The PDZ-interaction of the intestinal anion exchanger downregulated in adenoma (DRA; SLC26A3) facilitates its movement into Rab11a-positive recycling endosomes

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Lissner S, Hsieh CJ, Nold L, Bannert K, Bodammer P, Sultan A, Seidler U, Graeve L, Lamprecht G. The PDZ-interaction of the intestinal anion exchanger downregulated in adenoma (DRA; SLC26A3) facilitates its movement into Rab11a-positive recycling endosomes. Am J Physiol Gastrointest Liver Physiol 304: G980–G990, 2013. First published April 11, 2013; doi:10.1152/ajpgi.00132.2012.—Electroneutral NaCl absorption in the ileum and colon is mediated by downregulated Na+/HCO3− exchanger; SLC26A3) and Na+/H+ exchanger 3 (NHE3, SLC9A3). Surface expression of transport proteins undergoes basal and regulated recycling by endo- and exocytosis. Expression and activity of DRA in the plasma membrane depend on intact lipid rafts, phosphatidylinositol 3-kinase (PI3-kinase), and the PDZ interaction of DRA. However, it is unknown how the PDZ interaction of DRA affects its trafficking to the cell surface. Therefore, the (re)cycling pathway of DRA was investigated in HEK cells stably expressing enhanced green fluorescent protein (EGFP)-DRA or EGFP-DRA-ETKF minus (a mutant lacking the PDZ interaction motif). Early, late, and recycling endosomes were immunolabeled with mCherry-hemagglutinin (HA)-Rab5a, -7a, or -11a. EGFP-DRA and EGFP-DRA-ETKF minus were equally present in early endosomes. In recycling endosomes, wild-type DRA was preferentially present, whereas, in late endosomes, DRA-ETKF minus dominated. Correspondingly, EGFP-DRA colocalized with mCherry-HA-Rab11a in recycling endosomes, whereas EGFP-DRA-ETKF minus colocalized with mCherry-HA-Rab7a in late endosomes. Functionally, this different distribution was reflected by a shorter half-life of the mutant DRA. Transient expression of dominant-negative Rab11aS22N inhibited the activity (17%, P < 0.05) and the cell surface expression of DRA (−30%, P < 0.05). Transient transfection of Rab4a or its dominant-negative mutant Rab4aS22N was without effect and thus excluded participation of the rapid recycling pathway. Taken together, the PDZ interaction of DRA facilitates its movement into Rab11a-positive recycling endosomes, from where it is inserted in the plasma membrane. A scenario emerges where specific PDZ adaptor proteins are present along several compartments of the endocytosis-recycling pathway.

endocytosis; endosomes; Rab5a

THE INTESTINE IS RESPONSIBLE for the absorption of macro- and micronutrients as well as for electrolytes and water. Water transport occurs secondary to either of four modes of Na+ and Cl− movement: 1) nutrient-coupled Na+ absorption by various Na+-coupled transporters, 2) electroneutral NaCl absorption, 3) electrogenic Cl− secretion by the cystic fibrosis transmembrane regulator (CFTR) and the calcium-activated chloride channel, or 4) electrogenic Na+ absorption by the epithelial sodium channel (ENaC) (17). Electroneutral NaCl absorption in the distal ileum and proximal colon is mediated by the integrated actions of the Na+/H+ exchanger NHE3 (SLC9A3) and the Cl-/HCO3− exchanger downregulated in adenoma (DRA, SLC26A3) (17, 23). The intestinal phenotype of DRA and NHE3 knock-out mice, which display chronic diarrhea (38, 39), as well as the human genetic disorder congenital chloride diarrhea (14), in which a loss-of-function mutation of DRA leads to life-threatening diarrhea, are evidence for the importance of these transport proteins. Correspondingly, these transporters are tightly regulated under physiological conditions and are targeted by several enterotoxins and pathogenic bacteria (17). Both DRA and NHE3 interact with PDZ domain proteins of the NHE3 regulatory factor (NHERF) family [NHERF, NHE3 kinase A regulatory protein (E3KARP), PDZ domain protein kidney 1 (PDZK1), and intestine and kidney expressed PDZ protein (IKEPP)] by virtue of their PDZ interaction motifs located at the very COOH-terminus and internally in the COOH-terminal tail, respectively (23).

