hCASK and hDlg Associate in Epithelia, and Their Src Homology 3 and Guanylate Kinase Domains Participate in Both Intramolecular and Intermolecular Interactions

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Stacey L. Nix‡, Athar H. Chishti§, James M. Anderson‡, and Zenta Walther†**

From the Departments of Internal Medicine, ‡Cell Biology, and §Pathology, Yale University School of Medicine, New Haven, Connecticut 06520 and the Division of Hematology Research, St. Elizabeth’s Medical Center, Tufts University School of Medicine, Boston, Massachusetts 02135

Membrane-associated guanylate kinase (MAGUK) proteins act as molecular scaffolds organizing multiprotein complexes at specialized regions of the plasma membrane. All MAGUKs contain a Src homology 3 (SH3) domain and a region homologous to yeast guanylate kinase (GUK). We showed previously that one MAGUK protein, human CASK (hCASK), is widely expressed and associated with epithelial basolateral plasma membranes. We now report that hCASK binds another MAGUK, human discs large (hDlg). Immunofluorescence microscopy demonstrates that hCASK and hDlg colocalize at basolateral membranes of epithelial cells in small and large intestine. These proteins co-precipitate from lysates of an intestinal cell line, Caco-2. The GUK domain of hCASK binds the SH3 domain of hDlg in both yeast two-hybrid and fusion protein binding assays, and it is required for interaction with hDlg in transfected HEK293 cells. In addition, the SH3 and GUK domains of each protein participate in intramolecular binding that in vitro predominates over intermolecular binding. The SH3 and GUK domains of human p55 display the same interactions in yeast two-hybrid assays as those of hCASK. Not all SH3-GUK interactions among these MAGUKs are permissible, however, implying specificity to SH3-GUK interactions in vivo. These results suggest MAGUK scaffold assembly may be regulated through effects on intramolecular SH3-GUK binding.

The unique morphologies and specialized functions of terminally differentiated cells depend in large part on their ability to generate and maintain specialized plasma membrane domains. For example, in epithelial cells, interactions with the extracellular matrix and responses to growth factors are mediated by receptor proteins embedded in the basolateral plasma membrane. In recent years, it has become evident that signal transduction is facilitated by the organization of receptor proteins and many of their intracellular targets into macromolecular complexes anchored both to the membrane and to the underlying actin-based cortical cytoskeleton (1). Many signaling complexes are assembled upon scaffolding proteins which, by means of multiple protein-binding domains, serve to bring together and organize the various components of a particular signaling cascade. The study of such scaffolding proteins has begun to provide explanations for the remarkable speed, efficiency, and precision of signal transduction in certain systems and has offered exciting new insights into its regulation (2). Much remains to be learned, however, about the role of scaffolding proteins in the assembly, organization, and regulation of signal transduction complexes at the basolateral membrane of polarized epithelial cells.

Members of the MAGUK family of scaffolding proteins organize macromolecular complexes at different specialized regions of the plasma membrane (3). For example, the zonula occludens (ZO)-1, -2, and -3 proteins reside at epithelial tight junctions (4–6) and provide a scaffold which anchors many tight junction components including occludin (6–9) and claudins (10). A different MAGUK subfamily comprised of post-synaptic density-95 (PSD-95) and closely related molecules are concentrated in neuronal synapses, where they bind numerous synaptic channels, receptors, and other scaffolding molecules (1). MAGUKs possess a characteristic domain structure: one or more N-terminal PSD-95/Dlg/ZO-1 (PDZ) domain(s), an internal Src homology 3 (SH3) domain, and a guanylate kinase-like (GUK) domain at the C terminus. PDZ and SH3 domains are well established as protein-binding structures, the former recognizing peptide ligands, usually at the C terminus of the target protein (11), and the latter recognizing proline-rich peptide motifs (12). In addition, most MAGUKs contain a motif rich in basic residues which mediates binding to protein 4.1 and/or other ezrin-radixin-moesin family proteins (13), thus providing anchorage to the cortical actin network.

hCASK, the human homolog of Caenorhabditis elegans LIN-2 (14) and Drosophila camguk (15), is a MAGUK protein with widespread expression in human tissues (16). The name CASK (17) reflects a unique domain composition; there is an N-terminal region of homology to Ca2+/calmodulin-dependent protein kinase, followed by the three characteristic MAGUK domains (PDZ, SH3, and GUK). The mammalian protein was first identified in rat brain as a PDZ protein able to bind the C

