact with the second more distally located YxxØ sorting motif. Here, we show that ARH colocalizes and cooperates with AP-1B in REs.
endocytosis at the plasma membrane, and AP-1, AP-3, and AP-4 facilitate cargo sorting at the TGN or endosomes (Nakatsu and Ohno, 2003).

Epithelial cells co-express AP-1A and AP-1B, which differs only in the incorporation of their respective medium subunits μ1A or the tissue-specific μ1B (Fölsch et al., 1999; Ohno et al., 1999). Despite this close homology, AP-1A and AP-1B are functionally distinct (Fölsch et al., 2003). Whereas AP-1A localizes primarily at the TGN and functions in sorting to the endosomal/lysosomal system, AP-1B is localized in REs and operates in basolateral sorting of cargos with YxxØ motifs, including LDLR (Fölsch, 2005). Curiously, none of LDLR’s basolateral sorting determinants directly interacts with μ1B (Fields et al., 2007). Instead, the distal GYSY signal interacts weakly with μ2 and μ4 (Fields et al., 2007). Accordingly, basolateral sorting of LDLR(Y18A) is not dependent on AP-1B (Fields et al., 2007) but may rely on AP-4 (Simmen et al., 2002). In contrast, LDLR’s proximal FxNPxY motif failed to interact with any adaptor μ chain, though LDLR-CT27 depends on AP-1B for basolateral sorting (see Fig. 5 A; Fields et al., 2007).

During clathrin-mediated endocytosis, the proximal FxNPxY motif of LDLR is recognized by co-adaptors autosomal recessive hypercholesterolemia protein (ARH), Dab2, or numb (Traub, 2009). Whereas both Dab2 and numb incorporate LDLR into nascent AP-2 vesicles through interaction with the α subunit (Traub, 2003), ARH interacts with the platform subdomain of β2, a binding interface that is conserved in β1 of AP-1 (He et al., 2002; Mishra et al., 2005; Keyel et al., 2008). Besides the β1/β2-binding domain, ARH has two additional well-characterized domains: a clathrin box and a phosphotyrosine-binding domain that interacts with FxNPxY motifs. Membrane recruitment of ARH is facilitated by binding to phosphorylated lipids such as phosphatidylinositol 4,5-bisphosphate (PI[4,5]P2) in clathrin-coated pits at the plasma membrane (Mishra et al., 2002). We recently showed that REs in AP-1B–expressing epithelial cells are enriched in phosphatidylinositol 3,4,5-trisphosphate (PI[3,4,5]P3; Fields et al., 2010). Thus, the lipid environment in REs is similar to that found in clathrin-coated pits. Furthermore, ARH was shown to bind to AP-1 in vitro (Mishra et al., 2002). Therefore, we wondered whether ARH was the missing link needed for understanding basolateral exocytosis of LDLR.

In this study, we provide strong evidence that ARH cooperates with AP-1B in basolateral exocytosis of LDLR from REs, finally providing a comprehensive mechanism for LDLR sorting in polarized epithelial cells. This study expands ARH’s function and opens up the field for future work aiming at assigning potential roles for other endocytic proteins in REs of polarized cells, perhaps including but not limited to β-arrestin and epsin 1, which like ARH bind to the β1/β2 platform subdomain (Owen et al., 2000; Traub, 2003; Mishra et al., 2005).

**Results and discussion**

**ARH is expressed in epithelial cell lines**

Using an antibody directed against rat ARH, a previous study detected only trace amounts of ARH in the most common model cell lines used to study polarized membrane trafficking, MDCK and LLC-PK1 (Nagai et al., 2003). Here, we reinvestigated the expression of ARH in columnar epithelial cells. First, we determined whether ARH is transcribed in MDCK cells by performing RT-PCR on isolated RNA. Transcripts for Rab8 and Rab10, small GTPases involved in polarized sorting, served as positive controls, and heat inactivation of the RT step served as a negative control. Indeed, ARH transcripts are present in MDCK cells (Fig. 1 A, lane 1). This was confirmed by using a quantitative real-time RT-PCR (qRT-PCR) set up to determine relative mRNA levels of ARH, Rab8, and μ1B normalized to the transcript levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Fig. 2 B).

