Interaction of Two Actin-binding Proteins, Synaptopodin and α-Actinin-4, with the Tight Junction Protein MAGI-1*

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In an attempt to find podocyte-expressed proteins that may interact with the tight junction protein MAGI-1, we screened a glomerulus-enriched cDNA library with a probe consisting of both WW domains of MAGI-1. One of the isolated clones contained two WW domain-binding motifs and was identified as a portion of the actin-bundling protein synaptopodin. In vitro binding assays confirmed this interaction between MAGI-1 and synaptopodin and identified the second WW domain of MAGI-1 to be responsible for the interaction. MAGI-1 and synaptopodin can also interact in vivo, as they can be immunoprecipitated together from HEK293 cell lysates. Another actin-bundling protein that is found in glomerular podocytes and shown to be mutated in an inheritable form of glomerulosclerosis is α-actinin-4. We show that α-actinin-4 is also capable of binding to MAGI-1 in in vitro binding assays and that this interaction is mediated by the fifth PDZ domain of MAGI-1 binding to the C terminus of α-actinin-4. Exogenously expressed synaptopodin and α-actinin-4 were found to colocalize along with endogenous MAGI-1 at the tight junction of Madin-Darby canine kidney cells. The interaction and colocalization of MAGI-1 with two actin-bundling proteins suggest that MAGI-1 may play a role in actin cytoskeleton dynamics within polarized epithelial cells.

The ability of the mammalian kidney to produce a protein-free filtrate is predominantly due to specialized cells within the renal glomerulus known as visceral epithelial cells or podocytes. Mature differentiated podocytes are highly specialized cells whose many functions, including regulating glomerular permselectivity (1), depend on an elaborate and complex cellu-
lar morphology (2). Because of this distinct morphology, podocytes are found in glomerular podocytes and shown to be mutated in an inheritable form of glomerulosclerosis. α-actinin-4. We show that α-actinin-4 is also capable of binding to MAGI-1 in in vitro binding assays and that this interaction is mediated by the fifth PDZ domain of MAGI-1 binding to the C terminus of α-actinin-4. Exogenously expressed synaptopodin and α-actinin-4 were found to colocalize along with endogenous MAGI-1 at the tight junction of Madin-Darby canine kidney cells. The interaction and colocalization of MAGI-1 with two actin-bundling proteins suggest that MAGI-1 may play a role in actin cytoskeleton dynamics within polarized epithelial cells.

The ability of the mammalian kidney to produce a protein-free filtrate is predominantly due to specialized cells within the renal glomerulus known as visceral epithelial cells or podocytes. Mature differentiated podocytes are highly specialized cells whose many functions, including regulating glomerular permselectivity (1), depend on an elaborate and complex cellular morphology (2). Because of this distinct morphology, podocytes can be divided into three functionally and structurally different segments: cell body, major processes, and foot protrusions. Structural differences in the segments of podocytes are reflected in their different cytoskeletal foundation, with foot processes exhibiting an actin-based contractile apparatus (3). In glomerular diseases with massive proteinuria, podocytes may undergo dramatic structural changes resulting in loss of cell morphology and eventual effacement. Under some conditions, these changes are reversible, which illustrates the morphological dynamics of foot processes. Although the regulation of foot process dynamics is poorly understood at this time, it is apparent that an alteration in the actin-based cytoskeleton is a fundamental aspect.

Two actin-bundling proteins have recently drawn attention because of their expression within the podocyte. One of them, synaptopodin, was initially identified as an antigen to a monoclonal antibody that showed association with the actin system of podocyte foot processes (4). Its eventual cloning and characterization showed no significant homology to any other known proteins, and it was found in the dendritic spines of hippocampal neurons in addition to renal podocytes (5). The finding that synaptopodin associates with specialized actin-based compartments of renal podocytes and neuronal dendrites suggests that synaptopodin may play a role in the structural and/or functional dynamics of these cellular extensions. The non-muscle isoform of α-actinin is another actin-associated protein shown to be expressed in the podocyte (3, 6, 7). Of the two known non-muscle isoforms of α-actinin (α-actinin-1 and α-actinin-4), α-actinin-4 was recently identified as the isoform that is present in human podocytes, and mutations in the gene encoding this protein are responsible for an autosomal dominant form of focal and segmental glomerulosclerosis (FSGS)¹ in humans (8). Interestingly, both α-actinin-1 and α-actinin-4 are found in cultured mouse podocytes, but they exhibit differences in their spatial distribution within these cells (9). Originally identified as the antigen to a monoclonal antibody that exhibited a unique immunohistochemical reactivity, α-actinin-4 has also been implicated in cell motility and carcinogenesis (10).