1 The abbreviations used are: MAGUK, membrane associate guanylate kinase homologs; ZO, zonula occludens; PSD, post synaptic density; PDZ, PSD-95/Dlg/ZO-1; SH3, Src homology 3; GUK, guanylate kinase-like; hCASK, human CASK; hDlg, human discs large; NE-dlg, neurendocrine discs large; PCR, polymerase chain reaction; VSV-G, vesicular stomatitis virus glycoprotein; CB, calmodulin binding region; MBP, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis.astrological activity.
termini of neurexins (17), and it has subsequently been found to bind presynaptic N-type Ca\(^{2+}\) channels through its SH3 domain (18). In *C. elegans*, lin-2 was identified as a gene required for vulval development (14). Elegant genetic studies have shown that a protein complex containing LIN-2, LIN-7, and LIN-10 is required for proper localization of an epidermal growth factor receptor homolog to the basolateral membrane of precursor vulval epithelial cells (19). A mammalian counterpart to the LIN-2/7/10 complex has been identified in neurons (20, 21), but its involvement in synaptic assembly, organization, and/or vesicle exocytosis remains unclear. LIN-7 homologs have also been detected in mammalian epithelia (20, 22). In cultured epithelial cells, the basolateral localization of mLin-7 appears to depend upon its ability to bind CASK (23), again implicating CASK in the targeting/localization of membrane-associated complexes.

Although CASK-associated protein complex(es) in epithelial cells have not yet been fully characterized, we have shown previously that hCASK binds via its PDZ domain to the C terminus of syndecan, a cell surface proteoglycan that is a co-receptor for basic fibroblast growth factor (16). This finding suggests that hCASK may be associated with certain fibroblast growth factor receptor complexes. However, a detailed understanding of the role of CASK in protein targeting, membrane domain organization and/or signal transduction in mammalian epithelia awaits further elucidation of its scaffolding properties.

The GUK domain found in all MAGUKs is defined by its sequence similarity to yeast guanylate kinase. Although nucleotide binding sites are conserved in some MAGUKs, several investigators have been unable to detect guanylate kinase activity in recombinant MAGUK GUK domains (24, 25). Furthermore, there is evidence that guanylate kinase activity is not required for MAGUK function. For example, vulval development in *C. elegans* is not affected by mutations in the lin-2 gene that disrupt the guanine binding site (14).

GUK domains have recently been found to mediate protein-protein interactions. A family of neuronal proteins termed SAP90/PSD-95-associated proteins/GKAPs/DAPs (26–28) has been shown to bind the GUK domains of PSD-95 and closely related MAGUKs. A different neuron-specific protein which binds the GUK domain of PSD-95 has recently been identified and named BEGAIN (for brain-enriched guanylate kinase-associated protein) (29). Both SAP90/PSD-95-associated proteins and BEGAIN are enriched in synapses and presumably participate in a PSD-95-based post-synaptic scaffold. PSD-93 is also seen to colocalize with dendritic microtubules in some neurons, and its GUK domain binds microtubule-associated protein 1A (30).

Surprisingly, some GUK domains have been found to interact with MAGUK SH3 domains. McGee and Bredt (31) have shown that the PSD-95 GUK domain binds the PSD-95 SH3 domain in vitro and in brain in vivo, in what is most likely an intramolecular interaction, and Masuko et al. (32) report that the PSD-95 GUK domain binds the SH3 domain of NE-dlg (a MAGUK expressed in neuronal and endocrine tissues) in a Ca\(^{2+}\)- and calmodulin-dependent manner.

We report here that yeast two-hybrid screening of human liver cDNAs with a C-terminal hCASK bait has identified hDlg, a widely expressed member of the PSD-95 subfamily of MAGUKs (33, 34), as a binding partner of hCASK. We show that the GUK domain of hCASK binds the SH3 domain of hDlg in vitro and that hCASK and hDlg associate in vivo in human intestinal epithelial cells. In addition, we demonstrate that both hCASK and hDlg are capable of intramolecular SH3-GUK interactions. These data reveal an interaction between two MAGUKs not previously known to associate, and they imply a wealth of possible mechanisms that may regulate this mode of MAGUK scaffold assembly.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screen**—Yeast two-hybrid screening was performed as described (35). The bait construct was generated by PCR amplification of the C-terminal 846 base pairs of hCASK using sense primer 5'-CTGAAATCCAGTTGTTGATCATCA-3' and antisense primer 5'-TGGATCTCAATAGAC-CAAGAGAGA-3'. The PCR product was cloned into pBH5 (obtained from Dr. Morgan Sheng, Harvard University) in frame with the lexA DNA-binding domain. L40 yeast were cotransformed with the bait plasmid and a library of prey plasmids containing human liver cDNAs fused to the GAL4 activation domain in the pGAD10 vector (CLONTECH, Palo Alto, CA). Positive interacting yeast clones were determined by growth on selective medium (containing 5 mM 3-aminotriazole) and β-galactosidase activity. Prey plasmids were isolated from positive yeast clones, grown in KCl bacteria (CLONTECH, Palo Alto, CA), and purified. Purified prey plasmid and original bait plasmid were cotransformed into yeast to confirm positive interactions. Prey plasmids were also cotransformed with empty pBH5 vector to ensure that they were not able to interact with the lexA DNA binding domain alone (self-activators).

