Membrane-associated guanylate kinase (MAGUK) proteins participate in the assembly of multiprotein complexes on the inner surface of the plasma membrane at regions of cell-cell contact. MAGUKs are characterized by three types of protein-protein interaction modules: the PDZ domain, the Src homology 3 (SH3) domain, and the guanylate kinase (GuK) domain. The arrangement of these domains is conserved in all previously known MAGUKs: either one or three PDZ domains in the NH₂-terminal half, followed by the SH3 domain, followed by a COOH-terminal GuK domain. In this report, we describe the cDNA cloning and subcellular distribution of MAGI-1, a MAGUK with three unique structural features: 1) the GuK domain is at the NH₂ terminus, 2) the SH3 domain is replaced by two WW domains, and 3) it contains five PDZ domains. MAGI-1 mRNA was detected in several adult mouse tissues. Sequence analysis of overlapping cDNAs revealed the existence of three splice variants that are predicted to encode MAGI-1 proteins with different COOH termini. The longest variant, MAGI-1c, contains three bipartite nuclear localization signals in its unique COOH-terminal sequence and was found predominantly in the nucleus of Madin-Darby canine kidney cells. A shorter form lacking these signals was found primarily in membrane and cytoplasmic fractions. This distribution, which is reminiscent of that seen for the tight junction protein ZO-1, suggests that MAGI-1 may participate in the transmission of regulatory signals from the cell surface to the nucleus.

The prototypical members of the membrane-associated guanylate kinase (MAGUK) family are the Drosophila tumor suppressor protein DLG, the erythrocyte membrane protein p55, and the neuronal protein PSD-95/SAP90 (1, 2). These proteins share a common modular structure that consists of either one or three PDZ domains, a single Src homology 3 (SH3) domain, and a single region of homology to Saccharomyces cerevisiae guanylate kinase (GuK), known as the GuK domain. The MAGUK family includes the epithelial tight junction proteins ZO-1 and ZO-2, the Caenorhabditis elegans vulval protein LIN-2A, and the neurexin-binding protein CASK (3–5). The latter two represent a subfamily of MAGUKs characterized by an additional domain at the NH₂ terminus that is similar to calmodulin kinase II. All MAGUKs studied to date localize to regions of cell-cell contact, such as tight junctions in epithelial cells and synaptic junctions in neurons, where they nucleate the assembly of multiprotein complexes via their protein-protein interaction domains (6, 7). In addition, ZO-1 was found in the nucleus of cultured cells under certain growth conditions (8).

PSD-95/SAP90 is the prototype of a subfamily of neuronal MAGUKs that includes SAP97/hDLG, chapsyn-110/PSD-93, and SAP102 (9–15). The first and second PDZ domains of PSD-95 proteins bind to the cytoplasmic COOH termini of the Shaker-type K⁺ channel and N-methyl-D-aspartate receptor 2 subunits, resulting in clustering of these molecules on the neuronal surface (11, 16–18). DLG, the Drosophila homologue of the mammalian PSD-95 family, colocalizes with Shaker K⁺ channels in larval neuromuscular junctions, and these channels fail to cluster at synapses in Drosophila larvae that harbor mutant DLG alleles (19). In heterologous cells, the channel clustering activity of PSD-95 requires only the first PDZ domain and a region at the NH₂ terminus that is conserved among the other PSD-95 family members, suggesting that the second and third PDZ domains may recruit additional membrane or cytoplasmic proteins (20).

The name PDZ domain is derived from three members of the MAGUK family: PSD-95, DLG, and ZO-1 (21). However, PDZ domains are not restricted to MAGUKs. More than 50 proteins are known to contain PDZ domains; many of these lack the GuK and SH3 domains, and several contain domains that confer protein kinase, protein phosphatase, or other enzymatic activities (3, 7). PDZ domains are repeats of 80–100 amino acids in length that engage in either homotypic interactions (PDZ-PDZ dimers) or, more commonly, heterotypic interactions in which the ligand is the COOH terminus of a transmembrane or a cytoplasmic protein. The single PDZ domain in neuronal nitric-oxide synthase binds to PDZ domains in PSD-95, PSD-93, and α1-syntrophin (10, 22); these are currently the only known examples of homotypic interactions mediated by PDZ domains. In contrast, several heterotypic ligands for PDZ domains have been identified. In addition to the PSD-95 ligands: GST, glutathione S-transferase; MDCK, Madin-Darby canine kidney; bp, base pair(s); kb, kilobase pair(s); hYAP, human YAP65.
described above, these interactions include the following: 1) the COOH terminus of the FAS receptor binds to the third PDZ domain of the tyrosine phosphatase FAP-1 (23); 2) the COOH terminus of the APC tumor suppressor protein binds to the second PDZ domain of SAP97/hDLG (24); 3) the COOH terminus of the testis determining factor SRY binds to PDZ domains in the nuclear protein SIP-1 (25); and 4) the transient receptor potential protein, eye protein kinase C, and phospholipase C-