We recently reported on the localization of a protein, MAGI-1, in the podocytes of rat kidneys (11). In addition, we showed that MAGI-1 was found in the membrane fraction of mouse glomerular preparations and that it was insensitive to extraction with Triton X-100, which suggested to us that MAGI-1 might be associated with the actin cytoskeleton. The MAGI proteins consist of three members that together make up

¹ The abbreviations used are: FSGS, focal and segmental glomerulosclerosis; MAGI-1, membrane-associated guanylate kinase inverted-1; MAGUK, membrane-associated guanylate kinase; PDZ, PSD-95/DLG/ZO-1; AIP, α-trophin-1-interacting protein; GST, glutathione S-transferase; HA, hemagglutinin; HEK293, human embryonic kidney 293; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline.
a subfamily of a larger group of proteins known as the MAGUKs. MAGUK proteins share a common structural organization and are proposed to function as molecular scaffolds within cells (for recent reviews, see Refs. 12 and 13). Many of them are found at special subcellular regions such as postsynaptic densities within neurons as well as the tight and adherens junctions of epithelial cells and are believed to play a role in the structure and function of these specialized complexes. The MAGUK proteins exhibit a unique grouping of protein-protein interaction domains that is inverted in the MAGI family of proteins. In addition, two WW domains in the MAGI proteins take the place of the SH3 (Src homology 3) domain observed in the conventional MAGUKs. WW domains are small protein interaction modules of 30–40 amino acids in length and are often found in association with other protein interaction domains such as phosphotyrosine-binding and PDZ domains (14, 15). They have been found to bind polyproline-rich peptide sequences and can be classified into five distinct groups based upon current understanding of their binding specificity. Group I WW domains, like those found in the ubiquitin-protein ligase Nedd4 and Yes-associated protein, have been extensively studied and recognize “PXY” motifs (where P is proline, X is any amino acid, and Y is tyrosine; often referred to as PY motifs) (16, 17). The binding of a number of proteins to the PDZ domains and guanylate kinase domain of MAGI-1, MAGI-2, and MAGI-3 has been reported; however, there is a paucity of data on proteins that may interact with the WW domains of MAGI proteins. Although a screen to identify interacting partners for the DRPLA (dentatorubral and pallidoluysian atrophy) gene product atrophin-1 isolated partial cDNAs containing the WW domains of MAGI-1 (AIP-3) and MAGI-2 (AIP-1) (18), no further data on these interactions have yet been reported.

In an initial attempt to discover potential binding partners for MAGI-1 that are found in renal glomeruli, we screened a cDNA expression library made from glomerulus-enriched preparations of mouse kidney with the WW domains of MAGI-1. Among the clones that were isolated was a partial WW domain coding for the region of the actin-bundling protein synaptopodin whose C terminus contains a PDZ domain-binding motif, to bind to MAGI-1. We found that a-actinin-4 was also capable of binding to one of the PDZ domains of MAGI-1. The following MAGI-1 constructs were obtained using reverse transcription-PCR on total RNA derived from glomerulus-enriched preparations of mouse kidneys that was used to make a random-primed cDNA library, which was then modified at its ends with EcoRI adapters. The cDNA library was then cloned into aSSCREEN phage arms containing EcoRI sites at their ends (CLONTECH, Palo Alto, CA). The library had an initial complexity of $1-10^6$ plaque-forming units/ml prior to one round of amplification. The GST fusion protein GST-MAGI-1 WW12 (see above) was labeled with ($\gamma$-32P)ATP and used as a probe to screen the library (the pGSTag expression vector has a protein kinase A phosphorylation site placed in between the GST sequence and the downstream protein of interest). Plating and transferring of plaques to nitrocellulose membranes were performed as described in the aSSCREEN manual. The resulting membranes were blocked in Farwestern buffer (20 mm Hepes (pH 7.5), 1 mm KCl, 5 mm MgCl2, 5 mm dithiothreitol (DTT), 5% nonfat dry milk, and 0.02% sodium azide) for 2 h at room temperature with gentle agitation, followed by a 2-h incubation with the 32P-labeled probe ($2 \times 10^6$ cpm/ml of Farwestern buffer) at room temperature. The membranes were washed twice with Tris-buffered saline supplemented with Triton X-100 to 0.1% and then three times with Tris-buffered saline (5 min each wash) at room temperature. The membranes were exposed to x-ray film at $-80 \, ^\circ C$.

