Multiple Regions within the Coxsackievirus and Adenovirus Receptor Cytoplasmic Domain Are Required for Basolateral Sorting

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The coxsackievirus and adenovirus receptor (CAR) mediates attachment and infection by coxsackie B viruses and many adenoviruses. In human airway epithelia, as well as in transfected Madin-Darby canine kidney cells, CAR is expressed exclusively on the basolateral surface. Variants of CAR that lack the cytoplasmic domain or are attached to the cell membrane by a glycosylphosphatidylinositol anchor are expressed on both the apical and basolateral surfaces. We have examined the localization of CAR variants with progressive truncations of the cytoplasmic domain, as well as with mutations that ablate a potential PDZ (PSD95/dlg/ZO-1) interaction motif and a putative tyrosine-based sorting signal. In addition, we have examined the targeting of two murine CAR isoforms, with different C-terminal sequences. The results suggest that multiple regions within the CAR cytoplasmic domain contain information that is necessary for basolateral targeting.

The coxsackievirus and adenovirus receptor (CAR) mediates attachment and infection by coxsackie B viruses as well as by many human adenoviruses (1–3). Human CAR (hCAR) is a 46-kDa cell surface glycoprotein composed of an extracellular region with two immunoglobulin-like domains, a typical hydrophobic transmembrane region, and a cytoplasmic domain of 107 amino acids. A murine homolog of the human receptor has also been characterized (2, 4), and homologs in the rat, pig, dog (5), and zebrafish (6) have been reported. The murine and human proteins are very similar (91% amino acid identity (5), and zebrafish (6) have been reported. The murine and human proteins are very similar (91% amino acid identity (5), and zebrafish (6) have been reported. The murine and human proteins are very similar (91% amino acid identity (5), and zebrafish (6) have been reported. The murine and human proteins are very similar (91% amino acid identity (5), and zebrafish (6) have been reported. The murine and human proteins are very similar (91% amino acid identity

EXPERIMENTAL PROCEDURES

Cell Culture—MDCK type II cells were grown in Dulbeco’s modified Eagle’s medium with 10% fetal calf serum in 10% CO₂. For immuno-
fluorescence and adenovirus infection, 3 × 10⁵ cells/well were plated on 12-mm diameter polyester filters with a pore size of 0.4 μm (Transwell clear, Corning-Costar Corp., Cambridge, MA); for biotinylation experiments, 1 × 10⁵ cells/well were seeded onto Transwell 24-mm diameter filters. In both cases, the MDCK cells were cultured for 3–5 days at which point the cell monolayer was polarized, as demonstrated by “tight” transepithelial resistances (>700 ohms cm²) measured with an epithelial voltohmeter (World Precision Instruments, Inc., Sarasota, FL).

Expression Vectors Encoding Deletion and Chimeric Mutants of CAR—hCAR cytoplasmic deletion and substitution mutants were made using polymerase chain reaction (PCR)-based strategies to modify coding sequences cloned in the eukaryotic expression plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA). Tailless hCAR and GPI hCAR were previously described (30). For generation of deletion mutants, a forward primer was designed to anneal to sequences 5′ to a unique restriction site, BstWI, in the hCAR cDNA. The reverse mutagenic primers contained termination codons ~200–300 nucleotides downstream of the forward primer, as well as the unique restriction site XbaI. Mutants were named for the final three amino acids encoded by the truncated cDNA, as well as for the position of the final amino acid (Fig. 1). Stop codons were introduced so that the terminal amino acid was lysine 315 in YSK315, asparagine 344 in APN344, glycine 349 in RMG349, and serine 359 in AQS359. PCR products were digested with BstWI and XbaI, then inserted into the hCAR pcDNA3.1 plasmid cut with the same enzymes. The PCR-derived portion of each construct was sequenced to confirm that the correct mutation had been introduced.