Finally, we investigated ARH protein levels in several columnar epithelial cell lines. Indeed, a polyclonal antibody that was raised against human ARH detected ARH in MDCK cells (see also Cui et al., 2010) as well as in human bronchial epithelial (HBE) cells and LLC-PK1 cells that were stably transfected with μ1B (LLC-PK1::μ1B; Fig. 1 C).

**ARH pulls down AP-1B in vitro**

Mishra et al. (2002) have found that ARH precipitated AP-1A and AP-2 from brain and liver lysates. To investigate whether ARH might also precipitate AP-1B, we used affinity purified His6-tagged ARH (His-ARH) to pull down AP-1B from EFA47 cell lysates. EFA47 cells are murine μ1A−/− embryonic fibroblasts that express μ1B exogenously so that AP-1B is the only AP-1 complex present (Fölsch et al., 2001; Eskelinen et al., 2002). Lysates from 3T3 fibroblasts served as a positive control for AP-1A.

We found that ARH can pull down AP-1A and AP-1B (Fig. 1 D), and thus could potentially cooperate with both AP-1 complexes in vivo.

**ARH localizes in REs of AP-1B-positive epithelial cells**

To cooperate, ARH and AP-1B would have to colocalize in REs. To test this, we used LLC-PK1 cells stably expressing HA-tagged μ1B (LLC-PK1::μ1B-HA) or HA-tagged μ1A as a control (LLC-PK1::μ1A-HA; Fölsch et al., 2001). Because LLC-PK1 cells are μ1B negative (Ohno et al., 1999), these two cell lines can also be used to determine differences in localization between μ1B-expressing cells (LLC-PK1::μ1B-HA) and those that do not express μ1B (LLC-PK1::μ1A-HA).

LLC-PK1 cells were grown on coverslips and infected with defective adenoviruses encoding EGFP-tagged ARH (ARH-GFP), resulting in low levels of expression. 24 h after infection, specimens were labeled for AP-1A- or AP-1B-HA (Fig. 2 A), or for the RE marker transferrin receptor (TfnR; Fig. 2 B). Specimens were analyzed by confocal microscopy, and the percent overlap between ARH-GFP and AP-1A-HA, AP-1B-HA, or TfnR in the perinuclear region was determined according to Manders et al. (1993) using Velocity software as described in the Material and methods section. First, we analyzed ARH-GFP localization with respect to AP-1A or AP-1B.
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Collectively, our data show that ARH and AP-1B colocalize in TfnR-positive REs, which indicates that they may cooperate in basolateral sorting from this compartment. This preference of ARH for AP-1B in vivo is perhaps a reflection of the different lipid environments of the TGN (phosphatidylinositol 4-phosphate) and REs (PI[3,4,5]P3; Wang et al., 2003; Fields et al., 2010).