**Expression Cloning—**Total RNA isolated from glomerulus-enriched preparations of mouse kidneys was used to make a random-primed cDNA library, which was then modified at its ends with EcoRI adapters. The cDNA library was then cloned into aSSCREEN phage arms containing EcoRI sites at their ends (CLONTECH, Palo Alto, CA). The library had an initial complexity of $1-10^6$ plaque-forming units/ml prior to one round of amplification. The GST fusion protein GST-MAGI-1 WW12 (see above) was labeled with ($\gamma$-32P)ATP and used as a probe to screen the library (the pGSTag expression vector has a protein kinase A phosphorylation site placed in between the GST sequence and the downstream protein of interest). Plating and transferring of plaques to nitrocellulose membranes were performed as described in the aSSCREEN manual. The resulting membranes were blocked in Farwestern buffer (20 mm Hepes (pH 7.5), 1 mm KCl, 5 mm MgCl2, 5 mm dithiothreitol (DTT), 5% nonfat dry milk, and 0.02% sodium azide) for 2 h at room temperature with gentle agitation, followed by a 2-h incubation with the 32P-labeled probe ($2 \times 10^6$ cpm/ml of Farwestern buffer) at room temperature. The membranes were washed twice with Tris-buffered saline supplemented with Triton X-100 to 0.1% and then three times with Tris-buffered saline (5 min each wash) at room temperature. The membranes were exposed to x-ray film at $-80 \, ^\circ C$.

**Plasmid Constructs—**The following MAGI-1 constructs were obtained using reverse transcription-PCR on total RNA derived from mouse glomerulus-enriched preparations: full-length MAGI-1 (amino acids 1–1220; numbering of all MAGI-1 constructs is based on the full-length cDNA obtained from kidney and glomerular libraries previously described (11)), which contains the shorter “A” C-terminal tail as previously described (19); MAGI-1 WW12 (amino acids 219–416); MAGI-1 WW1 (amino acids 219–334); MAGI-1 WW2 (amino acids 315–414); MAGI-1 PDZ123 (amino acids 404–919); MAGI-1 PDZ324 (amino acids 607–1081); MAGI-1 PDZ45 (amino acids 905–1455), which contains the “C” C-terminal tail (19); MAGI-1 PDZ4 (amino acids 905–1081); and MAGI-1 PDZ5 (amino acids 1011–1202). The PCR products were initially cloned into the TA cloning vector pGEM-T-Easy (Promega, Madison, WI) and then subcloned into pGStag (20) and pHis-Myct (21) for production of GST fusion proteins in bacterial cells and expression of Myc-tagged proteins in mammalian cells, respectively. A human synaptopodin construct containing the two PXE motifs (amino acids 294–350) was obtained by PCR of a human expressed sequence tag cDNA using forward primer 5'-CATGCGGGAAGAGGAGGATGATGGGC-3' and reverse primer 5'-ACTTGGGTCGGGAGCGTGC-3'. The PCR product was first cloned into pGEM-T-Easy and then subcloned into pGStag to make GST-synaptopodin. A full-length mouse synaptopodin cDNA in the expression vector pRC/CMV was subcloned into HA-pcDNA3.1(+) (HA-synaptopodin) by PCR such that the flanking 5' and 3' untranslated sequences in the original cDNA were deleted. This HA-tagged synaptopodin cDNA was subcloned into pTRE2hyg for use in the Tet-Off inducible system. The Tet-inducible tagged mouse Nedd4 expression construct (T-Nedd4) was a kind gift from Dr. D. Rotin (Hospital for Sick Children, Toronto, Canada). The C-terminal tail of a-actinin-4 was obtained by PCR of a rat expressed sequence tag cDNA and consisted of the last 19 amino acids including the stop codon. The a-actinin-4 tail was subcloned into pGStag to make GST-a-actinin-4. A full-length mouse a-actinin-4 cDNA (a kind gift from Dr. S. R. Vincent, University of British Columbia, Vancouver, British Columbia, Canada) was cloned into the HA, pcDNA3.1(-) vector to make HA-a-actinin-4.

