System y⁺ localizes to different membrane subdomains in the basolateral plasma membrane of epithelial cells

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System y⁺ localizes to different membrane subdomains in the basolateral plasma membrane of epithelial cells. Am J Physiol Cell Physiol 284: C1784–C1794, 2003. First published August 22, 2002; 10.1152/ajpcell.00061.2002.—We report here that the system y⁺ cationic amino acid transporter ATRC1 localized to clusters within the basolateral membrane of polarized Madin-Darby canine kidney and human embryonic kidney (HEK) cells, suggesting that the transporters are restricted to discrete membrane microdomains in epithelial cells. Based on solubility in nonionic detergents, two populations of ATRC1 molecules existed: approximately half of the total ATRC1 in HEK cells associated with the actin membrane cytoskeleton, whereas another one-fourth resided in detergent-resistant membranes (DRM). In agreement with these findings, cytochalasin D reduced the amount of ATRC1 associated with the actin membrane cytoskeleton. Although some ATRC1 clusters in HEK cells colocalized with caveolin, the majority of ATRC1 did not colocalize with this marker protein for a type of DRM called caveolae. This distribution of ATRC1 is somewhat different from that reported for pulmonary artery endothelial cells in which transporters cluster predominantly in caveolae, suggesting that differences in the proportion of ATRC1 in specific membrane microdomains correlate with differences in the physiological role of the transporter in polarized kidney epithelial vs. vascular endothelial cells.

System y⁺; membrane microdomains; rafts; F-actin membrane cytoskeleton; retrovirus receptor

ATRC1 (amino acid transporter cationic-1) is a high-affinity, low-capacity transporter of cationic amino acids. ATRC1 is one of the three cationic amino acid transporters that are called system y⁺ (9, 22). This pH- and Na⁺-independent transporter is one of the principal transporters on most cell types except the liver (8, 9, 48). It is an essential member of the multigene solute carrier family 7 of transporters. Homozygous ATRC-1 (also called MCAT-1) knockout mice died shortly after birth (37). Neonates were grossly underweight and had kidney abnormalities. They were also severely anemic, apparently from failure of erythroid progenitor cells to complete maturation, enucleation, and hemoglobinization (37). The findings from the knockout mice indicate that although mammalian cells express other members of the system y⁺ family that transport cationic amino acids, their physiological function is not redundant with that of ATRC1.

ATRC1 is distributed in clusters on the plasma membrane of porcine pulmonary artery endothelial cells (PAEC), human fibroblasts, and rat hepatoma cells, suggesting that it segregates into specialized plasma membrane (PM) domains in these cells (49). Disruption of microtubules with nocodazole dispersed the transporter clusters (49). Intrigued by this observation of Woodard and coworkers, we asked whether the dispersion of system y⁺ upon depolymerization of microtubules in the presence of nocodazole affected the functions of ATRC1 in fibroblast and kidney epithelial cells. Because depolymerization of microtubules can cause rearrangement of the actin network (26), we also determined the effect of cytochalasin D, a specific inhibitor of actin polymerization. The rate of cationic amino acid transport remained unchanged in mouse NIH3T3 fibroblasts and human embryonic kidney (HEK) cells after as long as 3 h of exposure to either cytochalasin D or nocodazole (25).

In vascular endothelial cells, system y⁺ appears to play a unique physiological role in the production of nitric oxide by the endothelial nitric oxide synthetase (eNOS) (3, 10). In PAEC, eNOS localizes to caveolae, a type of detergent-resistant membrane (DRM) microdomain or lipid raft that is rich in signal transduction components. The majority of y⁺ transporter clusters on the PM of PAEC colocalized with caveolin, a marker protein for caveolae (31). Incubation of PAEC-derived plasma membrane lysates with an anti-eNOS antibody resulted in the immunodepletion of ATRC1-mediated cationic amino acid transport (31). The ability of an anti-eNOS antibody to immunodeplete ATRC1 indicates that ATRC1 and eNOS exist in a physical complex (31). In addition, eNOS and ATRC1 have each been shown to associate with caveolin (16, 17, 27), suggesting that eNOS and ATRC1 complexes form through mutual interaction with caveolin in caveolae of PAEC. Colocalization in caveolae and the existence of a physical complex are also consistent with the

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proposed role of ATRC1 as the source of L-arginine substrate for production of nitric oxide by eNOS (3, 10, 31).

