The coxsackievirus and adenovirus receptor (CAR) is a component of the epithelial cell tight junction. In a yeast two-hybrid screen we identified the multi-PDZ domain protein MUPP1 as an interaction partner for the CAR cytoplasmic domain. CAR and MUPP1 were found to colocalize at the tight junction, to coprecipitate from epithelial cells, and to interact in vitro. The interaction was found to specifically involve the PDZ-binding motif within the CAR C terminus and MUPP1 PDZ domain 13. In transfected cells, CAR recruited MUPP1 to cell–cell contacts. The inhibition of CAR expression with small interfering RNA inhibited MUPP1 localization to the tight junction. The results indicated that CAR interacts with MUPP1 and is involved in MUPP1 recruitment to the tight junction.

The coxsackievirus and adenovirus receptor (CAR)3 was first identified as a cellular protein involved in attachment and infection by group B coxsackieviruses and adenoviruses (1–4). CAR also functions as a transmembrane component of epithelial tight junctions (TJs) (5). The TJ is the apical-most component of the junctional complex of epithelia and endothelia and is critical in maintaining and restricting the paracellular flow of ions and solutes. Though CAR localizes to the TJ, the nature of its interactions with other TJ-associated proteins remains unclear.

Several PDZ domain-containing proteins have been localized to the cytoplasmic region of the TJ. These include the structurally related membrane-associated guanylate kinase proteins zonula occludens (ZO)-1, ZO-2, and ZO-3, the membrane-associated guanylate kinase inverted proteins, the partitioning protein 1 (ZO)-1, ZO-2, and ZO-3, the membrane-associated guanylate kinase inverted proteins, and the multi-PDZ domain protein 1 (MUPP1). These proteins likely function to link TJ-associated transmembrane proteins to intracellular signaling molecules.

The CAR C terminus resembles hydrophobic C-terminal peptide motifs known to interact with PDZ protein domains, such as those contained within membrane-associated guanylate kinase proteins and other scaffolding proteins localized to the TJ region. CAR associates with ZO-1, as demonstrated by coprecipitation from polarized epithelia and by the relocalization of ZO-1 that occurs in CAR-transfected Chinese hamster ovary (CHO) cells (5). CAR may also interact with a component of the adherens junction, β-catenin, as has been reported in A549 cells (6). In addition, yeast-two-hybrid studies have shown that CAR interacts with Ligand of Numb-X (LNX) (7), a PDZ protein believed to regulate Notch signaling in the central nervous system; however, LNX is not known to associate with TJs (8, 9).

To define the associations mediated by the cytoplasmic tail of CAR, we performed a yeast two-hybrid screen and identified MUPP1 as a CAR-interacting protein. The localization of MUPP1 to the area of the TJ was disrupted when CAR was absent, although the distribution of other TJ-associated components such as ZO-1 remained unchanged. Taken together, these data indicate that CAR interacts directly with MUPP1 and that this interaction is involved in restricting MUPP1 to the cytoplasmic region of the TJ.

MATERIALS AND METHODS

Cell Culture—CHO cells stably transfected with cDNA constructs encoding human CAR (CHO-CAR cells), CAR lacking its cytoplasmic tail (CHO-CARcyt), CAR with deletion of its hydrophobic C-terminal motif (CHO-CARΔcyt), or with vector alone (CHO-pcDNA) were cultured in nucleoside-free α-minimum essential medium containing 10% dialyzed fetal calf serum (5).

Caco-2 cells were cultured in high glucose Dulbecco’s modified Eagle’s medium containing 20% fetal bovine serum, 1% nonessential amino acids, and penicillin/streptomycin. Cells were plated in 12-mm Transwell-Col inserts (0.4-μm pore size) (Costar) at a density of 5 × 104 cells or in collagen-coated chamber slides at a density of 2 × 103 cells/well.

Plasmids, siRNAs, and Transfections—Plasmid GW1 containing a hemagglutinin (HA)-tagged full-length MUPP1 cDNA and the pGEX-MUPP1 (PDZ 12–13) plasmid were kindly provided by Ronald T. Javier (Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX) (10). pGEX-MUPP1 (PDZ10) was provided by Shoichiro Tsukita (Department of Cell Biology, Faculty of Medicine, Kyoto University, Japan) (11). CHO cells were transiently transfected with 2 μg of pG3W1-HAMUPP1 using FuGENE 6 according to the manufacturer’s protocol (Roche Applied Science) and assayed 48 h later.