**Antibodies**—The rabbit polyclonal anti-hCASK antibody has been described (16). A mouse monoclonal antibody recognizing the same region of hCASK (residues 316–415) was obtained commercially (Zymed Laboratories Inc. Laboratories, South San Francisco, CA) and used at 1:500 dilution on immunoblots. A mouse monoclonal anti-hDlg antibody was raised to amino acids 1–29 of hDlg in the laboratory of Dr. Athar Chishti; ascites fluid containing this antibody was used at 1:5000 to 1:7500 dilution on immunoblots. The monoclonal anti-ZO-1 antibody is commercially available (Transduction Labs, San Diego, CA). A rabbit polyclonal anti-HSV-V antibody was obtained commercially (MBI Laboratories, Japan) and used at 1:2000 dilution on immunoblots. The mouse monoclonal anti-HSV-V antibody used in immunoprecipitation experiments was prepared from the hybridoma P5D4 (originally a gift of Dr. Thomas Kreis, University of Geneva, Switzerland).

**Immunoprecipitation**—A confluent culture dish of Caco-2 cells was washed twice with ice-cold phosphate-buffered saline containing 2 mM MgCl\(_2\) and 0.2 mM CaCl\(_2\) and then incubated for 30 min in 3 ml of ice-cold lysis buffer (1% Nonidet P-40, 150 mM NaCl, 3 mM Mg\(_2\)Cl\(_2\), 50 mM Tris, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml trypsin-chymotrypsin inhibitor). The lysate was sheared by passing through a 30-gauge needle and centrifuged at 100,000 g for 30 min at 4 °C. Supernatants were preclarified by incubation with protein G-Sepharose beads (Amersham Pharmacia Biotech) at 4 °C for 1 h. Preclarified supernatants were incubated with either 0.5 μl of the monoclonal anti-hDlg antibody or 5 μl of the monoclonal anti-ZO-1 control antibody. Antibody complexes were recovered using protein G-Sepharose beads and washed extensively. Samples were needle aspirated, resuspended in 75 μl of 4X protein sample buffer that was preheated to 95 °C and stored at −80 °C.

**Immunofluorescence**—Confluent monolayers of Caco-2 cells grown on Costar Transwell filters (Corning, Inc., Corning, NY) were fixed in 2% paraformaldehyde for 1–2 h at room temperature and quenched in 50 mM NH\(_4\)Cl for 10 min. Cells were permeabilized for 15 min at room temperature in 0.2% Triton X-100, 0.5% bovine serum albumin, phosphate-buffered saline and blocked in 1% bovine serum albumin, phosphate-buffered saline, 5% normal donkey serum. Filters were double-labeled with primary antibodies polyclonal anti-hCASK (1:300) and monoclonal anti-hDlg (1:1500) and secondary antibodies Cy3-conjugated anti-rabbit (1:1000; Amersham Pharma Biotech) and Cy2-conjugated anti-mouse (1:100; Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Filters were mounted on glass slides in Mowiol (Hoechst, Frankfurt, Germany) containing 1% N-propyl gallate (Sigma-Aldrich). Cells were visualized by epifluorescent microscopy using a Nikon Microphot FX microscope. Images were captured using a SenSys cooled charge-coupled device camera (Photometrix) and Image Pro Plus acquisition software (Media Cybernetics, Silver Spring, MD). Images were processed using Adobe Photoshop 5.0 (Adobe Systems, Inc., Mountain View, CA).

Sections of formalin-fixed, paraffin-embedded normal human small intestine and colon tissue mounted on glass slides were obtained from the Critical Technologies Program of the Department of Pathology, Yale University School of Medicine. Antigen retrieval was performed by pressure cooker treatment in a dilute citric acid buffer as described (36).

Sections were double-labeled with the polyclonal anti-hCASK antibody.
Identification of an Interaction between hCASK and hDlg—
Initially motivated to identify potential binding partners for the GUK domain of hCASK, we conducted a yeast two-hybrid screen of a human liver cDNA library, using as bait the C-terminal 281 aa of hCASK. Eight positive clones were isolated from this screen, and, surprisingly, four of them were found to contain identical transcripts encoding nearly full-length hDlg (amino acids 109–875), another member of the MAGUK protein family (33).