**Antibodies—**Anti-Myc antibody 9E10 was obtained from mouse ascites fluid produced at the Hybridoma Core Facility of the University of Michigan. Anti-T7 antibody (T7-Tag) was from Novagen (Madison, WI). Anti-synaptopodin polyclonal antibody NT was described elsewhere (5). Anti-MAGI-1 polyclonal antibody UM209 was described previously (11). An additional anti-MAGI-1 polyclonal antibody (UM223) was produced by the Hybridoma Core Facility of the University of British Columbia, Vancouver, British Columbia, Canada) was cloned into pGSTag to make GST-MAGI-1 WW12 (see above) was labeled with (gamma)-32P and used as a probe to screen the library (the pGSTag expression vector has a protein kinase A phosphorylation site placed in between the GST sequence and the downstream protein of interest). Plating and transferring of plaques to nitrocellulose membranes were performed as described in the aSSCREEN manual. The resulting membranes were blocked in Farwestern buffer (20 mm Hepes (pH 7.5), 1 mm KCl, 5 mm MgCl2, 5 mm dithiothreitol (DTT), 5% nonfat dry milk, and 0.02% sodium azide) for 2 h at room temperature with gentle agitation, followed by a 2-h incubation with the 32P-labeled probe ($2 \times 10^6$ cpm/ml of Farwestern buffer) at room temperature. The membranes were washed twice with Tris-buffered saline supplemented with Triton X-100 to 0.1% and then three times with Tris-buffered saline (5 min each wash) at room temperature. The membranes were exposed to x-ray film at $-80 \, ^\circ C$.

**Tissue and Cell Lysates—**Adult mouse brains were placed in a Dounce homogenizer along with high salt Triton X-100 buffer (50 mm Hepes (pH 7.5), 500 mm NaCl, 1.5 mm MgCl2, 1 mm EDTA, 10% glycerol, and 1% Triton X-100) plus protease inhibitors (Complete protease inhibitor mixture tablets, Roche Molecular Biochemicals) and homogenized with 25–30 strokes. The lysates were centrifuged at 20,000 $\times g$ for...
30 min at 4°C, and the resulting supernatants were transferred to fresh tubes and stored at −20°C until used. Glomerular extracts were prepared in radioligand precipitation assay lysis buffer (50 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) plus protease inhibitors as described previously (11).

Transiently transfected HEK293 cells were washed once with ice-cold phosphate-buffered saline (PBS), scraped in 400 µl of Triton lysis buffer (with 150 mM NaCl), transferred to microcentrifuge tubes, vortexed briefly, and incubated on ice for 10 min. The lysates were centrifuged at 20,000 × g for 20 min at 4°C, and the resulting supernatants were transferred to fresh tubes and stored at −20°C until used. Cells transfected with synaptopodin expression constructs either alone or with other expression constructs were scraped and lysed in radioligand precipitation assay lysis buffer plus protease inhibitors.

**Gust Fusion Protein Precipitation (Pull-down) and Immunoprecipitations**—Gust fusion proteins were produced in DH5α bacteria cells as previously described (21). Tissue or cell lysates were combined with ~10 µg of GST fusion protein bound to glutathione-agarose beads and incubated on a rocker at 4°C overnight. The beads were then washed twice with ice-cold HNTG buffer (20 mM Hepes (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol) and once with ice-cold buffer containing 20 mM Hepes (pH 7.5), 150 mM NaCl, and 0.1% Triton X-100. Forty microliters of 1X SDS sample buffer was added to the beads and then placed at 100°C for 5 min. Proteins eluted off of the beads were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and blotted with the appropriate primary and horseradish peroxidase-conjugated secondary antibodies or protein A-horseradish peroxidase. Blots were then developed with chemiluminescence reagents (PerkinElmer Life Sciences) and exposed to x-ray film.

For immunoprecipitations, lysates (200–300 µl) from transfected HEK293 cells were brought up to a total volume of 1 ml with HNTG buffer and rocked overnight at 4°C with anti-Myc antibodies. The following day, protein A-Sepharose beads were added to the samples and rocked for an additional 2 h at 4°C. The beads were then washed and processed in the same manner as the GST pull-down samples above.