Constructs encoding chimeric proteins consisting of the hCAR extra- cellular and transmembrane domains fused to the cytoplasmic domain of each mCAR isoform were generated by splice overlap extension PCR (31). The constructs for Y318A and LSRM(A4) were also generated by splice overlap extension PCR, with primers designed to encode an alanine rather than a tyrosine at position 318 for Y318A or four alanine residues in place of the amino acids LSRM for LSRM(A4).

Cell Transfection and Isolation of CAR Expressing Cell Lines—Mutant cDNA constructs were transfected into MDCK cells by electroporation (Bio-Rad, Hercules, CA) or with LipofectAMINE 2000 (Life Technologies, Inc., Gaithersburg, MD), and stably transfected cells were selected with 500 μg/ml Geneticin (Life Technologies, Inc.). Cell populations with surface hCAR expression were isolated by two or three rounds of fluorescence-activated cell sorting with the anti-hCAR monoclonal antibody RmcB and fluorescein isothiocyanate-conjugated goat antibody to mouse immunoglobulin (Sigma-Aldrich, St. Louis, MO). The murine myeloma protein mineral oil plasmacytoma 195 (Sigma-Aldrich) was used instead of RmcB as a negative control.

Immunofluorescence and Confocal Microscopy—To test for apical expression using immunofluorescence, polarized cells were fixed in paraformaldehyde (1% in PBS) for 30 min, washed, and then stained only on the apical surface with RmcB followed by a fluorescein isothiocyanate-conjugated goat antibody to mouse immunoglobulin (Sigma-Aldrich). To examine the distribution of hCAR throughout a cell, polarized cultures were fixed but not permeabilized with 0.2% Triton X-100, and stained from both the apical and basal surfaces with RmcB followed by tetramethylrhodamine-conjugated goat antibody to mouse immunoglobulin (Sigma-Aldrich). Cells were then examined either by conventional immunofluorescence using a Nikon Eclipse 800 epifluorescence microscope or by confocal microscopy in both XY and XZ planes using a Leica TCS 4D confocal microscope. All immunofluorescence experiments were performed at least three times.

Selective Biotinylation of Polarized MDCK Cells—For biotinylation experiments, polarized cultures of MDCK were labeled with paraformaldehyde, and stained for hCAR with the monoclonal antibody RmcB. The first column (labeled permeabilized) shows representative immunofluorescence images of cell monolayers that were permeabilized with Triton X-100 before staining with RmcB. Monolayers in the next column (labeled apical staining) were fixed but not permeabilized, then exposed to RmcB only at the apical surface. The third column (labeled XZ plane) shows representative confocal images in the XZ plane of cells that had been permeabilized prior to staining with anti-hCAR.
washed with 1 mg/ml glycine in Dulbecco’s modified Eagle’s medium to quench unreacted biotin. The filters were excised to remove unpolarized cells from the edge, and cells were lysed at 4 °C in PBS containing 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.15 trypsin inhibitor units/ml aprotinin. The lysate was centrifuged at 4 °C for 30 min at top speed in a microcentrifuge and the supernatant was precleared twice, for 2 h at 4 °C, with mineral oil plasmacytoma 195 bound to protein G beads. To immunoprecipitate CAR protein, the precleared supernatant was agitated overnight at 4 °C with 15 μl of protein G beads covalently linked to RmcB antibody. The beads were washed on ice with PBS containing 1% Triton X-100 and 2 mM EDTA, then boiled for 5 min in 30 μl of Laemmli buffer. Beads were separated by brief centrifugation, then supernatant was run on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. For detection of biotinylated CAR, membranes were blocked overnight at 4 °C in PBS containing 2% bovine serum albumin, exposed for 20 min at room temperature in a 1:2000 dilution of horseradish peroxidase-conjugated streptavidin (Pierce) in PBS 0.1% Tween-20, then developed with ECL reagents (Amersham Pharmacia Biotech, Arlington Heights, IL) and an exposure was made to film. These experiments were performed at least two times for each cell line.