**ARH knockdown leads to specific apical missorting of LDLR-CT27**

To test whether ARH may function in exocytosis, we next depleted human ARH in HBE cells, which resemble MDCK cells with respect to AP-1B expression and other polarity features (Nokes et al., 2008). To this aim, we used a lentiviral vector that encodes GFP-tagged short hairpin RNA (shRNA) targeting the 3′ untranslated region (UTR) of human ARH to generate HBE cells stably depleted of ARH. Vectors targeting GAPDH were used as controls. ARH knockdown was measured using two different methods. First, we performed quantitative Western blot analysis using the LI-COR Odyssey system. On average, ARH protein levels were reduced by ~80% in knockdown cells (Fig. 3 A). This was confirmed in individual cells using a confocal microscopy–based assay as detailed in Materials and methods (Fig. S1, A and B).
Figure 2. ARH localization in TfnR-positive REs depends on AP-1B. LLC-PK1::µ1A-HA and LLC-PK1::µ1B-HA cells were grown on coverslips and infected with defective adenoviruses encoding ARH-GFP. After 24 h, cells were fixed and stained with anti-HA (A) or anti-TfnR (B) antibodies. Specimens were analyzed by confocal microscopy and representative images are shown. Insets show 2x magnifications of the boxed regions. A' and B' show representative fluorescence intensity profiles through a region in the boxed area where noncoincidental peaks are marked by arrowheads (ARH-GFP) or arrows (AP-1A or TfnR). Bars, 10 µm. (C) For quantitation, confocal raw data were analyzed using Volocity software to determine the degree of colocalization between ARH-GFP and AP-1A (n = 64) or AP-1B (n = 71) as well as TfnR in LLC-PK1::µ1A-HA (TfnR[A], n = 54) or LLC-PK1::µ1B-HA cells (TfnR[B], n = 60). Data represent mean values from at least three independent experiments, error bars indicate SD. *, P < 0.0001. (D) Filter-grown MDCK cells were infected with defective adenoviruses encoding ARH-GFP. 24 h later, cells were stained for TfnR. Specimens were analyzed by confocal microscopy and representative xy, xz, and yz images are shown. Bar, 5 µm.
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55

ARH (34 kD)

γ-adaptin (92 kD)

µg total protein

2.5 5.0 7.5

2.5 5.0 7.5

quantitative Western blot:

ARH KD = 79.2 ± 0.2%

We infected filter-grown HBE cells stably depleted of ARH or GAPDH with defective adenoviruses encoding various cargo proteins, and analyzed their steady-state localization. First, we examined LDLR-CT27, which has only the proximal FxNPxY sorting motif and thus might be a cargo for ARH during exocytosis. Indeed, upon knockdown of ARH, LDLR-CT27 localization was randomized in 92 ± 8% of the cells analyzed (Fig. 3 B). Importantly, in GAPDH knockdown cells, LDLR-CT27 was sorted correctly to the basolateral membrane in 68.3 ± 3% of the cells analyzed (P < 0.0001). This indicated that the expression of shRNAs in the cells did not lead to nonspecific missorting LDLR-CT27. Next we tested additional cargos with either YxxØ- or LL-based motifs. We found that LDLR(Y18A), which contains only the distal YxxØ sorting signal, was sorted to the basolateral membrane even when ARH levels were reduced (Fig. 3 C). Moreover, Fc receptors (FcRs), which contain LL-based sorting information and are sorted to the basolateral membrane independent of AP-1B (Matter et al., 1994; Roush et al., 1998), were also correctly sorted in the absence of ARH (Fig. 3 D). Finally, we analyzed the truncation mutant neuron-glia cell adhesion molecule (NgCAM)-CT43. NgCAM-CT43 contains a YxxØ sorting motif and requires AP-1B expression for basolateral sorting (Anderson et al., 2005). NgCAM-CT43 was correctly sorted in ARH knockdown cells (Fig. 3 E).

