THE MOLECULAR BASIS OF THE INTERACTION BETWEEN THE COXSACKIEVIRUS AND ADENOVIRUS RECEPTOR (CAR) AND MAGI-1

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

By

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I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Abimbola Olayinka Kolawole ENTITLED The Molecular Basis of the Interaction Between the Coxsackievirus and Adenovirus Receptor (CAR) and MAGI-1 BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

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ABSTRACT

Kolawole, Abimbola Olayinka. Ph.D., Biomedical Sciences Ph.D. Program, Wright State University, 2011. The Molecular Basis of the Interaction Between the Coxsackievirus and Adenovirus Receptor (CAR) and MAGI-1.

A major factor in virus entry into cells is localization and abundance of the primary receptor. The Coxsackievirus and adenovirus receptor (CAR) is the primary receptor for group B coxsackievirus and many serotypes of adenovirus. In most epithelia, a seven exon isoform of CAR (CAR\textsuperscript{Ex7}) is exclusively localized at the basolateral surface where it behaves as a homophilic adhesion protein and is inaccessible for viral infection.

However, in well-differentiated human airway epithelia, we recently discovered an alternatively spliced, low abundance isoform of CAR (CAR\textsuperscript{Ex8}) that is apically localized where it may initiate apical viral infection. The two isoforms differ only in the last 26 (CAR\textsuperscript{Ex7}) or 13 (CAR\textsuperscript{Ex8}) amino acids of the cytoplasmic domain, which suggests that some intracellular interactions may differ. One such differential interaction involves MAGI-1, an essential PDZ-domain containing protein known to be involved in cell polarization and cancer. In non-polarized COS7 cells, the CAR\textsuperscript{Ex8} protein level is regulated by MAGI-1b. CAR-MAGI-1 interactions were investigated by MAGI-1 siRNA knockdown, \textit{in vitro} translation, immunocytochemistry, co-immunoprecipitation-Western blot analysis, fluorescence resonance energy transfer, direct binding assays, and adenovirus infection. Data showed that both CAR isoforms were expressed in several cell lines with CAR\textsuperscript{Ex7} RNA consistently more highly expressed than CAR\textsuperscript{Ex8} and MAGI-1 siRNA knockdown improved adenovirus infection in polarized cells. Both isoforms strongly interacted with PDZ3. CAR\textsuperscript{Ex8} also interacted with PDZ1. Whereas co-
expression of PDZ1 with CAR\textsuperscript{Ex8} did not affect adenovirus infection, it could compete with full length MAGI-1 to protect CAR\textsuperscript{Ex8} from loss. Co-expression of CAR\textsuperscript{Ex8} with PDZ3 significantly reduced CAR\textsuperscript{Ex8} cell surface expression and adenovirus infection but did not reduce total CAR\textsuperscript{Ex8} proteins levels suggesting that this may be the MAGI-1 PDZ domain responsible for holding CAR\textsuperscript{Ex8} within the cell but it is likely that other MAGI-1 interacting proteins/domains are required for loss of CAR\textsuperscript{Ex8}. This dissertation provides the molecular basis for MAGI-1-mediated regulation of CAR\textsuperscript{Ex8} which will allow further investigation into the mechanisms of CAR\textsuperscript{Ex8} cell surface expression and hence viral infection of polarized epithelia.
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DEDICATION

This is dedicated to

My girls: *Abimbola & Anuoluwapo*

And

My boys: *Abimbola & Temidun*

*You make all the sacrifice worth it*
CHAPTER 1: INTRODUCTION

Understanding the development, maintenance, and composition of the lung epithelial barrier is of great importance to human respiratory health and disease. This barrier segregates the microbial infested external environment from the body’s sterile internal environment. Each epithelial cell has two distinct domains: an apical or mucosal surface exposed to the air, and a basolateral surface in close communication with the internal environment. Investigations into the mechanisms of pathogenic microbial penetration of the lung epithelial barrier provide insights into its structure and regulation. Interestingly, many viruses use basolateral adhesion proteins as their primary receptor. Two distinct groups of viruses, Coxsackie B viruses (CVB) and most serotypes of adenovirus interact with the Coxsackievirus and adenovirus receptor (CAR), a transmembrane adhesion protein found on the basolateral membrane. A major unanswered question is how these pathogenic viruses initiate infection when the receptor is safely segregated on the basolateral membrane.

Several protein isoforms have been described for CAR, including two transmembrane forms (CAR\textsubscript{Ex7} and CAR\textsubscript{Ex8}; Fig.1). These two isoforms differ only in their C-termini, suggesting that some of their interactions, and hence localization and regulation, may differ. In polarized epithelial cells, CAR\textsubscript{Ex7} resides on the basolateral surface and is thus sequestered away from potential viral interactions on the apical surface (Fig.2A). In contrast, although CAR\textsubscript{Ex8} is a less abundant isoform, recent work
Fig. 1 Schematic of the two transmembrane-containing CAR isoforms.

A) $\text{CAR}^{\text{Ex7}}$ and $\text{CAR}^{\text{Ex8}}$ are identical except for the C-terminal 26 or 13 amino acids.

B) The $\text{CAR}^{\text{Ex7}}$-specific 26 amino acid sequence is distinct from the sequence of $\text{CAR}^{\text{Ex8}}$.

Each isoform sequence is not present in the other isoform due to splicing within exon 7.
reveals that CAR\textsuperscript{Ex8}, under certain conditions, is localized apically where it can mediate initiation of adenovirus infection from the apical surface (Fig.2B)(1). This is a paradigm shift from the commonly held belief that there must be a transient or sustained break in the barrier for the virus to gain access to the receptor.

The apical and basolateral surfaces of lung epithelial cells are quite different in terms of lipid and protein composition, a result of intracellular sorting and trafficking. It is generally accepted that protein sorting pathways have key molecules that recognize specific protein, lipid, or post-translationally modified sequences. One large class of such key molecules is the family of PSD95/Dlg/ZO-1 (PDZ)-domain containing proteins. Membrane-associated guanylate kinase inverted-1 (MAGI-1) is an essential member of this group. Our recent work suggests a novel function for MAGI-1 isoform b (MAGI-1b) - the destabilization and degradation of CAR\textsuperscript{Ex8} (1). I hypothesized that receptor abundance and apical localization are regulated by a PDZ-based interaction with MAGI-1b. The objective of this study was to understand the molecular basis of this interaction through PDZ domain isolation, binding and competition, and ultimately the effects on viral infection. Understanding what causes this surface location and how this is regulated will provide insight into susceptibility to viral infections, and lead to strategies both for prevention of virus infection and facilitation of adenovirus-mediated gene therapy for the treatment of inherited and acquired respiratory diseases.

**Coxsackievirus and Adenovirus**

The Coxsackie B viruses, first discovered in Coxsackie, New York in 1948, are non-enveloped icosahedral single stranded RNA viruses about 30 nm in size. They are human picornaviruses (2) that are very resistant to various chemical treatments and cause
Fig. 2 Localization and accessibility of the Coxsackievirus and adenovirus receptor (CAR) on a polarized cell.

(A) CAR$^{\text{Ex7}}$ is absent from the apical surface of polarized cells and thus there is no adenovirus infection. (B)

(B) When CAR$^{\text{Ex8}}$ is present at apical surface, adenovirus is able to infect the cell from the apical surface.
diseases including myocarditis (3), cardiomyopathy and pancreatic inflammation (4). Adenoviruses were first identified from tonsils in 1953 (5, 6). In 1968 the Commission of Acute Respiratory Diseases of the U.S. Armed Forces found that adenovirus infection was one of the major causes of acute febrile respiratory illness in recruits (7).

Adenoviruses are non-enveloped, icosahedral viruses approximately 80 nm in size (Fig. 3). The adenovirus capsid is made up of 240 copies of the hexon proteins with a penton base with protruding fiber knobs at each of the 12 vertices of this icosahedral virus. The knob region of the fiber-knob is required to attach to the extracellular domain of CAR (8). Over the past 5 decades, adenovirus research has yielded impressive knowledge about the pathogenesis of viral pneumonias, vaccination, and basic aspects of molecular virology and cellular biology. Moreover, adenovirus has emerged as a potential vector for gene based therapeutics. However, improvement in therapeutic efficacy as a vector is required for clinical use, and wild type pathogenic adenovirus remains a significant military and civilian threat. This study focused on factors that influence adenovirus infection of cells.

The Coxsackievirus and Adenovirus Receptor

CAR was first described in 1997 as the primary receptor for Coxsackie B viruses (CVB) and most adenovirus serotypes (9). It is now known that CAR is a developmentally essential cell adhesion protein. Deletion of the murine CAR gene results in embryonic death around E11.5 with apparent cardiovascular complications (10-12). Although loss in adult tissues is less severe, specific knock-out of CAR within the adult heart leads to a block in arterio-ventricular transduction, apparently due to loss of
Fig. 3 Adenovirus and its interaction with CAR. A) Adenovirus capsid is made up of 240 copies of hexon proteins. Of note is the penton base and fiber-knob. A) Adenovirus contains ~38 kb of double-stranded DNA. B) Adenovirus attaches to cells through the binding of the knob region of the fiber knob to the D1 domain of CAR. The interaction between the penton base and cellular integrin aids internalization (13).
connexin 45, ZO-1, and β-catenin localization at the intercalated disc (14, 15). Recently, the essential nature of CAR for CVB-mediated pathogenesis in the heart and pancreas was established (16, 17). It was demonstrated that CVB-mediated pancreatic and cardiac pathology in the mouse model system is virtually abrogated in the tissue specific absence of CAR, revealing an amazing proof of principal for the importance of a primary receptor for viral pathogenesis. Although not addressed in this dissertation, the role of CAR in lung development and viral-mediated respiratory disease in the conditional knock-out mouse model will yield additional interesting insights into the importance of this protein.

**CAR in the airway**

CAR plays a central role in airway epithelial cell-cell adhesion (18-20) and adenoviral egress. The previously studied CAR isoform (CAR\textsuperscript{Ex7}) resides in the junctional adhesion complex of polarized epithelia where it plays a role in the maintenance of the epithelial adhesion complex (18-23). Disruption of CAR-CAR interactions by adenovirus, adenovirus fiber protein, or antibody against CAR, leads to a disruption of the tight junctions and increased transepithelial permeability. Accordingly, adenovirus is able to escape from the lung by disrupting the tight and adherens junctions through interactions with CAR\textsuperscript{Ex7}. In contrast to assisting with egress, the basolateral localization of CAR\textsuperscript{Ex7} is thought to provide an innate barrier against the initiation of adenoviral infection. The current model for initiation of viral infection requires a breach in the tight junction barrier allowing the virus access to the basolateral receptor (24, 25). For example, a small number of CVB interact with the decay accelerating factor (DAF) – a protein found at the apical surface of epithelia (26, 27). Interaction of CVB with DAF in Caco-2 cells, a polarized intestinal epithelial cell line, activates kinase signaling
cascades that result in viral translocation to epithelial cell junctions and a reduction of tight junction integrity. This breach in the junctions is required to allow an essential interaction with basolaterally localized CAR, and viral entry, likely by macropinocytosis. The requirement for junctional penetration may be overcome by apical CAR expression, as experimentally shown by substituting the transmembrane and C-terminus of CAR with a glycoposphatidylinositol (GPI) tail (21). Consistent with this, our recent work suggests that there is a direct pathway for adenovirus infection in the airway – adenovirus binding to endogenous CAR\textsuperscript{Ex8} on the apical membrane (1). While the biological reason behind apical localization of an adhesion protein is unknown, modulation of its expression levels presents an opportunity to affect viral susceptibility.

Splicing of CAR

Alternative splicing not only regulates protein expression, but also allows multiple proteins to be expressed from the same gene resulting in significant proteomic diversity. Human CAR was first described by Bergelson \textit{et al.} as a seven exon encoded protein (9). In contrast to other species, mouse CAR (mCAR) was initially cloned as a protein composed of eight exons (28). Transcripts for the seven-exon mouse and eight-exon human forms were subsequently identified (3). A detailed analysis of protein expression and localization in mice has revealed differential tissue dependent expression and localization for the mCAR\textsuperscript{Ex7} and mCAR\textsuperscript{Ex8} isoforms (29-31). This suggests that interactions and potentially the functional importance of these two isoforms may be distinct. Furthermore, considering the emerging importance of signal transduction originating from microdomains within the cell membrane, these two isoforms would be predicted to differentially regulate cellular biology. We are the first group to investigate
the importance of human CAR_{Ex8} (1). The alternative splicing event that creates CAR_{Ex8} occurs within the seventh exon. Thus, these two isoforms contain the same extracellular and transmembrane domains (Fig. 1). The majority of the cytoplasmic C-terminal domain is identical except for the last 26 (CAR_{Ex7}) or 13 (CAR_{Ex8}) amino acids. Although comprised of distinct sequences, the last 4 amino acids of both isoforms encode class I (PDZ domain binding sequences (-X-(S/T)-X-Φ, where X = any amino acid and Φ = hydrophobic amino acid) PDZ binding domain. PDZ-domain containing proteins known to interact with human CAR_{Ex7} include MAGI-1, PICK1, PSD-95, MUPP-1, LNX1, and ZO-1 (18-20, 32, 33). Both mCAR_{Ex7} and mCAR_{Ex8} interact with LNX2 and this interaction appears to be modulated by both the PDZ-binding domain of each isoform as well as an upstream sequence common to both isoforms (34).

**CAR isoform specific localization in primary airway epithelia**

In polarized primary human airway epithelia grown at the air-liquid interface, each of the CAR isoforms manifests distinctly different properties (Fig. 4D-H). Primary cultures of well-differentiated human airway epithelia, greater than two weeks of age, were stained for endogenous CAR_{Ex7} (Fig. 4D, green), CAR_{Ex8} (Fig. 4E, green) with antibodies raised against peptides composed of the last 13 amino acids of either CAR_{Ex7} or CAR_{Ex8}, or pre-immune serum control (Fig. 4F, green), and were co-stained for the tight junction protein ZO-1 (red). Distinct patterns of localization were observed for these two isoforms. As previously shown, CAR_{Ex7} localizes to the tight and adherens junctions of airway epithelia (18-20, 23). In contrast, CAR_{Ex8} does not overlap with ZO-1 at the tight junctions. Instead, CAR_{Ex8} localizes primarily to the upper region of the cytoplasm and a few puncta appear at the apical surface above ZO-1 (Fig. 4E, arrow). The low level
of localization above ZO-1 co-relates well with the low apical infection efficiency of adenovirus under endogenous conditions. However, the finding of apical localization suggests susceptibility to apical adenovirus infection may be related to \( \text{CAR}^{\text{Ex8}} \) expression and localization.

**\text{CAR}^{\text{Ex8}} \text{ mediates adenovirus infection of primary airway epithelia}**

We hypothesized that \( \text{CAR}^{\text{Ex8}} \) may be responsible for the inefficient albeit detectable level of adenovirus infection from the apical surface of airway epithelia. Augmenting expression of \( \text{CAR}^{\text{Ex7}} \) results in mainly junctional localization (Fig. 4G). In contrast, \( \text{CAR}^{\text{Ex8}} \) localizes diffusely within the cell (Fig. 4H). To determine whether this altered localization results in increased apical infection, cultures overexpressing \( \text{CAR}^{\text{Ex7}} \), \( \text{CAR}^{\text{Ex8}} \), or GFP, were infected from the apical surface with adenovirus containing the \( \beta \)-galactosidase gene (Ad-\( \beta \)-Gal, Fig. 4I). Cultures expressing GFP showed a baseline level of adenovirus-mediated gene transfer and represent the low albeit detectable infection observed in the airway. Although cultures expressing h\( \text{CAR}^{\text{Ex7}} \) showed increased apical infection, there was roughly a 2-fold increase in gene transfer to cultures expressing \( \text{CAR}^{\text{Ex8}} \) over cultures expressing \( \text{CAR}^{\text{Ex7}} \) \((p<0.01)\). This increase in infection is similar to previously published results for glycophosphatidylinositol-linked CAR, which is missing the transmembrane and cytoplasmic domains and localizes explicitly to the apical surface of polarized airway epithelia (19). These data also suggest that there may be a limit to receptor usage at the apical surface. Overall, this suggests that \( \text{CAR}^{\text{Ex8}} \) could be the endogenous apical receptor and raises the question why \( \text{CAR}^{\text{Ex8}} \) does not localize to the basolateral surface.
Fig. 4 In contrast to non-polarized cells, CAR\textsuperscript{Ex8} localizes and behaves distinctly from CAR\textsuperscript{Ex7} in well-differentiated primary airway epithelia. In non-polarized cells, both A) CAR\textsuperscript{Ex7} and B) CAR\textsuperscript{Ex8} localize to cell-cell junctions in transfected COS-7 cells (light brown arrow), and C) mediate-adenovirus infection similarly in transfected CAR-negative CHO-K1 cells. In polarized primary airway epithelia endogenous D) CAR\textsuperscript{Ex7} (green) overlaps (yellow) and is basolateral to the tight junction protein ZO-1 (red), E) CAR\textsuperscript{Ex8} (green) localizes to an apical compartment and is distinct from ZO-1 (red). The arrow indicates CAR\textsuperscript{Ex8}-specific staining above the ZO-1 delineated tight junction. F) Background staining with pre-immune rabbit serum (green) and ZO-1 (red). Over-expression results in G) CAR\textsuperscript{Ex7} localization primarily at the basolateral junctions and H) CAR\textsuperscript{Ex8} localization diffusely and at the apical surface of primary airway epithelia. I) Augmenting CAR\textsuperscript{Ex8} expression significantly increases apical adenovirus infection. *p<0.01 vs. CAR\textsuperscript{Ex7} or GFP. Dotted line represents support membrane; AP, Apical surface; BL, Basolateral surface. Confocal microscopy (60x oil immersion) (1).
**PDZ interactions**

Many diverse modular protein interacting domains have been described. The PSD-95/DlgA/ZO-1 or PDZ domain, named after the three prototypical proteins containing this type of domain, is a modular sequence approximately 90 aa in length (35, 36) that generally interacts with specific C-terminal motifs named PDZ binding domains. Despite intense study, the biological importance of these PDZ domains is still being deciphered. The potential complexity of PDZ-based interactions stems from the array of domains these “scaffolding” type proteins contain. In particular, membrane-associated guanylate kinases (MAGUKs) contain one or several PDZ domains along with other interacting domains that allow diverse interactions and often multimerization. MAGI-1 is a member of the MAGUK family for which three isoforms have been described (MAGI-1a, b or c; Fig. 5) (37, 38). Each isoform has distinct tissue distribution with MAGI-1b being present in the basolateral junctions of epithelial tissues. MAGI-1 contains an inactive guanylate kinase domain, two WW domains and six PDZ domains. Many important interactions have been described for MAGI-1b including tumor suppressor PTEN, several ion channels, and viral oncogenes, thus implicating it in epithelial junction composition and stability, epithelial-mesenchymal transition, and invasiveness (Table 1).

Whether PDZ-domain containing proteins are passive scaffolding proteins bringing a variety of other proteins into close proximity, resulting in functional interactions, or are active participants in discerning binding partners, and modulating the respective functions, remains debated. Studies such as the one present here will help elucidate the regulation, function and importance of a member of this important category of proteins and its relevance to normal and disease processes.
Fig. 5 Schematic of MAGI-1 isoforms. MAGI-1 has three distinct isoforms. MAGI-1b is the predominant lung epithelial isoform. PDZ – PSD95/DLG/ZO-1 domain; GuK – guanylate kinase domain; ww – ww domain; α/β alternatively spliced internal sequences; A/B/C – alternatively spliced C-terminal sequence which defines the isoform.
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CAR^{Ex7} and CAR^{Ex8} interactions with PDZ-domain-containing proteins

PDZ interactions may modulate localization as well as function. It is known that both the four C-terminal amino acids that directly interact with a PDZ domain and the upstream sequences affect PDZ domain interactions. Although CAR^{Ex7} and CAR^{Ex8} both have type 1 PDZ-binding domains at the C-terminus (GSIV in the case of CAR^{Ex7}, and ITVV in the case of CAR^{Ex8}), these and the upstream sequences are distinct. Thus, we hypothesized that differences in PDZ interactions may be responsible for their differential localization in human airway epithelia. We have previously shown that CAR^{Ex7} interacts with MAGI-1b via a PDZ-binding domain specific interaction (1). In contrast to the primarily junctional localization of CAR^{Ex7} (Fig. 4A), MAGI-1b is diffusely expressed within the cytoplasm of transfected COS-7 cells (Fig. 6A). However, when CAR^{Ex7} and MAGI-1b are co-transfected, CAR^{Ex7} and MAGI-1b co-localize at the junctions between cells (Fig. 6B, C, D). This interaction can also be observed by co-immunoprecipitation (19). In view of these results we hypothesized that CAR^{Ex8} also interacts with MAGI-1b.

COS-7 cells were co-transfected with CAR^{Ex8} and MAGI-1b-GFP and evaluated by immunocytochemistry. While cells transfected with CAR^{Ex8} alone exhibited junctional localization of CAR^{Ex8} (Fig. 6B), little to no CAR^{Ex8} staining (red, Figure 6E) was present in cells co-expressing MAGI-1b-GFP (green, Fig. 6F, merge G).

Most of the small amount of CAR^{Ex8} present within MAGI-1b-GFP positive cells appeared within vesicular structures and not at the junctions. A small percentage of cells appeared to express CAR^{Ex8} alone (Fig. 6H) and the expression was robust in comparison to neighboring MAGI-1b-GFP expressing cells (Fig. 6I, merge J). This was not the case after co-transfection with GFP or another PDZ-domain containing protein, PSD-95-GFP,
where abundant co-expression and CAR\textsuperscript{Ex8} junctional localization was evident (data not shown).