Surface proteins, including NHE3, undergo basal recycling by coordinated endo- and exocytosis (32). In addition, these mechanisms can be regulated, resulting in increased or decreased surface expression and activity of transport proteins. The epithelial sodium channel ENaC, aquaporin-2, and the glucose transporter GLUT-4 are widely known and well-characterized examples for this type of regulation of transcellular transport (3, 5, 46). The classic route of endocytosis and recycling involves clathrin-dependent or -independent endocytosis from the plasma membrane into early endosomes, subsequent transfer to recycling endosomes, and trafficking back into the plasma membrane. This intracellular vesicular traffic is a tightly organized process that is regulated by RabGTPases as well as by many other factors (13, 32, 44). Rab5 is localized in early endosomes, where it regulates early steps of the endocytic process. Rab11 is associated with recycling endosomes that mediate the trafficking of surface proteins from intracellular compartments back to the plasma membrane. Late endosomes are part of the degradative pathway, and several Rab proteins, including Rab7, are localized to this compartment (34). This characteristic expression allows Rab proteins to be used as marker proteins for these compartments.
Phosphatidylinositol (PI) 3-kinase may be involved in this cycle at the level of the early endosome, where its product PI 3-phosphate has been shown to interact with the downstream target early endosome antigen 1, as well as at various other levels with its other product PI 3,4,5-trisphosphate (6). This lipid might regulate the actin cytoskeleton by interaction with guanine nucleotide exchange factors for GTPases Arf6, Rac1, or Cdc42 (29).

The importance of an intact PDZ interaction in the endocytosis-recycling pathway has been demonstrated for several proteins, such as the thyrotropin receptor, the somatostatin receptor subtype 5 (SSTR5), and CFTR (20, 45, 48). While the molecular details of PDZ interaction-dependent NHE3 recycling are not fully understood, a number of studies indicate involvement of PDZ adaptor proteins in the basal and regulated expression of NHE3 in the plasma membrane. NHE3 is moved from the plasma membrane into endosomes in response to increased intracellular calcium concentrations, which induce membrane localization of protein kinase Ca, which in turn interacts with E3KARP and α-actinin 4, and finally phosphorylates NHE3 (18). Furthermore, treatment with carbacal followed by increased intracellular Ca\(^{2+}\) concentration leads to a PDZK1-dependent reduction of surface expression of DRA accompanied by the dissociation from PDZK1 (24). On the other hand, in PS120 cells, elevated intracellular calcium leads to accumulation of NHE3 in intracellular compartments and regulated recycling remains incompletely understood.

In a previous study, we have shown that the expression of DRA in the plasma membrane and thus its activity under basal conditions partly depends on the activity of PI3-kinase, on intact lipid rafts, and on the interaction of DRA with one or several PDZ adaptor proteins (30). Based on these findings, we concluded that the trafficking from an intracellular compartment into the plasma membrane is affected by these three factors and that this compartment may be part of the endocytosis recycling pathway.

Therefore, the current study addressed the question where along the endocytosis recycling pathway of DRA its PDZ interaction exerts this role. To this end, DRA has the advantage that a mutant that lacks the PDZ interaction motif (DRA-ETKminus) can be expressed and is functionally active (24). Here we show that the PDZ interaction of DRA facilitates its movement from Rab5a-positive early into Rab11a-positive recycling endosomes, thereby keeping it in the recycling pathway and prolonging its half-life compared with the mutant that lacks the PDZ interaction motif (DRA-ETKminus). Furthermore, we show that DRA activity and plasma membrane expression are under control of Rab11a, underscoring the functional importance of this compartment.

**METHODS**

**Materials.** Sulfo-NHS-SS-biotin and NeutrAvidin agarose were from Pierce (Rockford, IL). Nigericin, 4',6-diamidino-2-phenylindole (DAPI), cycloheximide (CHX), hemagglutinin (HA)-agarose, puromycin, and the monoclonal antibody against Rab7 were from Sigma-Aldrich (Steinheim, Germany). 2',7'-Bis(carboxyethyl)-5,6-carboxyfluorescein-acetoxyethyl ester was from Invitrogen (Paisley, Scotland). Complete protease inhibitor mixture was from Roche Applied Science (Mannheim, Germany). The monoclonal antibody against enhanced green fluorescent protein (EGFP) was from Clontech (Mountain View, CA). The monoclonal antibodies against Rab5 and Rab11 were from BD Transduction. Anti-mouse secondary antibody was from Millipore (Billerica, MA). FluorSave Reagent was from Merck (Darmstadt, Germany).

**Cell lines.** HEK293 cells stably transfected with EGFP-tagged DRA (HEK/EGFP-DRA) or EGFP-tagged DRA-ETKminus (HEK/EGFP-DRA-ETKminus), a DRA construct that lacks the COOH-terminal PDZ interaction motif (glutamate-threonine-lysine-phenylalanine, ETKF), were used as described previously (21).