System y\textsuperscript{+} appears to play another physiological role in polarized epithelia of the kidney and placenta: the transport of cationic amino acids across the basolateral membranes (4, 15, 42). In the kidney, system y\textsuperscript{+} participates in the regulation of nitrogen levels by moving excess nitrogen in the form of L-arginine through the basolateral membrane for rapid delivery to the liver where it is converted into urea (12). System y\textsuperscript{+} has also been implicated as the principal transporter on the basolateral membrane of the placental epithelia where it contributes to the concentrative transfer of cationic amino acids from maternal to fetal blood (15, 21). The localization of ATRC1 on kidney epithelial cells has not been determined.

In these studies, the distribution of ATRC1 in kidney epithelial cells was determined by using confocal microscopy, and the nature of the membrane microdomains observed was characterized by biochemical analysis of the detergent solubility of the transporters. ATRC1-green fluorescent protein (GFP) fusion protein localized to the basolateral PM of Madin-Darby canine kidney (MDCK) and HEK kidney epithelial cells, evenly arranged in distinct patches on the lateral cell surfaces. This finding is in agreement with the previous physiological localization of system y\textsuperscript{+}. In contrast to PAEC, the majority of the ATRC1 in HEK cells did not colocalize with caveolin or exhibit detergent solubility properties characteristic of membrane proteins residing in lipid rafts. Instead, the majority of transporters resided in clusters that colocalized with the filamentous-actin (F-actin) membrane cytoskeleton and exhibited solubility properties characteristic of actin-associated membrane proteins. From these and previously reported results, we propose that the plasma membrane localization of ATRC1 is not essential to its ability to transport amino acids per se but is instead related to accomplishing its cell type-specific physiological and developmental roles.

**MATERIALS AND METHODS**

cDNAs and cell lines. Plasmid pcDNA ATRC1–3HA encoding the ATRC1–3HA fusion protein was constructed by replacing nucleotides 2,064–2,420 of the ATRC1 cDNA, including the translation termination codon (nt 2,064–2,066), with a 121-bp sequence encoding 3 consecutive copies of the 9 amino acid epitope HA from influenza virus hemagglutinin protein (13) using oligonucleotide-directed mutagenesis. Plasmid pcDNA ATRC1-GFP was constructed by replacing the Scal-EcoRI fragment encoding the triple copy of HA epitope in pcDNA ATRC1–3HA with a Scal-EcoRI fragment encoding the GFP from *Aequoria victoria* (7). The vector for both plasmids was pcDNA3 (Invitrogen). HEK or MDCK cells were transfected by using the calcium phosphate technique (40) with plasmid pcDNA ATRC1–GFP or pcDNA ATRC1–3HA and then grown in medium containing 1 mg/ml G418 to select for the presence of the G418-resistance gene on the plasmid. Selection was maintained for 5 wk before analyses were performed. The original population of ATRC1-GFP cells was selected for green fluorescing cells by fluorescence-acti-

vated cell sorter (FACS). FACS enrichment of the ATRC1–3HA line was desirable as well, but because the COOH terminus of ATRC1 is cytoplasmic, the HA epitope tag was not accessible for use in live cell sorting.

**Fluorescence microscopy.** HEK and MDCK cell populations stably expressing the ATRC1-GFP fusion protein were grown on 22-mm glass coverslips for 48 h. For polarization of MDCK, cells were grown on Transwell (Costar) filters for 7 days. Unextracted cells were fixed at room temperature with 10% formalin for 25 min. For detection of caveolin, formalin-fixed cells were permeabilized by exposure to 0.2% Triton X-100 for 10 min before incubation with anti-caveolin-1 mouse monoclonal antibody clone 2297 (1:250 dilution). Live cells were extracted with detergents by washing three times with ice-cold 1× PEM (10 mM PIPES, pH 6.8, 5 mM EGTA, and 2 mM MgCl\(_2\)) extracting with 0.1, 0.5, or 1.0% Triton X-100 or 60 mM octyl glucopyranoside (OGP, Calbiochem) for 5 min at 4°C as described by McNeill et al. (32), and then fixation at room temperature with 10% formalin for 25 min. Cells exposed to 1 μg/ml cytochalasin D for 25 min before extraction were processed in the same manner. Hybirdoma cells that might have been detached during the drying stage were imaged on a Zeiss LSM510 laser scanning confocal microscope.