To determine a physiological response to the loss of CAR\textsuperscript{Ex8} in the presence of MAGI-1b-GFP, CAR-deficient non-polarized CHO-K1 cells were transfected with CAR\textsuperscript{Ex8} or MAGI-1b-GFP alone, or co-transfected with CAR\textsuperscript{Ex8}, at a constant dose, with MAGI-1b-GFP at increasing doses. All transfections were balanced with GFP plasmid to maintain equal amounts of DNA. Transfected cells were transduced with Ad-β-gal 48 hr later. Beta-galactosidase expression was determined 24 hr post-transduction. Similar to Fig. 4C, transfection of CHO-K1 cells with CAR\textsuperscript{Ex8} renders them susceptible to adenovirus infection while transfection with MAGI-1b-GFP does not. Co-transfection of CAR\textsuperscript{Ex8} with MAGI-1b-GFP resulted in a dose-related reduction of susceptibility to Ad-β-gal-mediated gene expression (1), suggesting that there was a dose-dependent reduction of cell surface CAR\textsuperscript{Ex8} available as a receptor. This was also reflected by immunofluorescence and Western blotting quantification (1). The observed difference in CAR\textsuperscript{Ex8} protein was not due to transfection or transcription since quantitative PCR for plasmid and RT-PCR for CAR\textsuperscript{Ex8} RNA were similar (data not shown). Taken together with the previous data, we concluded that in contrast to CAR\textsuperscript{Ex7}, co-expression of CAR\textsuperscript{Ex8} with MAGI-1b-GFP results in a reduction in CAR\textsuperscript{Ex8} and may explain the absence of CAR\textsuperscript{Ex8} in the junctions of airway epithelia.
Fig. 6 Co-expression of CAR$_{Ex8}$ and MAGI-1b-GFP results in the loss of CAR$_{Ex8}$. A) Expression of MAGI-1b-GFP alone results in diffuse localization of MAGI-1b-GFP. Co-expression of B) CAR$_{Ex7}$ (red) and C) MAGI-1b-GFP (green) results in co-localization of MAGI-1b-GFP and CAR$_{Ex7}$ (D, merge, yellow). In contrast, co-expression of CAR$_{Ex8}$ (E, red) and MAGI-1b-GFP (F, green) results in decreased levels of CAR$_{Ex8}$ (E, G). Rare cells devoid of MAGI-1b (I, green) showed abundant expression of CAR$_{Ex8}$ (H, red, merge J). Non polarized COS-7 cells. Confocal microscopy (60x oil immersion) (1).
**CAR\textsuperscript{Ex8} PDZ degradation requires both the PDZ-binding domain and the upstream sequence**

The interaction of CAR\textsuperscript{Ex8} with MAGI-1b requires the CAR\textsuperscript{Ex8} PDZ-binding domain. Deletion of the CAR\textsuperscript{Ex8} ITVV PDZ-binding domain sequence allows CAR\textsuperscript{Ex8} protein expression at cell junctions, where no co-localization with MAGI-1 is observed (1). However, the PDZ-binding domain is not sufficient and the degradation phenotype requires the upstream CAR\textsuperscript{Ex8} -specific sequence. Interestingly, swapping the CAR\textsuperscript{Ex7} GSIV sequence with the CAR\textsuperscript{Ex8} ITVV sequence does not swap the degradation phenotype (data not shown)(1). These data show that the ITVV sequence interacts with MAGI-1b but that the 9 amino acids upstream to the CAR\textsuperscript{Ex8} ITVV sequence play an important role in degradation. We hypothesize that this reflects a CAR\textsuperscript{Ex8} specific interaction with MAGI-1b directly. However, other proteins may modulate this direct interaction.

**Model for CAR\textsuperscript{Ex8} trafficking in polarized cells**

Remarkably little is known about the intracellular C-terminus of CAR. The C-terminus encodes many motifs potentially involved in processes such as phosphorylation and protein-protein interactions that may play a role in cell adhesion, cell division, and lead to signaling, such as NF-κB activation (19, 58, 59). This domain is also critical for basolateral localization of CAR\textsuperscript{Ex7} within the epithelium (20, 23). The surprising apical localization of CAR\textsuperscript{Ex8} suggests that although both CAR isoforms contain many of the same motifs, the CAR\textsuperscript{Ex8} 13 unique c-terminal amino acids are dominant over other key upstream sequences shown to direct CAR\textsuperscript{Ex7} basolateral targeting. I hypothesize that if
CAR\textsuperscript{Ex8} interacts with MAGI-1b during the translation process or within the basolateral targeting pathway, it will be degraded (Fig. 7-I). Thus, MAGI-1b regulates the levels of

**Fig. 7** Model for MAGI-1b as a master switch for CAR\textsuperscript{Ex8} abundance and localization in polarized epithelial cells. A) (I) Newly translated CAR\textsuperscript{Ex8} entering the basolateral trafficking pathway is destabilized upon binding to MAGI-1b, leading to degradation. (II) CAR\textsuperscript{Ex8} entering the apical trafficking pathway safely makes it to an apical compartment and mediates adenovirus infection. B) Increased MAGI-1b expression leads to additional CAR\textsuperscript{Ex8} degradation, and thus decreased apical CAR\textsuperscript{Ex8} and viral infection. C) Decreased MAGI-1b expression leads to increased CAR\textsuperscript{Ex8} apical expression and viral infection. ER, endoplasmic reticulum; G, Golgi; TJ, tight junction; AP, apical (air) surface; BL, basolateral surface.
CAR^{Ex8} within the cell and excludes CAR^{Ex8} from the basolateral junctions. However, I further hypothesize that CAR^{Ex8} escapes this regulation by entering an alternative pathway that leads to the apical compartment, and thus, it can (and does) survive within the polarized cell (Fig. 7-II). My major question is what clinically relevant molecular targets can be altered to decrease or increase apical CAR^{Ex8} expression levels. I hypothesize that MAGI-1b is the master switch and thus aim to understand the mechanism of interaction, and the effect of the critical domain(s) on CAR^{Ex8} expression and adenovirus infection.

This work is an essential first step to investigate the molecular basis of CAR^{Ex8} regulation, interactions, and hence localization. Fundamental regulatory mechanisms discovered in this research will be used to identify small molecules that may have clinically relevant implications such as the prevention of pulmonary viral infections and augmenting gene therapy approaches.

Objective

The over-arching hypothesis of this study was that CAR^{Ex8} protein levels and localization in epithelial cells are regulated by PDZ-based interactions with MAGI-1. My aim was to dissect these interactions and determine the impact on CAR^{Ex8} stability, localization and activity with respect to adenoviral infection. The three specific aims outlined below were used to achieve the objective.

Specific aims

The first goal of this study was to determine the effect of MAGI-1 knockdown on CAR^{Ex8} expression. Based on our previous data, which suggested that MAGI-1 degrades
CAR^{Ex8} and is involved in apical localization (1), I hypothesized that increasing MAGI-1 will result in the decrease of apical CAR^{Ex8} due to additional quality control, and conversely, decreasing MAGI-1 or interfering with the PDZ-based regulation will augment apical CAR^{Ex8} expression and apical localization since more CAR^{Ex8} will escape degradation. We already reported indirectly, by adenovirus infection, a reduction in surface CAR^{Ex8} level due to increasing MAGI-1 in cells (1), but there is no report of the effect of MAGI-1 knockdown on CAR^{Ex8} levels in cells. MDCK cells that expressed CAR isoforms, MAGI-1, and also displayed characteristics like primary airway epithelia were used for MAGI-1 siRNA knockdown assays. The results from MAGI-1 knockdown experiments were used to support the earlier report that MAGI-1 contribute to CAR^{Ex8} loss in airway epithelia.

The second goal was to determine the molecular basis of the interaction between CAR isoforms and MAGI-1. Our preliminary data also show that both isoforms of CAR interact with MAGI-1b in a PDZ dependent manner. However, it is not clear if they interact with the same or different PDZ domains. MAGI-1 contains 6 PDZ domains (PDZ0-PDZ5). A list of known interactions and the respective domain, if known, is given in Table 1. In order to understand the molecular basis of interaction between CAR and MAGI-1, identification of the specific MAGI-1 domain involved is an important step. There is, therefore, a need to investigate the domains and the affinity for CAR with the ultimate goal of understanding how this affects CAR^{Ex8} degradation in the presence of MAGI-1. Several components, beyond consensus sequence, may define a PDZ-based interaction, such as binding affinity, domain structure, other proteins present in the complex, and modifications such as phosphorylation. Considering the distinct sequences
and the difference in the result of interaction, I hypothesized that each isoform binds to a different PDZ domain in MAGI-1. It will be a significant advance to know which MAGI-1b PDZ domain(s) CAR\textsuperscript{Ex8} interacts with and if the two CAR isoforms interact with the same or different PDZ domains. This knowledge will allow us to dissect the functional consequences of these interactions, define the important residues in these interactions, and lead to a greater understanding of the role of these protein interaction domains within MAGI-1. Specifically, the specific MAGI-1 PDZ domains that interacted with CAR isoforms were determined by immunoprecipitation, pull down and binding assays. The ability of the isolated MAGI-1b PDZ domains to compete with the wild type domain to alter the interaction with CAR\textsuperscript{Ex8} in cells was also studied.

The third goal was to determine the physiological effects of CAR-MAGI-1 interactions on adenovirus infection. The physiological response to the loss of CAR\textsuperscript{Ex8} in the presence of MAGI-1b is demonstrated by reduced adenovirus infection in cells. If an isolated MAGI-1 PDZ domain competes with wild type domain to prevent CAR\textsuperscript{Ex8} degradation by MAGI-1, a resultant increase in adenovirus infection is expected. It is therefore necessary to investigate the possibility of an isolated PDZ domain outcompeting wild type MAGI-1. If this were discovered it becomes a step towards designing some small molecules that can be used to regulate MAGI-1-CAR interactions for the purpose of reducing adenovirus infection during an outbreak or increasing adenovirus infection for gene therapy.

Hypotheses

The hypotheses are that: 1) more CAR\textsuperscript{Ex8} will be expressed at the cell surface when MAGI-1 is knocked down, 2) each isoform binds to a different PDZ
domain in MAGI-1 and 3) compared to a control, isolated MAGI-1 PDZ domain(s) will prevent $\text{CAR}^{\text{Ex8}}$ degradation by out competing the full length interaction and allow adenovirus infection.
CHAPTER 2: MATERIALS AND METHODS

Materials

Cell Lines

Nine immortal epithelial cell lines were used for various experiments (Table 2). Nuli-1 and HEK 293 cell lines were obtained from the University of Iowa Cell Culture Core. Clontech Laboratories Inc., CA., supplied 293T cell line while the remaining cell lines were purchased from ATCC, Virginia. All cell lines were stored in liquid nitrogen in the Department of Biological Sciences at Wright State University Dayton OH.

Culture Media

The cells were cultured in appropriate sterile culture medium (Gibco™ Invitrogen Corporation, Grand Island, NY) supplemented with 10% fetal bovine serum (except MDCK, supplemented with 5% FBS) immediately after removing the cells from liquid nitrogen. After stimulation of growth, the culture medium was further substituted with a final medium-serum mix containing penicillin and streptomycin antibiotics. The powdered culture medium, containing L-glutamine and 25 mM HEPES buffer, was dissolved in distilled deionized water (ddH₂O) from a Nanopure Millipore Water System. All culture media were supplemented with 3.024g/L sodium bicarbonate. The solution was adjusted to pH 7.0 to 7.1 with 1 N HCl dropwise. The culture media solutions were sterilized through a 0.2 µm-pore cellulose nitrate Nalgene vacuum filtration apparatus. Sterile media were supplemented with 0.5% penicillin/streptomycin and the appropriate
heat inactivated fetal bovine serum by appropriate volume percentage. The final culture media was stored in 500-ml Pyrex bottles and kept at 4ºC.

**Competent Cells**

*Escherichia coli* competent cells were used for transformation experiments according to their unique properties (Table 3)

**Antibodies**

The antibodies used in this study are listed in Table 4.

**Primers**

The list of primers used in this study is shown in Table 5.
Table 2 Immortal cell lines.

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<th>Cell name</th>
<th>Cell type</th>
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Nine cell lines were investigated for expression and localization of both CAR isoforms. These cells were also studied for the ability to polarize similarly to primary human airway epithelia.
Table 3 Competent cells.

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*Escherichia coli* competent cells were used for transformation experiments according to their unique properties.
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<tr>
<td>Myc</td>
<td>Sigma</td>
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<td>V5</td>
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<td>R960CUS</td>
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<td>1:1000-1:2000</td>
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<td>Invitrogen</td>
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### Table 5A Primers

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<tr>
<td><strong>Regular PCR to determine the expression in cell lines</strong></td>
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<tr>
<td>CAR-4</td>
<td>F  GTCCCTCCTTCAAATAAAGGT</td>
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<td></td>
<td>R  CAGCTTTATTTGAAGGAGGCAC</td>
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<td>CAR-6</td>
<td>F  GTCCTTGTTAACGCTTCAGG</td>
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<td>R  CGGATCCCTATATACCTAGACCACATC</td>
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<tr>
<td>CAR&lt;sup&gt;Ex8&lt;/sup&gt;</td>
<td>R  GTGGATCCTTATACACTGTAATCCTCA</td>
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<td>R  GTGGATCCTTATACACTGTAATCCTCA</td>
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<tr>
<td>hMAGI</td>
<td>F  TGGATTTTGGACGCACGTAATTT</td>
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<td></td>
<td>R  AGCCACACGATGCATTGCCAAAG</td>
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<td>hMAGI</td>
<td>F  GCTGCAATGGATGGCAAGATG</td>
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<td></td>
<td>R  TGAGTGCTATGGAGGAAGC</td>
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<td>cMAGI</td>
<td>F  GCATAGGCCATGCAGGAAGATG</td>
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<td></td>
<td>R  TGTGTCTTCCGGTGATGTGGTC</td>
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<tr>
<td><strong>qPCR to determine relative expression in cells</strong></td>
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<tr>
<td>CAR&lt;sup&gt;Ex7&lt;/sup&gt;</td>
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<td>hHK</td>
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<tr>
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<td></td>
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<td><strong>MAGI knockdown (siRNA)</strong></td>
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<td>siRNA-A</td>
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<td>siRNA-B</td>
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<td>siRNA-C</td>
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<td>R  CAGCCACCGGAGGCUUGGCUUCAAA</td>
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F= forward, R=reverse, HK, housekeeping genes, h= human, c= canine
**Table 5B Primers**

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<th>Primer</th>
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<td>MAGI-1 PDZ domains cloning into pcDNA TOPO expression vector</td>
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<tr>
<td>PDZ1</td>
<td>F GACCCGAAATTCGCCACCGATGGAAACTCCACCTTCAGGGA R GGATCCGGCCACCCAGGAGTGCACAAAC</td>
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<td>PDZ2</td>
<td>F GACCCGAAATTCGCCACCGATGGAAACTTCCTTCAACAGC R GGATCCGTCTTTCCCTCCATGGACG</td>
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<tr>
<td>PDZ3</td>
<td>F GACCCGAAATTCGCCACCGATGGAAACTTCCTTCAACAGC R GGATCCGTCTTTCCCTCCATGGACG</td>
</tr>
<tr>
<td>PDZ4</td>
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<tr>
<td>PDZ5</td>
<td>F GACCCGAAATTCGCCACCGATGGAAACTTCCTTCAACAGC R GGATCCGTCTTTCCCTCCATGGACG</td>
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**Cloning into pHH2 vector (In-Fusion cloning)**

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<th>Forward primer</th>
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<td>GGGGGCCCCTTGGGATCC...</td>
<td>GTCGACCCTGGAAATTCGG....</td>
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**PDZ0**
- F ATGTCGAAATGGATCCAGAAGAAGAACC
- R TTACTTGGTGAGCCTTCCTCTTCTTGTC

**PDZ1**
- F AGGGAAAATCCCATTCAGGGAACAAAC
- R TTATGTGTTGAGCTTTCCTCCATAGG

**PDZ2**
- F ATGTCGAAATGGATCCAGAAGAAGAACC
- R TTACTTGGTGAGCCTTCCTCTTCTTGTC

**PDZ3**
- F ATGTCGAAATGGATCCAGAAGAAGAACC
- R TTACTTGGTGAGCCTTCCTCTTCTTGTC

**PDZ4**
- F ATGTCGAAATGGATCCAGAAGAAGAACC
- R TTACTTGGTGAGCCTTCCTCTTCTTGTC

**PDZ5**
- F ATGTCGAAATGGATCCAGAAGAAGAACC
- R TTACTTGGTGAGCCTTCCTCTTCTTGTC

**CARfull**
- F ATGTCGAAATGGATCCAGAAGAAGAACC
- R TTACTTGGTGAGCCTTCCTCTTCTTGTC

**CAREx7**
- F ATGTCGAAATGGATCCAGAAGAAGAACC
- R TTACTTGGTGAGCCTTCCTCTTCTTGTC

**CARTerm**
- F ATGTCGAAATGGATCCAGAAGAAGAACC
- R TTACTTGGTGAGCCTTCCTCTTCTTGTC

**GFPMAGI1b**
- F ATGTCGAAATGGATCCAGAAGAAGAACC
- R TTACTTGGTGAGCCTTCCTCTTCTTGTC

**MAGI1ctermR**
- F ATGTCGAAATGGATCCAGAAGAAGAACC
- R TTACTTGGTGAGCCTTCCTCTTCTTGTC

**GGATCC** BamHI restriction site

**GAATTC** EcoRI restriction site

In-Fusion cloning. Fifteen nucleotides that overlap with the vector nucleotides were designed to be included in each of the primers for any gene to be cloned into such vector.
Methods

Cell Culture Maintenance

Cell culture techniques were performed under a laminar flow hood using aseptic techniques. Cell cultures were stored and grown in 25- or 75-cm² tissue culture flasks within a humidified incubator at 37°C and 5% CO₂. Used media-serum mix was discarded and new media-serum mix was added, based upon a pH change from 7.1 to about 6.0, as indicated by a color change of the media from pink to yellow. As cells began to divide and reached about 80% confluency, as observed under 400X total magnification with an inverted microscope, the cell cultures were divided into new flasks using a ratio of between 1:5 and 1:20 cells to media after removing attached cells with 0.25% Trypsin-EDTA (Mediatech, Inc., Manassas, VA). All flasks were supplied with new media-serum mix to a final volume of 5 ml in a 25-cm² tissue culture flask and 10 ml in a 75-cm² tissue culture flask. Cell viability was verified before each experimental set using the trypan blue exclusion test (60). Dead cells appeared blue since the membranes became permeable to trypan blue dye. Cell viability above 80% was good for experiments.

Determination of Cell Concentration (Hemocytometry)

The technique reported was used during cell seeding before transfection and adenovirus infection. Adherent monolayers of cells were trypsinized from 1-2 flasks and combined in a 50 ml centrifuge tube. The tube was centrifuged at 150 rcf for 5 min. at 4°C. The supernatant was aspirated and the cells resuspended in 1 ml culture medium and mixed gently by pipetting up and down. A clean coverslip was centered on a hemocytometer between the outside railings over the two counting grids after wetting
rails with water. This helped to hold down the coverslip while loading the cells. A drop of well-mixed cell suspension was placed at each notch. The drop was added once to ensure even distribution of cells. Cells were counted with a push button counter using 400X total magnification. Cells were counted in the four corner (1mm X 1mm) squares of the cytometer. These squares were 1/400 mm$^2$. The average value of the total cells counted in the 4 squares was multiplied by 10$^4$ to determine the number of cells per milliliter. The volume of the mixture was adjusted by dilution with culture media to an appropriate concentration, depending on the particular assay.

**Total Cellular RNA Extraction**

Total cellular RNA was extracted from treated and control cells using TRIzol$^R$ Reagent (Invitrogen Life Technologies, Carlsbad, CA). Gloves were worn throughout the experiment with frequent changing and ribonuclease (RNase)-free materials were used in an RNase-free environment (61, 62). Cells were centrifuged for 5 min. at 12,000 rcf at room temperature. The supernatant was discarded. The cells were lysed with 1 ml cold Trizol reagent by repetitive pipetting. The contents of the tube were allowed to stand at room temperature for 5 min. to permit dissociation of nucleoprotein complexes. Chloroform (0.2 ml) was added to each tube and the tubes were capped securely. The tubes were shaken vigorously by hand and then allowed to sit for 3 min. at room temperature. The tubes were spun at 12,000 rcf for 15 min. at 4°C. The aqueous layer, which contains the RNA, was removed to new centrifuge tubes. Isopropanol (0.5 ml) was added into each tube. The contents were gently mixed and incubated at room temperature for 10 min. These were centrifuged at 12,000 rcf for 10 min. at 4°C. The supernatant was aspirated. The RNA pellet was washed with 1 ml 75% ethanol, vortexed, and centrifuged
at 7,500 rcf for 5 min. at 4°C. All ethanol was removed, without drying the RNA pellet completely. The RNA pellet was dissolved in 30 μl diethyl pyrocarbonate (DEPC)-treated ddH₂O by passing the solution through a pipette tip and incubating for 10 min. at 55-60°C. The solutions were then transferred to RNase-free Eppendorf tubes. The purity and concentration of the extracted RNA were determined with a nanodrop (Thermo Scientific). Pure RNA has a 260:280 nm reading equal to 2.0. Extracted RNA was stored at -70°C.

**Polymerase chain reaction (PCR)**

**RT-PCR**

The designed primers for CAR isoforms and MAGI-1 mRNA were purchased from Intergrated DNA Technologies (Coralville, IA) and used for RT-PCR in a two-step RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA). RNA samples taken from extracted total RNA from each cell line were used as templates for reverse transcription into cDNA using the reverse transcriptase enzyme (Invitrogen). 1μL cDNA and 10 μm of each primer were added to 0.2 ml, nuclease-free, thin-walled PCR reaction tubes on ice. The reaction mixtures were placed in a preheated and programmed personal Master Thermal cycler (Eppendorf, Westbury, NY) for gene amplification using Taq DNA polymerase (New England BioLab Inc. Ipswich, MA). The conditions that were programmed in the thermal cycler included 1 cycle of incubation for DNA synthesis at 94°C for 2 min. The PCR amplification was programmed for 35 cycles at 94°C for 15 sec (denaturation), 55°C for 15 sec (annealing), and 72°C for 45 sec (extension). The final extension cycle was programmed for 5 min. at 72°C.
Regular PCR

This was performed at several instances during the study to confirm correct inserts during cloning using DNA plasmids, bacterial colonies as templates, as described above.

Q-PCR

RNA was isolated using TRIzol (Invitrogen). cDNA was synthesized from same starting amount of RNA using Quanta First Strand Kit Quanta BioSciences (Gaithersburg, MD). The PCR amplification was programmed for 40 cycles at 95ºC for 30 sec (denaturation), 58ºC for 30 sec (annealing), and 74ºC for 1 min. (extension). The final extension cycle was programmed for 10 min. at 74ºC. qPCR was performed using SYBR Green with low ROX (Quanta, Gaithersburg, MD; 10 µl master mix qPCR reaction contained 2 µl cDNA, 2X reaction buffer with dNTPs, Accusat Taq DNA polymerase, MgCl₂ ROX reference dye and SYBR green dye that binds specifically to dsDNA) in Stratagene's Real Time PCR System (Agilent Technologies) using 3000p software v2.0.1 for data analysis. Primers used are listed in Table 5.

Analysis of PCR Products

PCR products were analyzed for DNA presence by running 20 µl of the sample on 1% agarose gels made in TBE buffer. The molecular weight marker (MWM) used were 1 kb and 2 log DNA (New England BioLab Inc. Ipswich, MA) because of the relative sizes of CAR and MAGI-1. The electrophoresis was run for 30 min at 110 V. DNA sizes in the PCR products were estimated by comparing the position of bands of interest to the closest band on the MWM.
Immunocytochemistry

Cells were kept on ice for 5 min, washed 3 times with ice cold PBS supplemented with Mg$^{2+}$ and Ca$^{2+}$ (PBS +/-). The cells were then fixed with methanol containing 1% paraformaldehyde for 20 min at -20°C. Cells were rinsed with ice cold PBS, allowed to come to room temperature, and blocked with 2% bovine serum albumin in SuperBlock (Pierce, Rockford, IL) for 45 min. Appropriate primary antibodies were added to cells for 2.5 hrs at 37°C or overnight at 4°C. Cells were rinsed 5 times with PBS with the last rinse greater than 10 min. Cells were blocked with 2% BSA in Superblock for 5 min and then incubated with appropriate Alexa-labeled secondary antibodies (Invitrogen) for 2 hrs at 37°C. The cells were rinsed 5 times with PBS and mounted onto glass slides using Vectashield mounting media with DAPI (Vector Laboratories Inc., Burlingame, CA). Staining was evaluated by laser scanning confocal microscopy (Olympus FV1000) with a 60X oil immersion lens; images are shown as either single X-Y or X-Z sections.