RESULTS

Basolateral Sorting Information between Residues 315 and 349—Full-length hCAR is targeted to the basolateral surface of MDCK cells, whereas hCAR lacking a cytoplasmic domain (tailless) or bound to the cell by a GPI anchor is expressed on both the apical and basolateral surfaces (10). To define the sequences responsible for basolateral localization, we generated a series of CAR mutants with truncations within the cytoplasmic domain (Fig. 1).

Mutant constructs were stably expressed in MDCK cells, which were selected for hCAR surface expression. Transfected cells were grown to confluence as polarized cultures, and examined by both fluorescence and confocal microscopy. These experiments were performed at least two times for each cell line. Infection of Polarized Monolayers with Adenovirus—Polarized monolayers of cells expressing mutant hCAR constructs were treated with neuraminidase type II (Sigma-Aldrich) to remove the glycocalyx as previously described (10). 1010 particles of adenovirus type 5 encoding green fluorescence protein (AdVGFP, a kind gift from Erik Falck-Pedersen, Cornell University) were added to the apical chamber of each well and incubated for 2 h at 37 °C. Monolayers were washed, incubated 48 h at 37 °C, then examined for GFP expression by epifluorescence microscopy. These experiments were performed at least three times for each cell line.
the primarily lateral localization of hCAR in the XZ plane observed by confocal microscopy. In contrast, tailless and GPI-linked hCAR were expressed at both the apical and basolateral surfaces of the polarized cells (Fig. 2).

The hCAR C-terminal peptide, SIV, closely resembles C-terminal motifs ((T/S)eX-hydrophobic) responsible for interaction with PDZ domains (27, 32). Because such PDZ interactions determine basolateral localization for some proteins (33), we tested a construct, AQS359, from which the putative PDZ recognition motif had been removed (Fig. 1). Like full-length hCAR, AQS359 was expressed exclusively at the basolateral surface, suggesting that PDZ interactions were not required for CAR targeting (Fig. 3). Mutant RMG349, which lacked 16 C-terminal residues, was also expressed exclusively on the basolateral surface, indicating that residues 350–365 do not contain essential targeting information (Fig. 3).

We next generated a mutant, YSK315, from which approximately half of the cytoplasmic domain had been deleted (Fig. 1). YSK315 was expressed on both the apical and basolateral surfaces of polarized cells (Fig. 3), suggesting that basolateral targeting information must be contained within the distal portion of the CAR cytoplasmic domain, between amino acids 315 and 349.

To define more precisely where this sorting information is located, we generated an additional deletion mutant, APN344, which lacked 21 C-terminal residues. Apical expression of APN344 was evident by both confocal and epifluorescence microscopy of transfected MDCK cells (Fig. 3). This suggested that the amino acids LSRMG, which are present in RMG349 but not APN344, might contain basolateral sorting information. We then replaced the LSRM residues with alanine residues to generate mutant LSRM(A4). This was also expressed on the apical surface of polarized MDCK cells (Fig. 4), indicating that these amino acids function in basolateral targeting.

Expression of CAR on the basolateral surface of polarized cells is not sufficient to permit adenovirus entry from the apical surface (8, 10). As another measure of apical CAR expression, we examined the susceptibility of the transfected cell lines to adenovirus infection. Polarized monolayers were exposed to AdVGFP, with transgene expression detected by fluorescent microscopy 48 h later. Only an occasional GFP-positive cell was found in those cell lines expressing full-length hCAR, RMG349, or AQS359 (Fig. 5). In contrast, cells expressing tailless CAR, YSK315, APN344, or LSRM(A4) all showed bright fluorescence 2 days after apical exposure to AdVGFP. Consistent with the results obtained by microscopy, these observations indicate that AQS359, YSK315, and LSRM(A4) are expressed on the apical surface of polarized MDCK cells but RMG349 and wild-type hCAR are not.