**Immunofluorescence**—Wild-type MDCK cells or stable MDCK cell lines were seeded onto Transwell filters (Corning, Inc., Cambridge, MA) and allowed to reach confluency. Cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature and then solubilized with 1% SDS in PBS for 5 min at room temperature. Blocking was performed in 50% goat serum diluted in PBS for 2 h at 30°C in a humidified chamber. Primary antibodies were diluted in 2% goat serum in PBS (PBS-G) and placed on cells overnight at 30°C in a humidified chamber. The slides were washed three times with PBS-G (5 min each wash). Fluorescein-conjugated anti-rabbit, anti-rat, or anti-mouse secondary antibodies diluted in PBS-G were incubated on the filters for 2 h at 30°C in a humidified chamber. The filters were then washed four times with PBS-G and mounted on glass slides with ProLong Antifade mounting medium (Molecular Probes, Inc., Eugene, OR). Immunofluorescence images were obtained with a confocal microscope.

**RESULTS**

In an effort to identify proteins in the kidney that interact with the MAGUK protein MAGI-1, we screened a cDNA expression library made from a glomerulus-enriched preparation of mouse kidneys. The library was probed with a radiolabeled GST fusion protein containing both WW domains of mouse MAGI-1. Screening a total of ~1 × 10⁶ plaque-forming units with the probe resulted in the initial isolation of 11 independent clones that survived additional rounds of selection and purification. Sequencing of the cDNA inserts from the 11 clones showed that five of them represented the extreme C terminus of synaptopodin (clone 11.2) contained a region of this protein encompassing amino acids 255–379 and harboring two potential WW domain-binding PY motifs. These two PY motifs are conserved in human synaptopodin, suggesting that they may have some physiological relevance.

In parallel with the screening of the glomerular expression library, we had PCR-amplified a region of human synaptopodin containing its PY motifs (amino acids 294–350) and fused it to GST to perform analysis on proteins that could potentially bind to the WW domains of MAGI-1. This fusion protein, GST-synpoPY, was then used in a pull-down assay with lysates from HEK293 cells expressing a Myc-tagged portion of MAGI-1 containing only its two WW domains, Myc-MAGI-1 WW12. We found that the GST-synpoPY fusion protein was able to pull-down Myc-MAGI-1 WW12, whereas GST alone could not (Fig. 1A). In addition, GST-synpoPY was also capable of pulling down a Myc-tagged full-length MAGI-1 construct expressed in HEK293 cells (Fig. 1B) as well as endogenous MAGI-1 from mouse brain lysates (Fig. 1C). The multiple bands observed for MAGI-1 in mouse brain lysates are most likely due to different forms of the protein that we (11) and others (19) have observed previously in brain and other tissues as well. A protein whose WW domains are very similar to those of MAGI-1 is the ubiquitin-protein ligase Nedd4. We expressed a T7-tagged full-length Nedd4 construct in HEK293 cells and tested its ability to interact with the GST-synpoPY fusion protein. GST-synpoPY was unable to pull-down Nedd4 from cell lysates in this assay (Fig. 1D), indicating that the PY motifs of synaptopodin do not interact with any of the three WW domains of this Nedd4 construct. In reciprocal experiments, the GST-MAGI-1 WW12 fusion protein that was used as a probe to screen the glomerular cDNA library was utilized in GST pull-down assays with lysates from HEK293 cells expressing a HA-tagged full-length mouse synaptopodin construct. As shown in Fig. 1E, GST-MAGI-1 WW12 could pull-down HA-synaptopodin from the lysates, whereas GST alone could not. In addition, using the same assay on mouse brain lysates, we found that GST-MAGI-1 WW12 could pull-down endogenous synaptopodin (Fig. 1F). We have observed that lysates from brain contain a form of synaptopodin that is much more stable than the one found in glomerular lysates; and therefore, brain lysates were used as our source of synaptopodin in these binding assays. These data suggest that a region in synaptopodin containing its two conserved PY motifs can interact with the WW domains in MAGI-1. Moreover, this interaction appears to be somewhat specific, as this region of synaptopodin is unable to interact with Nedd4, whose WW domains show a high degree of similarity to those in MAGI-1.