Cell Polarization

For polarization studies, 2 x 10$^4$ – 2 x 10$^6$ cells per well were seeded on 10 mm diameter polyester Millicell filters with a pore size of 0.4 µm (Millipore, Bedford, MA). Media on the apical surface of cells was removed every alternate day in order to establish and maintain an air-liquid interface (Fig. 8). Polarized cells actively transport fluid from the apical to the basolateral surface and thus maintain a defined apical surface fluid composition.
**Transepithelial resistance (TER) measurement**

Transepithelial electrical resistance was measured with a chopstick ohmmeter (World Precision Instruments, Sarasota, FL) every other day. Media was aspirated from the wells and replaced with 400 μL fresh media at the basolateral surface. The same amount of PBS supplemented with Ca$^{2+}$ and Mg$^{2+}$ was applied to the apical surface. The background electrical resistance was determined by adding media to the basolateral chamber and PBS to the apical chamber of a blank Millicell filter. The TER measurements were recorded as mΩ/cm$^2$. 
**Fig. 8** Polarization of cells.

A) $2 \times 10^4$ – $2 \times 10^6$ non-polarized cells were seeded on 10 mm diameter polyester Millicell filters with a pore size of 0.4 µm (Millipore, Bedford, MA). Media on the apical surface of cells was removed every alternate day in order to establish and maintain an air-liquid interface which leads to

B) polarized cells. TJ = tight junction
**Immunoprecipitation and Western blot**

Cell cultures were placed on ice for 5 min, washed with ice-cold PBS, and lysed in buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, protease inhibitors (20 µg/ml, leupeptin, aprotinin, 10 µg/ml pepstatin, and 17.4 µg/ml phenylmethylsulfonyl fluoride)) by rocking at 4°C for 10 min. Cells were scraped into a tube, sonicated five times with 5 pulses using Sonic Dismembrator (Fisher Scientific, model 100) and centrifuged at 14,000g for 10 minutes at 4°C. The supernatant was transferred to fresh tubes and subjected to protein estimation by Bio-Rad protein assay according to manufacturer’s instructions. Aliquots of each lysate were saved for Western blot analysis of total protein in immunoprecipitation (IP) or co-immunoprecipitation (co-IP) experiments. For IP or co-IP, an appropriate amount of primary antibody was added to approximately equal amounts of protein in centrifuge tubes and rotated at 4°C for 2 hours to overnight. Protein G Sepharose bead slurry (GE Healthcare) was prepared by washing 3 times with 2X volume of lysis buffer and once with lysis buffer minus Triton X-100, followed by resuspension in fresh lysis buffer (50% beads + 50% buffer). 50 µl washed beads was added to each sample tube with more rotation at 4°C for at least 60 min. Samples were washed 3 times with lysis buffer and once with lysis buffer minus Triton X-100 by centrifugation at 17000 rcf, 4°C, 1 min to pellet beads in between each wash. Each IP sample was resuspended in 100 µl 2X denaturing buffer with dye (for WB) and incubated in a heat block at 60°C with shaking at 300 rpm for 5-7 min. Samples were then immediately vortexed vigorously before spinning at 17000 rcf, 30-60 sec. Only supernatant was subjected to SDS polyacrylamide gel electrophoresis (10% unless otherwise indicated). Proteins were transferred to a polyvinylidene difluoride membrane.
(Millipore, Bedford, MA), blocked with 5% BSA in PBS, washed, probed with appropriate antibody diluted in 2% BSA in tris-buffered saline tween-20 (TBS-T) with sodium azide, followed by HRP-conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA) diluted in TBS-T (1:10,000). Protein bands were detected by adding ECL reagents (Pierce, Rockford, IL) for 1 min and imaged on a Fuji LAS 4000 and/or developed in an X-ray Medical film processor model Konica SRX 101.

**MAGI-1 knockdown by MAGI-1 siRNA**

Three different Stealth™ siRNA primer duplexes (identified as A, B and C, see Table 5) targeting different parts of MAGI-1 gene were purchased from Invitrogen Custom Laboratory Services (Carlsbad, CA) as dry pellets. Each siRNA duplex was resuspended in RNase-free water to make a 20 µM solution. The resuspension would reconstitute the original buffer solution that was used to prepare the siRNA to 10 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA. According to Lipofectamine™ RNAiMAX protocol, siRNA duplex was transfected at the time of cell seeding either singly or in various combinations (A, B, C, AB, AC, BC, ABC) and concentrations (6 – 36 pmol) into MDCK cells that were seeded with the appropriate number of cells that would give 30-50% confluence 24 hours after plating. Cells were incubated at 37°C, 5% CO₂. Control experiments include the use of primers that do not target MAGI-1 and non transfected MDCK cells. Validation of the level of MAGI knockdown and evaluation of CAR⁸⁺ abundance were conducted by using a combination of Western blotting, immunocytochemistry, and qPCR at 24, 48 and 72 hours after transfection. Adenovirus susceptibility after MAGI-1 knockdown and in the control cells was measured by β-galactosidase assay.
Adenovirus infection

Adenovirus serotype 5 containing the β-galactosidase gene (Ad-β-Gal) or CAR^{Ex7}/CAR^{Ex8} (University of Iowa Vector Core, Iowa City, IA) was diluted with OptiMem culture media (Gibco, Invitrogen) without serum to the multiplicity of infection (MOI) indicated in the text. Growth media was aspirated from cells that had been seeded for 24 to 48 hours, depending on the experiment, and the cells were rinsed with phosphate buffered saline (PBS+/+). 250 uL of diluted adenovirus was added to each well in a 24 well plate (3 ml in 10 cm^2 dish) at a MOI of approximately 100 plaque forming units/cell (PFU/cell), unless otherwise indicated, and incubated for 1 hr at 37°C and 5% CO_2 with gentle swirling every 15 min. The inoculum was then removed, cells were rinsed with OptiMem, and fresh media was added. Cells were incubated at 37°C, 5% CO_2 until further analysis.

Beta-galactosidase assay

The Galacto-Light Plus System (Applied Biosystems, USA) was used to analyze adenovirus-mediated beta-galactosidase activity according to manufacturer directions. Briefly, cells were lysed 24 hours after adenovirus infection. 50 µl lysis buffer was added to each well in a 24-well plate for 20 min at room temperature. Cells were scraped and 2 µL of lysate was added into a 96-well plate followed by 100 µL of a 100X dilution of Galacton and 60 min incubation at room temperature. 200 µL of accelerator was then added just before measuring β-galactosidase luminescence in Luminometer. Protein concentration was determined by adding 6 µl of lysate into a cuvette, followed by the addition of 1 ml Bio-Rad reagent diluted (1:10 with double distilled water (ddH_2O)). The mixture was incubated at room temperature for 10 min and the optical density determined.
with a spectrophotometer set to 595 nm. Each experiment was performed in 5-6 replicates and lysis buffer was used as control. Data was analyzed by the two-tailed Student’s T-test (Prism, GraphPad).

**Cloning of MAGI-1 PDZ domains into pcDNA directional TOPO expression vectors**

MAGI-1 isoforms for *Homo sapiens* and *Mus musculus* were aligned and the amino acid sequences for each of the 6 MAGI-1 PDZ domains were determined by domain conservation. Isolated MAGI-1 PDZ domains were created by cloning each individual PDZ domain (aa 20-110, 465-555, 630-730, 840-930, 990-1080, 1140-1230 for PDZ0 – PDZ5 respectively) into pcDNA/V5/GW/D-TOPO (Invitrogen) according to the manufacturer’s protocol. This vector is a eukaryotic expression vector that fuses a V5 epitope to the C-terminus and contains a T7 promoter. Each forward primer was designed to include EcoRI restriction site the 4 base pair sequences (CACC) necessary for directional cloning on the 5’ end and the reverse primer included a BamHI restriction site without any stop codon. This would allow the extension of each PDZ domain to include V5 during protein synthesis. Individual domains were PCR amplified using full length MAGI-1 as a template. Each PCR product was cloned into TOPO vector and then used to transform One Shot TOP10 chemically competent *E. coli*. Correct insert was confirmed by PCR, restriction digestion and plasmid DNA sequencing (Retrogen Inc., San Diego, CA). Plasmids for myc-tagged full length MAGI-1c and all the isolated domains (GuK, WW, PDZs and C-terminus) were also graciously provided by Dr. Zhigang Xu (Shandong University, Shandong, China).
Plasmid transfection techniques

Cells were transfected with plasmids 24 hours after seeding, when cultures reached over 60% confluence, with Xfect transfection reagent (Clontech Laboratories Inc, Mountain View, CA) according to the manufacturer’s protocol. Transfected cells were used for immunoprecipitation, Western blot, immunofluorescence, or infected with adenovirus after incubation for 48 hours. GFP plasmid was transfected into separate wells, under the same conditions with the cloned domains to monitor transfection efficiency. All experiments included both positive and negative controls, as described in the text, and were repeated a minimum of three times. COS7 cells were transfected with the cloned domain plasmids to confirm protein expression by immunocytochemistry and Western blotting. Subsequently, the plasmids were used for CAR-MAGI-1 PDZ interaction studies.

Cell surface biotinylation

Cell surface proteins were biotinylated with 3 ml per 100 mm plate of 1 mg/ml sulfo-NHS-biotin (Cat # 21331 Thermo Scientific, Rockford, IL) for 1 hour at 4°C after first cooling cells on ice for 10 min and washing with ice cold PBS +/-+. After washing the cells again with PBS +/-+, any unreacted biotin was quenched with 100 mM glycine for 20 min at 4°C. Washed cells were then incubated with lysis buffer for another 20 min at 4°C. Cell lysate was immunoprecipitated with NeutrAvidin ultra link resin (Cat # 53150 Thermo Scientific) and Western blotting with antibody of interest.
In vitro translation and pull down assay

The TNT T7 quick coupled transcription/translation system (Cat # L1170 Promega Corporation, Madison, WI) was used to study the CAR-MAGI-1 PDZ domains in vitro according to the manufacturer’s protocol. Briefly, COS-7 cells were transfected with individual V5-tagged MAGI-1 PDZ domains plasmid and FLAG-tagged CAR isoform plasmids individually and incubated at 37°C, 5% CO₂, for 48 hrs after which the cells were immunoprecipitated with either V5 or FLAG antibody and protein A Sepharose beads added to IP samples as described previously. [\(^{35}\)S, L-methionine-labeled (PerkinElmer, Waltham, MA Cat. # NEG709A) proteins were also synthesized using coupled in vitro reactions from isolated MAGI-1 PDZ domain plasmids and full length CAR isoforms plasmids for 90min. Respective in vitro translated MAGI-1 PDZ domain proteins were mixed with CAR-IP lysates and vice versa and incubated at 4°C for 2 hours with rotation, followed by washing, centrifugation and resuspension of beads in 2X denaturing buffer, as previously described. Incorporation of radioactive label was determined with scintillation cocktail. Reaction products were subjected to SDS-PAGE. Labeled proteins were visualized either by autoradiography of dried gels or by autoradiography after Western blotting after gel transfer onto PVDF membrane.

In-Fusion cloning of CAR C-terminus and MAGI-1 PDZ domains into pHH₂ vectors

A pGEX plasmid that was modified to contain both 6XHis and GST tag and a precision protease site (pHH₂) was kindly provided by Dr. Hostetler (Wright State University). In-Fusion HD cloning (Clontech) employs In-Fusion enzyme, which fuses PCR-generated sequences and linearized vectors precisely by recognizing a 15 base
overlap at their ends. The primers that were designed for each MAGI-1 PDZ domain and the C-terminus of each CAR isoform contain BamHI and EcoRI restriction sites and 15 base sequences of the pHH2 vector that was linearized with BamHI and EcoRI restriction enzymes. According to the manufacturer’s manual, primers for the respective domains were used to PCR amplify the DNA sequences of each MAGI-1 PDZ domain and CAR isoforms C-terminus using Phusion high fidelity DNA polymerase (Finnzymes, Thermo Scientific). Correctly amplified product was verified by gel electrophoresis, based on base pair size, and cloned into linearized pHH2 plasmid. The clones were transformed into Stellar competent cells (Clontech). Restriction digestion, PCR and DNA sequencing were used to confirm the desired inserts.

Testing for expression of proteins in E. coli

Confirmed clones of MAGI-1 PDZ domains and CAR C-terminus (pHH2 plasmids) were transformed into Rosetta cells (EMD chemicals, Gibbstown, NJ) competent cells. Rosetta competent cells are BL21 derivatives that supply tRNAs for AGG, AGA, AUA, CUA, CCC, GGA codons on a chloramphenicol-resistant plasmid and are therefore capable of expressing eukaryotic proteins that contain codons rarely used by E. coli. Transformation was done by adding 50 ng pHH2 plasmids into 25 μl competent cells in a 2.0 ml centrifuge tube and kept on ice for 30 min. The tube was heat-shocked for 45 sec at 42°C and again kept on ice for 2 min. 250 μl SOC medium was added to the cells and the tube incubated for 1 hr at 37°C with shaking at 200 rpm. Each vial of transformed cells was then spread on two ampicillin (100 μg/ml) and chloramphenicol (10 μg/ml) containing LB agar plates and incubated overnight at 37°C.
The next day, 3 - 9 colonies of each plasmid were grown overnight as 1 colony per 3 ml of LB broth containing ampicillin (50 µg/ml) and chloramphenicol (10 µg/ml).

Next day, 300 µl of each overnight culture was subcultured into three 3 ml of LB (Amp+, Chl+) broth and grown until OD₆₀₀ = 0.6 – 0.8, after which 1 ml of culture was transferred from each sample into another tube and stored at -20°C as the zero hour non-induced sample. 10 µl of 100 mM IPTG was added to each remaining 2 ml culture to induce protein expression and continued incubation at 37°C. 1 ml samples were taken every hour for a total of 6 hrs. All samples were centrifuged at 9,500 rpm, 4°C for 5 min. Each pellet was resuspended in 100 µl PBS and sonicated with 5 pulses to completely lyse the cells. The lysate was again centrifuged after which the supernatant was transferred into fresh tubes on ice and the protein concentration determined. Protein samples were mixed with 2X denaturing buffer and incubated at 75°C for 5 min with shaking at 300 rpm. 15 µg of protein from each sample was subjected to SDS-PAGE and the gels were stained with Coomassie blue to look for a protein band of appropriate size. The sample with the highest amount of protein after induction was chosen and this clone was used to make and purify the protein of interest. Desired clones were grown in LB broth and stored at -80°C in cryogenic tubes containing 50% glycerol (1:1 50% glycerol : cell culture ratio) to make the glycerol stock.

**Coomassie blue staining**

Gel containing 10% acrylamide was immersed in 0.2% stain solution (1 g brilliant blue R250, 100 ml ethanol, 2.5 ml methanol, 397.5 ml ddH₂O) for 60 min and then rinsed briefly with ddH₂O. To destain, the gel was placed in 100 ml of 30% methanol with
gentle agitation and changed to fresh destain solution every half hour until the gel was clean enough to see protein bands clearly. The gel was transferred into 0.5% acetic acid and subsequently soaked in 4% glycerol for 2 hr. The gel was dried at 70°C for 50 min in a gel dryer vacuum system (BioRad, model 583).

**Purification of proteins from BL21 bacteria**

On the evening of the first day of purification, Rosetta *E. coli* containing the appropriate pHH_{2} plasmid were taken from a glycerol stock and streaked on a LB (amp+/chlo+) agar plate and then grown at 37°C overnight. The plate was removed the next morning and kept at room temperature until evening. A 40 ml LB (amp+/chlo+) broth was inoculated with a single colony from the plate and incubated at 37°C and 200 rpm overnight in a shaker. The overnight culture was subcultured into 1 L of pre-warmed LB broth and incubated at 37°C for about 4 hours (until OD_{600} 1.5-1.9). 1 ml of uninduced sample was taken for gel analysis before adding 1 ml of 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and continued shaking for about 4 hours. 1 ml of induced sample was also taken for gel analysis. The 1 L culture was then centrifuged in 500 ml bottles at 8,500 rpm, 4°C for 10 min in a Sorvall centrifuge. The pellet was stored at -80°C with 400 μl of 100 mM PMSF.

Pellets from the 1 L culture were resuspended in 10 ml 2X L&C buffer (Tris-based elution buffer; 400 μl 1 M Tris, pH 8.0, 0.35 g NaCl, 2 ml glycerol in 10 ml solution), 10 ml 2X protease inhibitors cocktail (Sigma-Aldrich, St Louis, MO Cat # S8830), 40 μl 0.5 M EDTA and 20 μl 1 M DTT. The resuspended cells were sonicated on ice for 6 min with 30 sec pulses and centrifuged at 9,500 rpm at 4°C for 20 min. The
supernatant was filtered through 1.2 micron filters (Whatman, GE Healthcare REF 10 462 261) to remove any debris. The clarified lysate was pumped through a Fractogel GST-bind cartridge (EMD) using a BioRad pump in the cold box according to the manufacturer’s instructions. The Fractogel cartridge bound the GST-tagged proteins while other synthesized proteins pass through the column.

Briefly, after pumping the lysate through the cartridge, the column was washed with 2 ml 1X L&C buffer, followed by 5 ml ATP wash buffer (2X L&C buffer, 10 mM ATP, 50 mM MgCl$_2$, 1.3 ml ddH$_2$O) and finally, 12 ml 1X L&C buffer containing 1 mM DTT and 1 mM EDTA. To excise protein without any tag, PreScission protease mix (1 ml L&C buffer containing DTT and EDTA and 50-80 µl of protease) was pumped in the column and allowed to circulate through the system for 5 hours. Typically the protease was originally at 1.5 mg/ml and desalted. The material in the recirculated tube was collected as elution 1 (E1). L&C buffer containing DTT and EDTA was pumped through the column to collect at least 3 more fractions of about 1 ml each and labeled E2, E3 and E4 respectively. The fractions contained tag-free proteins which were quantified and the quality verified using SDS-PAGE. 3 ml of reduced glutathione buffer (0.01 g/ml of 1X L&C buffer) without DTT or EDTA was pumped into the column to remove GST tags and protease and the column was subsequently washed with 10 ml L&C buffer without DTT or EDTA to regenerate the column for future use. The column was stored at 4°C for other purifications while the purified protein was stored at -80°C. In any situation where GST-tagged protein was purified, the bound protein was eluted directly with reduced glutathione after washing thereby skipping the use of protease.
Protein dialysis

Proteins were purified with a Tris-based buffer that did not allow proper labeling of the proteins with fluorophores for subsequent experiments. Tris-based buffer was replaced by dialysis against 3 changes of 2 L dialysis buffer containing 10 mM HEPES (pH 8.0), 0.1 mM EDTA, 0.4 mM DTT, 400 mM KCl and 5% glycerol at 4°C for minimum of 16 hours (2 hrs; 2 hrs; overnight) using a Slide-a-lyzer dialysis cassette (Thermo scientific). Each cassette was removed aseptically from its pouch and immersed in dialysis buffer for 2 min to hydrate the membrane. 2 ml protein sample was added and the cassette was floated vertically in the dialysis buffer and stirred gently. A syringe was used to transfer protein samples into and to remove samples from the cassette after dialysis as directed by the manufacturer.

Protein labeling

Purified MAGI-1 PDZ domain proteins without GST tag were labeled with FluoroLinkTM Ab Cy3 labeling kit (GE Healthcare Cat # PA33000) while purified CAR isoforms C-terminus proteins were labeled with FluoroLink Ab Cy5 labeling kit (GE Healthcare Cat # PA35000) according to the manufacturer’s recommendations. Protein solution (1 mg/ml) was added to the vial of coupling buffer, mixed gently, then transferred to the vial of reactive dye and incubated at room temperature with mixing every 10 minutes. While the labeling reaction was incubating, 13 ml of fresh elution buffer was added to the column for priming. The antibody-labeling mixture was carefully added to the top of the column and allowed to enter the resin. 2 ml of elution buffer was added to the column to separate the mixture into a faster moving pink (Cy3) or blue
(Cy5) band of labeled protein from the unconjugated dye. An additional 2.5 ml of elution buffer was then added to the column that will help to elute the pink/blue band. The labeled protein was collected in a clean tube.

**Quantification and Calculation of D/P ratio**

<table>
<thead>
<tr>
<th>Cy3/Cy5 bisfunctional dye characteristics</th>
<th>Cy3</th>
<th>Cy5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula Weight</td>
<td>949.11</td>
<td>975.15</td>
</tr>
<tr>
<td>Absorbance max</td>
<td>550nm</td>
<td>649nm</td>
</tr>
<tr>
<td>Extinction max</td>
<td>150000M⁻¹cm⁻¹</td>
<td>250000M⁻¹cm⁻¹</td>
</tr>
<tr>
<td>Emission max</td>
<td>570nm</td>
<td>670nm</td>
</tr>
<tr>
<td>Quantum Yield</td>
<td>&gt;0.15*</td>
<td>&gt;0.28*</td>
</tr>
</tbody>
</table>

* = for labeled proteins, D/P = 2

An aliquot of the labeled protein was diluted such that the maximum absorbance is 0.5 to 1.5 AU. The absorbance of each labeled protein was measured at 280 nm, 552 nm (Cy3), and 650 nm (Cy5) after which protein concentration was determined with Bio-Rad reagent.

**Estimation of Cy3 final Dye/Protein (D/P) Ratios**

\[
[\text{Cy3 dye}] = \frac{(A @ 552\text{nm})}{150000} \quad (\text{[ ]}= \text{concentration})
\]

\[
[\text{protein}] = \frac{[A @ 280\text{nm} - (0.08 * A @ 552\text{nm})]}{\text{molecular weight of protein}}
\]

\[
\text{D/P final} = [\text{dye}]/[\text{protein}]
\]
**Estimation of Cy5 final Dye/Protein (D/P) Ratios**

\[
[\text{Cy5 dye}] = \frac{(A @ 650\text{nm})}{250000}
\]

\[
[\text{protein}] = \frac{A @ 280\text{nm} - (0.05 \times A @ 650)}{\text{molecular weight of protein}}
\]

D/P final = [dye]/[protein]

**Cy3/Cy5 Spectroscopy fluorescence resonance energy transfer (FRET)**

FRET from each Cy3-labeled MAGI-1 PDZ domain to each Cy5-labeled CAR isoform was done as described by Hostetler et al., (2005) with an ISS PC1 photon counting spectrofluorometer (SLM Aminco Instruments, Rochester, NY) using the following parameters:

- Excitation slit width (back) = 1.0
- Emission slit width (right) = 0.5
- Excitation band width = 8.00 nm
- Emission band width = 4.00 nm
- Excitation = 550 nm
- Emission scan = 560-700 nm, 1nm increments, 8 iterations.