Additional Sorting Information between Residues 261 and 315—We also used selective biotinylation to examine the distribution of CAR between the apical and basolateral surfaces. Polarized monolayers were exposed to a biotinylating agent at the apical or basolateral surfaces, CAR was immunoprecipitated from cell lysates, and biotinylated CAR was detected with streptavidin. Consistent with the immunofluorescence results shown above, full-length hCAR was detected only on the basolateral membranes of MDCK monolayers (Fig. 6). Two protein bands were visible when biotinylated full-length CAR, and several of the mutant constructs, were immunoprecipitated from MDCK cells (Fig. 6) as well as from transfected Chinese hamster ovary cells (data not shown). Western blot analysis of the immunoprecipitated protein revealed that both bands are forms of hCAR (data not shown), and the smaller form may represent a degradation product.

Mutants AQS359 and RMG349 were found only on the basolateral surface of polarized MDCK cells, which is in good agreement with the data obtained by immunofluorescence and adenovirus infection. Apical expression of APN344 was detectable, but the expression level, when compared with basolateral expression, was clearly lower than that seen in cells transfected with YSK315 (Fig. 6). This is similar to what was seen by
immunofluorescence, where apical staining of APN344 appeared dimmer than that of YSK315 (Fig. 3). Although residues between 345 and 349 (LSRMG) are required for basolateral targeting, additional information is contained between residues 315 and 345.

GPI-anchored CAR was detected predominantly on the apical surface, and tailless CAR was distributed equally between the apical and basolateral membranes. In contrast, YSK315 showed significantly higher expression on the basolateral than the apical surface (Fig. 6). This suggests that there is also sorting information contained between residues 261 and 315, in the region deleted from tailless CAR but not from YSK315.

Tyrosine 318 Is Important for Basolateral Targeting—As noted earlier, the amino acids between positions 315 and 345 appear to contain one or more basolateral sorting determinants. An examination of this region revealed the presence of a potential tyrosine-based sorting signal (YNQV) beginning at amino acid 318. To see if this sequence plays a role in hCAR sorting, the tyrosine residue at position 318 was changed to an alanine (Fig. 1). As seen in Fig. 7, Y318A could be detected on both the apical and basolateral membranes of polarized MDCK cells as determined by fluorescence and confocal microscopy and by selective biotinylation. Polarized cells expressing Y318A were easily infected by AdVGFP that had been added to the apical chamber of a Transwell filter, confirming that Y318A is expressed on the apical membrane (Fig. 7). Thus, Y318 is involved in basolateral targeting.

CAR Isoforms mCAR1 and mCAR2 Are Both Sorted to the Basolateral Membranes of MDCK Cells—The two isoforms of murine CAR (mCAR) have cytoplasmic domains very similar to that of human CAR, although the C-terminal peptide of mCAR2 is distinctly different from that of hCAR (Fig. 8) (2, 4). To determine whether the cytoplasmic domains of both isoforms contained basolateral targeting signals, chimeras were constructed that combined the extracellular and transmembrane regions of hCAR with the cytoplasmic domains of mCAR1 (hm1) or mCAR2 (hm2). Both chimeras were expressed solely on the basolateral membranes of MDCK cells as determined by immunofluorescence and biotinylation (Fig. 8).

**DISCUSSION**

Previous work demonstrated that wild-type hCAR is sorted solely to the basolateral membrane of polarized MDCK cells, and that essential sorting information is contained within the CAR cytoplasmic domain (10). In these experiments we have delineated CAR sequences involved in basolateral sorting. Our data suggest that multiple regions within the CAR cytoplasmic domain are required for basolateral sorting. The amino acids YNQV, beginning at amino acid 318, comprise a previously described basolateral sorting signal, and changing the tyrosine...
within this motif led to apical expression of hCAR. Deletion or mutation of the sequence LSRMG between residues 345 and 349 led to apical expression of hCAR, indicating that these amino acids may also be a distinct sorting motif. There also appears to be additional sorting information contained between residues 261 and 315 that functions independently of these other potential signals.