In summary, knockdown of ARH led to specific apical missorting of an AP-1B–dependent cargo with an FxNPxY sorting motif, but not of cargos that contained YxxØ- or LL-based sorting signals. These data support the hypothesis that ARH and AP-1B may cooperate in basolateral sorting of cargos with FxNPxY motifs.
This mutant protein still binds FxNPxY motifs and clathrin; however, the binding to β1/β2 adaptins is abolished (He et al., 2002). Thus, ARH(R266A) can no longer facilitate cargo selection into nascent clathrin-coated vesicles (Mishra et al., 2005). If our model is correct, we would expect that overexpression of ARH(R266A) using a microinjection-based assay should impair surface delivery of LDLR-CT27. We co-injected plasmids expressing ARH(R266A) with plasmids encoding LDLR-CT27 into polarized MDCK cells. Subsequently, cells were incubated at 20°C to block LDLR-CT27 exit from the TGN while ARH(R266A) accumulated in the cytosol followed by a 2-h chase at 37°C in the presence of cycloheximide to inhibit further protein synthesis. During this chase period, LDLR-CT27 will move from the TGN into REs for basolateral sorting along the AP-1B pathway (Fields et al., 2007; Nokes et al., 2008). Receptors at the surface were then stained with antibodies recognizing their ectodomains before fixation and total staining of LDLR-CT27 and ARH(R266A). Under control conditions, LDLR-CT27 was correctly sorted to the basolateral membrane. Correct sorting of LDLR-CT27 was further observed upon coexpression of wild-type ARH-GFP (Fig. S1 C). However, coexpression of ARH(R266A) completely abolished surface delivery in virtually every cell analyzed, and LDLR-CT27 accumulated within the cells (Fig. 4 A and Fig S1 C). In contrast, sorting of LDLR(Y18A), which does not involve movement through REs during biosynthetic delivery (Nokes et al., 2008), was not affected by ARH(R266A) coexpression (Fig. 4 B). Furthermore, basolateral sorting of vesicular stomatitis virus glycoprotein (VSVG) was also not influenced by the presence of ARH(R266A) (Fig. 4 C). Like LDLR-CT27, VSVG moves from the TGN into REs during biosynthetic delivery (i.e., during the chase period of our protocol) to be sorted to the basolateral membrane, dependent on AP-1B (Ang et al., 2004; Nokes et al., 2008). However, unlike LDLR-CT27, VSVG has a YxxØ sorting motif that interacts with µ1B/AP-1B (Fields et al., 2007). Thus, the dominant-negative action of ARH(R266A) did not disrupt global AP-1B–dependent sorting, but was specific for LDLR-CT27.

Conclusions

We propose that during biosynthetic delivery or endocytic recycling, the FxNPxY sorting determinant of LDLR-CT27 is recognized by ARH, which subsequently bridges LDLR-CT27 to AP-1B by interacting with AP-1B’s β1 subunit, thereby facilitating incorporation of LDLR-CT27 into nascent clathrin-coated vesicles (Fig. 5 B). Disruption of ARH binding to β1 may result in the capture of LDLR-CT27; however, incorporation of LDLR-CT27 into clathrin-coated vesicles may be stalled, leading to impaired surface delivery (Fig. 5 C). Successfully formed AP-1B vesicles with or without LDLR cargo are then thought to tether to the basolateral membrane via the exocyst complex followed by membrane fusion facilitated by the SNARE proteins cellubrevin and syntaxin 4 (Fölsch, 2005; Fields et al., 2007).

LDLR's FxNPxY motif is well conserved in different genes of the LDLR gene family, including the basolaterally localized LDLR-related protein (LRP) and the apically targeted...
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Materials and methods

DNA constructs and plasmids

To generate ARH with a C-terminal EGFP tag, we first PCR amplified ARH using full-length human ARH (clone ID 5197824; Thermo Fisher Scientific) as template and ARH-FL-N and ARH-FL-C as N- and C-terminal primers (Table S1). The resulting PCR products were cloned as EcoRI–XbaI fragments in frame into the pET28 expression vector, thereby introducing N-terminal His<sub>6</sub> tag.

Full-length T7-tagged human ARH including either short (UTR1) or long (UTR2) sections of its 3′ UTR were cloned from mRNA extracted from HBE cells using TRIzol (Invitrogen) followed by coupled RT-PCR using...
were grown in DME (10% fetal bovine serum) containing 200 µg/ml geneticin. EFA47 fibroblasts PK1::lin/streptomycin (except where otherwise stated). MDCK and HBE cells media were supplemented with 2 mM l-glutamine and 0.1 mg/ml penicillin, 1 mg/100 ml bovine serum albumin, 3 mg/100 ml bovine collagen I, and 1 mg/100 ml fibronectin.