To search for evidence that hCASK and hDlg are associated in vivo, we first performed immunofluorescence localization studies. Employing polyclonal anti-hCASK and monoclonal anti-hDlg antibodies, we observed that these proteins are colocalized at the lateral plasma membrane of confluent Caco-2 cells (human colon carcinoma-derived, (39)) in culture (Fig. 1 A). Next, we used the monoclonal anti-hDlg antibody in immunoprecipitation experiments and found that hCASK and hDlg co-precipitate from detergent lysates of confluent Caco-2 cells (Fig. 1 B). hDlg, which has a predicted molecular mass of 103 kDa, is recognized by the monoclonal antibody as a doublet at approximately 130 kDa in these cells (Fig. 1 B, left panel, Caco-2 lysate). hDlg and its rat homolog, synapase-associated protein 97, have been found previously to appear as doublets of higher than predicted molecular mass upon gel electrophoresis in several cell types (34, 40). hCASK is seen as a single band of approximately the predicted molecular mass (112 kDa) in Caco-2 cells (Fig. 1 B, right panel, Caco-2 lysate). The hDlg monoclonal antibody precipitates both hDlg (Fig. 1 B, arrowheads) and hCASK (Fig. 1 B, asterisk) from these cells. As a negative control in this experiment, we used a monoclonal antibody recognizing the tight junction MAGUK ZO-1, this antibody precipitated neither hDlg nor hCASK (Fig. 1 B, control IP). We next studied the subcellular localization of hCASK and hDlg in formalin fixed, paraffin-embedded human tissue sections. Immunohistochemistry revealed that hCASK and hDlg colocalize along the basal plasma membrane and the basal aspect of the lateral membrane in epithelial cells of both small intestine and colon (Fig. 2). Together these results strongly suggest that hCASK and hDlg are both located at the lateral plasma membrane in close proximity with the cortical actin cytoskeleton and that at least a fraction of each is physically associated with the other.

The hDlg SH3 Domain Binds the hCASK GUK Domain—To identify the binding sites between hCASK and hDlg, we employed directed yeast two-hybrid assays. Segments of hDlg were tested for their ability to bind the original hCASK bait (Fig. 4 A). We observed that hDlg fragments containing the SH3 domain are able to bind hCASK, whereas fragments encompassing the other domains (II, PDZ1–3, CB, CBGUK, and GUK) do not interact with the bait. The smallest hDlg construct capable of binding hCASK in this experiment (SH3–2) includes the SH3 domain as well as 22 downstream residues that comprise a putative Ca2+/calmodulin-binding motif (32).
Interestingly, not all constructs that encode the SH3 domain of hDlg are able to interact with hCASK. hDlg fragments containing both the SH3 domain and the full-length GUK domain (ΔPDZ and SH3GUK) do not bind hCASK (Fig. 3B), suggesting that the presence of the hDlg GUK domain is inhibitory to the interaction. To confirm this, we engineered a truncated hDlg protein in which the GUK domain is incomplete and compared this to full-length hDlg in ability to bind hCASK. As shown in Fig. 3B, full-length hDlg does not bind, but truncation of the hDlg GUK domain restores an interaction with the hCASK bait. These data suggest that an intramolecular SH3-GUK interaction occurs within hDlg and prevents intermolecular binding between certain hCASK and hDlg fragments in our yeast two-hybrid assays.

We next sought to reproduce the yeast two-hybrid interaction between the hCASK GUK and hDlg SH3 domains using an in vitro fusion protein binding assay (Fig. 5). For these experiments, the SH3 domain of hDlg, both with the putative Ca\(^{2+}\)/calmodulin-binding region (G-SH3CB) and without this region (G-SH3), were fused to GST. A GST fusion of the SH3 domain of cortactin (G-CrtSH3) and GST alone were used as controls. Various C-terminal fragments of hCASK were expressed as fusion proteins with MBP. GST fusion proteins bound to glutathione-agarose beads were incubated with crude bacterial lysates containing the MBP-hCASK fusion proteins or MBP alone, and the MBP fusion proteins co-isolated with the beads were identified by Western blotting with an anti-MBP antibody (Fig. 5). Both of the GST fusion proteins containing the hDlg SH3 domain (G-SH3 and G-SH3CB) were able to pull down all three GUK-containing hCASK fusion proteins with apparently equal efficacy. GST alone did not bind MBP or any of the MBP-hCASK fusion proteins. Calmodulin is not necessary for this SH3-GUK interaction in vitro, because 1) binding occurs in the absence of supplemental Ca\(^{2+}\) and calmodulin, and 2) the hDlg SH3 construct lacking a Ca\(^{2+}\)/calmodulin binding motif pulls down hCASK. The SH3 domain of cortactin (G-CrtSH3) did not bind any of the hCASK GUK fusion proteins, indicating a degree of specificity to this SH3-GUK interaction. It is interesting to note that the hCASK GUK protein sequence lacks a PXXP motif of the kind usually recognized by SH3 domains (41), implying a distinct mode of interaction.