Double-stranded siRNAs targeted against human CAR (sense, 5′-GGUGGAUAAGUGAUUUUUUAG-3′ and antisense, 5′-AAUAUACUUGAUCCACC-3′), GAPDH, and a control with no similarity to any human gene sequence were designed and synthesized by Ambion (Austin, TX). Caco-2 cells grown on collagen-coated chamber slides were transfected with duplex siRNAs using Oligofectamine (Invitrogen). Cells were assayed 24–48 h following transfection.

Antibodies—CAR-specific antiserum was generated in rabbits and affinity-purified as described (12). Rabbit polyclonal antibodies against MUPP1 were kindly provided by Shoichiro Tsukita and Ronald T. Javier (10, 11). Mouse anti-ZO-1 and rabbit anti-protaseome subunit antibodies were purchased from Zymed Laboratories, Inc. Antibodies to HA, GAPDH, early endosome antigen-1, Rab7, lysosomal integral membrane proteins II (LIMP II), and lysosome-associated membrane protein 1 (LAMP 1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CAR monoclonal antibody RmcB and sheep anti-MUPP1 were purchased from Upstate (Lake Placid, NY).

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1 The abbreviations used are: CAR, coxsackievirus and adenovirus receptor; TJ, tight junction; ZO, zonula occludens; MUPP1, multi-PDZ domain protein 1; CHO, Chinese hamster ovary; siRNA, small interfering RNA; HA, hemagglutinin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LAMP 1, lysosome-associated membrane protein 1; LIMP II, lysosomal integral membrane proteins II; GST, glutathione S-transferase; hCAR, human CAR; mCAR, mouse CAR.

2 This paper is available on line at http://www.jbc.org.

The Coxsackievirus and Adenovirus Receptor Interacts with the Multi-PDZ Domain Protein-1 (MUPP-1) within the Tight Junction*

48079
MOPC21 was purchased from Sigma. Oregon Green-conjugated anti- 
ti-HA antibody was purchased from Clontech. Fluorochrome-conju- 
gated secondary antibodies for immunofluorescence were purchased from 
Jackson Immonoresearch (West Grove, PA). Horseradish peroxi- 
dase-conjugated antibodies were obtained from Santa Cruz 
Biotechnology.

Yeast Two-hybrid Screening—DNA encoding the mCAR1 cytoplasmic 
domain (CCHRK → DGSIW) was inserted into plasmid pGBK7T 
(Clontech), in-frame with the yeast GAL4 DNA binding domain. The 
resulting plasmid (CAR-BD) was used as bait in a yeast two-hybrid 
screen of an 11-day mouse embryo library constructed in pGAD77 using 
protocols provided with the Clontech Matchmaker Two-Hybrid System 
3. Saccharomyces cerevisiae strain AH109 was transformed with both 
library and bait plasmids, and ~2.5 × 10^6 dual transformants were 
screened for growth on medium deficient in histidine and adenine and 
for production of β-galactosidase. Library plasmids were recovered from 
putative positive clones and further tested to eliminate any that in- 
duced GAL4 activation independently of any bait plasmid, in the 
presence of binding domain alone, or in the presence of binding domain 
fused to a nonspecific control protein (human lamin C). Of 11 cDNAs 
that were obtained, one encoded PDZ domain 13 of MUPP1; the other 
10 encoded a PDZ domain derived from another protein.

Immunofluorescence Microscopy—CHO cells plated on glass 
slides or siRNA-transfected Caco-2 cells grown on collagen-coated 
slides were washed in phosphate-buffered saline, and fixed in 1% 
paraformaldehyde in phosphate-buffered saline for 15 min at room 
temperature, washed, and permeabilized with 1% Triton X-100 for 10 
min at room temperature. Cells were incubated with the indicated 
primary antibodies for 1 h at room temperature, washed, incubated 
with fluorochrome-conjugated secondary antibodies for 30 min at 
temperature, mounted with Vectashield (Vector labora- 
tories, Burlingame, CA) containing 4,6-diamidino-2-phenylindole 
(DAPI). All cells were postfixed with 4% paraformaldehyde. Images 
were captured with an Olympus fluorescence microscope (Melville, NY).

Caco-2 cells grown on transwell inserts were permeabilized with 
methanol for 5 min at −20°C, washed briefly in phosphate-buffered 
saline, and blocked with 5% bovine serum albumin for 30 min at 37°C. 
Cells were incubated with monoclonal antibody RmcB, or control 
anti-MUPP1 for 1 h at room temperature. Cells were washed and 
centrifuged at 50 g for 3 min at 4°C. The supernatant was 
loaded directly onto a 10% gradient polyacrylamide gel containing 
10% glycerol. Following a brief centrifugation, the supernatant was 
run at 10°C for 15 min at 2700 rpm in a horizontal rotator. Beads were 
adsorbed overnight at room temperature with 500 ml of 0.1 M Tris-HCl, pH 8.0. 