**Immunoblot analysis.** HEK cells expressing ATRC1–3HA were grown in 100-mm tissue culture dishes and prepared as described by Scannevin et al. (41). Briefly, cells were washed three times with 1× PBS. Live cells were then extracted with 400 μl of 0.1, 0.5, or 1.0% Triton X-100, 60 mM OGP in extraction buffer [10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl\(_2\), and mammalian protease inhibitor cocktail (Sigma)] for 10 min on ice, or 60 mM OGP in extraction buffer for 6 min on ice. The extraction buffer was collected and centrifuged at 16,000 g for 10 min at 4°C. The supernatant (soluble fraction) was then removed to a fresh tube. The cells were scraped from the extracted plate and added to the pellet. This pool of the insoluble fraction was resuspended in 400 μl of extraction buffer containing the corresponding concentration of Triton X-100 or OGP. Both the soluble and insoluble fractions were adjusted to 0.5% deoxycholate and 1% NP-40 and then mixed with an equal volume of UTS buffer (2× Laemmlı buffer supplemented with 8 M urea) and incubated for 30 min at 37°C to completely solubilize the ATRC1 transporters as previously described by Davey et al. (11).

Live cells expressed to cytochalasin D before extraction were submitted to an additional step. Cytochalasin D or the solvent dimethyl sulfoxide (DMSO) alone was added to the cell culture medium to a final concentration of 1 μg/ml cytochalasin D and 0.1% DMSO or 0.1% DMSO alone at 37°C. At 45 min after addition of the drug or solvent, the culture medium was removed and centrifuged at 1,000 g for 2 min to pellet cells that might have detached during the drug exposure. These cell pellets were resuspended in the extraction buffer, which was then immediately applied to the original plate of cells, and the extraction procedure and soluble and insoluble fraction collections were performed as described above. In experiments where the effects of accessibility of the extrac-
tion buffer on solubilization of ATRC1 were tested, cells were detached from the plate by using phosphate-buffered saline without calcium (PBS−) after exposure to cytochalasin D or DMSO. The cells were then collected by centrifugation, resuspended in extraction buffer, extracted, and then separated into soluble and insoluble fractions. Both fractions were subjected to SDS-PAGE and immunoblotted by using either anti-HA epitope mouse monoclonal antibodies 12CA5 or HA (1:500 dilution; Berkeley Antibodies) and goat antimegu IgG conjugated to horseradish peroxidase. The band intensities in each fraction were quantified from digital scans made on a Hewlett-Packard Scanjet 4C scanner using ImageQuant software.

RESULTS

ATRC1 clustered on the lateral surface of epithelial cells. We constructed a cDNA encoding a mouse ATRC1 fusion protein in which the COOH-terminal residue lysine 622 was fused to the first amino acid of GFP (ATRC1-GFP). Polyclonal populations of HEK and MDCK cells stably expressing ATRC1-GFP were established. The ATRC1-GFP fusion protein exhibited amino acid transport comparable to wild-type ATRC1 (24). In addition to being amino acid transporters, the mouse and rat homologs of ATRC1 are also the host-cell receptor for a genus of retroviruses called the ecotropic murine leukemia viruses (1, 22). The ATRC1-GFP fusion protein also retained virus receptor properties, e.g., virus binding and infection (data not shown).

Laser scanning confocal microscopy of the ATRC1-GFP-expressing cell lines was used to determine the membrane localization of ATRC1. ATRC1-GFP localized predominantly to the basolateral membrane in the MDCK-derived population (Fig. 1A, left and bottom). Three-dimensional reconstruction of serial confocal images revealed a remarkably regular pattern of distribution in these cells; the majority of transporters arranged in evenly distributed, distinct patches or clusters on the lateral cell surfaces up to a region near the top of the cell where the interface between the apical and lateral membranes would be expected (Fig. 1A, right). In the HEK cells expressing ATRC1-GFP, the majority of mouse transporters also localized predominantly to small clusters in a basolateral-like re-