The crystal structures of the third PDZ domain of SAP97/hDLG and of the third PDZ domain of PSD-95 bound to a synthetic peptide ligand were recently determined (27, 28). This information, together with in vitro biochemical studies (29), has begun to reveal the mechanism by which specificity is achieved in heterotypic PDZ domain interactions. One group of PDZ domains, including those in the PSD-95 family, bind COOH-terminal sequences that conform to the consensus (S/T)X where X is any amino acid), such as those found in the Shaker K+ channel and N-methyl-D-aspartate receptor subunits. The hydroxyl group of the serine or threonine at the −2−position in this consensus forms a hydrogen bond with a histidine (His372 in PSD-95) that is conserved in most PDZ domains (28). The single PDZ domains in p55 and LIN-2A contain a valine in place of the semiconserved histidine, and these domains prefer ligands in vitro that contain aromatic residues in the −2−position (29). The PDZ domain of neuronal nitric-oxide synthase represents a third variant in which the histidine is replaced by a tyrosine. Besides forming homotypic complexes with other PDZ domains, the neuronal nitric-oxide synthase PDZ domain binds to peptides in vitro that terminate in the sequence DXV (30). Thus, a single residue in each PDZ domain (corresponding to His372 in PSD-95) may dictate which amino acid is found in the −2−position of its cognate ligand. Several other structural features of PDZ domains are predicted to contribute in a more subtle manner to the selection of a particular COOH-terminal tripeptide sequence as a ligand (27–29). Moreover, interactions with amino acids that are adjacent to the tripeptide consensus probably impart additional specificity.

GuK domains in MAGUKs were named because of their similarity to S. cerevisiae GuK, which uses ATP to phosphorylate AMP, producing guanosine diphosphate and adenosine diphosphate (31). Three classes of GuK domains have been found in MAGUKs: those that lack both the GMP- and ATP-binding residues (ZO-1 and ZO-2), those in which the GMP-binding residues are conserved but the ATP-binding motif is absent (PSD-95 family), and those that conserve both the GMP- and ATP-binding residues (p55, LIN-2A, and CASK) (32). However, none of the GuK domains in MAGUK proteins are known to be catalytically active. A clue to the function of the GuK domain in MAGUK proteins emerged recently when two groups identified a synaptic protein, designated GKAP by one group and SAPAP by the other, which binds to the GuK domains of the PSD-95 proteins (33, 34). GKAP was found in a complex with PSD-95 and Shaker K+ channels, suggesting that it may serve to anchor this complex in the postsynaptic density. Although it is not yet known if the interaction between GKAP and PSD-95 is regulated by guanine nucleotide binding, this finding suggests that the GuK domains in other MAGUK proteins may also function as sites for protein−protein interaction.

A protein−protein interaction module that has not previously been found in MAGUK proteins is the WW domain (35, 36). The WW domain was named for two conserved tryptophan residues and was first identified as two repeats in mouse YAP65 (Yes-associated protein of 65 kDa) (37). WW domains have been identified by sequence similarity in more than 30 proteins from various species, and interactions mediated by WW domains have been implicated in human diseases such as Liddle’s syndrome, muscular dystrophy, and Alzheimer’s disease (38). WBP1 and WBP2 are candidate ligands for the single WW domain in human YAP65 (39). These proteins bind to the YAP65 WW domain via polyproline sequences that conform to the consensus sequence PXXY, designated the PY motif. However, the WW domains in formin-binding proteins bind to a proline-rich sequence in formins that does not match the PY motif, indicating that not all WW domains bind to this consensus (40). The same study also demonstrated overlap between the WW domain binding site and a SH3 domain binding site, raising the possibility that these two domains may sometimes compete with one another for the same ligand. Phosphorylation of the tyrosine in a synthetic PY motif abolished its interaction with the YAP65 WW domain, suggesting a potential regulatory mechanism for some WW domain interactions (41).