RESULTS

Identification of MUPP1 as a CAR Binding Partner—To 
identify the proteins that interact with the C-terminal tail of 
mouse CAR1, we performed yeast two-hybrid screening. The 
mouse cytoplasmic domain was fused to the yeast GAL4 DNA 
binding domain, and used as bait to screen an 11-day mouse 
embryo library. A cDNA encoding the thirteenth PDZ domain 
of the multi-PDZ domain protein MUPP1 was isolated; the 
two-hybrid interaction with the CAR C terminus appeared to be 
specific (Table 1).

| Table 1 |
|----------|
| Specificity of MUPP1-CAR interaction in yeast |
| Binding domain | GAL4 activation |
| pGBK7T-MUPP1 | pGADTT-CAR |
| pGBK7T-MUPP1 | None |
| pGBK7T-MUPP1 | None |
| pGBK7T (no insert) | pGADTT-CAR |
| None | None |
| pGBK7T-p53 | None |
| pGBK7T-p53 | pGADTT-CAR |
| pGBK7T-p53 | pGADTT-TAg |

Caco-2 cells grown in LB were diluted 1:5 into a final culture volume of 25 ml. At an 
OD600 of 0.6, the culture was expanded to 1 l in isopropyl-1-thio-β-galac- 
topyranoside (IPTG, Fisher), and bacteria were collected after 4 h. The bacte- ical cell pellet was resuspended in phosphate-buffered saline containing 
1 mM phenylmethanesulfonyl fluoride, 0.5 μM NaCl, and 1% Triton 
X-100. The cells were lysed by sonication, and the cell debris was 
removed by centrifugation at 10,000 rpm for 10 min. The supernatant 
was incubated with 2 ml of a 50% slurry of Streptactin-MacroPrep 
beads (IBA-GmbH) for 2 h at 4°C. The beads were washed four times 
with cold phosphate-buffered saline, and the protein was eluted from 
the beads with 2.5 mM desthiobiotin. The desthiobiotin was removed by 
dialyzing the protein into 20 mM Hepes, pH 6.7. 

In Vitro Binding Assays—GST fusion proteins expressed in E. coli 
were purified using glutathione-Sepharose 4B beads (Amersham Biosciences). 
SUMO or SUMO-hCAR (1 μg) was added to a pull-down 
buffer (150 mM NaCl, 0.2 mM phenylmethanesulfonyl fluoride, 5 μM bovine serum 
component) and incubated with GST, GST-MUPP1PDZ10, or GST-MUPP1PDZ12–13 bound to glutathione-Sepharose 
4B beads for 1 h at 4°C. Beads were sedimented by centrifugation, 
followed by a brief centrifugation, the supernatant was run on a 4–15% Tris-HCl gel and transferred to a polyvinylidene diflu- 
ride membrane. The membranes were blocked overnight in 5% bovine 
saline buffer at 4°C and incubated with anti-Streptavidin mono- 
clonal antibody. Alternatively, samples were run on 4–15% gels and 
visualized with Coomassie Blue to control for loading of GST and 
GST fusion proteins.

RESULTS

Identification of MUPP1 as a CAR Binding Partner—To 
identify the proteins that interact with the C-terminal tail of 
murine CAR1, we performed yeast two-hybrid screening. The 
murine cytoplasmic domain was fused to the yeast GAL4 DNA 
binding domain, and used as bait to screen an 11-day mouse 
embryo library. A cDNA encoding the thirteenth PDZ domain 
of the multi-PDZ domain protein MUPP1 was isolated; the 
two-hybrid interaction with the CAR C terminus appeared to be 
specific (Table 1). 