Fig. 1. Confocal microscopy reveals the basolateral localization of ATRC1, a system y+ cationic amino acid transporter. A 3-dimensional reconstruction of serial confocal images of cells expressing the green fluorescent ATRC1-GFP fusion protein revealed a remarkably regular pattern of distribution in Madin-Darby canine kidney (MDCK) cells: transporters arranged in evenly distributed, distinct patches on the lateral cell surfaces. A: ATRC1-green fluorescent protein (GFP) in MDCK cells, a well-characterized polarized epithelial cell line. Left: XY section through the approximate midpoint of the cells. Right: a 3-dimensional reconstruction of serial confocal XY sections of the cells shown at left. Bottom: the XZ plane of view through cells shown in the XY plane at left. B: ATRC1-GFP fusion protein in human embryonic kidney (HEK) cells. Left: XY confocal section of direct cell fluorescence through the approximate midpoint of the cells. Right: zoom-in view of the cells in the rectangle at left shows the highly clustered appearance of the transporters. Bottom: the XZ plane of view through cells shown in the XY plane at left, revealing the lateral-like localization of ATRC1-GFP. Images were captured at ×630 magnification.
region of the plasma membrane (Fig. 1B). HEK are reportedly an epithelial-like cell line derived from human fetal kidney cells (19). Our results suggest that these cells are capable of segregating the plasma membrane in a manner typical of polarized cells. Together, these results indicate that mouse ATRC1 localize to the basolateral membrane of epithelial cells in discrete membrane microdomains.

ATRC1 appears to localize to at least two distinct membrane microdomains based on differences in detergent solubility. There are two well-characterized mechanisms that maintain membrane proteins in membrane microdomains. The first is association with the underlying membrane cytoskeleton, and the second is insertion into specialized membrane microdomains called DRMs, also called lipid rafts. Membrane proteins restricted by one of these mechanisms can be distinguished by distinct differences in their solubility in the nonionic detergents Triton X-100 and OGP (5, 28, 33, 36, 38, 41, 50). For example, the Na\(^{+}\)-K\(^{+}\)-ATPase resists extraction from the membrane at 4°C by Triton X-100 and OGP because it is physically associated with the ankyrin-fodrin-based actin cytoskeleton (28, 36, 38). In contrast, proteins residing in DRMs or rafts are resistant to Triton X-100 but are solubilized by OGP extraction at 4°C (33, 45).

We attempted to determine whether the clustered localization of ATRC1 in MDCK and HEK cells might result from one of these mechanisms. Initially, the solubility of ATRC1 in Triton X-100 was compared with that of the endogenous cellular Na\(^{+}\)-K\(^{+}\)-ATPase by extracting live cells with Triton X-100 on ice immediately before fixation. Soluble molecules were washed away, whereas insoluble molecules remained in the cells. Transporters on HEK and MDCK cells showed three distinct profiles of detergent solubility. The ATRC1-GFP fluorescence in the HEK-derived cell population diminished somewhat, at 0.5–1.0% Triton X-100, but the majority of molecules were as resistant as the Na\(^{+}\)-K\(^{+}\)-ATPase to removal by the detergent (Fig. 2). The majority of the transporters in MDCK cells were also resistant to Triton X-100 extraction at 4°C (Fig. 3, f and g). Exposure of live cells to 60 mM OGP solubilized substantial amounts of ATRC1-GFP in the HEK and MDCK cells 4°C (Fig. 3, c and h). These results suggest that some of the ATRC1 molecules associate with the membrane actin cytoskeleton (Triton X-100 and OGP resistant), whereas others are in DRMs (Triton X-100 resistant and OGP soluble). To control for changes in localization that might result when membranes were solubilized, parallel cultures of ATRC1-GFP expressing HEK and MDCK cells were fixed before detergent extraction. The localization of ATRC1-GFP in cells extracted after fixation was similar to that in cells extracted before fixation (data not shown), indicating that subcellular localization of ATRC1 was apparently not altered in live-extracted cells.

Cytochalasin D altered the Triton X-100 solubility of ATRC1. If the Triton X-100- and OGP-insoluble ATRC1 in HEK and MDCK cells was associated with the underlying membrane actin cytoskeleton, then disruption of F-actin with cytochalasin D should change the detergent solubility properties of ATRC1. We previously showed that exposure of mouse NIH 3T3 and rat XC cells to cytochalasin D for as long as 3 h, followed by withdrawal of drug, did not result in a significant difference in the number of cell divisions occurring within the next 48 h compared with parallel cultures of cells not exposed to the drug (25). In addition to retaining viability, cells repolymerized their actin network within 2 h of drug withdrawal (24). A similar assay was used in these studies to determine that exposure of ATRC1-expressing HEK cells to cytochalasin D for as long as 3 h did not reduce cell viability (data not shown).