Evidence of an Intramolecular SH3-GUK Interaction within Both hCASK and hDlg—As described above, the results of

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**Fig. 1.** hCASK and hDlg associate in vivo in the human colon cancer cell line, Caco-2. A, immunofluorescence. Confluent Caco-2 cells grown on filter inserts were fixed in 2% paraformaldehyde and labeled with polyclonal anti-hCASK and monoclonal anti-hDlg antibodies. hCASK and hDlg colocalize at the lateral plasma membrane (60×). B, co-immunoprecipitation. Immunoprecipitations were performed on detergent lysates of confluent Caco-2 cells with either a monoclonal anti-hDlg antibody or a control antibody (monoclonal anti-ZO-1). 15 µl of starting material (4% of total used for each immunoprecipitation; Caco-2 lysate) and 20 µl of each immunoprecipitate (control IP, hDlg IP) were separated by SDS-PAGE and transferred to nitrocellulose. Duplicate blots were probed with a monoclonal anti-hDlg antibody (left panel) or a monoclonal anti-hCASK antibody (right panel). The anti-hDlg antibody precipitates both hDlg (arrowheads, 130 kDa) and hCASK (asterisk, 112 kDa), whereas the control antibody does not precipitate either protein. The low molecular mass bands (IgG, brackets, 50–60 kDa) represent immunoglobulin and are recognized by the secondary antibody alone (data not shown).

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**Fig. 2.** hCASK and hDlg colocalize in human tissues at the basolateral plasma membrane of epithelial cells. Formalin fixed normal human small intestine and colon sections were double-labeled with polyclonal anti-CASK and monoclonal anti-hDlg antibodies and visualized by fluorescence microscopy. hCASK and hDlg colocalize at the basolateral plasma membrane and the basal aspect of the lateral membrane in epithelial cells of these tissues.
SH3-Guanylate Kinase Domain Interactions of hCASK and hDlg

FIG. 3. The SH3 domain of hDlg binds hCASK in the yeast two-hybrid system. A, hDlg constructs in pGAD were tested in yeast two-hybrid assays for interaction with the hCASK original bait construct in pBHA5. In addition to the nearly full-length hDlg fragment isolated in the original screen (Prey), hDlg constructs encoding the SH3 domain (SH3GUK, SH3-1, and SH3-2) interact with hCASK CB, putative calmodulin-binding region of hDlg. B, full-length hDlg (cDNA) and fragments of hDlg which contain both SH3 and GUK domains (ΔPDZ and SH3GUK) are unable to bind hCASK in the yeast two-hybrid system, whereas a construct with the SH3 domain but no GUK domain (SH3ΔGUK) binds hCASK. Truncation of the hDlg GUK domain (ΔGUK) allows an interaction with hCASK. Thus, the presence of the hDlg GUK domain in cis appears to inhibit the interaction between the hDlg SH3 domain and hCASK.

FIG. 4. The GUK domain of hCASK binds the SH3 domain of hDlg in the yeast two-hybrid system. hCASK constructs in pBHA5 were tested in yeast two-hybrid assays for interaction with the hDlg SH3–2 construct in pGAD. The smallest hCASK fragment able to interact with hDlg (GUK) is slightly larger than the defined GUK domain. Smaller hCASK GUK constructs are self-activators in the yeast two-hybrid system (not shown). A fragment of hCASK containing both the SH3 and GUK domains (SH3GUK) does not interact with hDlg SH3–2. *, because the hCASK GUK construct is a self-activator in pBHA5, it was subcloned into the pGAD vector and tested for interaction with hDlg SH3–1 in pBHA5.

In a similar experiment, GUK containing constructs of hCASK were tested in the yeast two-hybrid system for their ability to bind the hCASK SH3 domain (Fig. 7). We find that hCASK constructs that contain the GUK domain are able to bind the hCASK SH3 domain in trans. A construct that contains both the SH3 domain and the GUK domain of hCASK cannot bind the isolated SH3 domain, suggesting that SH3-GUK binding in cis within this molecule is preferred over binding in trans to the isolated SH3 domain.