Cy3-labeled protein = 30 nM

Cy5-labeled protein = 0-500 nM

Temp 25°C

Briefly, a cuvette was washed with ddH\(_2\)O and absolute ethanol and subsequently dried with air. A stir bar and 2.0 ml PBS, pH 7.4, were added to the cuvette and placed in the spectrophotometer for excitation and scanning so as to prime the equipment. Proteins were added into the cuvette, left to stir for 3 min and then scanned. The cuvette was washed after each round of experiment before repeating the whole process. The following rounds of experiments were run:
PBS buffer only (control)

PBS buffer plus Cy3-labeled protein

PBS buffer, Cy3-labeled protein, plus increasing concentrations of Cy5-labeled protein (0, 1, 2, 4, 6, 8, 10, 15, 20, 30, 40, 50, 75, 100, 150, 200, 300, 500 nM)

PBS buffer plus increasing concentrations of Cy5-labeled protein (no Cy3-labeled protein; Cy5 control)

Experiments were done in 5 replicates. FRET data were collected by smoothing curves and subtracting out buffer and/or Cy5-labeled protein effects. Maximum emission peaks at 570 nm (Cy3) and 670 nm (Cy5) were recorded. The change in fluorescence intensity (Fo-F) was calculated for each replicate at 570 nm / 670 nm. A plot of the change in fluorescence intensity with respect to increasing Cy5-labeled protein concentrations was made both for 570 nm and for 670 nm. Statistical software Sigma Plot – Statistics – Regression Wizard – Ligand Binding – One Site Saturation was used to calculate dissociation constant (K_d) values. If needed, the Two Site Saturation function was also tested to determine the number of binding sites. Linear plot y-axis = 1/(1-Fi/Fmax) and x-axis = [protein]/Fi/Fmax was also used to determine K_d values.
**Fluorescence Resonance Energy Transfer (FRET) Calculations**

To calculate FRET and intermolecular distance based upon donor quenching at 570 nm,

\[
E = 1 - \frac{F_{DA}}{F_D} \quad \quad E = \frac{R_0^6}{R_0^6 + r^6} \quad \quad R_0 = 50.0\text{Å} \text{ for Cy3/Cy5}
\]

To calculate FRET based upon acceptor emission increase at 670 nm,

\[
E = \frac{F_{AD}/F_A}{(e_A/e_D)} \quad \quad E = \frac{R_0^6}{(R_0^6 + r^6)} \quad \quad R_0 = 50.0\text{Å} \text{ for Cy3/Cy5}
\]

- \( E \) = FRET efficiency
- \( F_{DA} \) = Fluorescence Intensity of donor in the presence of acceptor
- \( F_D \) = Fluorescence Intensity of donor in the absence of acceptor
- \( F_A \) = Fluorescence Intensity of acceptor in the absence of donor
- \( e_A \) = Extinction coefficient of acceptor
- \( e_D \) = Extinction coefficient of donor

\[
E = 1 - \frac{F_{DA}}{F_D} \quad \quad E = \left( \frac{F_{DA}/F_A - 1}{(e_A/e_D)} \right)
\]

\[
E = \frac{R_0^6}{R_0^6 + r^6} \quad \quad R_0 = 9.79 \times 10^3 (\kappa^2 \cdot \eta^{-4} \cdot Q_D \cdot J)^{1/6} \text{ in } \text{Å}
\]

- \( R_0 \) = Förster distance = distance for 50% of the molecules to be quenched
- \( \kappa^2 \) = Orientation factor = 2/3
- \( \eta \) = Refractive index of medium (viscosity) = 1.4 for proteins in solution
\[ Q_D = \text{Quantum yield of donor (based upon trp, try, and phe content)} \]

\[ J = \text{Overlap integral of acceptor emission and donor excitation} \]

\[ r = \text{Distance between donor (D) and acceptor (A)} = \text{To be solved for} \]

\[ K_T = 8.71 \times 10^{23} \text{ sec}^{-1} \ (r^{-6} \cdot J \cdot \kappa^2 \cdot \eta^{-4} \cdot \lambda_d) \]

\[ K_T = \text{Rate of energy transfer} \]

\[ \lambda_d = \text{Emissive rate of donor} \]

\[ E = \left[ \frac{G(\lambda_2)}{G(\lambda_1) - \varepsilon_A(\lambda_2) / \varepsilon_A(\lambda_1)} \right] \times \frac{\varepsilon_A(\lambda_1)}{\varepsilon_D(\lambda_2)} \]

\[ G(\lambda_1) = \text{Magnitude of corrected excitation spectrum for A @} \lambda_1 \]

\[ \lambda_1 = \text{Low absorption of A (ex. 320nm)} \]

\[ G(\lambda_2) = \text{magnitude of corrected excitation spectrum for A @} \lambda_2 \]

\[ \lambda_2 = 280\text{nm for tyr or trp} \]

\[ \varepsilon_A = \text{Extinction coefficient of acceptor} \]

\[ \varepsilon_D = \text{Extinction coefficient of donor} \]

\[ J = \int F_d(\lambda) \varepsilon_d(\lambda) \lambda^4 \ d\lambda / \int \lambda \ F_d(\lambda) \ d\lambda \]

\[ \varepsilon_a = \text{extinction coefficient of the acceptor} \]

\[ \lambda = \text{wavelength of overlap} \]

\[ F_d = \text{Fluorescence Intensity of the donor} \]
ε (Extinction coefficient) of proteins

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp</td>
<td>5690 M⁻¹·cm⁻¹</td>
</tr>
<tr>
<td>Tyr</td>
<td>1280 M⁻¹·cm⁻¹</td>
</tr>
<tr>
<td>Cys</td>
<td>120 M⁻¹·cm⁻¹</td>
</tr>
</tbody>
</table>

Q (Quantum yield) of proteins (value between 0-1) (63)

**Direct binding assay**

This assay was done to verify any conformational change that could occur during interaction. The procedure was similar to FRET except that one of the interacting proteins was labeled and the second unlabeled. Only PBS control was run in this set of experiments. In addition, for Cy5-labeled proteins, excitation was 650 nm and fluorescent emission spectra were obtained between 660-700 nm while for Cy3-labeled proteins, excitation was 550 nm and emission was 560-650 nm. If the Cy3-labeled protein plus unlabeled other protein resulted in a decrease in fluorescence intensity at 570 nm, then the protein was undergoing a conformational change that could result in the K_d estimate to be high. If the Cy5-labeled protein plus unlabeled other protein resulted in a decrease in fluorescence intensity at 670 nm, then the protein was undergoing a conformational change that could result in the K_d estimate to be low. If the Cy5-labeled protein plus unlabeled other protein resulted in an increase in fluorescence intensity at 670 nm, then the protein was undergoing a conformational change which could result in the K_d estimate to be high.
CHAPTER 3: EFFECTS OF MAGI-1 KNOCKDOWN ON CAR EXPRESSION

Rationale

Even though $\text{CAR}^{\text{Ex7}}$ levels seem not to be affected by the presence of MAGI-1, Excoffon et al. reported a loss of $\text{CAR}^{\text{Ex8}}$ in non-polarized cells when both $\text{CAR}^{\text{Ex8}}$ and MAGI-1 were co-expressed (1). Consequently, increasing the protein expression of MAGI-1 leads to a corresponding reduction in adenovirus infection due to the loss of the receptor ($\text{CAR}^{\text{Ex8}}$). Based on this preliminary data, we proposed a model suggesting that MAGI-1b degrades $\text{CAR}^{\text{Ex8}}$ and that this affects $\text{CAR}^{\text{Ex8}}$ apical localization in polarized cells (Fig. 3). It is expected therefore, that $\text{CAR}^{\text{Ex8}}$ will escape degradation if MAGI-1 expression in cells is reduced allowing more $\text{CAR}^{\text{Ex8}}$ to traffic to the apical surface which in turn will augment viral infection. **I hypothesized therefore, that more $\text{CAR}^{\text{Ex8}}$ would be expressed at the cell surface when MAGI-1 was knocked down.**

Results

Due to the limitations of primary human airway epithelia (expensive, difficult to manipulate, high donor and culture variability), experiments were performed in model epithelial cells.

First, there was need to select epithelial cell lines that could polarize under suitable conditions and express CAR in a similar manner to primary airway epithelia. Such cell lines would provide an excellent opportunity to study $\text{CAR}^{\text{Ex8}}$ localization and regulation under reproducible conditions. Cells that are known to polarize were initially
investigated. I hypothesized that model epithelial cells would polarize and CAR\textsuperscript{Ex8}, similar to primary cells, would demonstrate apical localization.

**CAR is expressed in several cell lines except CHO-K1**

CAR\textsuperscript{Ex7} and CAR\textsuperscript{Ex8} expression was determined in nine different cell lines including human lung adenocarcinoma epithelia (A549), human colonic carcinoma (Caco2), human submucosal gland (Calu3), Chinese hamster ovary (CHO-K1), SV40 transformed African Green monkey kidney (COS-7), human embryonic kidney (HEK 293, 293T), Madin Darby canine kidney (MDCK) and normal lung (NuLi-1) cell lines (Table 2). Epithelial cells were cultured under standard conditions at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}. Cell lysate from each cell line was subjected to SDS-PAGE followed by Western blot. A CAR specific band was detected at approximately 46kDa when probed with a rabbit polyclonal antibody directed against the intracellular domain of both CAR isoforms (\(\text{r}\alpha1605\)). CAR was detectable, after loading 150 µg of cell lysate on the gel (Fig. 9A), in all cell lines except CHO-K1 (Fig. 9B). To verify that CAR protein was not present in CHO-K1 lysate, CAR was immunoprecipitated with a mouse monoclonal antibody against CAR extracellular domain (\(\text{m}\alpha\text{CAR RmcB}\)) followed by SDS-PAGE and Western blot with \(\text{r}\alpha1605\) to confirm CAR expression in all the cell lines but CHO-K1 (Fig. 9B). We have shown however that CAR expression levels vary in different cell lines with COS-7 cells expressing the highest level of CAR proteins and MDCK cells expressing more CAR than A549 cells (Sharma \textit{et al.} manuscript submitted). CAR\textsuperscript{Ex7} is the most abundant and widely studied CAR isoform (1). I decided then to investigate how much of the total CAR was specifically CAR\textsuperscript{Ex8}. I
CAR is expressed in many different cell lines but not CHO-K1. Lysate from several cell lines were subjected to SDS–PAGE using 10% denaturing gels. Separated proteins were transferred to PVDF membrane and analyzed by Western blotting. CAR expression was detected with a CAR-specific polyclonal antibody (rabbit anti-CAR-1605p) that recognizes the C-terminus of all CAR isoforms.

A) Total CAR was expressed in all cell lines investigated

B) Only CHO-K1 cells did not express CAR.

Protein molecular weight marker is indicated on the left.

*Polarized cells

**Fig. 9** CAR is expressed in many different cell lines but not CHO-K1. Lysate from several cell lines were subjected to SDS–PAGE using 10% denaturing gels. Separated proteins were transferred to PVDF membrane and analyzed by Western blotting. CAR expression was detected with a CAR-specific polyclonal antibody (rabbit anti-CAR-1605p) that recognizes the C-terminus of all CAR isoforms.

A) Total CAR was expressed in all cell lines investigated

B) Only CHO-K1 cells did not express CAR.

Protein molecular weight marker is indicated on the left.
was unable to detect CAR^{Ex8} isoform by Western blot in all the cell lines after using CAR^{Ex8} specific polyclonal antibody (rα5678). There were multiple non-specific bands detected with using the antibody that did not give me the confidence to confirm CAR^{Ex8}. This may be due to low sensitivity of the antibody for Western blot or low expression of CAR^{Ex8} in which case the band detected by rα1605 on the Western blot was predominantly CAR^{Ex7}. The CAR^{Ex8} antibody has now been purified in the Excoffon lab and it has been used to detect CAR^{Ex8} in Calu3 cells (data not shown). CAR^{Ex8} expression was shown, however, by immunocytochemistry (Fig. 12G-J). Purification of CAR^{Ex7} specific antibody (rα5490) is currently going on in Excoffon lab. This will aid the systematic separation of both CAR isoforms during future experiments that require the detection of a specific isoform.

**CAR^{Ex7} RNA is more abundant than CAR^{Ex8} RNA in cells expressing both isoforms**

Since the detection of the CAR^{Ex8} isoform could not be conclusively verified by Western blot, CAR^{Ex8} RNA level was investigated by quantitative PCR in the cell lines. The RNA isolated from each cell line using TRIzol was used to synthesize cDNA for quantitative PCR (qPCR). RNA levels were measured with respect to β-actin. All the cell lines, except CHO-K1 cells express RNA specific for each isoform (Fig. 10). CAR^{Ex7} RNA expression level was significantly higher than CAR^{Ex8} RNA across all cell lines. Among the cell lines expressing both CAR isoforms, CAR^{Ex7} RNA was 8 – 14 times more abundant than CAR^{Ex8} RNA (compare HEK 293 cells to 293T cells) (Fig. 10, Table 6). This relative abundance of CAR isoforms is similar to what was observed in primary airway epithelia (1) suggesting that all the cell lines except CHO-K1 are candidates for CAR expression studies.
**Fig. 10** CAR isoform-specific RNA expression. a) Cells were seeded in 24 well plates and incubated for 24 hours prior to total RNA extraction. Equal amounts of RNA (1 μg) were used for quantitative RT-PCR and expression was relative to β-actin. b) CAR<sup>Ex7</sup> specific RNA is about 8 to 14 times more abundant than CAR<sup>Ex8</sup> transcripts. CHO-K1 did not show any detectable expression of CAR RNA.

**Table 6**: Ratio of CAR<sup>Ex7</sup>: CAR<sup>Ex8</sup> RNA in cell lines as determined by qRT-PCR

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CAR&lt;sup&gt;Ex7&lt;/sup&gt;: CAR&lt;sup&gt;Ex8&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-K1</td>
<td>ND</td>
</tr>
<tr>
<td>A549</td>
<td>9:1</td>
</tr>
<tr>
<td>Caco2</td>
<td>10:1</td>
</tr>
<tr>
<td>Calu3</td>
<td>12:1</td>
</tr>
<tr>
<td>COS7</td>
<td>9:1</td>
</tr>
<tr>
<td>HEK 293</td>
<td>8:1</td>
</tr>
<tr>
<td>293T</td>
<td>14:1</td>
</tr>
<tr>
<td>MDCK</td>
<td>11:1</td>
</tr>
</tbody>
</table>
**Caco2, Calu3 and MDCK cells polarize**

Many cells transform from non-polarized to polarized forms during development. Polarization occurs by the formation of tight junctions, segregation into apical and basolateral surfaces with distribution of proteins into specific surfaces. $\text{CAR}^{\text{Ex7}}$ is segregated to the basolateral surface while $\text{CAR}^{\text{Ex8}}$ is found at the subapical and apical surface in primary airway epithelia. The integrity of epithelial tight junctions can be quantified by measuring transepithelial resistance (TER). Cells were seeded on 12 mm diameter polyester Millicell filters with a pore size of 0.4 µm. Media on the apical surface of cells was removed every alternate day in order to establish and maintain an air-liquid interface over a period of time. The resistance in A549 cell cultures grown on Millicells never increased above background levels (dotted line) suggesting that these cells fail to fully polarize (Fig. 11, blue). In contrast, MDCK cells displayed a rapid increase in TER after seeding (Fig. 11, purple). MDCK cells polarized within 2 days after seeding. Caco2 and Calu3 cells were also able to polarize but the polarization was not as rapid as MDCK cells. It took about 5 days for Calu3 to attain a resistance of 300 mΩ/cm² which is indicative of functional tight junctions and polarization.

**CAR is localized at cell junctions**

To determine the localization of CAR, cells were seeded on glass slides for 48 hours at a density low enough not to polarize fully, after which the cells were fixed and subjected to immunocytochemistry, as previously described (64), with total CAR stained with α1605 and $\text{CAR}^{\text{Ex8}}$ stained with α5678 (Fig. 12). Both CAR isoforms (green) were localized at the cell junctions of non-polarized A549, Calu3, Caco2, COS-7, MDCK and Nuli-1 (total CAR, Fig. 12A-F) even though $\text{CAR}^{\text{Ex8}}$ expression was very low (Fig. 12G-
Fig. 11 Cell polarization. Cells were seeded onto semi-permeable Millicell membranes and incubated at 37°C, 5% CO₂. Media from apical surface was removed every other day to create an air-liquid interphase that would aid cell polarization. The transepithelial resistance was measured at 2 day intervals over 3 weeks. Resistance above 300mΩ.cm² indicated polarized cells. MDCK cells achieved the highest resistance and polarized faster than Caco2 or Calu3 cells. A549 cells did not polarize.
J), which corresponds with the low RNA levels in the cells. The nucleus was stained with DAPI and is shown in blue. As demonstrated above, Caco2, Calu3 and MDCK cells polarize. To determine the localization of CAR in these cell lines after polarization, the cells were seeded onto Millicells and allowed to polarize as described in methods followed by immunocytochemistry. Specific staining for total CAR and tight junction specific ZO-1 (red), was observed in all the three cell types. The three polarized cell lines showed basolateral localization of CAR\textsuperscript{Ex7} (Fig. 13A-I). The smooth “chickenwire” outline surrounding the cells suggests that tight junctions are well formed in these cells. When viewed in the XZ plane, the apical junctions of Caco2 (Fig. 13B), Calu3 (Fig. 13E), and MDCK (Fig. 13H) cells were dotted with red ZO-1 staining followed by a region of ZO-1 and CAR overlap (yellow Fig. 13C, F, I) and green CAR staining extending along the basolateral junctions (Fig. 13A, D, G) suggesting a high degree of polarization. This is in contrast to A549 cells where CAR is much less organized and does not form a smooth chickenwire pattern around each cell suggesting a lack of polarization (Fig. 14; Sharma et al. manuscript submitted). It was difficult to observe CAR\textsuperscript{Ex8} in the polarized cells due to the low expression of the isoform. Sub-apical and some cytoplasmic presence of CAR\textsuperscript{Ex8} was found in Caco2 (Fig. 15A-C), Calu3 (Fig. 15D-F) and MDCK (Fig. 15G-I). In summary, Caco2, Calu3 and MDCK are able to polarize express both CAR isoforms similar to primary airway epithelia. The characteristics shown by these three cell lines make them candidates for investigating CAR isoform specific expression in polarized epithelia.
**Fig. 12** CAR expression and localization in multiple cell lines. Cells were cultured on glass slides for 48 hours and subjected to immunocytochemistry. Total CAR (rα1605) was localized at cell junctions for A) A549, B) Calu3, C) Caco2, D) COS7, E) MDCK and F) Nuli. CAR\textsuperscript{Ex8} (rα5678) expression was low and also localized at cell junctions in G) A549, H) Calu3, I) Caco2 but not J) MDCK.
**Fig. 13** CAR localizes at the basolateral surface in polarized cells. Cells were stained for CAR (rα1605, green), the tight junction protein ZO-1 (red), and nuclei (blue) four days after seeding onto Millicells™ to polarize. Endogenous CAREx7 (green) in A) Caco2, D) Calu3, and G) MDCK cells overlaps with ZO-1 (B, E, H; merge C, F, I, respectively) at the tight junctions and extends towards the basolateral surface. X-Z plane shown, 60X oil immersion confocal microscopy.
Fig. 14 A549 cells do not polarize. A549 and MDCK cells were seeded for 48 hours followed by immunocytochemistry for CAR (ra1605, green) and nucleus (DAPI, blue).

A, B) A549 cells showed discontinuous outline

C, D) Smooth outline surround MDCK cells

60X oil immersion confocal microscopy
**Fig. 15** CAR\textsuperscript{Ex8} expression in polarized cells. Cells were stained for CAR\textsuperscript{Ex8} (r5678, green), the tight junction protein ZO-1 (red), and nuclei (blue) four days after seeding onto Millicells\textsuperscript{TM} to polarize. Endogenous CAR\textsuperscript{Ex8} (green) expression in A) Caco2, D) Calu3, and G) MDCK cells was low with limited co-localization with ZO-1 (B, E, H; merge C, F, I, respectively) at the basolateral surface, while some puncta were cytoplasmic. X-Z plane shown, 60X oil immersion confocal microscopy.
Localization of exogenous CAR in polarized MDCK

To verify whether polarized MDCK cells were candidates for replicating CAR expression in human airway epithelia, each CAR isoform was overexpressed in MDCK cells. MDCK cells were examined because of the rapid polarization within two days after seeding thus allowing studies on surfaces other than Millicells. MDCK cells were either transfected with each CAR isoform or co-transfected with GFP-MAGI-1b and CAR and allowed to polarize. CAR expression and localization was inspected by immunocytochemistry with CAR antibody (rα1605) 48 hrs post transfection. Similar to what was reported for primary human airway epithelia (1), overexpressed CAR\textsuperscript{Ex7} is localized mainly to the basolateral surface (Fig. 16A-F) while CAR\textsuperscript{Ex8} is diffuse and localized mainly at the subapical and potentially the apical surface (Fig. 17A-F). In addition, MAGI-1 appears to degrade CAR\textsuperscript{Ex8}, as there was no cell expressing both proteins (Fig. 18A-C) unlike the case where CAR\textsuperscript{Ex7} drags MAGI-1b to the basolateral surface (Fig. 18D-F) suggesting that MDCK is a good model for investigating the interactions between MAGI-1b and CAR\textsuperscript{Ex8} upon polarization. In addition, the data verifies that MAGI-1 has effects on CAR\textsuperscript{Ex8} in polarized cells.

I hypothesized that overexpression of CAR\textsuperscript{Ex8} might lead to a CAR\textsuperscript{Ex8} traffic to the apical surface, rather than causing a transient reduction in TER, leading to apical adenovirus infection. To address this, CAR was overexpressed by infecting MDCK cells with adenovirus expressing CAR\textsuperscript{Ex7} (AdCAR\textsuperscript{Ex7}), CAR\textsuperscript{Ex8} (AdCAR\textsuperscript{Ex8}), or eGFP (AdGFP) as control, at the time of seeding on Millicells, and allowed to polarize. This was done since endogenous CAR is highly expressed and exposed upon trypsinization and using adenovirus to facilitate gene expression routinely results in nearly 100% of the
**Fig. 16** Overexpressed CAR$_{Ex7}$ is localized at the basolateral surface of polarized MDCK cells. MDCK cells were seeded and infected with adenovirus encoding CAR$_{Ex7}$. These cells were allowed to polarize for 2 days and stained for CAR (r1605, green, A, B), and ZO-1 (red, C, D) as the reference point for tight junctions. E and F) Merged. 60x oil immersion confocal images (X-Y plane, A, C, E and X-Z plane B, D, F). F) Similar to primary airway epithelia, CAR$_{Ex7}$ is largely at the basolateral surface.
**Fig. 17** Overexpressed CAR$^{\text{Ex8}}$, unlike overexpressed CAR$^{\text{Ex7}}$, is localized throughout the cytoplasm of polarized MDCK cells. MDCK cells were seeded and infected with adenovirus expressing CAR$^{\text{Ex8}}$. These cells were allowed to polarize for 2 days and stained for CAR (rα1605, green, A, B) using ZO-1 (red, C, D) as the reference point for tight junctions. Merged 60x oil immersion confocal images (X-Y plane, E and X-Z plane, F) showed CAR$^{\text{Ex8}}$ filling up the cytoplasm and localizing at the sub-apical surface.
**Fig. 18** CAR localization in polarized MDCK cells cotransfected with GFP-MAGI-1b. In polarized MDCK cells exogenous A) CAR$^{Ex7}$ (red) overlaps (yellow) with MAGI-1b (green) primarily at the basolateral region while D) CAR$^{Ex8}$ (red) localizes diffusely to the apical compartment and does not appear in the same cells as MAGI-1b (green). The lack of both MAGI-1b and CAR$^{Ex8}$ in any cell after checking several fields of view is consistent with the hypothesis that MAGI-1 degrades CAR$^{Ex8}$ as observed in non-polarized COS-7 cells. Confocal microscopy (60x oil immersion).
cells expressing the transgene, as opposed to non-viral methods which achieve much lower rates. MDCK cells without any overexpression were used as another control. Apical viral infection was performed on three samples per condition 48 hours post transfection and incubated for another 24 hours, followed by β-galactosidase assay. The TER for the remaining cells in Millicells was measured over a period of time to monitor cell polarization. Cells overexpressing GFP had delayed polarization unlike the other control cells that polarized within two days post seeding, suggesting that potentially adenovirus infection may have a negative effect on polarization. There was no significant difference between polarization in cells overexpressing CAR\textsuperscript{Ex7} and CAR\textsuperscript{Ex8} expressing cells (Fig. 19A). However, there was a consistent higher viral infection among cells overexpressing CAR\textsuperscript{Ex8} than any other condition (Fig. 19B). Cells expressing GFP did not show any increase in apical adenoviral infection over the negative control cells despite having lower TER (Fig. 19B). This suggests that the apical localization of CAR\textsuperscript{Ex8}, rather than lowering of TER, augments apical adenoviral infection in MDCK cells overexpressing CAR\textsuperscript{Ex8}.