**Expression constructs.** DsRed2-HA-Rab5a (plasmid 13050), DsRed-HA-Rab7a (plasmid 12661), DsRed-HA-Rab11a (plasmid 12679), and DsRed-HA-Rab11aS25N (plasmid 12680) were obtained from Addgene. pcDNA3.1[puro]/mCherry was generated as the common vector backbone for the various Rab constructs as follows: a puromycin cassette was amplified from pMSCVpuro (Clontech) using forward primer CAA GGA TAT CAT GAC GCA GTA CAA GCC CAC and reverse primer TCG GAT ATC GAT CGT CAG GCA CCG GCC GTG, each incorporating an EcoRV restriction site. As for all PCR amplifications, the PCR was performed using pfu (Stratagene), and the product was cloned into pCRII-TOPO (Invitrogen), sequenced in both directions, and then used for further cloning. The Hygromycin cassette of pcDNA3.1+[hygro] (Invitrogen) was replaced with the Puromycin cassette after cutting with PvuII and dephosphorylation with Shrimp Alkaline Phosphatase (Fermentas). The orientation of the Puromycin cassette was confirmed by restriction digestion with PvuI.

An mCherry cassette was amplified from pmCherry (Clontech) using forward primer CAC CAC CGG TCG CCA CCA TGG TGG AC and reverse primer AGA GTC GCC GTC TCT GAG ACT TGT A, incorporating an AgeI and a XhoI site. These sites were used to replace the EGFP cassette from pEGFP-C1 (Clontech), resulting in a customized pmCherry. The mCherry cassette was further moved into pcDNA3.1[puro] using NheI and BamHI, resulting in pcDNA3.1[puro]/mCherry. HA-Rab5a, HA-Rab7a, HA-Rab11a, and HA-Rab11aS25N were then subcloned into pcDNA3.1[puro]/mCherry.

HA-Rab4a and HA-Rab4aS22N were amplified from pcDNA3/Rab4a and pcDNA3/Rab4aS22N (a kind gift from Peter van der Sluijs, Department of Cell Biology, University Medical Center Utrecht, Utrecht, The Netherlands) using forward primer GCT GTA CAA GTA AGC TTC CAT GATA CCC ATGA TGT TCC GGA TTA CGC TTC GCA GAC GGC CAT GTC GTA AAC C and reverse primer CGC TTC GCA GAC GGC CAT GTC GTA AAC C, incorporating a HindIII site plus the sequence of the HA-tag and a BamHI restriction site. These restriction sites were used to subclone HA-Rab4a and HA-Rab4aS22N into pcDNA3.1[puro]/mCherry.

**Cell transfection.** For stable transfection, subconfluent (~70% confluence, 25-cm\(^2\) tissue culture flask) HEK/EGFP-DRA or HEK/EGFP-DRA-ETKminus cells were transfected using 5 μg expression construct of the Rab proteins and 10 μl Lipofectamine 2000 according to the recommendations of the manufacturer (Invitrogen). For transient transfection, subconfluent (~70% confluence) HEK/EGFP-DRA cells grown in six-well plates were transfected using 2.5 μg expression construct of the Rab proteins and 5 μl Lipofectamine 2000/well. After transfection (24 h), the cells were used for functional studies or selected with Puromycin.

**CHX treatment.** For inhibition of protein biosynthesis, confluent monolayers of HEK/EGFP-DRA or HEK/EGFP-DRA-ETKminus...
cells were treated with 25 μg/ml CHX in DMEM plus 0.2% BSA at 37°C for 1–9 h.

SDS-PAGE, western blotting, and immunodetection. For Western blot analysis, proteins were separated by 8.5% SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Billerica, MA). Membranes were blocked with 5% milk in 20 mM Tris-base, 7.5 mM pH, 150 mM NaCl, and 0.1% Tween 20 and subsequently incubated with anti-EGFP monoclonal antibody (1:500), anti-Rab5 monoclonal antibody (1:500), anti-Rab7 monoclonal antibody (1:2,000), or anti-Rab11 monoclonal antibody (1:1,000) followed by incubation with fluorescently conjugated goat anti-mouse IgG (1:10,000) in the dark. The immunoblots were detected by the Odyssey infrared imaging system (LI-COR, Bad Homburg, Germany).

DRA activity. DRA activity was assayed as changes in the intracellular pH (pH) upon removal and readdition of extracellular chloride as previously described (21, 24). The pH, traces were analyzed as described previously (25). This nonlinear curve-fitting algorithm allows the calculation of the initial slope after the change of the buffer solutions as well as the estimation of the maximal pH change from baseline. Thus two parameters are calculated for each trace, which describe the dynamics of the intracellular alkalinization after removal of extracellular chloride and which can be compared between groups using t-test statistics (25).

Fluorescence microscopy. For fluorescence microscopy, cells were grown on glass cover slips. After several washings with PBS, the cells were fixed for 15 min with 4% formaldehyde in PBS, the nucleus was labeled for 10 min with DAPI (0.3 μg/ml), and the cover slip was mounted with FluorSave Reagent. Cover slips were examined using an oil immersion objective (Plan-Apochromat; ×63 magnification, numerical aperture 1.40; Zeiss, Oberkochen, Germany). Structured illumination was used as an alternative to confocal laser scanning microscopy (ApoTome; Zeiss) to obtain optical sections for the colocalization of EGFP-DRA or EGFP-DRA-ETKFminus (emission 610 nm) (33). The resulting pixel size was 100 × 100 nm, which correlates well with the theoretical maximal resolution of EGFP and mCherry (90 and 109 nm, respectively) as well as the size of endosomal vesicles (100–200 nm).