In Vivo—To investigate the mechanism of binding between hCASK and hDlg in vivo, we asked whether the GUK domain of hCASK is required for its association with hDlg in transfected cells. Initial experiments revealed that endogenous hCASK and hDlg are associated in human embryonal kidney 293 cells (as assayed by co-immunoprecipitation; data not shown). To test for dependence of this interaction on the GUK domain of hCASK, 293 cells were transiently co-transfected with full-length hDlg and either full-length or truncated VSV-G epitope-tagged hCASK constructs. The truncated hCASK construct is missing the C-terminal 185 aa that encode the GUK domain. As shown in a representative experiment in Fig. 8, epitope-tagged full-length hCASK can be co-immunoprecipitated with hDlg from transfected cells using either the anti-hDlg monoclonal antibody or an anti-VSV-G monoclonal antibody (first and second lanes). Epitope-tagged truncated hCASK (V-CΔG) is expressed in transfected cells and can be immunoprecipitated with the anti-VSV-G antibody (fifth lane, bottom panel). However, hDlg is not detectable in these immunoprecipitates (fifth lane, top panel), despite the high sensitivity of the anti-hDlg antibody on Western blots. By comparison, hDlg is readily detectable in anti-VSV-G immunoprecipitates of full-length hCASK (second lane, top panel). Consistent with these findings, truncated hCASK also fails to co-precipitate with hDlg when the anti-hDlg antibody is used for immunoprecipitation (fourth lane). The ability of full-length hCASK but not the truncated protein to associate with hDlg in these experiments indicates that the interaction between these two molecules in vivo requires the GUK domain of hCASK. These data support the model, suggested by our in vitro findings, that hCASK and hDlg associate via an intramolecular SH3-GUK interaction.

Specificity of SH3-GUK Interactions among MAGUKs—Because hCASK and hDlg each have both SH3 and GUK domains, it is theoretically possible that there are two sites of SH3-GUK binding between these proteins. Having shown that the hCASK GUK domain binds the hDlg SH3 domain, we next asked whether the hCASK SH3 domain could bind the hDlg GUK domain. As shown in Fig. 6, none of the hDlg GUK-encoding constructs that display interaction with the hDlg SH3 domain are able to bind the hCASK SH3 domain. This result is not due to inactivity of the hCASK SH3 domain construct in the yeast two-hybrid system because, as shown in Fig. 7, it is able to interact with hCASK GUK domains. Thus, it appears that in heterodimers there can be only one site of SH3-GUK interaction between hCASK and hDlg. This is consistent with our findings in transfected 293 cells that a truncated hCASK pro-
Fig. 5. The hCASK GUK domain binds specifically to the SH3 domain of hDlg in an in vitro fusion protein binding assay. A, GST fusion proteins. Two fragments of hDlg containing the SH3 domain were fused to GST: one encompassing the putative Ca\(^{2+}\)/calmodulin-binding motif (G-SH3CB) and one containing only the SH3 domain (G-SH3). GST alone (GST) and a GST fusion of the cortactin SH3 domain (G-CrtSH3) were used as controls. B, in vitro binding assay. Isolated, immobilized GST fusion proteins were incubated with a molar excess of MBP-hCASK fusion proteins in crude bacterial lysates. Three different MBP-hCASK constructs (M-hCASK-A, B, and C), all encoding the entire GUK domain, were used. Crude lysates containing these MBP proteins (1% of the total used for each pulldown; lysate) and proteins from these lysates co-purifying with the indicated GST fusion proteins (+GST, +SH3CB, +SH3, and +CrtSH3) were separated by SDS-PAGE and blotted to nitrocellulose. Blots were probed with an anti-MBP antibody. Both of the hDlg SH3 fusion proteins but not the cortactin SH3 fusion protein are able to pull down all three GUK-containing hCASK constructs. GST alone does not interact with MBP alone or any of the MBP fusion proteins. The Ca\(^{2+}\)/calmodulin-binding region of hDlg is not required for interaction with the hCASK GUK domain in this assay.

The GUK domain of hDlg binds the hDlg SH3 domain in trans, but it does not bind the hCASK SH3 domain. pGAD constructs encoding C-terminal domains of hDlg were tested in yeast two-hybrid assays for interaction with hDlg and hCASK SH3 domains in pBHA5. The hDlg GUK domain (CBGUK, GUK-1, and GUK-2) binds the hDlg SH3 domain in trans in these experiments, whereas an hDlg fragment encompassing both the SH3 and GUK domains (SHGUK) does not bind the isolated hDlg SH3 construct. Thus, the presence of the hDlg SH3 domain in cis with the GUK domain appears to inhibit binding to the hDlg SH3 domain in trans. None of the hDlg GUK domain constructs interact with the SH3 domain of hCASK.

To further investigate the specificity of these interactions, we determined the binding properties of the SH3 and GUK domains of the human erythrocyte MAGUK, p55, in yeast two-hybrid assays. p55 was first identified in red blood cells as a plasma membrane-associated protein with a single PDZ domain that binds the cytosplamatic tail of glycoporphin C (42–44). It is expressed in many tissues, including epithelia (45). Although p55 lacks an N-terminal Ca\(^{2+}\)/calmodulin-dependent protein kinase domain, the remainder of the molecule displays very high sequence similarity with hCASK (Fig. 8A). In pairwise combination with SH3 and GUK domains of hCASK and hDlg, the p55 SH3 and GUK domains behave exactly as do the SH3 and GUK domains of hCASK (Fig. 8B). That is, the p55 SH3 domain interacts with the hCASK GUK domain but not with the hDlg GUK domain. p55 SH3 and GUK domains also interact with each other in this assay, suggesting that p55 is capable of the same type of intramolecular SH3-GUK interaction previously reported for PSD-95 (31) and shown above for hDlg and hCASK.