**MAGI-1 is expressed in MDCK cells**

Calu-3 cells are a well studied homogeneous lung epithelial model system that develops tight junctions and microvilli (65, 66). Similarly, our data have shown that MDCK and Caco2 also possess the necessary characteristics to be used for polarized epithelial studies. MAGI-1 expression should be detected in any cell line suitable for studying the effect of MAGI-1 knockdown on CAR\textsuperscript{Ex8} expression. I hypothesized that the cell types will express endogenous MAGI-1. All the three cell lines express MAGI-1 RNA (Fig. 20) as detected by qRT-PCR. To determine if MAGI-1 protein could be
Fig. 19 Overexpression of CAR in MDCK cells does not reduce cell polarization. MDCK cells were seeded with adenovirus encoding CAR^{Ex7}, CAR^{Ex8}, or GFP into semipermeable Millicells and allowed to polarize for 4 days before apical adenovirus infection. Non-infected cells polarize very quickly. A) Cells overexpressing GFP (purple) have significantly delayed polarization while there was no significant difference between cells overexpressing CAR^{Ex7} (red) and CAR^{Ex8} (green). B) CAR^{Ex8} expressing cells support higher apical adenovirus infection than CAR^{Ex7} expressing cells and mock infected cells 4 days after seeding. (*LU/mg = luminescence/mg protein*)
Fig. 20 MAGI-1 RNA is expressed in several cell lines. Cells were seeded in plates and incubated for 24 hours prior to total RNA extraction. Equal amounts of RNA (1 μg) were used for quantitative RT-PCR and expression was relative to β-actin. A549, HEK 293 and MDCK cells express relatively high levels of MAGI-1 RNA.
detected, 3 commercial anti-MAGI-1 antibodies were investigated and a single mouse anti MAGI-1 antibody (m\(\alpha\)MAGI) was found that could detect MAGI-1 by Western blot. COS-7 cells transfected with GFP-MAGI-1 and blotted for GFP served as a positive control to test the m\(\alpha\)MAGI effectiveness. The m\(\alpha\)MAGI antibody could not detect any endogenous MAGI-1 from lysates obtained from 293T, Caco2, A549, HeLa, Calu3 and COS-7, except in COS7 cells overexpressing GFP-MAGI-1 (Fig. 21A). However, MAGI-1 was detected with this antibody in lysate obtained from MDCK cells (Fig. 21B). Polarized MDCK cells were then subjected to immunocytochemistry for MAGI-1 (Fig. 22A, B green) and CAR (Fig. 22C,D red). Just like in primary human airway epithelia, MAGI-1 and CAR localize to the basolateral surface of polarized MDCK (Fig. 22E, F). Since MDCK was the only cell line with detectable MAGI-1 expression by Western blot, coupled with the fact that the cells show similar characteristics to primary airway epithelia, MAGI-1 knockdown experiments were conducted using MDCK cells.

**MAGI-1 is knocked down by siRNA in MDCK cells**

Previous data have shown that MAGI-1 leads to loss of CAR\(^{Ex8}\) (1). I hypothesized that reduction of MAGI-1 expression would increase CAR\(^{Ex8}\) protein levels thereby increasing adenovirus infection. Three different MAGI-1 siRNA duplexes were designed to target different regions of MAGI-1. MDCK cells were transfected at the time of seeding with MAGI-1 siRNA either singly or in various combinations (A, B, C, AB, AC, BC, and ABC) and concentrations for 24-72 hours using Lipofectamine RNAiMAX. Cell lysates were subjected to SDS-PAGE and Western blot to determine the level of MAGI-1 knockdown, relative to \(\beta\)-actin.). Two of the siRNAs (A and B) and all the combinations significantly decreased MAGI-1 expression by 40-90%, however, as
Fig. 21 Testing of MAGI-1 antibody.

A. Endogenous MAGI-1 expression was too low to be detected by Western blot when cell lysates were blotted with mαMAGI antibody after SDS-PAGE. Actin blot was used confirm transfer of proteins. The antibody was able to detect MAGI expression in COS-7 cells transfected with GFP-MAGI (COS7*).

B. The antibody however detected MAGI-1 in MDCK cells. Molecular weight sizes are indicated on the left.
**Fig. 22** Endogenous MAGI-1 co-localizes with CAR in polarized MDCK cells. MDCK cells were polarized and subjected to immunocytochemistry. MAGI-1 (A, B green, mαMAGI) and total CAR (C, D red, rα1605) overlap (E, X-Y plane, orange) at tight junctions (F, X-Z plane; nucleus stained blue). A, C, E: XY plane; B, D, F: XZ plane. 60X confocal microscopy.
observed for many other proteins, 100% knockdown of MAGI-1 was not achieved (Fig. 23A,B). The third siRNA (C) did not decrease total MAGI-1 significantly. MAGI-1 knockdown was also detected by immunocytochemistry (Fig. 24B) as compared to the control MDCK cells not transfected with any siRNA (Fig. 24A).

The effect of MAGI-1 knockdown was also investigated on MAGI-1 RNA relative abundance using qRT-PCR. MDCK cells were transfected at the time of seeding with MAGI-1 siRNAs and incubated for 48 hours before RNA extraction. Equal amounts of RNA from each extract were used for qPCR. Interestingly, MAGI-1 RNA level was significantly reduced by individual siRNA A and siRNAB with respect to control cells (Fig. 25).

**MAGI-1 knockdown increases adenovirus infection in MDCK cells**

Two siRNAs decreased the protein expression of MAGI-1. I hypothesized that the knockdown might lead to an increase in CAR<sup>Ex8</sup> expression and possible augmentation of apical adenovirus infection. CAR<sup>Ex8</sup> expression was not detectable by Western blot with our CAR<sup>Ex8</sup> specific polyclonal antibody (rα5678) possibly due to the generally low expression of this isoform in MDCK cells or effectiveness of the antibody. However adenovirus infection in the MAGI-1 knockdown cells was used to evaluate CAR expression at the apical surface. MDCK cells were transfected with 18pmol each of three MAGI-1 siRNAs (A, B, C) and incubated for 48 hours before infecting with adenovirus expressing β-galactosidase. An siRNA duplex that was not directed against MAG-1 (R) was also transfected into MDCK cells as a control. Another control experiment was also conducted with cells not transfected with any siRNA. There was significant increase in adenovirus infection in cells transfected with MAGI-1 siRNA-A and siRNA-B (Fig 26).
**Fig. 23** MAGI-1 siRNA reduces MAGI-1 expression in MDCK cells. A) MDCK cells were transfected with different combinations of three MAGI-1 siRNAs and incubated for 48 hours prior to Western blot analysis of total lysate with mouse anti-MAGI. MAGI-1 was transfected into some cells as a positive control for the MAGI-1 antibody while the negative control consisted of cells not transfected with any siRNA (mock). B) The quantitation of protein bands, relative to actin, with respect to non-transfected cells (100%). These results are representative of five experiments. Note that later experiments used a non-specific siRNA as a control and results were similar to levels in untransfected (mock) control.
Fig 24 MAGI-1 siRNA knockdown reduces endogenous MAGI-1 levels. MDCK cells were seeded with MAGI-1 siRNA and incubated for 48 hours. Cells were then subjected to immunocytochemistry with mαMAGI-1. A. Control cells B. MAGI-1 siRNA-B treated. 60X confocal microscopy.
**Fig. 25** MAGI-1 specific siRNA reduces MAGI-1 RNA expression. MDCK cells were seeded in plates with MAGI-1 siRNA and incubated for 48 hours prior to total RNA extraction. Equal amounts of RNA (1 μg) were used for quantitative RT-PCR and expression was relative to β-actin. MAGI-1 RNA level is significantly reduced by single siRNA-A and siRNA-B but not by siRNA-C.
Fig. 26 MAGI-1 siRNA knockdown leads to increased adenovirus infection. MDCK cells were transfected with 18 pmol each of three MAGI-1 siRNAs (A, B, C) and incubated for 48 hours before infecting with adenovirus expressing β-galactosidase. Control experiments (Non-transfected cells (mock), and cells transfected with non-specific siRNA that was not directed against MAGI-1 (siRNA-R) were also infected with adenovirus at the same time. Beta-galactosidase assay 24 hours post-infection shows that both MAGI-1 siRNAs-A and -B significantly increases adenovirus infection (* p<0.05) with siRNA-B causing a higher increase than siRNA-B. The effect of siRNA-C on adenovirus infection was not significantly different from controls.
suggesting that MAGI-1 knockdown resulted in elevated CAR, likely CAR{\textsuperscript{Ex8}}, at the apical surface of MDCK cells, thereby increasing adenovirus infection. Similar to Western blot results, MAGI-1 siRNA C did not cause any significant increase in adenovirus infection.

**Conclusions**

Based on the data presented, two major conclusions were reached. First, MDCK cells are good candidates for replicating CAR-MAGI-1 interactions in human airway epithelia. MDCK cells are easy to grow in the lab, quickly polarize, extensively studied and commercial antibodies are readily available for studies. Secondly, MAGI-1 knockdown leads to increase in adenovirus infection in MDCK cells. This suggests our mechanism of CAR{\textsuperscript{Ex8}} apical localization and degradation by MAGI-1 may be correct. I believe that MAGI-1 knockdown increases CAR{\textsuperscript{Ex8}} thereby increasing the apical availability of CAR{\textsuperscript{Ex8}} and subsequent viral infection (Fig 27). However, any increase in CAR{\textsuperscript{Ex8}} protein levels as a result of MAGI-1 siRNA knockdown still needs to be confirmed.
Fig. 27 Model for MAGI-1 knockdown allowing CAR$^{Ex8}$ abundance. Decreased MAGI-1 expression reduces CAR$^{Ex8}$ degradation which leads to increased CAR$^{Ex8}$ apical expression and viral infection. ER, endoplasmic reticulum; G, Golgi; TJ, tight junction; AP, apical (air) surface; BL, basolateral surface.
CHAPTER 4: CAR\textsuperscript{Ex8} INTERACTS WITH MAGI-1 PDZ DOMAINS 1 AND 3

Rationale

The over-arching hypothesis of this thesis was that CAR\textsuperscript{Ex8} protein levels and localization in polarized epithelial cells are regulated by PDZ-based interactions with MAGI-1. Our preliminary data suggest that MAGI-1b is responsible for the loss of CAR\textsuperscript{Ex8} through a PDZ-based interaction but the specific MAGI-PDZ domain involved in the interaction has not been reported. The aim of this chapter was to dissect this interaction by determining the specific MAGI-1 PDZ domain(s) involved. MAGI-1 contains 6 PDZ-binding domains (PDZ0-PDZ5). A list of known interactions and the respective domain, if known, is given in Table 1. Several factors, beyond consensus sequence, may define a PDZ-based interaction. Examples include binding affinity, domain structure, other proteins present in the complex, and modifications such as phosphorylation. Investigation of the PDZ interactions was initially done \textit{in vitro} by using isolated individual MAGI-1 PDZ domains to determine which domain(s) interact with CAR. The advantage of using isolated domains as opposed to MAGI-1 deletion constructs was to confirm the specificity of binding by each of the domains and to avoid any confusion that may arise from more than one domain interacting with CAR or the effects of one domain on another. However, the use of deletion constructs may provide additional information to using isolated PDZ domains since CAR-MAGI-1 may require co-operation between multiple PDZ domains. CAR\textsuperscript{Ex7} drags MAGI-1 to basolateral
surface of polarized epithelia while CAR\textsuperscript{Ex8} is not present in the presence of MAGI-1 (1).

Considering the distinct sequences in both CAR isoforms and the difference in the result of interaction of each CAR isoform with MAGI-1, I hypothesized that each isoform binds to a different PDZ domain in MAGI-1.

**Results**

**Expressing Isolated MAGI-1 PDZ domains in COS-7 cells**

To determine the specific MAGI-1 PDZ domain(s) that interact with CAR, isolated MAGI-1b PDZ domains were created by designing PCR primers to individual PDZ domain (aa 20-110, 465-555, 630-730, 840-930, 990-1080, 1140-1230) for cloning by PCR and inserting into pcDNA/V5/GW/D-TOPO (Invitrogen). This vector is a eukaryotic expression vector that fuses a V5 epitope to the C-terminus and contains both a CMV and a T7 promoter. Interestingly, upon fully sequencing the GFP-MAGI-1b construct, originally from Dobrotskya et al., it was discovered that only the complete nucleotide sequence for PDZ1, PDZ2, PDZ3 and PDZ5 were present. Initially, only these four domains were cloned into pcDNA plasmid (isolated domains). Dr. Zhigang Xu (Shandong University, Shandong, China) kindly provided us with myc-tagged MAGI-1c, which included all of the PDZ domains (PDZ 0-5 and C-terminus), as well as myc-tagged individual domains and the isolated C-terminus in pcDNA plasmids. Although MAGI-1c contains all PDZ domains, the ones in common with MAGI-1b have the same PDZ domain sequence as MAGI-1b so the clones were collected for completeness of the PDZ domains. The clones were confirmed by PCR and restriction digestion. Sequencing also confirmed that nucleotide and protein sequences for the V5-tagged PDZ domains and myc-tagged PDZ domains were identical except for the tags.
Protein expression of the individual PDZ domains was measured by Western blotting of cell lysates, loaded in equal concentrations, after transfecting the clones into COS-7 cells and incubating for 48 hours. COS-7 cells were used for ease of transfection and the high levels of protein production obtained with this cell line. The plasmids were not toxic to the cells since there was no significant difference in cell viability between the non-transfected and transfected COS-7 cells. The V5 tagged proteins were identified by Western blot with mouse-αV5 antibody and were present at the expected molecular weight between 20 and 30 kDa (Fig. 28A). Similarly, myc-tagged PDZ domains and the C-terminus were expressed and identified at the expected weight (20-30 kDa) after blotting with mouse-αmyc (Fig. 28B). Since PDZ expression was easily observed by Western blot, the CAR interaction with each of the PDZ domains by co-immunoprecipitation and in vitro pull down assay was investigated next.

**CAR<sup>Ex8</sup> interacts with MAGI-1 PDZ1 and PDZ3 domains**

Co-immunoprecipitation was used to investigate CAR-MAGI-1 PDZ interactions. Each FLAG-tagged CAR isoform was co-transfected with individual isolated myc-tagged MAGI-1 PDZ domains in COS-7 cells and incubated for 48 hours. A control experiment was also set up with COS-7 without any transfected CAR or PDZ plasmids. Interestingly, no PDZ domain could be pulled down with rabbit anti-CAR polyclonal antibody (ra1605). This may be due to the fact that this polyclonal antibody is directed to the C-terminus of CAR and may prevent the interaction between CAR and the PDZ domains. Transfecting different concentrations of the plasmids along with the use of another CAR antibody, not directed against the C-terminus, for immunoprecipitation (IP)
**Fig. 28** Expression of isolated MAGI-1 domains. A) V5 tagged and B) myc-tagged isolated MAGI-1 PDZ domains and C-terminus were transfected into COS-7 cells. Protein expression was measured 48 hours later after SDS-PAGE and Western blot of the cell lysates. rαV5 (rabbit anti-V5); mαmyc (mouse anti-myc).
**Fig. 29** Both CAR isoforms co-immunoprecipitate with MAGI-1 PDZ3 from COS-7 cell lysates. COS-7 cells were co-transfected with MAGI-1 PDZ3, 4, or 5 with CAR\textsuperscript{Ex7}, while MAGI-1 PDZ1, 3, 4, or 5 were co-transfected with CAR\textsuperscript{Ex8} and incubated for 48hrs before extracting cell proteins. A plate of cells was not transfected with any plasmid (Mock). CAR was immunoprecipitated with maCAR RmcB, an antibody directed against the extracellular domain of CAR and thus common to both isoforms and endogenous CAR, followed by Western blotting. The membrane was cut into two and probed with rα1605 (CAR, ~46 kDa) and ramyc (MAGI-1 PDZ domains; 20-28kDa). A-D) Adequate transfection of cells was confirmed by blotting lysates only. MAGI-1 PDZ3 immunoprecipitated with both F) CAR\textsuperscript{Ex7} and H) CAR\textsuperscript{Ex8}. 

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<th>myc-PDZ:</th>
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### Blot

- **BLOT**
  - maFLAG
  - ramyc
  - rα1605
  - ramyc

- **lysate**

- **IP**
  - maCAR RmcB
was more effective. After monoclonal mouse-αCAR (RmcB) IP for over 2 hours to overnight at 4°C, both CAR isoforms pulled down MAGI-1 PDZ 3 domain (Fig. 29F,H).

To verify transfection and protein expression of all PDZ domains, aliquots from cell lysates were subjected to SDS-PAGE. CAR was blotted with mouse-αFLAG antibody for CAR<sup>Ex7</sup> (Fig. 29A) and CAR<sup>Ex8</sup> (Fig. 29C). There was no CAR band for nontransfected cells (Fig. 29A, lane Mock) since FLAG antibody only identified exogenous, and not endogenous, CAR. Similarly, rabbit α myc antibody detected PDZ 1, 3, 4, 5 expressions (Fig. 29B,D) and no bands were observed in the control lysate (Fig. 29B, Mock). After IP of lysates with mαCAR (RmcB), α1605 was used to blot for total CAR and αmyc for MAGI-1 PDZ domains. CAR did not pull down PDZ0 or PDZ2 (data not shown). Since α1605 detects all forms of CAR, including endogenous CAR, CAR<sup>Ex7</sup> (Fig. 29E) and CAR<sup>Ex8</sup> (Fig. 29G) were pulled down in all cells including non-transfected cells using mαCAR (RmcB) antibody. The amount of CAR pulled down in CAR transfected conditions was significantly higher than untransfected suggesting that most CAR in these conditions was the transfected version. Both isoforms pulled down MAGI-1 PDZ 3 (Fig. 29F, H) suggesting that both CAR isoforms interact with MAGI-1 at the PDZ 3 domain.

Since CAR antibody (RmcB) can pull down both CAR isoforms, there was need to ensure that the PDZ3 band that obtained was not as a result of endogenous CAR<sup>Ex7</sup>. CAR and individual MAGI-1 PDZ domains were overexpressed in COS-7 cells with the highest concentration of each plasmid permissible for transfection without being toxic to the cells. The IP was done with FLAG antibody to ensure that only exogenous CAR was immunoprecipitated. Interestingly, in addition to both CAR<sup>Ex7</sup> (Fig. 30A) and CAR<sup>Ex8</sup>
**Fig. 30** CAR^{Ex8} interacts with MAGI-1 PDZ1 and PDZ3 while CAR^{Ex7} interacts with MAGI-1 PDZ3. COS-7 cells co-transfected with MAGI-1 PDZ domain and FLAG-tagged CAR plasmids and immunoprecipitated with mαFLAG followed by Western blot with CAR antibody (α1605) or PDZ antibody (αmyc). B) CAR^{Ex7} immunoprecipitated with PDZ3 while D) CAR^{Ex8} interact with both PDZ1 and PDZ3.
(Fig. 30B) pulling MAGI-1 PDZ3, CAR^{Ex8} was able to also pull down MAGI-1 PDZ1 (Fig. 30B). This suggests CAR^{Ex8} can also interact with MAGI-1 at PDZ1 domain. I hypothesized that the interaction at PDZ1 domain would account for the difference between the CAR isoform-specific interactions with MAGI-1. Dr. Priyanka Sharma in the Excoffon lab verified interactions between both CAR isoforms and the MAGI-1 PDZ3 domain and an interaction between CAR^{Ex8} and MAGI-1 PDZ1 with a yeast 2-hybrid assay. In this experiments, the MAGI-1 PDZ domains were used as prey and the PDZ-binding domain of each CAR isoform was the bait to arrive at the same conclusions with the results in this study.

For further confirmation of CAR-MAGI-1 PDZ interactions, an *in vitro* pull down assay was performed. Isolated MAGI-1 PDZ domains were generated *in vitro* using the TNT Quick Reticulocyte Lysate system (Promega) in the presence of $^{35}$S-methionine. Lysates from FLAG-CAR isoform-specific transfected COS-7 cells were bound to different aliquots of glutathione sepharose 4B beads (GE) in ice cold PBS. Aliquots of isolated MAGI-1b PDZ domains were then mixed with the beads followed by rotation at 4°C for 1-2 h. Beads were washed, bound proteins eluted, run on SDS-PAGE and analyzed by autoradiography. Results showed that CAR^{Ex7} interacts with MAGI-1 PDZ3 (Fig. 31A) while CAR^{Ex8} interacts with both MAGI-1 PDZ 1 and PDZ3 (Fig. 31B).

**Isolated MAGI-1 PDZ domains are localized in the cytoplasm of COS-7 cells**

A change in localization of MAGI-1 from the cytoplasm to cell junctions has been reported when cells were co-transfected with MAGI-1 and CAR^{Ex7}. It was necessary therefore to identify the localization of each isolated PDZ domain before co-transfecting
**Fig. 31** $\text{CAR}^{\text{Ex7}}$ pulled down MAGI-1 PDZ1 and PDZ3. Isolated MAGI-1 PDZ domains generated *in vitro* with the TNT Quick reticulocyte system in the presence of $^{35}\text{S}$-methionine and added to lysates from COS-7 cells transfected with $\text{CAR}^{\text{Ex7}}$ or $\text{CAR}^{\text{Ex8}}$ for pull down assay. B, D) both isoforms pulled down PDZ3 but only D) $\text{CAR}^{\text{Ex8}}$ was able to pull PDZ1.
with CAR isoforms. COS-7 cells seeded into chamber slides were transfected with each of the isolated MAGI-1 PDZ domains and incubated for 48 hours, followed by immunocytochemistry with V5 or myc antibodies. The localization of V5-tagged and myc-tagged PDZ1, PDZ2, PDZ3 and PDZ5 domains were identical. All the MAG-1 PDZ domains were localized in the cytoplasm of COS-7 cells (Fig. 32A-D, V5: PDZ1, PDZ2, PDZ3, PDZ4; Fig. 33A-F, myc: PDZ0 – PDZ5; respectively). Only the myc-tagged C-terminus of MAGI-1 was localized in the nucleus (Fig. 33G). The C-terminus contains a nuclear localization signal, which explains why it is localized in the nucleus.