In this report, we describe a MAGUK with three features that distinguish it from all other known members of the family: 1) the GuK domain is at the NH2 terminus rather than at the COOH terminus; 2) the SH3 domain is replaced by two WW domains; and 3) it contains five PDZ domains rather than the usual one or three. This protein has been designated MAGI-1, for membrane-associated guanylate kinase with an inverted arrangement of protein−protein interaction domains.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid System**—The yeast strain L40, the plasmids pBTM116 and pVP16, and the mouse embryo cDNA library in pVP16 (42) were obtained from Dr. Michael White of the University of Texas Southwestern Medical Center at Dallas. Bait constructs were prepared by polymerase chain reaction amplification of the desired fragment containing 5′-BamHI and 3′-PstI restriction sites. The product of each reaction was subcloned into the corresponding sites in pBTM116, and each plasmid was verified by DNA sequencing. For β-galactosidase assays the protein concentration of each yeast strain was grown for 16 h in −Trp− Leu medium and then diluted 10-fold with the same medium and grown for an additional 3 h. Cells were harvested by centrifugation, resuspended in buffer Z (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, pH 7.0), and lysed by two freeze/thaw cycles, followed by vortexing in the presence of glass beads. The lysate was centrifuged for 10 min at 1.5 × 104 g, the supernatant was transferred to a new tube, and protein concentration of each supernatant was determined with the BCA protein assay reagent (Fierce). Five μg of protein from each lysate was adjusted to a total volume of 30 μl with buffer Z and mixed with 200 μl of reaction buffer from the Luminescent β-Galactosidase Detection Kit II (CLONTECH). After a 5-min incubation at room temperature, at which time each reaction was progressing in a linear manner, the relative light units emitted by each reaction were measured in a luminometer. Blank values were obtained by measuring the β-galactosidase activity in 5 μg of protein from a lysate of nontransformed yeast.

cDNA Cloning—The 478-bp cDNA insert was excised from pVP16-K65 by digesting with NotI and radiolabeled with [α-32P]dCTP using the PrimeIt II random primer labeling kit (Stratagene). This probe was used to screen an oligo dT/random-primed mouse lung cDNA library in Lambda ZAP II (Stratagene), according to the manufacturer’s instructions. Eleven MAGI-1 cDNAs were isolated from a total of 3 × 107 plaque-forming units. Additional MAGI-1 cDNAs were isolated in a similar manner from an oligo dT-primed mouse brain library. The size of each cDNA insert was determined by restriction mapping, and selected clones were analyzed by DNA sequencing using T3, T7, and specific internal primers. The entire 5,296-bp MAGI-1b cDNA was sequenced on both strands.

**Blot Hybridization of RNA**—A 678-bp XhoI restriction fragment, spanning nucleotides 759–1437 of MAGI-1, was radiolabeled with [α-32P]dCTP and used to probe a mouse multiple tissue Northern blot (CLONTECH). The probe was used at a concentration of 2 × 106 cpm/ml in Rapid-hyb buffer (Amersham Corp.), according to the manufacturer’s
instructions. After exposure to Kodak X-Omat AR film for the indicated time, the blot was stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

**Antibodies**—A 417-bp fragment that encodes amino acids 2–140 of MAGI-1 was amplified by polymerase chain reaction and subcloned into the vectors pRSETA (Invitrogen) and pGEX-2TK (Pharmacia). The resulting plasmids, designated pRSETA-MAGI2–140 and pGEX-2TK-MAGI2–140, were transformed into BL21(DE3) bacteria (Novagen). Recombinant His6-MAGI2–140 protein was expressed and purified by Ni2+-Sepharose affinity chromatography and used to immunize rabbits as described previously (43, 44). GST-MAGI2–140 was expressed and purified by glutathione-agarose affinity chromatography, and the glutathione-Sepharose affinity chromatography and used to immunize rabbits as described previously (43). Preimmune IgG was isolated from crude serum obtained from the same rabbit by purification on protein A-agarose. A polyclonal anti-MAGI-1 polyclonal IgG was affinity-purified as described previously (46).

A 4,680-bp psDNA-MAGI-1c. Monolayers of human embryonic kidney 293 cells were transfected with 1 μg of plasmid DNA/10-mm dish as described previously (43). After a 16-h incubation, whole-cell lysates were prepared in buffer A (0.05 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 2 mM urea, 1% (v/v) SDS, pH 7.5), and the protein concentration of each lysate was determined as described above. An equal amount of protein from each lysate was subjected to SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis with the indicated antibody as described previously (46).

**Cell Culture and Subcellular Fractionation**—MDCK cells were maintained in Dulbecco's modified Eagle's medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal bovine serum. Cells were plated on day 0 at a density of 5 × 105 cells/100-mm dish and harvested on day 3 by scraping in phosphate-buffered saline. Cell pellets were resuspended in ice-cold buffer B (10 mM Tris-HCl, 0.1 M sucrose, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1% (v/v) aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, pH 7.5) and lysed by 10 strokes in a Dounce homogenizer on ice. The homogenate was centrifuged at 4°C for 10 min at 105,000 g. The supernatant from this step was discarded, and the pellet was resuspended in buffer B and designated the nuclear fraction. The protein concentration of each fraction was determined, and an equal percentage of each fraction was subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis with the indicated antibodies.