HBE cell lines stably knocking down either ARH or GAPDH were generated using lentiviruses encoding ARH or GAPDH shRNA constructs and selected with 8 µg/ml puromycin exactly as described previously (Anderson et al., 2005). They were maintained in 8–12 µg/ml puromycin added to the growth medium.

For Western blot analysis of total cell lysates, cells were seeded at a 1:1 dilution onto 10-cm plates and grown for 1 d. Cells were then lysed in 600 µl RIPA buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1.0% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, and 1× protease inhibitors [Boehringer Ingelheim]), incubated for 15 min on ice, and subsequently passed five times through a 22.5-gauge needle and 1-ml syringe. Samples were then centrifuged at 15,000 rpm for 25 min at 4°C (microcentrifuge; Eppendorf). Supernatants were transferred to a new tube, and total protein concentrations were quantified using the BCA protein assay (Thermo Fisher Scientific). Protein lysates were analyzed by SDS-PAGE and Western blot analysis using HRP-labeled secondary antibodies and SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific).

For quantitative Western blot analysis, we used an Odyssey infrared imaging system (Odyssey application software v2.0; LI-COR). Specifically, to determine ARH knockdown, we analyzed cell lysates of HBE cells stably depleted of GAPDH or ARH by SDS-PAGE and Western blotting using anti-ARH primary antibodies and IRDye 680-labeled secondary antibodies. For quantitation of ARH knockdown, ARH expression in cells depleted of GAPDH was set as 100%. Experiments were repeated three times. Each experiment had three data points: 2.5, 5, and 7.5 µg of total protein. First, we determined the percent knockdown of ARH for each data point. We then calculated the mean knockdown for each experiment. Finally, we calculated the mean values of the independent experiments. Errors are SD. Note that the value for γ-adaptin expression in ARH knockdown cells in comparison to GAPDH knockdown cells was 88.8 ± 5%.

Full-drown assay
Histidine-binding proteins were expressed in BL21 bacteria that were subsequently lysed using a French press. His-ARH was then batch purified using the His•Bind protein purification kit (EMD), and desalted using a PD10 desalting column (GE).

For pull-down experiments, 24 µg of purified His-ARH was immobilized on HisLink protein purification resin (Promega). Subsequently, immobilized His-ARH was incubated with cell lysates (1 mg/ml) generated as follows: 3T3 and EFA47 cells were each grown in 2 × 15-cm plates and washed twice with PBS•+ (PBS [0.2 g/liter KCl, 0.2 g/liter NaH2PO4, 8 g/liter NaCl, and 2.17 g/liter NaHPO4 · 7 H2O]) plus 0.1 g/liter CaCl2 and 0.1 g/liter MgCl2 · 6 H2O) on ice, scraped in 1 ml of ice-cold buffer A (20 mM Heps-KOH, pH 7.6, 320 mM sucrose, 100 mM NaCl, 25 mM imidazole, 0.5% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, and 1× protease inhibitors). Samples were then centrifuged at 15,000 rpm for 25 min at 4°C (microcentrifuge; Eppendorf). Supernatants were transferred to a new tube, and protein concentrations were quantified using the BCA protein assay (Thermo Fisher Scientific). Samples were adjusted to the same final concentration of 0.1% [wt/vol]. Pull-down reactions were incubated for 4 h at 4°C with gentle rotation. Subsequently, samples were washed three times in buffer A plus 0.1% Triton X-100. Bound proteins were eluted by vigorously shaking for 20 min and boiling in SDS sample buffer. Samples were analyzed by SDS-PAGE and Western blot analysis.

Immunofluorescence assays
For immunofluorescence experiments with coverslip-grown cells, we seeded LLC-PK1 cell lines on Alcian blue–coated coverslips and HBE cells on collagen- and fibronectin-coated coverslips. Cells were grown for 3–4 d.