Colocalization was determined by calculating Pearson’s correlation coefficient (PCC) for the AxioVision software (release 4.8.2; Zeiss) (40). This value can vary between 1 (perfect colocalization), 0 (random association), and −1 (specific dissociation).

Immunoisolation of different endosomal membrane vesicles. Preparation of early, late, and recycling endosomes was performed using a modification of the protocols first described by Gorvel et al. (12) and by Trischler et al. (47). Briefly, cells grown on 60-cm² tissue culture dishes (8 dishes corresponding to about 80 mg of protein) were lysed with 750 μl/ml CHX in DMEM plus 0.2% BSA at 37°C for 1–9 h. The cushions of the cells were treated with 25 μg/ml CHX in DMEM plus 0.2% BSA at 37°C for 1–9 h.

For Western studies. Care was taken to select clonal cell lines of EGFP-HA-Rab5, -Rab7, -Rab11, and endogenously expressed Rab proteins. Bands were quantified using ImageMaster 1D, version 3.0 (Amersham Biosciences), with manual background subtraction. The relative amount of EGFP-DRA and EGFP-DRA-ETKFminus as well as of mCherry-HA-Rab5, -Rab7, and -Rab11 and their endogenously expressed counterparts bound to the beads (i.e., present in mCherry-HA-Rab5, -Rab7, and -Rab11) were found not to be suitable for the immunoisolation of endosomal fractions (data not shown). Therefore, we cloned mCherry-HA-Rab5, -Rab7, and -Rab11 into HEK cells already expressing EGFP-DRA or EGFP-DRA-ETKFminus. RabGTPases are associated with membranes by geranylgeranyl moieties at the COOH-terminus, whereas the NH2-terminus faces the cytosol. In the fusion proteins, the mCherry-HA-tag is conjugated to the NH2-terminus. Therefore, intact endosomes could be isolated by HA-agarose.

Figure 1 shows expression of the Rab fusion proteins and their native counterparts in clonal cell lines used for further studies. Care was taken to select clonal cell lines of EGFP-HA-Rab5, -Rab7, and -Rab11 into HEK cells already expressing EGFP-DRA or EGFP-DRA-ETKFminus. RabGTPases are associated with membranes by geranylgeranyl moieties at the COOH-terminus, whereas the NH2-terminus faces the cytosol. In the fusion proteins, the mCherry-HA-tag is conjugated to the NH2-terminus. Therefore, intact endosomes could be isolated by HA-agarose.

Subcellular localization of DRA depends on its PDZ interaction motif. Previously, we had found that the trafficking of DRA from an intracellular pool into the plasma membrane required 1) intact lipid rafts, 2) the activity of PI3-kinase, and 3) the presence of the COOH-terminal PDZ interaction motif of DRA, ETKF (30). Thus the data indicated that an interaction takes place, we immunoisolated different endosomal fractions (data not shown). Therefore, intact endosomes could be isolated by HA-agarose.

RESULTS

Subcellular localization of DRA depends on its PDZ interaction motif. Previously, we had found that the trafficking of DRA from an intracellular pool into the plasma membrane required 1) intact lipid rafts, 2) the activity of PI3-kinase, and 3) the presence of the COOH-terminal PDZ interaction motif of DRA, ETKF (30). Thus the data indicated that an interaction takes place, we immunoisolated different endosomal fractions (data not shown). Therefore, intact endosomes could be isolated by HA-agarose.

Figure 1 shows expression of the Rab fusion proteins and their native counterparts in clonal cell lines used for further studies. Care was taken to select clonal cell lines of EGFP-
Furthermore, these data indicate that the endocytic pathway of EGFP-DRA from early endosomes to recycling endosomes, characterized by the presence of Rab11a, wild-type DRA dominated over DRA-ETKFminus (31 \( \pm \) 0.5% of EGFP-DRA vs. 27% of HEK/EGFP-DRA-ETKFminus). In contrast, EGFP-DRA was significantly more colocalized with Rab5a (PCC 0.6 \( \pm \) 0.05) and Rab7a (PCC 0.7 \( \pm \) 0.003) than with Rab11a (PCC 0.5 \( \pm \) 0.1). Thus the reason for this variation in the colocalization with Rab5a and Rab11a coisolated with Rab5a was much higher than that isolated with Rab7a or Rab11a. This was due to the larger variations in the amount of mCherry-HA-Rab5a isolated or Rab5a coisolated. Therefore, it is of note that the variation in the amount of EGFP-DRA and EGFP-DRA-ETKFminus coisolated with Rab5a was much higher than that isolated with Rab7a or Rab11a. This was due to the larger variations in the amount of mCherry-HA-Rab5a isolated or Rab5a coisolated. Thus the reason for this variation in the colocalization with Rab5a and Rab11a coisolated with Rab5a-positive vesicles is currently unknown.