**RESULTS**

Localization of K-RasB to the plasma membrane requires a COOH-terminal farnesylated cysteine plus a polybasic domain, a stretch of six contiguous lysines (amino acids 175–180) that are separated from the farnesylated cysteine by four amino acids (47). The molecular mechanism by which these structural features target K-RasB to the plasma membrane is not known. We used the yeast two-hybrid system to search for proteins that interact with the COOH terminus of K-RasB. Preliminary studies revealed that full-length K-RasB failed to interact with Raf in the yeast two-hybrid system, whereas a mutant that was truncated after cysteine 185, thus preventing its farnesylation, interacted efficiently with Raf (data not shown). Farnesylated K-RasB is apparently unable to be transported into the nucleus, where it must reside to function properly in the yeast two-hybrid system. Thus, the bait used in subsequent cDNA library screens, designated K-RasB1–185, contained a segment from the COOH terminus of K-RasB that was truncated after cysteine 185.

A 478-bp cDNA, designated K65, was isolated from a mouse embryonic library. K65 interacted with K-RasB1–185 and with an essentially full-length bait, K-RasB1–185. K65, like Raf, failed to interact with a farnesylated K-RasB bait (data not shown). However, K65 also failed to interact with nonfarnesylated versions of H-Ras and Rac1, a Ras-related protein that also has a polybasic domain, suggesting that its interaction with K-RasB1–185 was specific (data not shown). A comparison of the deduced amino acid sequence of K65 with sequences in GenBank™ revealed a region of ~100 amino acids that represented a novel PDZ domain, which was designated MAGI1 (Fig. 1). MAGI1 retained the ability to interact with K-RasB1–185 when removed from the flanking sequences present in K65, suggesting that K-RasB was behaving as a heterotypic ligand for this novel PDZ domain (Table I, line a).

To investigate the structural features of K-RasB1–185 responsible for its interaction with MAGI1, various mutant K-RasB1–185 baits were prepared, and the β-galactosidase activity resulting from their interaction with MAGI1 was measured in a quantitative assay using a chemiluminescent substrate (Table I). Deletion of the COOH-terminal cysteine in K-RasB1–185 caused a 45-fold decrease in β-galactosidase activity (Table I, line b). The activity decreased by similar magnitudes when the COOH-terminal cysteine was replaced by serine, methionine, or alanine (Table I, lines c–e, respectively).

**FIG. 1.** Sequence alignment of MAGI1, and the third PDZ domain of PSD-95. The amino acid sequences of the third PDZ domain of PSD-95 (PSD-953) and MAGI1 were aligned by the Jotun-Hein method in MegAlign (DNASTAR). MAGI1 amino acids that are identical to the corresponding position in PSD-95, are shaded black; conservative substitutions, which were assigned using the chemical grouping option in MegAlign, are shaded gray. The numbers correspond to the sequence of PSD-95. The boundaries of secondary structure elements in the crystal structure of PSD-953, are indicated by brackets; filled circles denote amino acids that contacted a peptide ligand in the crystal structure (28). Amino acid substitutions in two MAGI1 point mutants, designated Mut. 1 and Mut. 2, are indicated below the MAGI1 sequence.

A Structurally Unique MAGUK

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FIG. 2  A Structurally Unique MAGUK
Likewise, substitution of histidine in MAGI1 with leucine (Fig. 183 in K-RasB1–185 was replaced with alanine (Table I, line f); k). All of the mutant K-RasB baits interacted equally well with was as detrimental as substitution of lysines 179 and 180 (line glutamine resulted in 20- and 90-fold decreases in activity, 200). Substitution of lysines 175 and 176 in the poly-Galactosidase activity (Table I, line h). Substitution of lysines PDZ domains. Substitution of lysines 175 and 176 in the poly-

Many heterotypic PDZ domain ligands contain a threonine or serine three amino acids from the COOH terminus (referred to as the −2-position), which forms a hydrogen bond with a histidine in their cognate PDZ domain (28), corresponding to histidine 372 in the third PDZ domain of PDZ-95 (PDZ-95, Fig. 1). β-Galactosidase activity decreased 140-fold when threonine 183 in K-RasB1–185 was replaced with alanine (Table I, line i); likewise, substitution of a histidine in MAGI1, with leucine (Fig, 1, Mut. 2) abolished its interaction with K-RasB1–185 (Table I, line a). Most known PDZ domains prefer ligands in which the COOH-terminal amino acid is a valine (29). The β-galactosidase activity increased by 22-fold when cysteine 185 in K-RasB1–185 was replaced by valine (Table I, line l).

Insertion of three alanines between lysine 180 and serine 181 in K-RasB1–185 caused a 34-fold decrease in β-galactosidase activity, suggesting that one or more lysines in the polybasic domain might participate in the interaction with MAGI1, (Table I, line g). Insertion of three alanines resulted in a similar reduction in activity when cysteine 185 was replaced by valine (Table I; compare lines l and m), indicating that the spacing between the polybasic domain and the COOH terminus was important even in the context of a COOH-terminal tripeptide that conformed to the STXV consensus preferred by most PDZ domains. Substitution of lysines 175 and 176 in the polybasic domain with glutamine caused a 4-fold reduction in β-galactosidase activity (Table I, line h). Substitution of lysines 177 and 178 (line i) and lysines 179 and 180 (line j) with glutamine resulted in 20- and 90-fold decreases in activity, respectively. A single substitution of lysine 180 with glutamine was as detrimental as substitution of lysines 179 and 180 (line k). All of the mutant K-RasB baits interacted equally well with