The intra- and intermolecular SH3-GUK interactions described here give rise to a variety of possible MAGUK complex configurations (Fig. 10). Binding between hCASK and hDlg may be direct, resulting in a heterodimer, or it may be indirect, with p55 interposed between hCASK and hDlg, forming a heterotrimer. Larger MAGUK complexes are also possible (not shown). Thus, intermolecular SH3-GUK interactions may greatly amplify the scaffolding potential of MAGUK proteins.
In this study, we identify a novel interaction between hCASK and hDlg by means of yeast two-hybrid screening. We showed previously that hCASK resides at the basolateral membranes of epithelial cells in several tissues (16). hDlg was shown independently to be localized at the basolateral membranes of epithelial cells in choroid plexus and small intestine (34). We now demonstrate directly that these two proteins colocalize at epithelial basolateral membranes in human small and large intestine. In addition, co-immunoprecipitation of hCASK and hDlg from a human colonic epithelial cell line (Caco-2) demonstrates that hCASK and hDlg associate in vivo. Using yeast two-hybrid and fusion protein binding assays, we show the binding sites between these two proteins in vitro to be the GUK domain of hCASK and the SH3 domain of hDlg. Immunoprecipitation experiments show that in vivo, in transfected HEK293 cells, the interaction between these two proteins requires the GUK domain of hCASK. In yeast two-hybrid assays, the hDlg SH3 domain is the only region of hDlg able to interact with the GUK domain of hCASK. Thus, the simplest interpretation of our data, taken together, is that the in vivo interaction between these MAGUKs is mediated by binding of the GUK domain of hCASK to the SH3 domain of hDlg.

MAGUK proteins are thought to act as molecular scaffolds that organize macromolecular complexes at specialized regions of the plasma membrane. Several MAGUKs have been found to cluster their transmembrane ligands through homotypic MAGUK-MAGUK interactions (46). Such homotypic interactions presumably increase the speed and efficiency of signal transduction by concentrating components of a signaling cascade within a small region of the plasma membrane. Heterotypic MAGUK interactions, like the hCASK-hDlg interaction described here, may also serve to increase signaling efficiency by increasing local concentrations of signaling components that are associated with both MAGUKs. In addition, interactions between different MAGUKs might allow for cross-regulation between different scaffold-associated signal transduction cascades.

There are several distinct mechanisms by which MAGUKs have been shown to interact. The N-terminal domains of PSD-95 (47, 48) and hDlg/synapse-associated protein 97 (49) have each been implicated in homotypic interactions, and the N-terminal region of PSD-95 also appears to bind the N-terminal domain of PSD-93/Chapsyn110 (50). In synapses, these MAGUKs bind an overlapping set of receptor/channel proteins, and the N-terminal interactions between them seem to facilitate receptor clustering and may contribute to overall synaptic organization (46). The MAGUK proteins of epithelial tight junctions, ZO-1, ZO-2, and ZO-3, have also been found to associate (6, 8, 9, 51). Binding between ZO-1 and ZO-2 is PDZ-dependent (8, 9) and presumably occurs through intermolecular PDZ dimerization, as described recently in other PDZ containing proteins (52). The third possible mechanism for MAGUK-MAGUK interactions is binding between SH3 and GUK domains. We present here evidence of an intermolecular SH3-GUK interaction between two different MAGUKs, hCASK and hDlg. A similar interaction has recently been observed between two neuronal MAGUKs; the SH3 domain of NE-dlg binds the GUK domain of PSD-95 in a Ca\(^{2+}\) and calmodulin-dependent manner (32). The hCASK-hDlg interaction we describe here differs in that it is not Ca\(^{2+}\) and calmodulin-dependent in vitro. Binding between fusion proteins containing the hCASK GUK domain and the hDlg SH3 domain occurs in the absence of Ca\(^{2+}\) and calmodulin, and it does not require the presence of the putative Ca\(^{2+}\)/calmodulin-binding site that overlaps the SH3 domain in hDlg.

In addition to intermolecular binding between the SH3 domain of hDlg and the GUK domain of hCASK, we have observed intramolecular SH3-GUK interactions within the hCASK, hDlg, and p55 molecules. In the yeast two-hybrid system, the SH3 domain from each of these MAGUKs is able to interact directly with the GUK domain from the same protein. Furthermore, fragments of hCASK and hDlg containing both...
the SH3 and GUK domains cannot interact with isolated SH3 or GUK domains from either protein, suggesting that intramolecular SH3-GUK interactions predominate over intermolecular SH3-GUK interactions in these yeast two-hybrid assays. Although our experiments do not rule out the possibility of homodimerization in which there are two SH3-GUK contacts per dimer (Fig. 10), we believe that a model in which intramolecular interactions are favored in vitro provides the simplest explanation for all of the binding data.