To assure that intact endosomes were isolated, a control experiment was performed using HEK/EGFP-DRA/mCherry-HA-Rab11a cells. The interphase enriched in endosomes was divided in two, and one part was incubated with 1% Triton X-100. The immunoisolation was then performed as usual. As expected, only mCherry-HA-Rab11a was recovered from the beads after the incubation with Triton X-100, but endogenous Rab11a and EGFP-DRA were not coisolated anymore because the vesicles had been disrupted by the detergent (Fig. 3).

Colocalization of DRA and DRA-ETKFminus with Rab5a, Rab7a, and Rab11a. To ascertain the association of EGFP-DRA and EGFP-DRA-ETKFminus with specific vesicles of the endocytic recycling pathway, we investigated their colocalization with mCherry-HA-Rab5a, -7a, and -11a by fluorescence microscopy. PCC was used as described in METHODS to quantify the colocalization. Figure 4 shows that both EGFP-DRA and EGFP-DRA-ETKFminus were partially colocalized with mCherry-HA-Rab5a (EGFP-DRA: PCC 0.6 \( \pm \) 0.2; EGFP-DRA-ETKFminus: PCC 0.6 \( \pm \) 0.3; P > 0.05). Colocalization of the DRA construct lacking the PDZ interaction motif, EGFP-DRA-ETKFminus, with the late endosome marker mCherry-HA-Rab7a was markedly increased compared with EGFP-DRA (PCC 0.5 \( \pm \) 0.1 and 0.1 \( \pm \) 0.1; P < 0.05). In contrast, EGFP-DRA was significantly more colocalized with DRA to early endosomes is independent of the interaction of DRA with PDZ adaptor proteins. Finally, it cannot be deduced from our data whether the movement of DRA from recycling endosomes back to the plasma membrane depends on the interaction with one or several PDZ adaptor proteins.
mCherry-HA-Rab11a than EGFP-DRA-ETKFminus (EGFP-DRA: PCC 0.6 ± 0.2; EGFP-DRA-ETKFminus: PCC 0.2 ± 0.1; P < 0.05). The predominant colocalization of wild-type DRA with recycling endosomes as opposed to that of DRA-ETKFminus with late endosomes corresponds to the coisolation data and further supports the interpretation that the PDZ interaction motif of DRA is important for its movement into Rab11a-positive recycling endosomes.

The PDZ interaction motif of DRA affects its half-life. Based on the finding that wild-type DRA is associated with recycling endosomes destined to the plasma membrane but DRA-ETKFminus is largely associated with late endosomes destined to the lysosome, we next asked whether this translates into different half-lives of DRA vs. DRA-ETKFminus. HEK/EGFP-DRA and HEK/EGFP-DRA-ETKFminus cells were incubated with CHX (25 μg/ml) for 1–9 h and lysed. Starting after 3 h, there was significantly less EGFP-DRA-ETKFminus detectable than the wild-type construct EGFP-DRA (Fig. 5), indicating faster degradation of DRA-ETKFminus than DRA. The apparent half-life of EGFP-DRA was about 4 h, whereas that of DRA-ETKFminus was reduced to about 2 h. These data suggest that the PDZ-binding motif keeps DRA in the recycling pathway and thereby protects it from lysosomal degradation.

Rab11a controls the activity and the plasma membrane expression of DRA. Given that EGFP-DRA is associated with Rab11a-positive vesicles, we investigated whether Rab11a affects the transport activity and the cell surface expression of DRA.

For functional studies, HEK/EGFP-DRA cells were additionally transfected with mCherry-HA-Rab11a or with a dominant-negative Rab11a mutant (mCherry-HA-Rab11aS25N). Transient transfection was chosen to use cells with the same basal activity of DRA and thus only to address the effect of Rab11a and Rab11aS25N. DRA activity was assayed and analyzed as changes in the pH₄ upon removal and readdition of extracellular chloride as previously described (21, 24). This approach results in calculated absolute values for the maximal pH₄ change and the initial slope of the pH₄ trace after the change of the buffer solutions (25). These absolute values were then analyzed statistically to assess the effect of Rab11a compared with Rab11aS25N. The maximal pH₄ change in the Rab11a-transfected cells was significantly higher than in the
mCherry-HA-Rab11aS25N-transfected cells (maximal ΔpH, 0.54 ± 0.09 vs. 0.45 ± 0.05, -17%, P < 0.05; and initial slope 0.60 ± 0.20 pH/min vs. 0.56 ± 0.12, -7%, P > 0.05; Fig. 6A). The transfection efficiency of the mCherry-HA-Rab11a constructs was estimated at about 30% by comparing only green fluorescent EGFP-DRA-expressing cells with also red fluorescent mCherry-HA-Rab11a (or mCherry-HA-Rab11aS25N)-expressing cells. Thus the “real effect” of dominant-negative Rab11a may be as much as three times larger than actually seen in the experiment.