**FIG. 2. Mouse MAGI-1 cDNAs.** Nucleotide and predicted amino acid sequence of MAGI-1b (A) and partial sequences of MAGI-1a (B) and MAGI-1c (C). Nucleotides are numbered on the left, and amino acids are numbered on the right. A, nucleotides 1–5,296 were derived from a single MAGI-1b cDNA, and nucleotides 5,297–5,371 were from a separate, overlapping cDNA. The region of similarity to S. cerevisiae GuK is indicated by a dashed underline, the WW domains are indicated by a box, and the PDZ domains are underlined. The triangle after nucleotide 2,852 denotes the mRNA splice site. B, the last 1,011 bp of a 3,770-bp MAGI-1a cDNA are shown with the predicted amino acid sequence. The first 2,852 bp of this cDNA are identical to MAGI-1b; only the last 93 bp of overlapping sequence are shown, and the numbering scheme assumes that the 5′-ends of MAGI-1a and MAGI-1b are identical. The underlined acidic amino acids denote the end of the fifth PDZ domain; the triangle denotes the mRNA splice site. C, the last 1,900 bp of a 3,038-bp MAGI-1c cDNA are shown with the predicted amino acid sequence. The first 1,231 bp of this cDNA are identical to MAGI-1b; as in panel B, only the last 93 bp of overlapping sequence are shown, and the numbering scheme assumes that the 5′-ends of MAGI-1c and MAGI-1a are identical. Bipartite nuclear localization signals are boxed.
other members of the MAGUK family. Amino acids 152–348 of MAGI-1b, a segment that begins within the GuK domain and ends just before the second WW domain, are 93% identical to the predicted amino acid sequence of a partial cDNA that was isolated from a human cDNA expression library via its interaction with synthetic peptides that contained PY motifs (48).

Two additional classes of MAGI-1 cDNAs were isolated whose sequences diverged from MAGI-1b downstream of an apparent mRNA splice site at nucleotide 4,293 (triangle in Fig. 2, A–C), just beyond the fifth PDZ domain. These cDNAs are predicted to encode proteins of 1,139 amino acids (124 kDa; Fig. 2B) and 1,374 amino acids (152 kDa; Fig. 2C) and were designated MAGI-1a and MAGI-1c, respectively. The longest MAGI-1a cDNA isolated to date contains 3,770 bp and was found in a brain library. The sequence of this cDNA begins within the GuK domain and ends with a poly(A) tail. If its 5'-end is assumed to be identical to MAGI-1b, it would be 5,212 bp in length, which may correspond to a transcript of ~5 kb detected in brain mRNA on a Northern blot (see Fig. 4). The longest MAGI-1c cDNA is 3,038 bp in length; its sequence begins between the second and third PDZ domains and ends in the 3'-untranslated region. Since it lacks a poly(A) tail, it is not yet possible to predict the total length of the transcript from which it originated, but it may correspond to either the ~6.9- or the ~7.8-kb transcript detected on a Northern blot (see Fig. 4).

The COOH-terminal 145 amino acids of MAGI-1c consist of 37% lysine plus arginine and 19% aspartate plus glutamate. This region contains three bipartite nuclear localization signals (49), the second and third of which are overlapping (boxes in Fig. 2C).

The tissue distribution of MAGI-1 mRNA was examined by Northern blot analysis of RNA from various mouse tissues. A probe derived from near the 5'-end of the MAGI-1b cDNA hybridized to transcripts of ~5.4, ~6.9, and ~7.8 kb in mRNA from kidney, liver, and lung (Fig. 4, top, lanes 2, 4, and 5). The 5.4- and 7.8-kb transcripts were also detected in testis and heart (lanes 1 and 8); prolonged exposures of the blot to film revealed the 5.4- and 6.9-kb transcripts in skeletal muscle (lane 3) and transcripts of ~5.0 and ~5.6 kb in brain (lane 7), indicating that expression of MAGI-1 in adult tissues is widespread.