Because MAGI-1 contains two WW domains and synaptopodin harbors two PY motifs that are relatively close together, it is conceivable that both WW domains of MAGI-1 bind to the two PY motifs of synaptopodin, thereby providing an enhanced interaction, or alternatively, that only one WW domain of MAGI-1 is responsible for this interaction. To address this possibility, we performed a Farwestern assay using the WW domains of MAGI-1 together or by themselves as 32P-labeled probes (Fig. 2A). As expected, the probe with both WW domains of MAGI-1 bound to the GST-synpoPY fusion protein (Fig. 2B, second lane). When the second WW domain of MAGI-1 was used as a probe, nearly the same degree of binding to GST-synpoPY was observed (Fig. 2B, sixth lane). In contrast, the degree to which the first WW domain bound to GST-synpoPY was significantly less and is most likely only residual in nature.
MAGI-1 interacts with actin-associated proteins

To investigate whether MAGI-1 and synaptopodin interact in vivo, HEK293 cells were transfected with full-length expression constructs of Myc-tagged MAGI-1 and HA-tagged synaptopodin either alone or together, and the resulting cell lysates were used in co-immunoprecipitation assays. Using anti-Myc antibodies to precipitate MAGI-1 from the cell lysates, we found that synaptopodin was precipitated along with MAGI-1 in cells transfected with both expression constructs (Fig. 2C), indicating that these two proteins are able to interact together in cells.

We have previously shown that MAGI-1 is found in Triton X-100-insoluble fractions of mouse glomerular preparations (11), which is suggestive of MAGI-1 association with the actin cytoskeleton. Although we have not ascertained whether MAGI-1 is present in lipid rafts, the above finding that MAGI-1 interacts with the actin-bundling protein synaptopodin lends support to its actin cytoskeletal association as the reason for its Triton X-100 insolvency. An additional actin-bundling protein expressed in the glomerular podocytes is one of the non-muscle isoforms of α-actinin, viz. α-actinin-4. Recent data provide evidence that mutations in the gene for α-actinin-4 cause a hereditary form of FSGS in humans. In addition, both non-muscle isoforms of α-actinin, α-actinin-1 and α-actinin-4, contain a consensus binding motif for PDZ domains (ESDL) at their extreme C termini. These data and observations provided the impetus to determine whether α-actinin-4 could interact with any of the PDZ domains of MAGI-1. For our initial binding assay, the last 19 amino acids of α-actinin-4 (Fig. 3A) were fused to GST and used in pull-down assays with lysates of HEK293 cells expressing different Myc-tagged constructs of MAGI-1. The GST-α-actinin-4 fusion protein was able to efficiently pull-down full-length MAGI-1, whereas GST alone was not (Fig. 3B). Of the MAGI-1 deletion constructs tested, only (Fig. 2B, fourth lane). None of the three probes bound to GST alone (Fig. 2B, first, third, and fifth lanes). Taken together, these data indicate that MAGI-1 and synaptopodin interact with each other in a direct manner in vitro and that the second WW domain of MAGI-1 specifically mediates this interaction.

Fig. 1. GST pull-down assays involving MAGI-1 and synaptopodin. Lysates of HEK293 cells expressing epitope-tagged constructs of MAGI-1, synaptopodin, or Ned4 and lysates of mouse brain were incubated with GST and the GST-synypoPY and GST-MAGI WW12 fusion proteins as indicated. The resulting blots were incubated with anti-Myc, anti-MAGI-1 (UM209), anti-T7, anti-HA, or anti-synaptopodin (NT) antibody and the appropriate secondary antibodies and then developed with chemiluminescence reagents. The following lysates were used: lysates from HEK293 cells transiently transfected with Myc-MAGI-1 WW12 (A), Myc-tagged full-length MAGI-1 (myc-MAGI-1 FL) (B), T-Nedd4 (D), and HA-synaptopodin (E) and mouse brain for endogenous (endo) proteins (C and F). Lysate lanes represent 10% of that which was used in the pull-down assays. Molecular mass markers (in kilodaltons) are indicated on the left of each panel.