In addition to the Rab11a-dependent slow recycling, a fast recycling route back to the plasma membrane directly from early endosomes exists (13). Rab4a plays a critical role in this fast recycling (34). To examine whether DRA is recycled back to plasma membrane by Rab4a-containing endosomes, we analyzed whether Rab4a affects the transport activity of DRA as well. HEK/EGFP-DRA cells were transiently transfected with mCherry-HA-Rab4a or with a dominant-negative Rab4a mutant (mCherry-HA-Rab4aS22N). The transfection efficiency of the mCherry-HA-Rab4a constructs was comparable to the mCherry-HA-Rab11a constructs (about 30%). The activity of DRA was not inhibited by overexpression of wild-type Rab4a or by expression of dominant-negative Rab4aS22N (maximal ΔpH, 0.59 ± 0.06 vs. 0.57 ± 0.07, -3%, P > 0.05; and initial slope 0.50 ± 0.07 pH/min vs. 0.46 ± 0.07, -8%, P > 0.05; Fig. 6B). Thus Rab4a-dependent rapid recycling does not play a role for DRA trafficking.

To investigate whether the Rab11aS25N-induced decreased activity of DRA is associated with a reduced plasma membrane expression, we determined the fraction of DRA at the cell surface by biotinylation of control and mCherry-HA-Rab11a- or mCherry-HA-Rab11aS25N-transfected cells; control cells were transfected with mCherry. Again, transient transfection was chosen to avoid the effects of clonal selection (Fig. 7). Figure 7C shows that, in control cells, 17.6 ± 0.8% of EGFP-DRA was detected at the cell surface, whereas 26 ± 7% (P < 0.05) was at the surface of cells transfected with mCherry-HA-Rab11a, corresponding to a 36% increase. In contrast, transfection of the cells with mCherry-HA-Rab11aS25N results in a 30% reduction of the cell surface expression (control 17.6 ± 0.8% vs. Rab11aS25N transfected cells 12.4 ± 2.3%, P < 0.05).

Together these data indicate that the activity and plasma membrane expression of DRA depends on the small GTPase Rab11a.

**DISCUSSION**

The current study addressed the question which step of the endocytosis-recycling pathway of DRA is dependent on its PDZ interaction. Previously, we have shown that the expression of DRA in the plasma membrane requires the activity of PI3-kinase, intact lipid rafts, and an intact PDZ interaction motif, i.e., the interaction with one or several PDZ adaptor proteins. A number of studies have demonstrated the importance of a PDZ interaction for the expression of various transport proteins in the plasma membrane, mostly suggesting that such an interaction is important for keeping these proteins in this location (9, 48, 51). However, the molecular details of the intracellular vesicular trafficking of DRA and in particular the importance of its PDZ interaction for such trafficking have not been studied. We addressed this question in transfected HEK cells expressing EGFP-tagged DRA constructs because this system allows us to measure DRA activity at low background of Cl−/HCO3− exchange and because we can specifically compare wild-type DRA with DRA-ETKFMinus, a mutant that lacks the PDZ-binding motif (21, 24). The expression of wild-type DRA and DRA-ETKFMinus was compared in early endosomes, recycling endosomes, and late endosomes. DRA and DRA-ETKFMinus were equally present in early endosomes. In recycling endosomes, on the other hand, wild-type DRA was preferentially present, indicating that the PDZ interaction of DRA affects its movement into Rab11a-positive recycling endosomes. If, on the other hand, the PDZ interaction motif was missing, DRA-ETKFMinus preferentially appeared in late endosomes, which are probably further processed to lysosomes. The functional consequence of this PDZ motif-dependent targeting of DRA to recycling as opposed to late endosomes was the longer half-life of wild-type DRA compared with DRA-ETKFMinus. Together these data suggest that, under physiological conditions, the PDZ interaction keeps DRA in the recycling pathway, whereas, in the absence of the PDZ interaction, DRA is preferentially targeted to degradation. Newly synthesized proteins traffic from the endoplasmic reticulum and the Golgi to Rab11-containing vesicles (13). In our experiments, EGFP-DRA predominated in Rab11a-containing vesicles, and EGFP-DRA-ETKFMinus reciprocally predominated in Rab7a-containing vesicles, suggesting that the PDZ interaction of DRA mainly affected the handling of DRA in the endocytosis recycling pathway. Nevertheless, an additional role in the targeting from the endoplasmic reticulum/Golgi to Rab11a-containing vesicles cannot be excluded. In addition, our findings suggest that endocytosis of DRA from the plasma membrane is independent of...
its PDZ-binding motif. It is currently unknown whether this occurs by a clathrin-dependent or -independent mechanism.