Mammalian expression vectors were prepared that contained the complete open reading frames of MAGI-1b and MAGI-1c, and these plasmids were transiently transfected into human embryonic kidney 293 cells. Lysates of these cells were analyzed by immunoblotting with anti-MAGI-1, a polyclonal antibody directed against amino acids 2–140 of MAGI-1. This antibody detected proteins of ~122 and ~142 kDa in cells transfected with MAGI-1b and MAGI-1c, respectively (Fig. 5A, top panel, lanes 2 and 4). These proteins were not detected in lysates of cells that were transfected with expression vector alone (lane 1), nor were they detected with preimmune IgG (Fig. 5A, bottom panel). Proteins of slightly slower mobilities (~137 and ~148 kDa) were detected in a whole-cell lysate of MDCK cells with anti-MAGI-1 IgG (top panel, lane 3) but not with preimmune IgG (bottom panel). Proteins with molecular masses identical to those seen in MDCK cells were also detected in lysates of mouse lung and liver (Fig. 5B, top panel, lanes 7 and 8), suggesting that they represent posttranslational modifications of viral MAGI-1 and MAGI-1c, respectively, rather than mRNA splice variants unique to MDCK cells. Although transfected MAGI-1a has not yet been analyzed on SDS gels, its mobility is expected to be similar to that of MAGI-1b, since their predicted molecular masses differ by only 3.5 kDa. Thus, the 137-kDa protein will be tentatively referred to as MAGI-1a/b and the 148-kDa protein as MAGI-1c. MAGI-1a/b was the predominant version expressed in mouse brain, whereas liver expressed primarily MAGI-1c (Fig. 5B, compare lanes 6 and 8); the two forms were present in approximately equal levels in mouse lung (lane 7).
domains from mouse MAGI-1 (MAGI 1–13) in the nuclear fraction (Fig. 5). Fractions were analyzed on replicate blots with antibodies against the nuclear envelope protein lamin B (Fig. 5). The amount was detected in the P100 fraction (lane 12), with a small amount in the S100 fraction (lane 11), and a large amount was found almost entirely in the nuclear fraction (lane 13). These same fractions were analyzed on replicate blots with antibodies against the nuclear envelope protein lamin B (Fig. 5, A, middle panel) and against the α-subunit of farnesyltransferase (FTα), a cytoplasmic enzyme (Fig. 5, B, bottom). Lamin B was detected almost entirely in the nuclear fraction (lane 13) and was also detected in the whole-cell lysates of transiently transfected 293 cells (lanes 10 and 14). In contrast, FTα was detected only in the S100 fraction (lane 11).

An amino acid sequence alignment revealed that the GuK domain of MAGI-1 is 27% similar to S. cerevisiae GuK over a 104-amino acid region (Fig. 6A). The similarity increased to 42% if the alignment was restricted to the region corresponding to amino acids 34–99 of S. cerevisiae GuK (amino acids 142–205 of MAGI-1). Although the MAGI-1 GuK domain lacks an ATP-binding motif, 7 of 11 amino acids required for GMP binding in yeast GuK (31) are conserved, and two of the four mismatches represent conservative substitutions. Thus, the MAGI-1 GuK domain is similar to the GuK domains in PSD-95 and DLG, which also conserve the GMP-binding residues but lack an ATP-binding motif (32).

Amino acid sequences corresponding to the five PDZ domains in MAGI-1, designated MAGI−MAGI5, were aligned with the sequences of the third PDZ domains in PSD-95 (PSD-95), and hDLG/SAP97 (hDLG), as described in the legend to Fig. 6B. Amino acids that are identical to the corresponding position in S. cerevisiae GuK are shaded black; conservative substitutions are shaded gray; numbers correspond to the sequence of S. cerevisiae GuK. Amino acids in S. cerevisiae GuK that bind ATP are indicated by a bracket; residues that contact the guanine ring (filled circles) and phosphate group (inverted triangles) of GMP and its bound Mg2+ ion (open circle) are also indicated. B, amino acid sequences of the third PDZ domains from rat PSD-95 (PSD-95), human DLG (hDLG), and the five PDZ domains from mouse MAGI-1 (MAGI1–MAGI5) were aligned as described in the legend to Fig. 1B. Amino acids that are identical to the corresponding position in PSD-95, are shaded black; conservative substitutions are shaded gray. The numbers correspond to the sequence of PSD-95. Amino acids that contacted a peptide ligand in the crystal structure of PSD-953 are indicated by filled circles (28).

To determine the subcellular distribution of MAGI-1 proteins in MDCK cells, S100, P100, and nuclear fractions were prepared, and an equal percentage of each fraction was subjected to immunoblot analysis with anti-MAGI-1 IgG (Fig. 5). MAGI-1a/b was found primarily in the P100 and S100 fractions (lanes 12 and 11, respectively), with a small amount in the nuclear fraction (lane 13). In contrast, MAGI-1c was found almost entirely in the nuclear fraction (lane 13); a small amount was detected in the P100 fraction (lane 12), but it was completely absent from the S100 fraction (lane 11). These same fractions were analyzed on replicate blots with antibodies against the nuclear envelope protein lamin B (Fig. 5, A, middle panel) and against the α-subunit of farnesyltransferase (FTα), a cytoplasmic enzyme (Fig. 5, B, bottom). Lamin B was detected almost entirely in the nuclear fraction (lane 13) and was also detected in the whole-cell lysates of transiently transfected 293 cells (lanes 10 and 14). In contrast, FTα was detected only in the S100 fraction (lane 11).