Intramolecular SH3-GUK binding has been described by McGee and Bredt (31) in the neuronal MAGUK, PSD-95. These authors used binding studies with wild type and mutant SH3 and GUK domains to show that SH3-GUK binding in PSD-95 is likely intramolecular, both in vitro and in transfected cells. This domain interaction is unusual in that the PSD-95 GUK domain does not contain a PXXP motif of the kind commonly recognized by SH3 domains (12). Similarly, we observe that the hCASK, p55, and hDlg GUK domains all lack PXXP motifs. The finding of this novel mode of intramolecular SH3-GUK binding in four different human MAGUK proteins now suggests that this interaction may be a general feature of MAGUKs, perhaps accounting for the evolutionary conservation of domain organization within this protein family.

Although intramolecular SH3-GUK interactions appear common in MAGUK proteins, intermolecular SH3-GUK interactions between different proteins seem to be highly specific. For example, our in vitro fusion protein binding data show that the cortactin SH3 domain cannot substitute for the hDlg SH3 domain in binding the hCASK GUK domain. Furthermore, only one of the two possible SH3-GUK interactions between the hCASK and hDlg proteins can be demonstrated in yeast two-hybrid assays; the hDlg SH3 domain can interact with the hCASK GUK domain, but the hCASK SH3 domain cannot bind the hDlg GUK domain. We believe this to be a meaningful result because the hCASK SH3 and hDlg GUK constructs that cannot interact with each other are both able to participate in other yeast two-hybrid interactions (hCASK SH3 + hCASK GUK and hDlg SH3 + hDlg GUK), indicating that these constructs are functionally active. Lastly, we find that the p55 SH3 domain shows the same binding preferences as the SH3 domain of hCASK; it can bind the GUK domain of hCASK but not the GUK domain of hDlg.

We observe that native hDlg is complexed with hCASK in colonic epithelial cells in vivo and that hDlg and full-length hCASK bind each other in transfected HEK293 cells. However, full-length hDlg does not bind our hCASK bait in the yeast two-hybrid system. These findings can be reconciled if in vitro, intramolecular SH3-GUK binding within hDlg predominates over the inter-MAGUK interaction. This would imply, conversely, that something about the in vivo condition favors an intermolecular interaction. One possibility is that full-length hCASK is able to induce a conformational change in hDlg that releases intramolecular SH3-GUK binding and allows the SH3 domain to bind hCASK. We note that we have thus far tested only fragments of hCASK in our in vitro assays; analysis of the binding properties of the full-length molecule is currently underway. Another possibility is that phosphorylation or some other post-translational modification of either hCASK or hDlg in vivo inhibits intramolecular interactions and promotes complex formation. Finally, it is likely that several other proteins are bound to the hCASK-hDlg complex in vivo, and some of these other proteins may alter SH3-GUK interactions so as to...
promote inter-MAGUK binding. Similar types of intramolecular regulation of intermolecular binding have been described for other cytoskeletal adapter proteins, such as members of the Wiskott-Aldrich syndrome protein (53) and ezrin-radixin-moesin (54) protein families. This may be a common feature of multidomain proteins involved in the assembly of large protein complexes.

The exact nature of the hCASK-hDlg complex in epithelia remains to be elucidated. We do not yet know, for example, whether p55 is associated with the complex or whether MAGUK complexes are heterogeneous in epithelial cells in vivo. In addition, there may be other MAGUK family members involved; two additional p55-related human MAGUK genes have been found in the course of chromosome mapping studies and shown to be expressed in small and large intestine (55, 56). Two further p55-related MAGUKs have recently been identified on the basis of their ability to bind mammalian Lin-7, and these are also expressed in some epithelia (23). All of these proteins contain SH3 and GUK domains, and the many combinatorial possibilities for interactions between them have not yet been tested. There may be a complex network of these scaffolding proteins at lateral plasma membranes of human epithelial cells. It is intriguing to speculate that the assembly of such a network might be regulated through effects on SH3-GUK interactions. There are many candidate regulators of SH3-GUK binding, including Ca\(^{2+}\)/calmodulin, protein 4.1, and proteins that bind MAGUK SH3 or GUK domains. In addition, there is recent evidence that in several MAGUK proteins, engagement of the PDZ domain by a peptide ligand influences the binding properties of the GUK domain (30). The ability to regulate MAGUK scaffolding properties may be important for epithelial cells during processes in which membrane polarity is altered, such as epithelial morphogenesis or wound healing.

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