Direct In Vitro Interaction between CAR and MUPP1—The 
yeast two-hybrid screen indicated that the CAR cytoplasmic 
protein interaction domain vector pGBK7T, and the mCAR cyto- 
plasmic domain in the binding domain vector pGADTT, or with control 
plasmids containing no insert, human lamin C, SV40 T antigen (T Ag), 
or murine p53. GAL4 activation was determined by the identification of 
Ade- /His-/LacZ+ transformants.
CAR Associates with MUPP1 and Recruits MUPP1 to Intercellular Junctions—In a human polarized epithelial cell line, Caco-2, both CAR and MUPP1 were localized to the apical region of the lateral cell membrane as determined by confocal immunofluorescence microscopy (Fig. 2A). ZO-1 colocalized with both CAR and MUPP1 (not shown), consistent with previous reports that all three proteins are components of the epithelial tight junction. When CAR was precipitated from Caco-2 cell lysates with monoclonal antibody RmcB, MUPP1 was detected in the precipitates by immunoblotting with a polyclonal anti-MUPP1 antibody (Fig. 2A). GST alone, or a control fusion protein containing MUPP1 PDZ domain 10, did not precipitate SUMO-hCAR. These results confirm that the CAR C terminus interacts directly with MUPP1.

Association with MUPP1 Depends on the CAR C Terminus—HA-MUPP1 introduced into CHO cells expressing truncated human CAR, with deletion of the entire cytoplasmic domain (CAR_{tail}) or deletion of the C-terminal DGSIV motif (CAR_{Cys}), was expressed throughout the cytoplasm, with no localization to cell-cell contacts (Figs. 4A). Unlike full-length CAR, CAR_{tail} and CAR_{Cys} did not coprecipitate with MUPP1, and MUPP1 did not coprecipitate with truncated CAR (Fig. 3). Thus, the association between CAR and MUPP1, and recruitment of MUPP1 to sites of cell contact, depends on the presence of the putative PDZ-binding motif at the CAR C terminus. Given the results of the yeast two-hybrid screen, and the in vitro pull-down experiment shown above, it is likely that CAR association with MUPP1 involves an interaction between the C-terminal motif and MUPP1 PDZ domain 13.

Localization of MUPP1 in the Absence of CAR—The experiments in CHO cells indicated that CAR recruits MUPP1 to sites of contact between transfected CHO cells. To determine whether endogenous CAR is involved in MUPP1 recruitment to the tight junction of polarized epithelial cells, we used RNA interference technology to silence CAR expression. siRNAs specific to CAR were transiently transfected into Caco-2 cells. RT-PCR was performed at 48-h post-transfection to determine the extent of CAR mRNA knockdown by siRNA transfection. Caco-2 cells transfected with a control siRNA with no sequence similarity to any human gene sequence showed a high level of CAR expression, indicating that transfection of cells with a nonspecific siRNA had no effect on CAR mRNA expression (Fig. 5A, right). However, transfection of cells with a siRNA specific for CAR significantly reduced endogenous CAR mRNA levels (Fig. 5A, left). To determine whether the decrease of mRNA levels resulted in a reduction in protein expression, control and CAR siRNA-transfected lysates were subjected to Western blot analysis. CAR protein expression was significantly reduced in CAR siRNA-transfected cells compared with controls (Fig. 5B). Immunoblots were stripped and reprobed for GAPDH to control for equal protein loading. The residual levels of CAR mRNA and protein most likely
reflect the limitation of silencing by transient transfection in this cell type.

To establish whether the loss of CAR expression by siRNA transfection would affect the localization or expression of TJ-associated proteins, particularly MUPP1, we performed immunostaining and Western blot analysis. Lysates from CAR or control siRNA-transfected Caco-2 were collected and probed with antibodies to several TJ proteins including ZO-1 and MUPP1. Down-regulation of CAR had no effect on the expression of either ZO-1 or MUPP1 (Fig. 5B). Loss of CAR expression also did not affect the distribution of ZO-1 as assessed by immunostaining (Fig. 6A). Several other TJ and adherens junction proteins were unaffected by CAR silencing, including ZO-2, junctional adhesion molecule-1, claudin-1, and β-catenin (data not shown).

In cells transfected with a nonspecific siRNA, MUPP1 distribution remained intact, as characterized by a continuous ring circumscribing each cell (Fig. 6B). In contrast, MUPP1 was dramatically redistributed to punctate bodies within the cytoplasm in cells transfected with a siRNA directed against CAR (Fig. 6B). As determined by serial confocal cross-sections, these areas of punctate MUPP1 staining resided in the apical-most domain of the epithelium (within 1.75 μm) with little localization below the level of the apical TJ (data not shown).