I hypothesized that there would be a change in localization of either the CAR isoforms or the isolated MAGI-1 PDZ domains upon co-expression. To test this COS-7 cells were co-transfected with the plasmids for the interacting partners. Surprisingly, immunocytochemistry of the PDZ domains (rpmyc) and CAR (mFLAG) did not show any drastic change in localization. Both CAR isoforms were localized at cell junctions (Figs. 34A,D; 35A,D) while the PDZ domains were localized in the cytoplasm (Figs. 34B,E; 35B,E). CAR\textsuperscript{Ex7} did not appear to drag MAGI-1 PDZ3 to the junctions as in the case of full length MAGI-1 (Fig. 34F). This may suggest that other components of MAGI-1 are required for MAGI-translocation. CAR\textsuperscript{Ex7} did not co-localize with PDZ1 as expected (Fig. 34C). Many cells showed both CAR\textsuperscript{Ex8} and PDZ1 in same cells (Fig. 35C) but not with PDZ3 (Fig. 35F) suggesting that PDZ3 may be required for CAR\textsuperscript{Ex8} loss during the CAR\textsuperscript{Ex8}-MAGI-1 interaction. Future experiments with full length MAGI-1 containing deletions in the PDZ1 and/or 3 domains along with each CAR isoform will be used to confirm these results. It should be noted that in a few cases it appeared that CAR\textsuperscript{Ex8} and PDZ3 co-localized at the junctions but the rarity of this occurrence could not
**Fig. 32** Isolated MAGI-1 PDZ domains are localized diffusely in the cytoplasm of COS-7 cells. V5-tagged isolated MAGI-1 PDZ domains were transfected into COS-7 cells. Protein localization was determined by immunocytochemistry with mαV5 48 hours later. A) PDZ1, B) PDZ2, C) PDZ3 and D) PDZ5. 60x oil immersion confocal microscopy.
Fig. 33 Isolated MAGI-1 C-terminus is localized in the nucleus of COS-7 cells. Myc tagged isolated MAGI-1 PDZ domains and the C-terminus were transfected into COS-7 cells. Protein localization was determined by immunocytochemistry with myc antibody 48 hours later. A) PDZ0, B) PDZ1, C) PDZ2, D) PDZ3 E) PDZ4 and F) PDZ5 are localized in the cytoplasm. G) Only the C-terminus is localized in the nucleus. 60x oil immersion confocal microscopy.
**Fig. 34** \(\text{CAR}^{\text{Ex7}}\) does not drag isolated MAGI-1 PDZ1 to junctions in COS-7 cells. Myc tagged isolated MAGI-1 PDZ1 was co-transfected with \(\text{CAR}^{\text{Ex7}}\) into COS-7 cells. Protein localization was determined by immunocytochemistry with ramyc antibody (PDZ) and m\(\alpha\)FLAG (CAR) 48 hours later. A) \(\text{CAR}^{\text{Ex7}}\) red is localized at the junctions while B) PDZ1 green is localized in the cytoplasm. C) Merge, nuclei in blue. 60x oil immersion confocal microscopy.
Fig. 35 CAR\textsuperscript{Ex8} does not drag isolated MAGI-1 PDZ1 to junctions of COS-7 cells. Myc tagged isolated MAGI-1 PDZ1 or PDZ3 domains were co-transfected into COS-7 cells. Protein localization was determined by immunocytochemistry with r\textsuperscript{Myc} antibody (PDZ) and m\textsuperscript{FLAG} (CAR) 48 hours later. A, D) CAR\textsuperscript{Ex8} red is localized at the junctions while B) PDZ1 green and E) PDZ3 green are localized in the cytoplasm. C, F) CAR\textsuperscript{Ex8} does not drag the PDZ domains to the junctions. 60x oil immersion confocal microscopy.
be used to conclude this interaction with immunocytochemistry. More experiments with PDZ domain deletion mutants will shed more light on the sufficiency of the deleted domains on interaction and localization.

**Expression of CAR C-terminus and MAGI-1 PDZ domains in *E. coli***

Co-immunoprecipitation, *in vitro* pull down and yeast 2-hybrid assays were only able to qualitatively confirm interactions between CAR and the MAGI-1 PDZ domains. Ultimately, the goal is to be able to find a way to regulate the MAGI-1 interaction with CAR$^{Ex8}$ for the purpose of augmenting gene therapy or preventing viral infection. Knowing the affinity of the PDZ domains with each CAR isoform is a step towards understanding what is required to achieve this goal since it may allow us to design peptides or small molecules that may disrupt or enhance these interactions. Moreover, purified protein binding assays provide the best evidence that these interactions are direct. Binding assays between purified MAGI-1 PDZ domains and CAR were used to determine the affinity between the molecules. Proteins were expressed in BL21 bacteria and purified for binding assays and fluorescence resonance energy transfer (FRET).

Isolated MAGI-1 PDZ domains were cloned into a modified pGEX plasmid (pHH$_2$) using In-Fusion cloning (Clontech). The C-terminus of each CAR isoform was also cloned into the plasmid. The plasmid, provided by Dr. Hostetler, has an IPTG regulated promoter, both 6xHis and GST as protein tags, and a precision protease site to cleave these tags off of the purified protein during purification. After confirming MAGI-1 PDZ domain and CAR C-terminus clones with PCR, restriction digestion and sequencing, the clones were transformed into Rosseta2 competent cells for protein
expression. Rosetta competent cells are modified BL21 bacteria that are capable of utilizing human codons which bacteria will ordinarily not use. The plasmid conferring tRNA specificity requires chloramphenicol selection. These “humanized” bacteria have an increased capacity to synthesize eukaryotic proteins. The transformed bacteria were grown in LB broth and protein synthesis was induced with IPTG.

Nine colonies were selected from each MAGI-1 PDZ3 and CAR\textsuperscript{Ex8} C-terminus clone to inoculate LB broth and incubate at 37°C. Culture samples were taken from each broth before IPTG induction of protein synthesis for analysis of baseline protein expression. The purpose was to identify the colony that would show the highest protein expression after induction. The cells were lysed followed by Coomasie staining of gels after SDS-PAGE. Interestingly, all the nine colonies from both PDZ3 (Fig. 36B) and CAR\textsuperscript{Ex8} C-terminus (Fig. 36D) showed a new band at the expected molecular weight (\(\sim 40\text{kDa}\)) after just 1 hour of protein induction suggesting that they were able to express the desired proteins. Lysates from non-induced cells did not show an intense band at the expected molecular weight suggesting that there was no expression of the desired proteins (Fig. 34A,C). These data suggesting that the Rosetta cells could express abundant levels of MAGI-1 PDZ3 and CAR C-terminus.

To extend this finding, three colonies were each selected from the remaining clones and treated in a similar manner to MAGI-1 PDZ3. As expected MAGI-1 PDZ0, PDZ1, PDZ2, PDZ4, PDZ5, or CAR\textsuperscript{Ex7} C-terminus (Fig. 37A-F respectively) expression was detected after 1 hour of protein induction in all the clones. Although full length CAR isoforms were successfully cloned into pHH2 vector, the proteins did not appear to be
**Fig. 36** Expression of CAR\textsuperscript{Ex8} C-terminus and MAGI-1 PDZ3 in *E. coli*. CAR\textsuperscript{Ex8} C-terminus and MAGI-1 PDZ3 pH\textsubscript{2} clones were transformed into Rosetta competent (BL21) bacteria and grown in LB broth. After 1 hour of induction by IPTG, all nine colonies selected per clone expressed a new protein band for B) PDZ3 and D) CAR\textsuperscript{Ex8} C-terminus that were not found in A or C) non-induced cultures, respectively.
**Fig. 37** Expression of CAR$^{\text{Ex7}}$ C-terminus and MAGI-1 PDZ domains in *E. coli* CAR$^{\text{Ex7}}$ C-terminus and MAGI-1 PDZ domain clones were transformed into Rosetta competent (BL21) bacteria and grown in LB broth. Three colonies were selected per domain and all expressed a new protein band (red arrowhead) upon IPTG induction. A) PDZ0, B) PDZ1, C) PDZ2, D) PDZ4, E) PDZ5 and F) CAR$^{\text{Ex7}}$ C-terminus.
expressible in bacteria. Full length CAR is likely in vesicles or non-soluble inclusions due to the transmembrane domain. Future experiments should involve trying conditions that allow protein purification from vesicles or inclusion bodies.

After confirming that Rosetta cells could express the protein of interest, large scale amount of the proteins was purified. One clone from each of the PDZ1, PDZ3, CAR\textsuperscript{Ex7}, or CAR\textsuperscript{Ex8} C-terminus constructs was grown in 1 L LB culture and purified using GST-Bind fractogel cartridge and eluted with 6xHis-GST tags (Fig. 38A-D) or without tags (Fig. 38E-H). Over 2 mg protein was purified from each of the clones in 3 ml of elution with 6xHis-GST tagged CAR\textsuperscript{Ex8} C-terminus yielding as much as 10 mg protein from 1 L culture (Table 7). Tris base does not allow proper fluorophore labeling of the proteins according to the manufacturer so the purified proteins were dialyzed in HEPES buffer to remove the Tris base that was used for purification. 2mg of each purified MAGI-1 PDZ and CAR C-terminus proteins were labeled with Cy3 fluorophore and Cy5 fluophore respectively.

**FRET between Cy3- and Cy5-labeled MAGI-1 PDZ domain proteins and CAR isoform specific C-terminus**

To establish the direct binding and an intermolecular distance between MAGI-1 PDZ domains and CAR, Cy3/Cy5 spectroscopy FRET was done between each Cy3-labeled MAGI-1 PDZ domain and each Cy5-labeled CAR isoform using an ISS PC1 photon counting spectrofluorometer. FRET is based on the use of excitation energy from one molecule to excite another molecule in close proximity. Cy3/Cy5 labels form a donor/acceptor pair for FRET that is used to examine protein-protein interactions (67). In
**Fig. 38** Purified proteins. BL21 bacteria containing clones of isolated MAGI-1 PDZ1, PDZ3, or CAR-isoform specific C-termini were grown in 1 L cultures and induced with IPTG to express the desired soluble proteins. Samples of purified proteins with or without GST tags (indicated by red arrows) were stained with Coomassie blue to determine purity and molecular weight of the proteins. Properties of the purified proteins are shown below.

**Table 7** Proteins purified from BL21 bacteria

<table>
<thead>
<tr>
<th>Protein</th>
<th>Yield (mg/ml)</th>
<th>Yield (mg/ml)</th>
<th>pI</th>
<th>MW, kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>His-Gst tag</td>
<td>no tag</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDZ1</td>
<td>1.00</td>
<td>0.75</td>
<td>5.48</td>
<td>19.28</td>
</tr>
<tr>
<td>PDZ3</td>
<td>3.54</td>
<td>1.93</td>
<td>5.93</td>
<td>15.80</td>
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<tr>
<td>CAR&lt;sup&gt;Ex7&lt;/sup&gt;</td>
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<td>9.23</td>
<td>11.89</td>
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<tr>
<td>CAR&lt;sup&gt;Ex8&lt;/sup&gt;</td>
<td>5.06</td>
<td>1.88</td>
<td>9.15</td>
<td>10.79</td>
</tr>
</tbody>
</table>
a Cy3/Cy5 pair, there is an overlap between the emission spectrum of Cy3 (peak 570 nm) and absorption spectrum of Cy5. MAGI-1 PDZ domains were labeled with Cy3 and excited at 550 nm. Cy5-labelled CAR C-terminus was added to the PDZ protein in the cuvette. If there is interaction between the two proteins, they will be in close enough proximity (less than 100 Å) for Cy5 to absorb the excitation energy from Cy3 and subsequently emit this energy at a wavelength average of 670 nm. An emission scan was taken in 1nm increments from 560-700 nm to allow the fluorescence signal from Cy3 and Cy5 to be detected (Figs. 39A,C; 40A,C). The maximum peak values were recorded at 570 nm for Cy3 and 670 nm for Cy5. Initially 30 nM of Cy3-PDZ protein was used for FRET with the addition of increasing concentrations of Cy5-labeled C-terminus protein from 0-500 nM at a temperature of 25°C.

Repeated excitation of Cy3 with light has the ability to quench the fluorophore. To account for this quenching effect, PBS buffer (pH 7.4) only was added, in an identical manner as the CAR-Cy5 molecules, as a control condition. On the other hand, Cy5 emission peak can increase with increasing concentration of the protein either by absorbing excitation energy or by the constant light in the equipment. To control for the effect of Cy5 excitation from light, the Cy5 proteins without any Cy3 protein was added but replaced by PBS. The change in fluorescent intensity was calculated after deducting both the PBS and Cy5 controls. Sigmaplot statistical software was used for data analysis. FRET assay was used to determine CAR affinity for each of the PDZ domains and intermolecular distance between the binding proteins.

Plot of change in fluorescence as a function of PDZ concentration showed a saturable ligand binding curve indicating high affinity binding for PDZ1-CAREx8 (Fig.
Fig. 39  Fluorescence resonance energy transfer (FRET) from Cy3-labeled MAGI-1 PDZ1 domain to Cy5-labelled CAR C-terminus. Isolated MAGI-1 PDZ domain and CAR C-terminus proteins were labeled with fluorescent Cy3 and Cy5 fluorophores, respectively. All spectra were corrected for background fluorescence. Emission spectra of Cy3-PDZ1 upon excitation at 550nm at 0, 10, 50, 200, 500 nM A) Cy5-CAREx7 or C) Cy5-CAREx8 respectively. FRET from donor Cy3-PDZ1 to acceptor Cy5-CAR was detected as quenching of Cy3 fluorescence emission (≈ 570 nm) and as the appearance of sensitized Cy5 emission (≈ 670 nm). Saturable binding curves were obtained for B) CAREx7 and D) CAREx8 by plotting change in fluorescence intensity due to the quenching of Cy3 fluorescence as a function of CAR concentration from which protein binding affinity was calculated. Values represent the mean ± SE, n = 5
FRET from Cy3-labeled MAGI-1 PDZ3 domain to Cy5-labelled CAR C-terminus. Isolated MAGI-1 PDZ3 domain and CAR C-terminus proteins were labeled with fluorescent Cy3 and Cy5 fluorophores, respectively. FRET was measured from 30nM PDZ3 in the presence of increasing concentrations (0-500nM) of CAR. All spectra were corrected for background fluorescence. Emission spectra of Cy3-PDZ1 upon excitation at 550nm at 0, 10, 50, 200, 500 nM A) Cy5-CAR$^\text{Ex7}$ or C) Cy5-CAR$^\text{Ex8}$ respectively. FRET from donor Cy3-PDZ3 to acceptor Cy5-CAR was detected as quenching of Cy3 fluorescence emission ($\approx 570$ nm) and as the appearance of sensitized Cy5 emission ($\approx 670$ nm). Saturable binding curves were obtained for B) CAR$^\text{Ex7}$ and D) CAR$^\text{Ex8}$ by plotting change in fluorescence intensity due to the quenching of Cy3 fluorescence as a function of CAR concentration from which protein binding affinity and intermolecular distance were calculated. Binding curve values represent the mean $\pm$ SE, $n = 5$

$F.I. = $fluorescence intensity
Table 8 Binding affinity and intermolecular distance between MAGI-1 PDZ domains (1&3) and CAR C-terminus: FRET results

<table>
<thead>
<tr>
<th>FRET pair</th>
<th>Donor emission</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Donor</td>
</tr>
<tr>
<td>MAGI-1 PDZ1</td>
<td>CAR$^{\text{Ex7}}$ C-terminus</td>
</tr>
<tr>
<td></td>
<td>CAR$^{\text{Ex8}}$ C-terminus</td>
</tr>
<tr>
<td>MAGI-1 PDZ3</td>
<td>CAR$^{\text{Ex7}}$ C-terminus</td>
</tr>
<tr>
<td></td>
<td>CAR$^{\text{Ex8}}$ C-terminus</td>
</tr>
</tbody>
</table>

Values were calculated based upon the decrease in donor fluorescence (PDZ domain) with respect to increasing acceptor concentration (CAR). Values represent the mean value ± standard error, $n = 5$

$ND$ Not detected
PDZ3- CAR\textsuperscript{Ex7} (Fig. 40B) and PDZ3-CAR\textsuperscript{Ex8} (Fig. 40D) with dissociation constant (K\textsubscript{d} values) less than 10 nM (PDZ3-CAR\textsuperscript{Ex8} > PDZ3-CAR\textsuperscript{Ex7} > PDZ1-CAR\textsuperscript{Ex8}; Table 8). These reactions suggest direct interaction between the proteins since the FRET for each reacting combination yielded intermolecular distance less than 100Å (40 < R< 60; Table 8). MAGI-1 CAR\textsuperscript{Ex7} did not show significant interaction with PDZ1 (Fig. 39B) with no significantly detectable K\textsubscript{d} value confirming the specificity of the assay.

**Direct fluorescent binding assay Cy3-labeled MAGI-1 PDZ domain proteins and unlabeled CAR C-terminus**

A direct binding assay was done to account for any conformational changes that could occur during an interaction. The procedure was similar to FRET except that one of the interacting proteins was labeled and the second unlabeled. Only PBS control was run in this set of experiments. Cy3-labeled proteins were excited at 550 nm and emission was 560-650 nm. Cy3 fluorescence on PDZ was quenched with increasing concentrations of non-labeled CAR C-terminus. In good correlation with the FRET studies, binding curves showed high affinity binding (Fig. 41B-D) with PDZ3-CAR\textsuperscript{Ex8} > PDZ3-CAR\textsuperscript{Ex7} >PDZ1-CAR\textsuperscript{Ex8} (K\textsubscript{d} = 2.6-42 nM, Table 9). No detectable binding was found between PDZ1 and CAR\textsuperscript{Ex7} C-terminus (Fig. 41A). The affinities detected by direct binding assay were weaker than those determined by FRET, although the trends were constant, likely due to greater sensitivity of FRET (67). Similar FRET and direct binding assays also confirm that CAR\textsuperscript{Ex7} and CAR\textsuperscript{Ex8} directly bind MAGI-1 PDZ3 specifically without the requirement of a helper or intermediate protein and only CAR\textsuperscript{Ex8} directly interacts with the PDZ1 domain. These results with K\textsubscript{d} values in the nM range also confirm the in vivo data of co-immunoprecipitation that involves overexpression of proteins. The results also
**Fig. 41** Fluorescent ligand binding assays of MAGI-1 PDZ domains and CAR C-terminus. Direct binding of Cy3-PDZ1 by A) CAR<sup>Ex7</sup> and B) CAR<sup>Ex8</sup> C-terminus or Cy3-PDZ3 by C) CAR<sup>Ex7</sup> and D) CAR<sup>Ex8</sup> c-terminus was determined by quenching of Cy3 fluorescence. PDZ domain protein was titrated with increasing concentrations of CAR. Emission was measured at 570nm upon excitation at 550nm. Emission spectra were corrected for background signal PDZ alone in buffer (PBS) and CAR alone in buffer. Change in fluorescence was plotted with respect to CAR concentration. Binding curve values represent the mean ± SE, n = 5.

*F.I.* = fluorescence intensity
**Table 9** Binding affinity of MAGI-1 PDZ domains (1&3) for CAR C-terminus

<table>
<thead>
<tr>
<th>Binding pair</th>
<th>Quenching</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>Acceptor</td>
</tr>
<tr>
<td>MAGI-1 PDZ1</td>
<td>CAR&lt;sup&gt;Ex7&lt;/sup&gt; C-terminus</td>
</tr>
<tr>
<td></td>
<td>CAR&lt;sup&gt;Ex8&lt;/sup&gt; C-terminus</td>
</tr>
<tr>
<td>MAGI-1 PDZ3</td>
<td>CAR&lt;sup&gt;Ex7&lt;/sup&gt; C-terminus</td>
</tr>
<tr>
<td></td>
<td>CAR&lt;sup&gt;Ex8&lt;/sup&gt; C-terminus</td>
</tr>
</tbody>
</table>

Values represent the mean value ± standard error, n = 5
confirm that the C-terminus of CAR is enough to interact with the PDZ1 and/or PDZ3 domain of MAGI-1, and vice versa. In addition, the intermolecular distances were consistent with close molecular interaction in PDZ3- CAR and PDZ1-CAR\textsuperscript{Ex8} C-terminus rather than non specific interaction as observed in PDZ1-CAR\textsuperscript{Ex7} C-terminus.

**Conclusions**

The results from these interactions have confirmed that CAR directly interacts with MAGI-1 PDZ domains through the C-terminal domain. Surprisingly, both CAR isoforms interact with the PDZ3 domain of MAGI-1 (Fig. 42A, B). However, only CAR\textsuperscript{Ex8} interacts with MAGI-1 PDZ1 (Fig. 42A). The difference in response of each isoform to MAGI-1 interaction is not yet understood. We are currently looking at candidate phosphorylation sites on both CAR isoforms to determine whether this may modulate or alter the PDZ-based interaction. We are also studying the effect of mutating candidate tyrosine residues on CAR\textsuperscript{Ex8} in order to understand the molecular mechanisms involved. In addition, both CAR\textsuperscript{Ex7} and CAR\textsuperscript{Ex8} have a high affinity for MAGI-1 PDZ3 with \( K_d \) values in the nM ranges. This suggests that if CAR and MAGI-1 are in proximity, they can bind strongly in cells. Future work should involve transfecting the purified labeled proteins directly into cells using protein transfection reagent in order to determine interactions and localization *in vivo*. In addition, only CAR\textsuperscript{Ex8} interacts with MAGI-1 PDZ1, albeit at a lower affinity. Moreover, it is possible that CAR\textsuperscript{Ex8} may simultaneously bind to both PDZ1 and 3. The physiological implications of this are currently unknown. Finally, in some cases, other PDZ domains are required for physiological interactions to occur. It will thus be necessary in the future to use PDZ deletion mutants of MAGI-1 to verify these interactions.
**Fig. 42** Model of CAR interaction with MAGI-1 PDZ.