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Amino acid sequences corresponding to the five PDZ domains in MAGI-1, designated MAGI−MAGI5, were aligned with the sequences of the third PDZ domains in PSD-95 (PSD-95), and hDLG/SAP97 (hDLG), as described in the legend to Fig. 6B. MAGI−MAGI5 are 34, 21, 32, 18, and 36% similar, respectively, to PSD-95. The highest similarity among the PDZ domains within MAGI-1 is 36% between MAGI1 and MAGI2, suggesting that each domain may interact with distinct ligands. Only MAGI1 contains four acidic residues in the region that is predicted to form the loop between the second and third β strands, consistent with the observation that K-RasB1-185 failed to interact with the other four PDZ domains in MAGI-1.2 The most divergent MAGI-1 PDZ domain is MAGI4, showing only 18% similarity to PSD-95. However, 7 of the 12 amino acids that contacted a peptide ligand in the crystal structure of PSD-953 are conserved in MAGI4, and three of the five mismatches are conservative substitutions.

Leucine 323 and leucine 379 in PSD-95 are on opposite sides of a hydrophobic pocket that binds the COOH-terminal amino acid in heterotypic ligands. All five MAGI-1 PDZ domains contain a phenylalanine in the position corresponding to leucine 323, and only MAGI2 contains a leucine at the position corresponding to leucine 379 in PSD-95 (Fig. 6B). This position is occupied by phenylalanine in MAGI1, and MAGI4, methionine in MAGI5, and isoleucine in MAGI3. Whether these subtle substitutions in the MAGI-1 PDZ domains result in unique binding properties will await identification of ligands for each domain. Four of the five MAGI-1 PDZ domains contain a histidine at the position corresponding to histidine 372 in PSD-95, and the other (MAGI5) contains a lysine at this position, suggesting that each of these domains bind to ligands that contain serine, threonine, or tyrosine at the −2-position (28, 29).

The amino acid sequences corresponding to the WW domains in MAGI-1, designated MAGIWW1 and MAGIWW2, were aligned with the WW domain in human YAP65 (hYAP), both PDZ domains in mouse YAP65 (mYAP1 and mYAP2), and the first WW domain in NEDD4 (NEDD41, Fig. 7). MAGIWW1 and MAGIWW2 are 60 and 36% similar, respectively, to hYAP. The solution structure of hYAP revealed a three-stranded β-sheet that adopts a slightly curved conformation (50). Prolines 174 and 202 interact with tryptophan 177 on the convex side of the sheet, stabilizing the domain. These three positions are conserved in both MAGI-1 WW domains (Fig. 7). The side chains of

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tyrosine 188, leucine 190, histidine 192, and tryptophan 199 in hYAP form a hydrophobic surface on the concave side of the domain that interacts with the principal elements of the PY motif. The corresponding positions in the MAGI-1 WW domains are either conserved or substituted with very similar amino acids (Fig. 7), and these domains both bind to a PY motif in vitro. However, the side chains corresponding to amino acids 194–198 of hYAP are also predicted to contribute to the specificity of a given WW domain-ligand interaction (50), and several of these positions in MAGIWW1 and MAGIWW2 differ from the corresponding positions in hYAP. Thus, the ligand binding specificity of each MAGI-1 WW domain remains to be determined.

**DISCUSSION**

The first PDZ domain of MAGI-1 was isolated on the basis of its interaction with the COOH terminus of K-RasB in the yeast two-hybrid system. This interaction required lysine 180, threonine 183, and cysteine 185 of K-RasB (Table I). These structural features are similar to those required for localization of K-RasB to the plasma membrane (47), consistent with a possible role for MAGI-1 in this process. However, K-RasB in mammalian cells is covalently modified by farnesyl and methyl groups on its COOH-terminal cysteine, modifications that apparently preclude the ability of K-RasB to function in the yeast two-hybrid system. While it is possible that these modifications increase the affinity of the interaction between MAGI1 and K-RasB, we have been unable to demonstrate an interaction between K-RasB and full-length MAGI-1b in lysates of transfected mammalian cells, in which K-RasB contains its full complement of posttranslational modifications. Moreover, threonine 183 of K-RasB is not required for plasma membrane localization or transformation of Rat-1 fibroblasts by oncogenic K-RasB, suggesting that if K-RasB does interact with MAGI-1 in cultured cells, the interaction is not required for either of these processes. Thus, despite the specificity of the interaction between MAGI1 and K-RasB in the yeast two-hybrid system, at this time we have no evidence that the interaction occurs in mammalian cells.