**Fig. 2.** ATRC1 resists extraction from the membrane of HEK cells by the nonionic detergent Triton X-100 similar to the resistance of the Na\(^{+}\)-K\(^{+}\)-ATPase. HEK cells expressing ATRC1-GFP were extracted with 0.1% (a), 0.5% (b), or 1.0% (c) Triton X-100 at 4°C before fixation and fluorescence microscopy. The endogenous cellular Na\(^{+}\)-K\(^{+}\)-ATPase was detected by using αs monoclonal antibody and anti-mouse antibody conjugated to Texas red (right). At left, phase-contrast images of the cells shown at middle and right. Middle: the green fluorescence of ATRC1-GFP. Images were captured at ×400 magnification.
Fig. 3. Differences in nonionic detergent solubility of ATRC1 molecules suggest that they are distributed in 2 distinct populations, one of which exhibits the characteristics of proteins in an actin-dependent plasma membrane microdomain [Triton X-100 and octyl glucopyranoside (OGP) insoluble], and the other that exhibits characteristics of detergent-resistant membrane (DRM) resident proteins (Triton X-100 insoluble, OGP soluble). Live ATRC1-GFP HEK, HEK-derived cell population sorted for stable expression of green-fluorescing-ATRC1-GFP by fluorescence-activated cell sorter (FACS) (right), or ATRC1-GFP Madin-Darby canine kidney (MDCK), unsorted population of MDCK-derived cells stably expressing the pcDNA ATRC1-GFP plasmid (left) were extracted with 0.5% Triton X-100 or with 60 mM OGP at 4°C before fixation. For comparison, cells that were not extracted were included. Some cells were exposed to 1 μg/ml cytochalasin D in 0.1% DMSO before extraction. 2-s exposures of green fluorescence are shown for all cells except those in e and j; 4-s exposures were required to visualize the ATRC1-GFP remaining after Triton X-100 extraction of cytochalasin D-treated cells. a and f: unextracted cells. b and g: Triton X-100 extracted cells. c and h: OGP extracted cells. d and i: unextracted cells exposed to cytochalasin D. e and j: Triton X-100 extracted cells exposed to cytochalasin D. k: OGP extracted MDCK cells exposed to cytochalasin D. No data were obtained for OGP extraction of HEK cells exposed to cytochalasin D because these cells detached from glass coverslips after even brief OGP exposure. Images were captured at ×400 magnification. Cyto D, cytochalasin D.
Upon exposure to cytochalasin D for 45 min, HEK cells rounded and arborized, losing their apparent segregation of the PM into basolateral and apical membranes. The majority of ATRC1 localized to dense clusters at regions where cells appeared to contact (Fig. 3d). MDCK cells did not arborize, and substantial amounts of ATRC1-GFP consistently remained distributed along the cell periphery (Fig. 3i). Extraction with Triton X-100 reduced the amounts of transporter remaining such that exposure times had to be doubled from that used to capture the images in all other panels, indicating that exposure times had to be doubled from that used to capture the images in all other panels, including those extracted in the absence of cytochalasin D (Fig. 3, e and j).

We were unable to determine the effect of cytochalasin D on OGP solubility of transporters in the HEK cell populations because these cells lost adhesion to the glass coverslips during OGP extraction. No detectable transporters remained after OGP extraction of cytochalasin D-treated HEK cells (data not shown).

**Half of exogenous ATRC1 in HEK cells resided in the membrane actin cytoskeleton-associated fraction, and one-fourth resided in DMVs.** To estimate the percentage of transporters that were soluble vs. insoluble in each detergent, HEK cells were extracted live on plastic culture dishes with Triton X-100 or OGP. Extracted cells were then lysed and fractionated into soluble and insoluble portions, and the fractions were submitted to immunoblot analysis. Anti-GFP antibody (Clontech) did not recognize the ATRC1-GFP fusion protein when cell lysates were blotted to nitrocellulose membrane (data not shown). Because Masuda et al. (30) and Lu and Silver (27) detected ATRC1-GFP by reacting anti-GFP with lysates transferred to polyvinylidene difluoride (PVDF) membranes, we presume that the epitopes on the fused sequences are inaccessible after transfer onto nitrocellulose. As an alternative, we constructed another mouse ATRC1 fusion protein in which the last residue, lysine 622, was fused to three consecutive copies of a nine amino acid epitope (HA) from influenza virus hemagglutinin protein (ATRC1-HA). The amino acid transport and virus receptor properties of the ATRC1-HA fusion protein were also comparable to wild-type ATRC1 (24). A polyclonal population of HEK cells stably expressing ATRC1-HA was established. ATRC1-HA fusion protein were also comparable to wild-type ATRC1 (24).