A. \( \text{CAR}^{\text{Ex8}} \) interacts with both PDZ1 and PDZ3

B. \( \text{CAR}^{\text{Ex7}} \) interacts with only PDZ3
CHAPTER 5: BIOLOGICAL IMPLICATIONS OF MAGI-1 MEDIATED LOSS OF CAR^{Ex8}

Rationale

MAGI-1b PDZ domain 3 interacts with both CAR^{Ex7} and CAR^{Ex8}, while PDZ1 interacts only with CAR^{Ex8}. There were no detectable interactions between CAR and PDZ0, 2, 4, or 5, thus, the focus of this chapter was on CAR^{Ex8} and full length MAGI-1, isolated PDZ1 or isolated PDZ3. This knowledge now allows the dissection of the functional consequences of these interactions (i.e. build a model of multiple simultaneous scaffolding interactions, Table 1), define the important residues in these interactions, and potentially lead to a greater understanding of the role of these protein interaction domains within MAGI-1. It was verified that both MAGI-1 PDZ1 and PDZ3 interact with CAR^{Ex8} with high affinity by in vitro FRET and binding assays (chapter 4). The next goal was to determine whether the MAGI-1-mediated loss of CAR^{Ex8} was due to one specific domain, both domains, or required the whole MAGI-1 proteins. Moreover, whether competition between the isolated MAGI-1 PDZ1 and PDZ3 domains would alter the loss of CAR^{Ex8}. I hypothesized that an isolated interacting PDZ domain would not compete with the wild type interaction to allow co-existence of CAR^{Ex8} and MAGI-1. The preliminary data (1) show that the physiological response to the loss of CAR^{Ex8} upon co-expression with MAGI-1 is reduced adenovirus infection. If an isolated MAGI-1 PDZ domain competed with the wild type domain to prevent CAR^{Ex8} degradation by MAGI-1,
a resultant increase in adenovirus infection was expected. I hypothesized that isolated MAGI-1 PDZ domain(s) would not prevent CAR\textsuperscript{Ex8} degradation by MAGI-1 interaction and therefore would not allow adenovirus infection.

Results

\textbf{CAR\textsuperscript{Ex8} increases adenovirus infection}

The effect of expressing individual CAR\textsuperscript{Ex8}, full length MAGI-1, or isolated MAGI-1 PDZ domains on adenovirus infection was confirmed in CHO-K1 cells that do not express endogenous CAR (Fig. 43). CHO-K1 cells were transfected with equal amounts of plasmid encoding CAR\textsuperscript{Ex8} or MAGI-1, or isolated PDZ1, or PDZ3 domain, alone. Some cells were transfected with empty pcDNA3.1 plasmid as controls. The transfected cells were infected with adenovirus after 48 hours of incubation. A second control experiment was pcDNA3.1 plasmid transfected cells that were not infected with any adenovirus. Control cells that were infected with adenovirus showed no significant difference in adenovirus infection from cells without adenovirus infection. As expected, MAGI-1 did not cause any significant increase in adenovirus infection as compared to pcDNA3.1 plasmid transfected control cells. This represents the baseline amount of infection. Similar results were obtained with isolated PDZ domains. Both MAGI-1 PDZ1 and PDZ3 did not increase adenovirus infection from the base value. This confirms that neither MAGI-1 nor the isolated domains by themselves are adenovirus receptors. Expression of CAR\textsuperscript{Ex8}, on the other hand, shows approximately a 6 fold increase in adenovirus infection confirming its adenovirus receptor function.
Fig. 43 CAR<sup>Ex8</sup> behaves as an adenovirus receptor but MAGI-1 does not. CHO-K1 cells were transfected with CAR or MAGI-1b plasmids followed by adenovirus infection and β-galactosidase assay. While CAR<sup>Ex8</sup> expression allows adenovirus infection, isolated PDZ1 and PDZ3 domains did not increase viral infection. Control experiments include pcDNA3.1 empty plasmid transfected cells with/without adenovirus infection.
**Co-transfection of MAGI-1 PDZ3, but not PDZ1, with CAR\textsuperscript{Ex8} reduces adenovirus infection**

Knowing that isolated MAGI-1 PDZ domains do not increase adenovirus infection in CHO-K1 cells, I hypothesized that these PDZ domains would not affect adenovirus infection when co-transfected with CAR\textsuperscript{Ex8}. Equal amount of full length MAGI-1 or each of the interacting MAGI-1b domain constructs, PDZ1 or PDZ3, was co-transfected with CAR\textsuperscript{Ex8}, followed by adenovirus infection and β-galactosidase assay, as previously described. All transfections were balanced with empty pcDNA3.1. As previously shown, co-expression of CAR\textsuperscript{Ex8} and MAGI-1 reduces adenovirus infection in comparison to cells transfected with CAR\textsuperscript{Ex8} alone (Fig. 44). Interestingly, isolated MAGI-1 PDZ1 co-transfected with CAR\textsuperscript{Ex8} did not significantly alter adenovirus infection, in comparison to CAR\textsuperscript{Ex8} alone. However, co-expression of PDZ3 with CAR\textsuperscript{Ex8} reduced adenovirus infection to a similar degree as MAGI-1b plus CAR\textsuperscript{Ex8} suggesting that MAGI-1 PDZ3 domain may be the “death domain” contributing to CAR\textsuperscript{Ex8} loss (Fig. 44).

**MAGI-1 PDZ1 competes with full length MAGI-1 in the presence of CAR\textsuperscript{Ex8} to increase adenovirus infection**

One important goal of this work was to find a way of regulating the MAGI-1-CAR\textsuperscript{Ex8} interaction towards increasing adenovirus infection for gene therapy or decreasing cell surface expression during viral outbreaks. Therefore, we asked whether there were small molecules that could compete with MAGI-1 to prevent loss of CAR\textsuperscript{Ex8}, thereby increasing adenovirus infection. The first step was taken by using isolated PDZ 1 and PDZ3 domains to determine if either of these domains might compete with MAGI-
**Fig. 44** MAGI-1 PDZ3 domain causes a loss of adenovirus infection. $\text{CAR}^{\text{Ex8}}$ plasmid co-transfected with empty pcDNA 3.1 plasmid, full length MAGI-1, isolated MAGI-1 PDZ1 domain, or PDZ3 domain in CHO-K1 cells followed by adenovirus infection and β-galactosidase assay. $\text{CAR}^{\text{Ex8}}$ increases adenovirus infection, MAGI-1 (green) and isolated PDZ3 domain (blue) both reduce adenovirus infection, while PDZ1 domain (red) has no effect on adenovirus infection in the presence of $\text{CAR}^{\text{Ex8}}$. 
1b. CHO-K1 cells were triple transfected with CAR\textsuperscript{Ex8}, MAGI-1b, and each isolated PDZ domain, at a constant dose. All transfections were balanced with empty pcDNA3.1 plasmid. Transfected cells were transduced with Ad-β-gal 48 hours later. Beta-galactosidase expression was determined 24 hours post-transduction. CAR\textsuperscript{Ex8} increased adenovirus infection and MAGI-1 reduced adenovirus infection when co-transfected with CAR\textsuperscript{Ex8}, as expected (Fig. 45). Expression of PDZ3 with CAR\textsuperscript{Ex8} at the same time as full length MAGI-1 reduced infection to a similar degree as MAGI-1 alone or PDZ3 alone suggesting that these proteins are not additive. Interestingly, PDZ1 rescued adenovirus infection when triple transfected with CAR\textsuperscript{Ex8} and MAGI-1b suggesting that PDZ1 can compete with MAGI1-b to prevent CAR\textsuperscript{Ex8} degradation (Fig. 45). This was surprising in consideration that the relative affinity of PDZ3 is roughly four times greater than PDZ1 by FRET (Table 8). This suggests that either the affinity of PDZ3 is different when in the context of full length MAGI-1b or the small isolated domain is somehow able to interact and protect CAR\textsuperscript{Ex8}. It is also possible that the protein required for destruction of CAR\textsuperscript{Ex8} binds to the PDZ1 domain of MAGI-1 and overexpression of PDZ1 sequesters this molecule from MAGI-1b. Future experiments will involve binding assays to study the competition between full length MAGI-1 and each isolated PDZ domain when both are in the presence of CAR\textsuperscript{Ex8}. Mutation of the PDZ binding site on PDZ1, followed by competition experiments, may also provide illumination as to the molecular mechanism of reaction between CAR\textsuperscript{Ex8} and MAGI-1.
Fig. 45 Isolated MAGI-1 PDZ1 domain, in the presence of full length MAGI-1, allows adenovirus infection in presence of CAR<sup>Ex8</sup>. CAR<sup>Ex8</sup> and the MAGI-1 plasmids were triple transfected with isolated MAGI-1 PDZ1 domain or PDZ3 domain in CHO-K1 cells followed by adenovirus infection and β-galactosidase assay. CAR<sup>Ex8</sup> expression allows adenovirus infection unless in the presence of MAGI-1 (green). Isolated PDZ3 domain (blue) reduced adenovirus infection to a similar degree as either MAGI-1 or PDZ3 alone. Interestingly, isolated PDZ1 domain (red) is able to rescue adenovirus infection in the presence of MAGI-1 and CAR<sup>Ex8</sup>.
**CAR\textsuperscript{Ex8} is degraded in the presence of MAGI-1**

Adenovirus infection normally proceeds rapidly when adenovirus binds to CAR at cell surface. The localization of CAR at the cell surface is therefore the important factor in adenovirus infection. We have not been able to verify whether CAR\textsuperscript{Ex8} is degraded in the cell or prevented from getting to cell surface during the CAR-MAGI-1 interaction. It is possible that MAGI-1 causes CAR loss by targeting it for degradation at the time of synthesis, or MAGI-1 targets a chaperone or cofactor required for CAR\textsuperscript{Ex8} translation, or MAGI-1b simply holds CAR within the cell. To answer this question, CHO-K1 cells were co-transfected with MAGI-1, isolated PDZ1, or PDZ3, with CAR\textsuperscript{Ex8} and incubated at 37°C for 48 hours. Total cell lysates was then used for SDS-PAGE and Western blot and, using actin to control for protein load during PAGE, proteins were blotted for CAR (m\textsubscript{a}FLAG) and PDZ domains (m\textsubscript{a}V5). There was no total loss of CAR\textsuperscript{Ex8} after co-transfection with the MAGI-1 PDZ domains (Fig. 46). CAR appeared as three bands; (mature (~46 kDa), hemi-glycosylated (~43 kDa), immature (~40kDa)) when electrophoresis was allowed to proceed for a long time. The three bands put together showed equal expression intensity for CAR\textsuperscript{Ex8} transfected cells with the PDZ3 domain (Fig. 46). On the other hand, there was more mature CAR\textsuperscript{Ex8} when co-transfected with MAGI-1 PDZ1. This likely reflects a shift in the glycosylation state of CAR\textsuperscript{Ex8}. Glycosylation is known to reflect CAR\textsuperscript{Ex8} maturity and may therefore play a role in adenovirus infection. Future experiments should involve confirming the glycosylation by transfecting cells with glycosylation deficient CAR and monitor adenovirus infection in the presence of PDZ domains and MAGI-1.
Fig. 46 MAGI-1 leads to a loss of CAR$^{Ex8}$ protein. CAR$^{Ex8}$ was co-transfected with full length MAGI-1, isolated MAGI-1 PDZ1, or PDZ3 in CHO-K1 cells. Cell lysate obtained after 48 hours incubation and Western blot for CAR (m$\alpha$FLAG), MAGI-1 PDZ domain (m$\alpha$V5) and actin (ractin) showed A) Loss of CAR$^{Ex8}$ in the presence of MAGI-1, B) Percent CAR$^{Ex8}$ loss in the presence of MAGI-1. C) Isolated PDZ domains do not lead to loss of CAR$^{Ex8}$ but may lead to a shift in the glycosylation state of CAR.
Since no significant reduction in the total amount of CAR\textsuperscript{Ex8} was observed in cells in the presence of either isolated MAGI-1 PDZ domain, the surface amount of CAR\textsuperscript{Ex8} in the presence of PDZ1 and PDZ3 was investigated. I hypothesized that, in correspondence with the amount of adenoviral infection, there would be significantly more CAR\textsuperscript{Ex8} at the cells surface when co-expressed with PDZ1 than PDZ3. CAR\textsuperscript{Ex8} was co-transfected with MAGI-1 or isolated PDZ1 or PDZ3 in 100mm culture dishes and incubated for 48 hours. Cells were moved to ice in order to reduce endocytosis and Easy-link biotin (Thermo Scientific, Rockford, IL) was added to the cells for 1 hour with rocking at 4°C. This type of biotin binds to all cell surface proteins. Cell lysates were then incubated with Neutravidin, which has avidin conjugated to beads, followed by SDS-PAGE and Western blot. NeutrAvidin precipitates biotin, which is conjugated only to membrane proteins (68). A significant decrease in surface levels of CAR\textsuperscript{Ex8} in the presence of full length MAGI-1 or PDZ3 was observed suggesting that in contrast to the absence of CAR\textsuperscript{Ex8} when co-expressed with MAGI-1b, PDZ3 holds CAR within the cell (Fig. 47). Replicate experiments are still needed to confirm these data.

**MAGI-1 PDZ1 competes with full length MAGI-1 to prevent CAR\textsuperscript{Ex8} loss in COS-7 cells**

Adenovirus infection was used to show that isolated MAGI-1 PDZ1 prevents full length MAGI-1 from reducing adenovirus infection when CHO-K1 cells were triple-transfected with CAR\textsuperscript{Ex8} plasmids. Moreover, this data suggested that PDZ1 is not involved in CAR\textsuperscript{Ex8} loss whereas PDZ3 is (Fig. 45). To determine CAR\textsuperscript{Ex8} localization in cells transfected with full length MAGI-1 in the presence of PDZ1 or PDZ3, COS-7 cells
**Fig. 47** MAGI-1 PDZ3 reduces cell surface CAR<sup>Ex8</sup> expression. CAR<sup>Ex8</sup> was co-transfected with empty pcDNA3.1 plasmid, full length MAGI-1, isolated MAGI-1 PDZ1, or PDZ3 in CHO-K1 cells and incubated for 48 hours. Cell surface proteins were labeled with biotin, isolated on Neutravidin beads, and were subsequently A) Western blotted with CAR antibody (mαFLAG) or for actin. B) ImageJ quantitation of Western blot, relative to actin. PDZ3 and full length MAGI reduced amount of surface CAR<sup>Ex8</sup> by about 70% in comparison to co-transfection with empty plasmid or PDZ1.
were triple transfected with full length GFP-tagged MAGI-1, FLAG-tagged CAR^{Ex8} and either myc-tagged PDZ1 or myc-tagged PDZ3 and incubated for 48 hours before immunocytochemistry. Confluent cells expressed green MAGI-1 (Fig. 48A, green), CAR^{Ex8} (Fig. 48C; mFLAG, grey), and PDZ1 (Fig. 48B; rmyc, red). With the confocal microscope, using a 60x oil immersion lens, majority of cells expressed PDZ1, MAGI-1 and CAR^{Ex8} in the same cell (Fig. 48D; nuclei shown in blue). This suggests that PDZ1 prevents MAGI-1 from causing CAR^{Ex8} loss and also prevents decrease adenovirus infection due to MAGI-1 (Fig. 45). It was also observed that PDZ1 seemed to overlap with MAGI-1 more than CAR^{Ex8} and CAR^{Ex8} was localized at the junctions. The effect of PDZ3 on CAR^{Ex8} in cells expressing PDZ3 and/or MAGI-1 could not be concluded by immunocytochemistry (Figure 49). The only few cases where there appeared to be co-expression of CAR^{Ex8} and PDZ3 or MAGI-1, the expression level of CAR^{Ex8} was very low and appeared sequestered within the cell although in a small number of cells, CAR^{Ex8} was at the junctions. The results suggest that CAR^{Ex8}-MAGI-1 interactions leading to CAR^{Ex8} loss occurs at the PDZ3 domain.
Fig. 48 Isolated PDZ1 domain, in the presence of full length MAGI-1, prevents CAR<sub>Ex8</sub> loss. COS-7 cells were triple transfected with myc-tagged MAGI-PDZ1 domain, full length GFP-tagged MAGI-1 and FLAG-tagged CAR<sub>Ex8</sub> and subjected to immunocytochemistry two days post seeding. Confluent cells expressed A) green fluorescent MAGI-1, and were stained for B) PDZ1 (red, rmyc), and C) CAR<sub>Ex8</sub> (grey, mFLAG), and D) merge, nuclei shown in blue. PDZ1, full length MAGI-1 and CAR<sub>Ex8</sub> were co-expressed in same cells. 60x oil immersion confocal microscopy. Results represent several cells co-expressing PDZ1, MAGI-1 and CAR<sub>Ex8</sub>.
**Fig. 49** Isolated PDZ3 domain and full length MAGI-1 contribute to CAR\textsuperscript{Ex8} loss. COS-7 cells were triple transfected with myc-tagged MAGI-PDZ3 domain, full length GFP-tagged MAGI-1 and FLAG-tagged CAR\textsuperscript{Ex8} and subjected to immunocytochemistry two days post seeding. Confluent cells expressed A) green fluorescent MAGI-1, and were stained for C) CAR\textsuperscript{Ex8} (grey; m\textsuperscript{#}FLAG) and B) PDZ3 (red; r\textsuperscript{#}myc), D) merge (nuclei shown in blue). PDZ3, full length MAGI-1 and CAR\textsuperscript{Ex8} did no co-express in same cells except in few cases where CAR\textsuperscript{Ex8} expression is reduced. 60x oil immersion confocal microscopy.
Conclusions

Thy physiological effect of CAR\textsuperscript{Ex8}-MAGI-1 interaction was measured by the effect on total CAR expression, cell surface levels, and adenovirus infection. Results showed that isolated MAGI-1 PDZ1 did not prevent adenovirus infection, whereas MAGI-1 PDZ3 reduced adenovirus infection, like full length MAGI-1 (1), when co-transfected with CAR\textsuperscript{Ex8}. While PDZ3 reduced adenovirus infection to a similar extent as MAGI-1 (Fig. 45), PDZ1 was able to compete with MAGI-1 to retain adenovirus infection. These data also confirmed that PDZ1 prevented CAR\textsuperscript{Ex8} loss when co-expressed with both CAR\textsuperscript{Ex8} and MAGI-1 in COS-7 cells. I propose a model (Fig. 50) where PDZ1 binds CAR\textsuperscript{Ex8} and prevents MAGI-1 binding leading to more CAR\textsuperscript{Ex8} at cell surface. CAR\textsuperscript{Ex8} increase at cell surface leads to a corresponding increase in adenovirus infection. Alternatively, PDZ1 binds a degradation protein that would ordinarily take CAR\textsuperscript{Ex8}/MAGI-1 to the proteosome, thereby preventing CAR\textsuperscript{Ex8} degradation. Future experiments will involve competition between PDZ1 and PDZ3 during CAR\textsuperscript{Ex8} interaction and subsequently performing the experiments in polarized epithelia.
Fig. 50 A model for a potential mechanism of PDZ1 preventing CAR^{Ex8} loss due to MAGI-1. PDZ1 preferentially binds CAR^{Ex8} thereby preventing full length MAGI-1 from binding. MAGI-1 co-expresses with CAR^{Ex8} in the cell but cannot target CAR^{Ex8} for degradation. This allows more surface expression of CAR^{Ex8} thus allowing increased adenovirus infection. ER, endoplasmic reticulum; G, Golgi; TJ, tight junction; AP, apical (air) surface; BL, basolateral surface.
CHAPTER 6: DISCUSSION

This report describes the molecular interaction between the PDZ domain containing protein MAGI-1 and two distinct isoforms of CAR. Here, I have demonstrated that two CAR isoforms (CAR$^{\text{Ex7}}$ and CAR$^{\text{Ex8}}$) are expressed in several cell lines with CAR$^{\text{Ex7}}$ consistently more highly expressed than CAR$^{\text{Ex8}}$. Knockdown of MAGI-1 with siRNA improves adenovirus infection in polarized cells. Both isoforms bind to MAGI-1 PDZ3 domain with high affinity while CAR$^{\text{Ex8}}$ also binds to PDZ1 domain with a slightly lower affinity. I also have demonstrated that PDZ3 causes a loss of adenovirus infection (Fig. 44), however it differs from MAGI-1 in that MAGI-1 causes a loss of CAR protein while a significant amount of CAR protein is still detectable in the presence of PDZ3 (Fig. 46). Finally, I have also demonstrated that PDZ1 can compete with full length MAGI-1 and protect CAR from loss (Fig. 48).

I conclude that CAR isoforms are expressed in various tissues from the Western blot (Fig. 9), qRT-PCR (Fig. 10), and immunocytochemistry (Fig. 12-17) experiments. This data is supported by differential tissue dependent expression and localization for the mCAR$^{\text{Ex7}}$ and mCAR$^{\text{Ex8}}$ isoforms demonstrated in mouse (29-31). COS-7 produced more CAR than other cell lines. We have observed that COS-7 produces high levels of many proteins (data not shown) in other experiments suggesting that this cell line is a good source of proteins for Western blot experiments. Although transfected FLAG-tagged CAR$^{\text{Ex8}}$ was easily detectable, I was not able to convincingly detect endogenous
CAR$^{\text{Ex8}}$ levels by Western blot in these experiments. The most likely reasons for the inability to detect CAR$^{\text{Ex8}}$ by Western blot are the low level of this isoform or that the isoform specific polyclonal antibody may not be sensitive enough for Western blot. It is common that antibodies may function well for some assays but not others. Future experiments will involve the development of a monoclonal antibody specific for CAR$^{\text{Ex8}}$.

The alternative splicing event that creates CAR$^{\text{Ex8}}$ occurs at a cryptic splice site within the seventh exon whereas the transcript for CAR$^{\text{Ex7}}$ reads through this potential splice site and terminates at the end of the seventh exon. It is possible that since the splice site is not typical, it is more likely to produce CAR$^{\text{Ex7}}$ than CAR$^{\text{Ex8}}$ in cells under normal circumstances. Alternatively, there may be specific splicing factors to recognize this site that are in low abundance. Low levels of human CAR$^{\text{Ex8}}$ have been reported in primary airway epithelia (1), thus, I hypothesized that RNA transcript levels for CAR$^{\text{Ex7}}$ would be more abundant than CAR$^{\text{Ex8}}$ in most cell types. I have shown that CAR$^{\text{Ex7}}$ RNA transcripts are about 8 times more prevalent than CAR$^{\text{Ex8}}$ RNA transcripts in Calu3 cells to as high as 14 times more in 293T cells. These results suggest that although the CAR$^{\text{Ex7}}$ transcript level is consistently higher, the difference between the two isoforms vary between different cell lines. The reasons for the variability in isoform ratio of RNA transcript are not yet known. This may be due to the type of cells (29-31) or variability within the cells during growth. Although each cell line grew at a different rate, I did not synchronize the cells before RNA extraction for qPCR to check the effect of cell cycle on RNA ratios of CAR isoforms. Cell confluence and polarization may also alter that ratio of CAR isoforms. Future experiments should also involve extracting RNA from polarized cells and see the possibility of any change in RNA transcripts as the cells polarize. A time
course experiment should be conducted in the future. This may suggest whether CAR\textsuperscript{Ex8} apical localization in polarized cells is due to increases in RNA synthesis with respect to CAR\textsuperscript{Ex7} RNA or not.