These results are consistent with studies addressing other membrane proteins. A mutant of the thyrotropin receptor that is lacking the PDZ-binding motif is localized in late endosomes, whereas the wild-type receptor is associated with recycling endosomes (20). Mutation of the PDZ-binding motif of the platelet P2Y₁₂ purinoreceptor attenuates receptor internalization.

Fig. 4. Wild-type DRA is preferentially localized in Rab11a-positive recycling endosomes. The colocalization of EGFP-DRA and EGFP-DRA-ETKFminus with mCherry-HA-Rab5a, -7a, or -11a in stably transfected HEK cells was examined with an ApoTome (Zeiss, Oberkochen, Germany) and quantified using Pearson’s correlation coefficient. A: wild-type DRA and Rab5a as well as Rab11a are partially colocalized, whereas wild-type DRA is not colocalized with Rab7a. The DRA mutant (DRA-ETKFminus) is also partially colocalized with Rab5a, and its colocalization with Rab7a dominates over wild-type DRA. DRA-ETKFminus barely colocalizes with Rab11a. Representative micrographs. B: summary of 4 independent fluorescence microscopic analyses from 50 to 100 cells each (1 high-power field).
tion and receptor recycling back to the membrane, thereby blocking receptor resensitization (36). NHERF binds with its first PDZ domain to the cytoplasmic tail of the $\beta_2$-adrenoreceptor and with its ERM-binding domain to the actin cytoskeleton. Disruption of the PDZ interaction or depolymerization of the actin cytoskeleton itself causes missorting of the receptor (4). The internalization of the SSTR5 and its targeting to Golgi-associated compartments are PDZ independent, but the recycling of the receptor to the plasma membrane depends on PDZ adaptor proteins PDZK1 and PDZ domain protein interacting specifically with TC10 (PIST, also known as GOPC or CAL) (48). Finally, Swiatecka-Urban et al. have shown that CFTR trafficking to the cell surface but not retrieval from the plasma membrane requires the PDZ interaction (45).

We have used HEK cells as a model system, which was transfected with either EGFP-DRA or EGFP-DRA-ETKFminus as well as with the fluorescent fusion constructs of the Rab proteins. These cells have successfully been used before to study the function of Rab proteins (8, 10). Transfection of tagged Rab proteins was found necessary because the available antibodies to native Rab proteins proved insufficient for the immunoisolation, as noted by others (7). Notably, the overexpression of tagged Rab proteins has been shown not to alter their function or their distribution (43). Most importantly, in our experiments, EGFP-DRA and EGFP-DRA-ETKFminus behaved differently with regard to their association with the different Rab proteins, indicating that the effects are specific.

Fig. 5. Disruption of the PDZ interaction of DRA shortens its half-life. HEK/EGFP-DRA and HEK/EGFP-DRA-ETKFminus cells were incubated with cycloheximide (CHX; 25 $\mu$g/ml; 0, 1, 2, 3, 5, 6, 7, 9 h; 37°C) and lysed. The lysates were analyzed using SDS-PAGE, Western blotting, and immunodetection using anti-EGFP antibody. The expression of EGFP-DRA and EGFP-DRA-ETKFminus was calculated as the percentage of total EGFP-DRA or EGFP-DRA-ETKFminus at 0 h (=100%). A and B: data of 3 experiments. C: $n=3$.

Fig. 6. DRA activity is inhibited by dominant-negative Rab11a (Rab11aS25N) but not by dominant-negative Rab4a (Rab4aS22N). HEK/EGFP-DRA cells were transiently transfected with mCherry-HA-Rab11a or mCherry-HA-Rab11aS25N as well as with mCherry-HA-Rab4a or mCherry-HA-Rab4aS22N before the functional experiment. A: dominant-negative Rab11aS25N led to a reduction of Cl⁻/HCO₃⁻ exchange mediated by DRA (maximal $\Delta$pH $0.54 \pm 0.09$ vs. $0.45 \pm 0.05$, $-17\%$, $P < 0.05$; and initial slope $0.60 \pm 0.20$ pH/min vs. $0.56 \pm 0.12$, $-7\%$, $P > 0.05$). B: dominant-negative Rab4aS22N did not reduce DRA-mediated Cl⁻/HCO₃⁻ exchange (maximal $\Delta$pH $0.59 \pm 0.06$ vs. $0.57 \pm 0.07$, $-3\%$, $P > 0.05$; and initial slope $0.50 \pm 0.07$ pH/min vs. $0.46 \pm 0.07$, $-8\%$, $P > 0.05$).
This study was done using HEK cells. Future studies will try to address the DRA recycling in a differentiated intestinal cell line such as Caco-2 cells.