**OGP and Triton X-100 solubility results.** Homogenization also solubilized the endogenous Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit (data not shown).

**ATRC1 colocalizes with clusters of residual F-actin in cell treated with cytochalasin D.** Because quantification of OGP-extracted cells suggested that about half the total transporters were associated with the actin insoluble but OGP soluble. The remaining one-fourth of transporters does not appear to associate with either the cytoskeleton or DRM (soluble in Triton X-100 and OGP). Because the ATRC1-HA was overexpressed in the HEK cells, this population might include newly synthesized transporters or transporters recycling in intracellular membranes.

Cytochalasin D exposure decreased the Triton-insoluble population to 52 ± 6\% (\(n = 5\)), an increase in solubility of about 30\% of ATRC1 (Fig. 5). In two replicate experiments, extraction of suspended cells detached after exposure to DMSO alone or without additive did not increase transporter solubility (Fig. 5), indicating that the cytochalasin D-induced increase in solubility was not the result of greater accessibility of detergent to cell membranes when the exposed cells were rounded and partially detached. Surprisingly, exposure to the solvent DMSO decreased the Triton-insoluble transporters to 78 ± 18\% (\(n = 5\)), a lesser extent than cytochalasin D but substantial. These results suggested that about 5–10\% of the 30\% of transporters that became soluble after exposure to cytochalasin D might be due to effects of the solvent, not the cytoskeletal inhibitor. Taken together, these studies indicate that at least 20\% but not more than 50\% of total ATRC1 is associated with the cytoskeleton. The existence of a large population of transporters whose insolubility is dependent on the cytoskeleton is also supported by observations that conditions that destabilize the HEK cell actin network, i.e., loss of cell adherence, resulted in solubilization of the majority of ATRC1 when prolonged or when detached HEK cells were homogenized in the cold (data not shown). The HEK cell cytoskeleton appears to be particularly dependent on adherence because detachment followed by homogenization also solubilized the endogenous Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit (data not shown).

**ATRC1 colocalizes with clusters of residual F-actin in cell treated with cytochalasin D.** Because quantification of OGP-extracted cells suggested that about half the total transporters were associated with the actin insoluble but OGP soluble. The remaining one-fourth of transporters does not appear to associate with either the cytoskeleton or DRM (soluble in Triton X-100 and OGP). Because the ATRC1-HA was overexpressed in the HEK cells, this population might include newly synthesized transporters or transporters recycling in intracellular membranes.
cytoskeleton, the 20% of ATRC1 that became Triton soluble after exposure to cytochalasin D was much lower than expected. We next visualized cells that were exposed to cytochalasin D to determine whether the F-actin was completely depolymerized. Live HEK cells expressing ATRC1-GFP were exposed to cytochalasin D and then extracted with 0.5% Triton X-100. The F-actin in these cells was visualized by using phalloidin and then extracted with 0.5% Triton X-100. The expressing ATRC1-GFP were exposed to cytochalasin D to determine whether the lower than expected. We next visualized cells that were soluble after exposure to cytochalasin D was much cytoskeleton, the 20% of ATRC1 that became Triton resistant transporters in these cells are associated with F-actin. We propose that the ATRC1-GFP clusters colocalizing with clusters of cytochalasin D-resistant F-actin account for the lower than expected amount of ATRC1 solubilized by the drug in Fig. 5 and suggest that 50% of the transporters are associated with the actin membrane cytoskeleton.

After Triton X-100 extraction, few cytochalasin D-exposed cells in almost every field retained transporters that colocalized with residual F-actin, but ones that did not colocalized with the cytoskeletal remnants. Because these cells consistently exhibited the highest levels of transporter expression, we propose that their distribution of transporters is not different from other cells; rather, the minority of ATRC1 residing in DRMs are visualized because their total numbers are increased. However, we cannot rule out the possibility that a subset of HEK cells contain more ATRC1 in DRMs than associated with F-actin.