For experiments with polarized cells, we seeded 4 × 104 cells on 12-mm filter supports (0.4-µm pore size; Corning) and cultured for 3–4 d with changes of the medium in the basolateral chambers daily. For microinjection experiments, clear filter supports were used. The microinjection assay was performed exactly as described previously [Nokes et al., 2008] using a Femtotjet (Intraman N2; Eppendorf) mounted on a microscope (Axiovert 200; Carl Zeiss, Inc.) equipped with a heated stage. Microinjected cells were processed for immunofluorescence microscopy essentially as described previously [Nokes et al., 2008] with the following alterations.
for LDLR proteins: LDLR proteins at the cell surface were stained for 1 h on ice with anti-LDLR antibodies (C7, IgG2b), fixed in 3% PFA for 15 min at room temperature followed by incubation in BSA† for 5 min. Subsequently, cells were incubated for 1 h in a blocking/permeabilization solution (BPS) (2% [wt/vol] bovine serum albumin, 0.4% [wt/vol] saponin in PBS†) plus 2% [vol/vol] goat serum, then incubated for 1 h with goat anti-mouse IgG2b antibodies labeled with Alexa Fluor 594 in BPS. Cells were then fixed again in 3% PFA for 15 min at room temperature followed by incubation in BSA† for 5 min. Subsequently, cells were incubated in BPS plus 2% [vol/vol] goat serum for 1 h, followed by a 1-h incubation with C7 antibodies to stain for LDLR that was not delivered to the surface and anti-V5 antibodies (IgG2a) to detect ARH(R266A)-V5 proteins produced in the cytosol. Subsequently, cells were washed five times with BPS with PBS 3D finally, cells were incubated with secondary antibodies (Alexa Fluor 488-labeled anti-IgG2b and Cy5-labeled anti-IgG2a) in BPS, followed by five washes in BPS over 30 min. Cells were mounted in a solution containing 10% [wt/vol] 1,4-diazabicyclo[2.2.2]octane (DABCO) and 50% [wt/vol] glycerol in water.

Infection of cells with defective adenoviruses was performed in growth media without fetal bovine serum by gently rocking cells with the viruses applied to the optical surface in a 37°C incubator. After 2 h, media were exchanged with regular growth media and cells were incubated for 24 h before fixation and immunofluorescence staining as described in the previous paragraph. MDCK and HBE cells on filters were infected with ARH-GFP and AP-1A-HA, AP-1B-HA, or TfnR was performed on confocal microscopy.

Images were acquired at room temperature using a confocal microscope (Microsystem LSM S10 with ConfoCor 3 software) equipped with a C-Apochromat 63×/1.2 NA oil immersion objective (all from Carl Zeiss, Inc.). The images were processed using Photoshop CS3 (Adobe), and combined using Illustrator (Adobe).

Statistical analysis and quantitative immunofluorescent assays

For the quantitation of shRNA knockdown in individual cells, HBE cells were grown on coverslips. 24 h after seeding, cells were cotransfected with a construct expressing shRNA targeting ARH (shARH), and the second transcribed T7-ARH with its original 3′ end (shARH*) and T7-ARH. ARH-GFP images were acquired at room temperature using a confocal microscope (Microsystem LSM S10 with ConfoCor 3 software) equipped with a C-Apochromat 63×/1.2 NA oil immersion objective (all from Carl Zeiss, Inc.). The images were processed using Photoshop CS3 (Adobe), and combined using Illustrator (Adobe).

Online supplemental material

Fig. S1 shows a quantitation of ARH knockdown in HBE cells and corrected for T7-LC27 sorting in polarized MDCK cells in the presence of ARH. Video 1 is a Quicktime interactive movie of a 3D reconstruction of a fully polarized MDCK cell expressing ARH-GFP. ARH-GFP localizes to the plasma membrane and colocalizes with TfnR in the perinuclear region. The individual xy sections used to build the 3D model are shown in the JCB DataViewer. Table S1 shows the primer sequences used for cloning, RT-PCR, and qRT-PCR. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.20101212/DC1.

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