We found that the deletion of a putative PDZ interaction motif (SIV) from the hCAR C terminus did not alter the exclusively basolateral expression pattern, indicating that interaction with PDZ proteins is not essential for basolateral sorting of hCAR. This contrasts with evidence that C-terminal PDZ interaction motifs (with the consensus sequence (T/S)-hydrophobic) are involved in polarized expression of such proteins as the cystic fibrosis transmembrane conductance regulator (29) and the receptor tyrosine kinase let 23 (33). However, our results are similar to those obtained with the γ-aminobutyric acid transporter BGT-1, in which deletion of a PDZ-interacting motif did not prevent localization to the basolateral membranes of MDCK cells despite the motif’s function as a retention signal (34). It is likely that hCAR’s PDZ recognition motif is important for interaction with other cellular proteins, but these interactions do not drive the polarization of CAR expression.

An examination of the cystolic domain of hCAR does not reveal other known sorting signals. Nevertheless, there is likely to be additional targeting information contained within the cystolic domain. Tailless hCAR was expressed in equal amounts on the apical and basolateral membranes of polarized MDCK cells. In contrast, mutant YSK315, which lacks approximately half the cytoplasmic domain and contains neither tyrosine residue 318 nor the potential basolateral sorting sequence LSRMG between amino acids 345 and 349, was preferentially, but not exclusively, distributed to the basolateral surface. This suggests that other basolateral sorting information is contained within the proximal half of the hCAR cytoplasmic domain. Although these regions are distant from each other in the linear amino acid sequence of hCAR, they may be in close proximity with one another in the tertiary structure of this protein. Therefore, deletion or mutation of any one of these regions may impair the interaction of the hCAR cytoplasmic domain with cellular sorting machinery.

For some proteins, such as the low density lipoprotein and epidermal growth factor receptors (18, 24), deletion of basolateral targeting determinants within the cytoplasmic domain results in expression that is almost exclusively apical, perhaps because of strong apical sorting determinants in the transmembrane or extracellular domains. In contrast, deletion of the entire CAR cytoplasmic domain resulted in an equal distribution of tailless CAR on the apical and basolateral surfaces, and even GPI-anchored CAR showed significant basolateral expression; these results suggest that the CAR extracellular domain does not contain strong apical sorting signals.

There are two isoforms of murine CAR: the cytoplasmic domain of mCAR1 is nearly identical to that of hCAR; mCAR2 differs from hCAR and mCAR1 at the C terminus (2, 4). To see if these differences in the cytoplasmic domain affected the targeting of these receptors, we constructed chimeric receptors containing the extracellular portion of hCAR fused to the cytoplasmic domains of the two mCAR isoforms. This was necessary, because the anti-CAR monoclonal antibody RmcB recognizes the extracellular region of hCAR, but not of mCAR. Like hCAR, both mouse isoforms were targeted exclusively to the basolateral membrane of polarized MDCK monolayers. We found that deletion of 21 residues from the C terminus of hCAR interfered with basolateral sorting; these residues are not present in mCAR2, which lacks 26 C-terminal residues present in hCAR and mCAR1 but which nonetheless is sorted to the basolateral surface. The mCAR2 C terminus must contain sorting information that compensates for loss of information from mCAR1, even though its sequence is quite dissimilar.

CAR’s role in virus infection is well established, but its cellular function remains to be determined. Recent evidence suggests that CAR may be involved in homotypic cell adhesion (35). It is concentrated at sites of cell-cell contact in both polarized (10, present study) and non-polarized cells.2 Consistent with this, CAR’s N-terminal immunoglobulin-like domain forms a homodimer, and residues at the homodimer interface are more highly conserved in evolution than is the rest of the extracellular domain (6).

The CAR cytoplasmic domain shows greater sequence conservation than does the extracellular domain. At the C terminus, 23 of 23 residues are identical in human and zebrafish CAR.3 This sequence conservation most likely reflects conservation of biological function. We find that information required for basolateral sorting is present within this highly conserved region; although the implicated sequences are absent from CAR’s variant isoform, the basolateral targeting is preserved. These observations suggest that expression at the basolateral surface of polarized epithelium may be important to CAR’s primary function.

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