The majority of ATRC1 in HEK cells did not colocalize with caveolin. Because ATRC1 had been shown to colocalize with caveolin-1 in the caveolae of PAEC (31), to coimmunoprecipitate with caveolin in transfected BHK cells (27), and some of the ATRC1 in HEK cells exhibited the detergent solubility properties characteristic of DRM proteins, we asked whether any of the ATRC1 clusters colocalized with caveolin. Anti-caveolin-1 monoclonal clone 2297 was used to detect caveolin. Because it recognizes an epitope that encompasses a peptide sequence (FEDVIAEP) conserved in caveolin-2 (NP_001224) (18) and caveolin-3 (P56539) (35), the antibody is essentially a pan-caveolin antibody that detects all three proteins. Figure 7 shows laser scanning confocal micrographs of unextracted HEK cells expressing ATRC1-GFP. The limited amount of overlap (yellow fluorescence) between the transporters (green fluorescence) and caveolin (red fluorescence) indicates that only a portion of transporters colocalized with caveolin. In addition, a substantial amount of the anti-caveolin-reactive species was intracellular, consistent with data implicating caveolin-1 in intracellular lipid transport between the endoplasmic reticulum and the plasma membrane (2, 14). This result is in agreement with the estimates that ~25% of the transporters in HEK cells were Triton X-100 insoluble but OGP soluble.

**DISCUSSION**

The demonstration presented here that the majority of ATRC1 localize to the basolateral membrane of MDCK and HEK cells agrees with the previous physiological localization of system y+ transport activity to the basolateral surface of polarized MDCK and human placental epithelia (4, 15, 42). Localization obtained by confocal microscopy indicates that it is predominantly on the lateral membrane. The signals that localize...
ATRC1 to the basolateral membrane in polarized epithelial cells are not known. The first intracellular domain of the transporter predicted by hydropathy plots contains the sequence Tyr-Gly-Glu-Phe that fits the consensus sequence for the tyrosine-based basolateral targeting motif Tyr-X-X-H, where X is any amino acid and H is any residue with a bulky hydrophobic side group, described for MDCK cells (20). In addition, the amino terminus and another intracellular domain of ATRC1 contain dihydrophobic residue motifs (Leu-Leu or Leu-Val), sequences identical to those that signal basolateral localization of the Fc receptor (20, 43). Genetic analysis of these potential targeting sequences should reveal whether they are responsible for the distribution of ATRC1 in polarized kidney epithelial cells.

Strikingly, we observed that ATRC1 was not evenly dispersed on the basolateral membrane but clustered in discrete regions of the lateral cell surface. This membrane distribution contrasts with the localization reported previously by Masuda et al. (30). Because these authors reported studies of ATRC1 in its capacity as the cellular receptor for ecotropic murine retroviruses, they characterized cells under conditions used for a typical virus infection, that is, cells at low density the next day after passage. In this case, they observed ATRC1-GFP distributed over the entire plasma membrane of HEK cells, including along numerous filopodia that extended from the bottom. Substantial amounts of presumably newly synthesized ATRC1-GFP were also seen colocalizing with a Golgi marker (30). We observed a similar distribution in HEK cells at low cell density and immediately after passage (data not shown). However, we found that the distribution of ATRC1-GFP changes as the HEK cells become confluent monolayers. At high densities, we observed segregation of the plasma membrane into apparent apical and basolateral membranes. In these polarized cells,
ATRC1-GFP localized to clusters in the lateral membrane. Thus the differences between ATRC1-GFP localization reported by Masuda et al. (30) and this study apparently arose from the differences in density of cells expressing the fusion protein.

To identify the basis for ATRC1 clustering, its solubility in the detergents Triton X-100 and OGP was determined. Approximately half of the ATRC1 in the HEK cell population were insoluble in Triton X-100 and OGP, a profile consistent with their association with the F-actin network that underlies the PM. One-fourth of the transporters in the HEK population were insoluble in Triton X-100 but soluble in OGP, suggesting that they reside in DRMs such as caveolae. These data are the first evidence that system y⁺ transporters can reside in more than one type of membrane microdomain in a single type of cell.

Although the detergent insolubility of multimembrane-spanning proteins such as the Na⁺-K⁺-ATPase has been used to determine that the protein is associated with the cytoskeleton (28, 36, 38), it is possible that other multimembrane-spanning proteins might display similar properties due to inherent insolubility. One observation supports that the detergent insolubility of ATRC1 is not an inherent property but results from its association with the cytoskeleton. That is, the amount of ATRC1 solubilized in Triton X-100 was increased by treatment of cells with cytochalasin D. Because depolymerizing the actin network should not affect the inherent solubility of membrane proteins, these results argue that the insolubility observed resulted from association of ATRC1 with the cytoskeleton or with DRM.