The interaction between MAGI1 and K-RasB in the yeast two-hybrid system required four acidic residues in the PDZ domain (Asp170, Glu171, Asp173, and Glu174 of MAGI-1b) and lysine 180 in K-RasB, which is 5 positions removed from the COOH-terminal cysteine (Table I). These sequence requirements were maintained when cysteine 185 of K-RasB was replaced by valine. The clustered acidic residues in MAGI1 are in a region that is predicted to lie in close proximity to amino acids in the −5 and −6-positions in a bound ligand (27, 28). Consistent with this observation, we recently isolated a candidate ligand for MAGI1 whose sequence terminates in a TXV consensus and contains lysines in the −5 and −6-positions.

This novel protein, designated Mbp-L, binds to MAGI1 but not to the other four PDZ domains in MAGI-1,2 all of which lack the four clustered acidic residues found in MAGI1, (Fig. 6B). MAGI1 is currently the only known PDZ domain that contains four acidic residues in this region, but several PDZ domains contain two adjacent acidic residues in this loop (27, 28). Although no previous studies have directly demonstrated a requirement for these residues in a PDZ domain-ligand interaction, the third PDZ domain in mouse DLG binds preferentially to a synthetic peptide that contains five lysines upstream of a TXV consensus (29). Thus, amino acids in the loop between β5 and β6 may be required in other PDZ domain interactions as well.

MAGI1 mRNA undergoes alternative splicing to produce three transcripts that encode proteins with unique COOH termini (Fig. 2). The COOH termini of MAGI-1a and MAGI-1b contain 16 and 48 amino acids of unique sequence, respectively, and their molecular masses differ by only 3.5 kDa. A protein that corresponds to MAGI-1a and/or MAGI-1b was found primarily in the S100 and P100 fractions of MDCK cells, with a small amount in the nucleus (Fig. 5C). The COOH terminus of MAGI-1c extends 251 amino acids beyond the splice site and is highly charged. The last 145 amino acids consist of 37% lysine plus arginine, 19% aspartate plus glutamate, and 15% serine plus threonine, which could contribute additional negative charge via phosphorylation. This region contains three segments of 17 amino acids each (residues 1251–1267, 1293–1309, and 1299–1315) that conform to the consensus sequence for a bipartite nuclear localization signal (Fig. 2C). This consensus sequence consists of two adjacent basic residues, followed by a spacer of 10 amino acids, followed by a second cluster of basic residues in which three of the five next residues are basic (49). Consistent with the presence of nuclear localization signals in the COOH terminus of MAGI-1c, it was found primarily in the nucleus of MDCK cells (Fig. 5C). Seven repeats of the sequence SPXIR/K (where X is glutamate, serine, or threonine), which matches the consensus sequence for phosphorylation by p34cdc2 (51), are clustered in and around the three nuclear localization signals, suggesting a potential regulatory mechanism for nuclear localization of MAGI-1c.

ZO-1, a MAGUK that was originally described as a component of the tight junction, was recently found in the nucleus of subconfluent, but not confluent, MDCK cells (8). ZO-1 also undergoes alternative splicing, resulting in two proteins that are distinguished by the presence or absence of an 80-amino acid segment, designated the α domain (1). The two isoforms, ZO-1α+ and ZO-1α−, are expressed in different cell types in a pattern that correlates with junctional plasticity, but both variants were able to localize to the nucleus of subconfluent cultured cells (8). In contrast, at least two of the three MAGI-1 isoforms were co-expressed in MDCK cells and in mouse lung, and only MAGI-1c was found preferentially in the nucleus (Fig. 5). Confluent MDCK cells were used in this experiment, suggesting that MAGI-1c is present in the nucleus at a time when ZO-1 is excluded from the nucleus. Whether the subcellular distribution of MAGI-1c is influenced by changes in cell density is not yet known.

In contrast to lung, mouse brain expressed almost entirely MAGI-1a/b, whereas liver expressed primarily MAGI-1c (Fig. 5B). This differential expression of MAGI-1c in mouse tissues, together with its nuclear localization, implies that its function is distinct from that of MAGI-1a/b. The short COOH-terminal sequences unique to MAGI-1a and MAGI-1b do not contain any features that would suggest a functional difference between the two. Antibodies that recognize the unique COOH termini of these two MAGI-1 proteins will reveal whether they are co-expressed in various tissues and cell types and, if so, whether

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they have distinct subcellular distributions. MAGI-1 is currently the only known protein in which a GuK domain is combined with five PDZ domains and two WW domains (Figs. 2 and 3). Based on sequence comparisons with similar domains from other proteins, the PDZ and WW domains in MAGI-1 are each predicted to interact with distinct proteins. If the MAGI-1 GuK domain also functions as a site for protein-protein interaction, as it does in the PSD-95 proteins (33, 34), MAGI-1 would have the potential to interact with up to eight different proteins simultaneously. Although the role of ZO-1 and MAGI-1 in the nucleus is not yet known, their presence there suggests that they may participate in the transfer of information directly from the cell surface to the nucleus. Future studies will seek to identify ligands for the various protein-protein interaction domains in MAGI-1, which will provide clues to the function of this newest member of the MAGUK family.

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