I measured the transepithelial resistance of cells seeded in Millicells to check the possibility of polarization among the cell lines. Cell polarization results in tight junction formation that separates apical from basolateral surfaces of the cell. This was observed after an air-liquid interphase was formed and the apical surface was dry because no media could pass across the newly formed tight junctions in the cells from basolateral surface where the media was added, to the apical surface. The higher the TER (>300mΩ/cm\textsuperscript{2}), the tighter the junctions and the more polarized the cells on the Millicells. From the measurement of high TER values, I conclude that MDCK, Calu3 and Caco2 cells polarize (Fig. 11). This is similar to primary airway epithelia (1) and other polarized epithelia cells (18, 20, 69, 70). The resulting morphological and biochemical changes lead to distribution of proteins and lipids to different parts of membranes (71, 72) as demonstrated by ZO-1 localization at tight junctions and CAR\textsuperscript{Ex7} sequestration at the basolateral surface (Figs. 13, 15). The localization of CAR\textsuperscript{Ex7} at the basolateral surface makes it inaccessible for apical viral infection (1). A549 cells on the other hand, do not attain any measure of transepithelial resistance above background (Fig. 11) and do not show continuous CAR staining around each epithelial cell (Figure 14) suggesting that the tight junctions are not formed and it is accessible for adenovirus infection (73). CAR\textsuperscript{Ex8} expression was very low in the polarized cell lines. Interestingly the isoform showed largely punctate staining found at the subapical surface and some at the junctions (Figs. 15, 17). The localization does not conform directly to the observation in primary airway
epithelia (1). Immortal cell lines are aneuploid cells so they may not necessarily express
CAR like primary cells as a result of undergoing several changes during culturing.
Future experiments should therefore involve the use of primary airway epithelia or the
development of regulatable cells that express CAR isoforms.

Excoffon et al., (1) suggested that adenovirus binding to endogenous $\text{CAR}^{\text{Ex8}}$ on
the apical membrane is a direct pathway for adenovirus binding and entry in the airway.
The current model for initiation of viral infection requires a breach in the tight junction
barrier allowing the virus access to the basolateral receptor (24, 25). For example, a small
number of CVB interact with the decay-accelerating factor (DAF) – a protein found at the
apical surface of epithelia (26, 27). Interaction of CVB with DAF in Caco-2 cells, a
polarized intestinal epithelial cell line, activates kinase signaling cascades that result in
viral translocation to epithelial cell junctions and a reduction of tight junction integrity.
This breach in the junctions is required to allow an essential interaction with basolaterally
localized CAR, and viral entry, likely by macropinocytosis. The requirement for
junctional penetration may be overcome by apical CAR expression, as experimentally
shown by substituting the transmembrane and C-terminus of CAR with a
glycophosphatidylinositol (GPI) tail (22). Zhong et al. (73) also observed a 10-fold
increase in transduction in MDCK cells treated with EDTA during adenovirus infection
as a reflection of tight junction disruption. Even though a breach in tight junction
integrity may play a role in adenovirus infection, results suggest that $\text{CAR}^{\text{Ex8}}$ is a direct
pathway to apical initiation of adenovirus infection in polarized cells. I did not observe a
significant difference in transepithelial resistance between cells overexpressing $\text{CAR}^{\text{Ex7}}$
and $\text{CAR}^{\text{Ex8}}$ (Fig. 19A), however, there was an increased adenovirus infection in $\text{CAR}^{\text{Ex8}}$
expressing cells than CAR\textsuperscript{Ex7} expressing cells (Fig. 19B). Consistent with earlier observation (1) and corresponding to expectations due to adenovirus infection, overexpressed CAR\textsuperscript{Ex7} is localized more at the basolateral surface (Fig. 16) while overexpressed CAR\textsuperscript{Ex8} is localized at the apical and sub apical surface of polarized MDCK cells (Fig. 17). Adenovirus infection in CAR\textsuperscript{Ex7} transduced cells was higher than control cells. This may be as a result of the overexpression of the isoform within the cell thereby allowing some access to the protein or some proteins may reach the cell surface with overexpression. The movement of CAR\textsuperscript{Ex8} towards the sub apical surface clearly demonstrates a difference in trafficking between the two isoforms. The difference in adenovirus infection as a result of the two isoforms is less than 10 fold. This may be a result of receptor saturation by adenovirus during infection. In future experiments, there is a need to develop isoform-specific regulatable cells so that the level of expression of each isoform can be carefully controlled during experiments. This will solve the problems of how much overexpression is achieved (i.e. not grossly overexpressed) and how many cells are expressing exogenous CAR (i.e. transfection/transduction efficiency).

While the biological reason behind apical localization of an adhesion protein is unknown, modulation of its expression levels presents an opportunity to affect viral susceptibility. Excoffon \textit{et al.} (1) have previously reported that MAGI-1 plays an important role in CAR\textsuperscript{Ex8} levels and localization by proposing that the loss of CAR was due to MAGI-1 after demonstrating reduction in adenovirus infection due to expression of MAGI-1. However, since the study only hypothesized that reducing the endogenous expression of MAGI-1 would affect CAR\textsuperscript{Ex8} expression and adenovirus infection in polarized epithelia cells, I decided to knock down MAGI-1. I hypothesized therefore, that
siRNA knockdown of MAGI-1 will increase adenovirus infection in epithelia cells. Due to limitations to the use of primary human airway epithelia (expensive, difficult to manipulate, high donor and culture variability) and low expression of CAR\textsuperscript{Ex8} that is difficult to detect except by immunocytochemistry, siRNA experiments were performed in model epithelial cells MDCK. Even though, MDCK is a canine cell line, it was chosen for siRNA study because it demonstrated several key characteristics including expression of CAR isoforms, polarization (Figs. 9-15), and MAGI-1 expression that was detectable by Western blot and immunocytochemistry (Figs. 20-22). Caco2 and Calu3 cells are human epithelia cells and also possess the necessary characteristics. They would have provided the excellent cell lines for MAGI-1 siRNA study but the only MAGI-1 antibody able to detect endogenous MAGI-1 was specific for MDCK cells. Future studies should involve developing an antibody that will recognize human MAGI-1 so that MAGI-1 knockdown can be performed in human primary airway epithelia.

I was able to consistently knockdown MAGI-1 protein expression (Figs. 23, 24) and RNA transcript levels (Fig. 25) with two MAGI-1 siRNAs targeting different parts of MAGI. Even though I was able to knockdown MAGI-1, I was not able to detect a significant increase in CAR\textsuperscript{Ex8} levels as consequence of MAGI-1 knockdown. I did not even detect CAR\textsuperscript{Ex8} by Western blot in control cells. This may be due to the low CAR\textsuperscript{Ex8} expression levels in these cells and/or the sensitivity of our CAR\textsuperscript{Ex8} polyclonal antibody. We are currently purifying the CAR\textsuperscript{Ex8} antibody for future experiments that will also include the use of regulatable cells expressing this isoform. A surrogate marker for the protein levels of CAR is adenovirus infection. Interestingly, I observed an increase in adenovirus infection of MDCK cells with MAGI-1 knockdown (Fig. 26) suggesting that
indeed MAGI-1 suppresses cell surface CAR expression. Since it has been shown that
CAR^{Ex7} is sequestered at the basolateral surface and CAR^{Ex8} is sub-apical or apical, and
CAR^{Ex7} is dominant over MAGI-1 (1) while CAR^{Ex8} protein levels are controlled by
MAGI-1, it is therefore reasonable to hypothesize that the CAR isoform which
expression level increases at the cell surface as a consequence of MAGI-1 knock down is
CAR^{Ex8}. Future confirmation is required to prove this hypothesis.

CAR upregulation has been achieved by treating cells with inhibitors of RAS-
MEK(74), of TGF-β signaling (75), or with HDAC inhibitors (76, 77) or knockdown of
ZEB1(78) suggesting that many factors play roles in CAR expression and localization.
The results here support observations (1) and our model of CAR localization at the apical
surface of polarized epithelia (Fig. 7) that MAGI-1 is one of the proteins involved in
isoform specific CAR expression. However, the underlying mechanisms of MAGI-1
regulation of CAR^{Ex8} expression have not yet been discovered. MAGI-1 may simply be a
passive scaffolding protein that brings a variety of other proteins into close proximity.
One of such proteins, for instance may be a repressor of CAR^{Ex8} such that it degrades
CAR^{Ex8} when both bind to MAGI-1. This is supported by the PDZ3 results where co-
transfection of PDZ3 with CAR^{Ex8} leads to less apical expression of CAR^{Ex8} and reduced
adenovirus infection. Alternatively, MAGI-1 may be an active participant that discerns
binding partners, modulates the protein interactions and functions, and directs trafficking.
Future studies should include studying CAR^{Ex8} trafficking in regulated MAGI-1
knockdown and overexpressing cells. This will help elucidate the regulation, function and
importance of MAGI-1 and its relevance to normal and disease processes.
MAGI-1 contains six PDZ-binding domains (PDZ0-PDZ5), any of which may bind to each CAR isoform. Since our preliminary data shows that both isoforms of CAR interact with MAGI-1b in a PDZ dependent manner with distinct outcomes, I hypothesized that each isoform would interact with a different MAGI-1 PDZ domain. Contrary to my expectation, by using coimmunoprecipitation, pull down assays (Figs. 29-31) and FRET (Figs 39, 40) with isolated MAGI-1 PDZ domains, I conclude that both isoforms interact with the PDZ3 domain of MAGI-1. The overlap in the PDZ3 domain, despite CAR-MAGI-1 interaction yielding different results with each isoform, suggests that unique sequence(s) at the C-terminus (13aa) of CAR\textsuperscript{Ex8} may somehow cause CAR\textsuperscript{Ex8} to be targeted for degradation after interaction with MAGI-1. It will be important to verify the specific amino acid(s) that are required for targeted degradation. This can be done by site-directed mutagenesis of CAR\textsuperscript{Ex8} specific amino acids to monitor CAR expression and regulation. No report suggesting such amino acid has been published. Potentially, CAR\textsuperscript{Ex8} is a target for MAGI-1 mediated ubiquitylation at the two unique lysines found in the CAR\textsuperscript{Ex8} amino acid sequence.

I also concluded that, in addition to PDZ3, CAR\textsuperscript{Ex8} interacts with the MAGI-1 PDZ1 domain. The result is similar to proteins that bind more than one domain, as observed for JAM4 (46). It is possible that the degradation of CAR\textsuperscript{Ex8} may occur during interaction with MAGI-1 PDZ1. Whether the degradation is pre-, co-, or post-translational needs to be verified. Since CAR\textsuperscript{Ex7} and CAR\textsuperscript{Ex8} interact with the same overlapping PDZ3 domain, and CAR\textsuperscript{Ex8} interacts with two PDZ domains, I determined the specificity and affinity of each interaction. We have confirmed these conclusions with yeast 2-hybrid assay in Excoffon lab where PDZ1 interacts with CAR at a lower affinity.
than PDZ3. Since both isoforms can bind distinct domains, it will be interesting to
determine if they can bind MAGI-1 at the same time and whether co-expression of
CAR^{Ex7} and CAR^{Ex8} in the presence of MAGI-1b may rescue CAR^{Ex8} expression or alter
CAR^{Ex7}-mediated junctional localization of MAGI-1. The main advantages of initially
investigating single PDZ domain interactions are that a positive interaction will indicate a
direct interaction not dependent on overall protein context (i.e. synergistic binding). I
used the results obtained for domain competition experiments within cells as other
investigators have successfully done (Table 1). My data suggests that PDZ3 and/or PDZ1
may be necessary for CAR-MAGI-1 binding but I am yet to confirm if either isolated
PDZ domain is essential for CAR^{Ex8} expression and regulation by MAGI-1.

Although autonomy of isolated MAGI-1b PDZ domains has been shown in vitro
(Table 1), other PDZ-based interactions have been shown to require appropriate
consecutive domains (i.e. some domain interactions require the presence of another
domain) (36, 79). Future experiments should involve making fusions of multiple PDZ
domains (e.g. 13, 123, etc.) or create MAGI-1 deletion mutants lacking each of PDZ1
domain or PDZ3 domain sequentially or both PDZ1 and PDZ3 domains to determine if
the domains are essential for CAR regulation. It is also necessary to consider whether the
MAGI-1 interaction is modulated by modification (e.g. phosphorylation) (19). The main
advantage of analyzing these interactions by FRET is to avoid potential degradation of
CAR^{Ex8} if performed in live cells. It is unlikely that our purified domains contain post-
translational modifications which occur in cells. Thus, the next logical step is to verify
interactions in the cellular milieu as indicated below. These experiments will provide a
major step forward in understanding the autonomy of these domains in a biological
context, for example, to address if we are able to put these domains into other protein backbones to engineer proteins of functional consequence (i.e. proteins or peptides able to augment or inhibit CAR$^{\text{Ex8}}$ degradation).

Reports have shown that PDZ domains bind the extreme C-terminus of proteins and binding takes place in a groove at the surface of PDZ domain (34). In agreement with earlier observations, binding assays and fluorescent energy transfer experiments confirmed strong affinity and direct binding between MAGI-1 PDZ3 and the C-terminus of CAR isoforms while CAR$^{\text{Ex8}}$ additionally interacts with PDZ1 with lower affinity (Figs. 39-41; Tables 8,9). This confirms that the C-terminus of CAR is important for directly interacting with MAGI-1. Although there is no published data for physiological concentrations of CAR in cells, the nM $K_d$ values obtained from CAR-MAGI-1 interactions is far less than the amount of proteins needed for co-immunoprecipitation and it is comparable to the range of protein concentrations of other proteins in the cytoplasm. FRET is inversely proportional to the sixth-power of the distance between donor and acceptor (67). FRET is therefore useful to determine direct binding between Cy3/Cy5 donor/acceptor pair when the molecular distance between the interacting molecules is less than 100Å. FRET between MAGI-1 PDZ3 and the C-terminus of both CAR isoforms and between MAGI-1 PDZ1 and CAR$^{\text{Ex8}}$ C-terminus yielded intermolecular distance < 100Å (Table 8) confirming a close proximity between these interacting proteins. This suggests that close molecular interactions occur between these proteins and not just any random non-specific binding displayed in MAGI-1 PDZ1 and CAR$^{\text{Ex7}}$ interactions. Future experiments should involve using full length CAR and MAGI to account for folding of proteins during interaction. SigmaPlot analysis of FRET
data suggests a single binding site in CAR C-terminus (Figs. 39, 40). I hypothesize that only one PDZ binding site will be present in full length CAR since the PDZ binding domain is at the extreme end of the C-terminus. This needs to be verified since Mirza et al. (34) observed two LNX2-interacting regions in CAR C-terminus.

CAR^{Ex8} interacts with both MAGI-1 PDZ1 and PDZ3 directly and with strong affinity but CAR^{Ex7} interacts only with MAGI-1 PDZ3. Since only CAR^{Ex8} seems degraded during CAR-MAGI-1 interactions, I hypothesized that the PDZ1 domain may be the “death domain” of MAGI-1 contributing to CAR^{Ex8} degradation. Contrary to my hypothesis, co-expression of PDZ1 and MAGI-1 in COS-7 cells (Fig. 35) suggests that PDZ1 is not the “death” domain while the lack of PDZ3 and CAR^{Ex8} in same cells suggest PDZ3 may be the “death” domain. Western blot and cell surface biotinylation experiments provide evidence that PDZ3 reduces the cell surface protein expression level of CAR^{Ex8} while PDZ1 increases the surface amount of CAR^{Ex8}. Surprisingly, I did not observe a significant reduction in the total cellular amount of CAR^{Ex8} upon co-expression with PDZ3. This suggests that CAR^{Ex8} degradation may involve multiple MAGI-1 domains in the cell. However, more immature CAR^{Ex8} was apparent in the presence of PDZ3 suggesting that CAR^{Ex8} may be degraded but new proteins synthesized. More experiments such as CAR^{Ex8} conditional knockdown, use of CAR^{Ex8} without glycol, CAR^{Ex8} labeling to monitor trafficking, need to be done to confirm whether CAR^{Ex8} is sequestered in vesicles or actually degraded and what other proteins are involved in degradation. Since PDZ1 is not the “death domain” I sought to determine if by competing full length MAGI-1 with isolated MAGI-1 PDZ domains, the MAGI-1-mediated loss of CAR^{Ex8} observed in non-polarized cells could be altered. I hypothesized that an isolated
interacting PDZ domain can compete with the wild type interaction and allow co-existence of \( \text{CAR}^{\text{Ex8}} \) and MAGI-1. Immunocytochemistry experimental data suggests that PDZ1 may compete with MAGI-1 and prevent \( \text{CAR}^{\text{Ex8}} \) degradation thereby allowing co-localization of MAGI-1 and \( \text{CAR}^{\text{Ex8}} \) (Fig. 48). The competition may occur by PDZ1 preferentially binding to \( \text{CAR}^{\text{Ex8}} \) as a result of its smaller molecular size thus preventing MAGI-1 binding and \( \text{CAR}^{\text{Ex8}} \) degradation. Another possibility is that binding of PDZ1 binds strongly to \( \text{CAR}^{\text{Ex8}} \) and prevents the binding of a protein that MAGI-1 recruits for \( \text{CAR}^{\text{Ex8}} \) degradation. This situation does not occur when PDZ3 domain is used because MAGI-1 is still able to recruit the protein that degrades \( \text{CAR}^{\text{Ex8}} \). Future experiments should involve competition between PDZ1 and PDZ3 for \( \text{CAR}^{\text{Ex8}} \) interaction to determine whether any of the domains is dominant over the other.

The physiological relevance of MAGI-1 mediated \( \text{CAR}^{\text{Ex8}} \) loss is the subsequent effect on adenovirus infection. MAGI-1 reduces adenovirus infection by contributing to \( \text{CAR}^{\text{Ex8}} \) loss (1). Isolated PDZ1 and PDZ3 alone do not support adenovirus infection while \( \text{CAR}^{\text{Ex8}} \) does (Fig. 43). CHO-K1 cells used in this study do not express MAGI-1 and CAR so adenovirus infection observed is a result of exogenous CAR. Similar to MAGI-1, the isolated PDZ3 domain reduces adenovirus infection suggesting that this is the “death” domain of MAGI-1. However, our data do not support this conclusion. Instead PDZ3 holds CAR captive within the cell (Fig. 46) although surface levels of \( \text{CAR}^{\text{Ex8}} \) is reduced in the presence of PDZ3 (Fig. 47). Just as observed by immunocytochemistry, isolated MAGI-1 PDZ1 did not prevent \( \text{CAR}^{\text{Ex8}} \) from increasing adenovirus infection. Surprisingly, immunocytochemistry data shows that PDZ1 and \( \text{CAR}^{\text{Ex8}} \) do not co-localize (Fig. 48), PDZ1 still prevents \( \text{CAR}^{\text{Ex8}} \) loss in the presence of
MAGI leading to increased surface CAR\textsuperscript{Ex8} expression (Fig. 47). This confirms that CAR\textsuperscript{Ex8} coexists with PDZ1 without degradation thus allowing adenovirus infection. A potential pitfall to modulating MAGI-1b expression and PDZ domain interactions is the fact that MAGI-1 interacts with many important proteins (Table 1). Interrupting other interactions, while targeting CAR\textsuperscript{Ex8} interactions, may have deleterious effects on the cell. I observed no cellular toxicity in CHO-K1 cells transfected with PDZ domains, CAR and/or full length MAGI-1 during triple transfection experiments. When the isolated PDZ domains were co-expressed with MAGI-1, I expected that each isolated PDZ domain could interact with CAR\textsuperscript{Ex8} in non-polarized cells and compete with the full length MAGI-1 interaction protecting CAR\textsuperscript{Ex8} from degradation and allowing high adenovirus infection. Beta-galactosidase assay confirmed that isolated PDZ1 prevents MAGI-1 mediated CAR\textsuperscript{Ex8} degradation by allowing adenovirus infection despite the presence of MAGI-1 (Figure 46). The possible mechanisms may involve PDZ1 preferentially binding CAR\textsuperscript{Ex8} instead of the large MAGI-1 thus preventing CAR\textsuperscript{Ex8} degradation. Alternatively, PDZ1 may prevent the recruitment of CAR\textsuperscript{Ex8} degrading proteins by blocking the binding of such proteins to either MAGI-1 and subsequently CAR\textsuperscript{Ex8}. This will facilitate the development of small molecule inhibitors that mimic PDZ1 or activators directed against MAGI-1b which could yield therapeutic benefit briefly during times when it is desirable to manipulate CAR\textsuperscript{Ex8} prevalence and localization. One important consideration is whether endogenous levels of CAR\textsuperscript{Ex8} and our current tools are sufficient for dissection. Thus I suggest considering developing regulatable FLAG-epitope tagged CAR\textsuperscript{Ex8} expressing Calu-3 cells using a retroviral regulatable expression system These studies
will also allow a greater understanding of MAGI-1b as a pivotal scaffolding protein in cellular regulation, trafficking, and signaling within airway epithelial cells.

The next step will be to perform these experiments in primary airway epithelia. There are at least three possible results. First, the results obtained in vitro are recapitulated in primary airway. This will be excellent because it means small molecules can easily be developed that can be used to block MAGI-1 expression, facilitate CAR\textsuperscript{Ex8} expression and hence adenovirus infection. It is also possible that the results are not replicated in primary airway. This may be a result of other factors that exist in primary cells that may have been altered in immortal aneuploid cells. Although, this situation is not desirable, a good way to overcome this problem is to use domain-deletion as an alternative for studying interactions. The third possible result is if only some of the in vitro results are reproduced in primary airway. In this case the factors that may be responsible for the difference should be investigated. Other proteins that participate in trafficking should be studied to see if any of the proteins affect MAGI-1 activity.

In summary I have shown that CAR\textsuperscript{Ex7} is more highly expressed than CAR\textsuperscript{Ex8} in many cell lines, and that MAGI-1 knockdown is possible and increases adenovirus infection in MDCK cells. I have demonstrated that PDZ1 and PDZ3 bind CAR\textsuperscript{Ex8} with strong affinity while only PDZ3 binds CAR\textsuperscript{Ex7}. I also showed that PDZ1 co-localizes with MAGI-1 and CAR\textsuperscript{Ex8}, preventing CAR\textsuperscript{Ex8} degradation and increasing adenovirus infection while PDZ3 prevents adenovirus infection like wild type MAGI-1 albeit through a distinct mechanism.
Conclusions

My findings suggest that CAR$^{Ex7}$ binds to the PDZ3 domain of MAGI-1 and drags MAGI-1 to the basolateral surface of epithelia. CAR$^{Ex8}$ on the other and binds tightly to PDZ3 domain of MAGI-1 where it is either degraded directly by MAGI-1 or MAGI-1 recruits a protein that probably binds to another PDZ domain, potentially PDZ1, and degrades CAR$^{Ex8}$. This binding may be overcome by isolated PDZ1 molecule which prevents CAR$^{Ex8}$ degradation, leading to increased cell surface CAR$^{Ex8}$ levels and increased apical adenovirus infection. This effect may be used to improve effective adenovirus gene therapy.

Future studies

This study is an essential first step towards understanding the regulation of a novel isoform of the primary receptor for two distinct pathogenic viruses. Future experiments will refine the identified interactions to define small molecules that will alter CAR$^{Ex8}$ localization and hence alter viral susceptibility. Studies will also focus on how cells target CAR$^{Ex8}$ for degradation and on the mechanisms and consequences of apical viral infection. For example, does interaction with an apical co-receptor, such as Coxsackievirus with DAF, augment CAR$^{Ex8}$ apical localization and aid in apical infection? Investigations into the correlation between splicing and altered localization of other viral receptors in polarized cells will provide an alternative view to viral susceptibility, particularly in the face of a diseased state. Finally studies should explore the involvement of PDZ-domain containing proteins in ER quality control mechanisms. This will provide a greater understanding of these processes and likely lead to novel therapeutics potentially applicable to a myriad of ER-processing diseases.
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