Many of the Triton-insoluble transporters became soluble upon exposure to cytochalasin D, supporting the observation that the insolubility of some ATRC1 results from their association with the actin-based cytoskeleton and not from inherent insolubility of ATRC1. However, the proportion of transporters solubilized was lower than would be expected if half of the transporters are associated with F-actin. Because the cortical actin network lying under the PM is more resistant to disruption by cytochalasin D than are other actin filaments (6), the lower than expected increase in solubility might have resulted from incomplete depolymerization of F-actin. In agreement with this possibility, micrographs of HEK cells exposed to cytochalasin D showed clusters of F-actin along the cell perimeters that partially resisted disruption.

Zharikov et al. (52) demonstrated in PAEC that prolonged disruption of F-actin microfilaments using the marine toxins sphingolide A, latrunculin A and B, or halichondramide A for 24 h reduces ATRC1-mediated cationic transport, whereas stabilization of the microfilament network enhances cationic amino acid transport. In our earlier studies, we found that a shorter exposure to cytochalasin D for up to 3 h did not reduce transport significantly but inhibited the function of ATRC1 as the receptor for ecotropic murine retroviruses (25). We did not examine the effect at longer time points or with other F-actin inhibitors because cell viability was critical to those studies. In a separate study in PAEC, Zharikov and Block (51) showed by reciprocal immunoprecipitate that ATRC1 forms a physical complex with fodrin, an actin-binding protein. The two PAEC studies provided evidence for a functional physical association of ATRC1 with the cellular actin network in vascular endothelial cells. Our findings that the majority of ATRC1 in epithelial cells are associated with the actin cytoskeleton suggest that the cytoskeletal association is common to both cell types.

How might localization of ATRC1 to different membrane microdomains be accomplished? Localization of ATRC1 to different membrane subdomains is likely mediated through its association with proteins that specifically reside in these membrane subdomains. Although we observed only a portion of transporters colocalizing with caveolin (this report), the majority of ATRC1 localized to caveolae in PAEC. In addition, ATRC1 in PAEC has been shown to coimmunoprecipitate with the actin-binding protein fodrin, as well as with caveolin. It is possible that the association of ATRC1 with each of these proteins is not mutually exclusive, in which case the populations of actin-associated and DRM-associated ATRC1 need not be mutually exclusive in HEK cells. For example, actin-associated ATRC1 might reside in caveolae. Recently, Rodgers and Zavzavadjian (39) reported evidence of precedence for this possibility. These authors showed that a DRM-resident protein, LCK, resides in two types of rafts. The first type colocalized with F-actin and contained the majority of LCK, whereas the second type did not colocalize with F-actin. Upon exposure to cytochalasin D, LCK was found exclusively associated with actin clusters on the cell membrane. Rodgers and Zavzavadjian proposed that some DRM are associated with the cytoskeleton.

A similar localization of ATRC1 might occur in endothelial cells, which express large amounts of both fodrin and caveolin. Because HEK cells express relatively low levels of caveolin (14), a smaller fraction consistent with our observations of ATRC1 might reside in this type of microdomain. That is, levels of expression of ATRC1-binding proteins might be limiting factors in determining ATRC1 membrane microdomain localization. If a method for dissociating transporters from the membrane cytoskeleton can be identified, then the localization of ATRC1 under these conditions would reveal whether DRM resident transporters are also associated with F-actin.

Alternatively, the DRM localization of ATRC1 in HEK cells could be mediated by DRM-targeting amino acid motifs on the transporter. Some Src family kinases and heterotrimeric G protein subunits contain an NH₂-terminal Met-Gly-Cys amino acid motif that serves as a DRM localization signal when the Gly is myristoylated and Cys is palmitoylated. A Met-Gly-Cys motif exists at the NH₂ terminus of the ATRC1, raising the possibility that ATRC1 might localize in rafts if the glycine and cysteine residues are modified by acylation. It is not known whether the...
potential sites on ATRC1 are modified. Genetic and biochemical analyses of this putative DRM localization sequence should determine whether they are involved in the membrane distribution of ATRC1.

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