NHE3 may serve as a paradigm for DRA because it colocalizes with and functionally interacts with it. NHE3 colocalizes with PDZK1 in the plasma membrane. After treatment with carbachol, NHE3 is moved into intracellular compartments, whereas PDZK1 remains in the plasma membrane (51). On the other hand, NHE3 colocalizes with IKEPP in Rab11-positive vesicles. After increasing intracellular calcium with A-23187, NHE3 moves from this compartment to the plasma membrane. The current results indicate that NHE3 is primarily present in Rab11-positive vesicles and that IKEPP remains colocalized with Rab11 (50).

Another study (unrelated to NHE3) has demonstrated PDZK1 to be present in Rab11a-containing vesicles (8, 10). The importance of E3KARP for calcium-mediated inhibition of NHE3 has been demonstrated in the plasma membrane in response to a calcium signal. Sorting nexin 27 (SNX27) has a single PDZ domain that has specific similarity with the first PDZ domain of NHERF and the second PDZ domain of E3KARP (26). We have previously shown that DRA specifically interacts with the second PDZ domain of E3KARP (22). SNX27 was initially thought to mediate intracellular traffic to the late endosome and thus to degradation (15, 31), but more recent data suggest that it rather mediates transport from Rab5- to Rab11-positive vesicles, while it remains in the Rab5-positive compartment (26). Taken together, a scenario emerges where PDZ adaptor proteins may specifically be present along several compartments of the endocytosis-recycling pathway. It will be challenging to characterize how DRA (and other transport proteins) are handled from one PDZ adaptor protein in one intracellular compartment to another PDZ adaptor protein in another intracellular compartment.

We have demonstrated the functional importance of the Rab11a-positive recycling compartment by the finding that overexpression or inhibition of Rab11a resulted in an increased or decreased activity and surface expression of DRA, respectively. Furthermore, the alternative Rab4a-mediated, so-called rapid recycling pathway to the plasma membrane was excluded to play a role for DRA recycling. DRA-ETKFminus is present and active in the plasma membrane (24) despite the fact that little DRA-ETKFminus (having a shorter half-life) is present in Rab11-containing endosomes. Thus, alternative or additional routes to the plasma membrane or different membrane retention times may exist at least in transfected cells. Interestingly, the amount of CFTR present in the plasma membrane of transfected cells is also not affected by the absence of its COOH-terminal PDZ interaction motif (2, 37).

Our data and our interpretation of the role of Rab11 in the process of recycling are in good agreement with previous studies demonstrating a role for Rab11 in the regulation of transport proteins. Overexpression of Rab11a with ENaC results in an increased channel activity. Brefeldin A, an inhibitor of intracellular protein translocation, blocked the stimulatory action of Rab11a on ENaC activity. Thus the trafficking of ENaC channels from an intracellular pool occurs in a Rab11-dependent manner (16). Silvis et al. demonstrated that CFTR is localized in Rab11a- as well as Rab11b-positive recycling endosomes. Rab11a is essential for the maintenance of apical membrane CFTR expression in the absence of agonist. Expression of dominant-negative Rab11b, but not dominant-negative Rab11a, results in an inhibition of forskolin-stimulated chloride secretion (41). The present studies were done under basal conditions, i.e., in the absence of extracellular stimuli. Thus Rab11b may be involved in regulated recycling of DRA and will be addressed in future studies.

The PI3-kinase-dependent exocytosis of DRA depends on intact lipid rafts (30). Interestingly, recycling endosomes are enriched in the raft lipids sphingomyelin and cholesterol as well as in the raft-associated proteins caveolin-1 and flotillin-1 (11). Disruption of raft integrity affects the turnover of the potassium channel Kv1.5, an effect that is prevented by transfection with dominant-negative Rab11 (1). It is therefore attractive to speculate that the selective incorporation of raft components from recycling endosomes into the plasma membrane contributes to DRA sorting and trafficking. PI3-kinase and several of its lipid products are also part of the intracellular vesicle.
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trafficking and its regulation (29). Inhibition of PI3-kinase prevents the transfer of recycling endosomes to the plasma membrane. Furthermore, a constitutive active PI3-kinase stimulates the endosomal fusion of vesicles, whereas a mutant lacking the kinase domains prevents the fusion (28). The transfer of aquaporin-2 from early endosomes to recycling endosomes depends on PI3-kinase and is sensitive to inhibitors of PI3-kinase (46). Therefore, it is apparent that additional studies are required to further characterize the interplay between PI3-kinase, lipid rafts, PDZ adaptor proteins, and the intracellular traffic of DRA.

Taken together, our data show that the PDZ interaction of DRA is necessary for its movement from Rab5a-positive early into Rab11a-positive recycling endosomes. The latter are an important compartment for DRA function and plasma membrane expression. Whereby, the PDZ interaction keeps DRA in the constitutive endocytosis-recycling pathway and protects it from early degradation.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