To determine whether these bodies resulted from the relocalization or recycling of MUPP1 through endocytic vesicles or from its being targeted for degradation, we attempted to stain MUPP1 intracellular bodies with antibodies specific for endosomal markers and for components of the ubiquitin degradation pathway. MUPP1-positive bodies did not express the early endosome marker early endosome antigen 1 (13), the late endosome marker Rab7 (14), the lysosomal markers LIMP II (Fig. 7), or the late endosome/lysosomal marker LAMP I (data not shown) (15). In addition, MUPP1-positive bodies did not colo-

**Fig. 2.** Association between CAR and MUPP1 in epithelial cells. A, CAR and MUPP1 colocalize in Caco-2 cells by confocal microscopy. Cells were stained with anti-CAR RmcB and polyclonal anti-MUPP1 antibodies and examined by confocal microscopy. Areas of colocalization appear as yellow. B, Caco-2 cell lysates were immunoprecipitated with anti-MUPP1, control antibody (CON), or anti-CAR antibody. Immunoprecipitates (IP) were electrophoresed in SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and probed with a polyclonal anti-MUPP1 antibody.

**Fig. 3.** Association between transfected CAR and MUPP1. CHO cells stably transfected with plasmid alone (CHO-pcDNA), full-length human CAR (CHO-hCAR), CAR lacking its cytoplasmic domain (CHO-hCAR₉₀₉₀), or CAR with a deletion of its C-terminal hydrophobic motif (CHO-CARAQS) were transiently transfected with HA-MUPP1, and 48 h later lysates were immunoprecipitated (IP) with anti-CAR antibody RmcB, anti-HA, polyclonal anti-MUPP1, or control (CON) MOPC21 antibody. Immunoprecipitates were electrophoresed in SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and probed with a polyclonal anti-CAR antibody (A) or sheep polyclonal anti-MUPP1 antibody (B).
calize with internalized fluorochrome-labeled dextran (data not shown), further indicating that MUPP1 was not relocalized to endocytic compartments. MUPP1-positive intracellular bodies did not stain with antibodies specific for ubiquitin or the 26 S proteasome (data not shown). These data indicated that CAR is involved in the localization of MUPP1 to the TJ and that in the absence of CAR, MUPP1 may be retargeted to specialized vesicles within the cytoplasm.

**DISCUSSION**

We found that the CAR cytoplasmic domain interacts with the multi-PDZ domain protein MUPP1. The interaction specifically involves the PDZ-binding motif within the CAR C terminus and MUPP1 PDZ domain 13. In transfected cells, localization of CAR to cell-cell contact appears to recruit MUPP1. In polarized epithelial cells, endogenous CAR and MUPP colocalize and are physically associated within the tight junction. Inhibition of endogenous CAR expression in polarized epithelia by siRNA transfection leads to relocalization of MUPP1. MUPP1 localizes to the TJ and interacts with junctional adhesion molecule and with members of the claudin family of transmembrane proteins (11, 16). The loss of CAR within the TJ by siRNA transfection led to pronounced relocalization of MUPP1 from the apical TJ complex to punctate bodies within the apical domain. We were unable to identify or define the nature of the MUPP1 bodies, and it remains unclear whether they result from recycling of MUPP1 from the TJ or the whether targeting of MUPP1 to the TJ is disrupted in the absence of CAR. MUPP1 is known to interact with the junctional adhesion molecule and claudin-1, and it is not known whether loss of these components would have a similar effect. The evidence in CHO cells indicates that CAR is capable of recruiting MUPP1 to sites of cell contact and therefore may indicate that loss of CAR leads to improper targeting of MUPP1 to the TJ. Other junctional proteins including junctional adhesion molecule, claudin-1, and ZO-1 have been shown to reside in unique storage organelles following a calcium switch (17).
It has been reported that intercellular junctions are disrupted when adenovirus fiber is applied to the basal surface of a polarized epithelial monolayer (6). The mechanism by which this occurs has not been determined, and it will be interesting to learn whether CAR interactions with TJ proteins are affected during the process of virus entry into cells.

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In addition to TJ proteins, MUPP1 interacts with several important mediators of cellular signaling, which may indicate its role as an adaptor for intracellular signaling molecules. By yeast two-hybrid screening, MUPP1 interacts with serotonin 5-hydroxytryptamine type 2C receptors (18, 19), kinase negative c-Kit (20), the tandem plextin homology domain protein (21), and the membrane-spanning NG2 proteoglycan (22).

In several human tumors, CAR expression is down-regulated during progression to malignancy (23–25), and the expression of transfected CAR in CAR-deficient tumor cells leads to alterations in cell cycle regulation and decreased proliferation (23). It is interesting to note that MUPP1 is targeted by viral transforming proteins and that other junctional PDZ proteins regulate cell proliferation (26, 27). It remains to be determined whether the effects of CAR on cell proliferation are mediated through its interaction with MUPP1 or other junctional proteins.