Fig. 2. Farwestern and co-immunoprecipitation analyses for interactions of MAGI-1 with synaptopodin. A. Shown is a diagram illustrating the GST-MAGI-1 constructs used as radiolabeled probes. Filled boxes denote the WW domains of MAGI-1 and are indicated as such above each. Probe 1, GST-MAGI-1 WW1; Probe 2, GST-MAGI-1 WW1; Probe 3, GST-MAGI-1 WW2. B, 100 ng of GST (first, third, and fifth lanes) and 100 ng of GST-synypoPY (second, fourth, and sixth lanes) were resolved on a 12% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was cut into three strips (each strip containing a lane of GST and GST-synypoPY), which were blocked in Farwestern buffer and then incubated with separate radiolabeled probes. After washing, each strip was exposed to X-ray film at −70 °C. The probing used on each strip is indicated at the bottom of the autoradiograph. Molecular mass markers (in kilodaltons) are indicated on the left. C, lysates from HEK293 cells transiently transfected with full-length (FL) HA-synaptopodin and Myc-MAGI-1 either alone or together were incubated with anti-Myc antibodies and protein A-Sepharose beads. Proteins bound to the beads were eluted off, separated on a 7% SDS-polyacrylamide gel, and blotted onto a nitrocellulose membrane. The blot was developed with anti-Myc or anti-HA antibodies and visualized with chemiluminescence reagents. Input control lysate lanes (lower two panels) represent 5% of the amount that was used in the immunoprecipitation (IP).
those with an intact fifth PDZ domain retained binding to GST-α-actinin-4 (Fig. 3B). We next performed the reciprocal experiment, in which the MAGI-1 construct containing only the fifth PDZ domain was fused to GST (GST-MAGI-1 PDZ5), and used this fusion protein in pull-down assays with HEK293 cells expressing a HA-tagged full-length α-actinin-4 construct. GST-MAGI-1 PDZ5 was sufficient to pull-down HA-α-actinin-4, whereas GST alone was not (Fig. 4A). In addition, we found that GST-MAGI-1 PDZ5 was able to pull-down endogenous α-actinin-4 from lysates made from mouse glomerulus-enriched preparations (Fig. 4B). As with synaptopodin and MAGI-1, we wished to explore whether α-actinin-4 and MAGI-1 could form a complex when expressed together exogenously in HEK293 cells. Lysates from cells expressing a Myc-tagged full-length MAGI-1 construct and a HA-tagged full-length α-actinin-4 construct either alone or together were used in immunoprecipitation assays with anti-Myc antibodies. As shown in Fig. 4C, HA-α-actinin-4 can be precipitated along with Myc-MAGI-1 from lysates expressing both constructs, but not from lysates expressing either construct alone. Together, these data strongly suggest that an additional actin-bundling protein (α-actinin-4) is able to bind to the MAGUK protein MAGI-1.

The data presented thus far using in vitro binding assays and co-immunoprecipitation analyses indicate that MAGI-1 is able to bind to two distinct actin-bundling proteins, synaptopodin and α-actinin-4. To further strengthen these observations, we stably expressed full-length HA-synaptopodin in Tet-Off MDCK cells and HA-α-actinin-4 in regular MDCK cells to determine whether they would colocalize with endogenous MAGI-1 in the cells. When normal MDCK cells were stained with an antibody against MAGI-1 and ZO-1, we found that there was complete colocalization of the two endogenous proteins (Fig. 5C), confirming the previous results of others (22) that MAGI-1 is a tight junction-associated protein in these cells. MDCK cells stably expressing HA-α-actinin-4 showed a localization pattern that was found all along the lateral membrane of most cells that extended up to and overlapped with MAGI-1 at the tight junction (Fig. 5H and I). This localization pattern mimics the pattern observed for endogenous α-actinin-1 in these cells.² Because our initial attempts to express HA-synaptopodin in regular MDCK cells were unsuccessful, we chose to express this construct in the Tet-Off MDCK inducible system. When stably transfected cells of this system are main-

² K. M. Patrie and B. Margolis, unpublished data.
MAGI-1 Interacts with Actin-associated Proteins

Protein MAGI-1 is found in the glomerular podocytes of rat kidneys (11). MAGI-1, like most MAGUK proteins that contain numerous protein-protein interaction domains, is envisioned as filling a scaffolding role in cells that would facilitate the nucleation of a multiprotein complex. In an effort to identify proteins in the glomerulus that interact with MAGI-1, we screened a cDNA expression library made from glomerulus-enriched preparations of mouse kidneys with a probe containing both WW domains of MAGI-1. One clone that was isolated in this screen contained a region of synaptopodin harboring two PY motifs, which are potential binding sites for Group I WW domains. Additional in vitro and in vivo binding data using full-length expression constructs as well as endogenous proteins confirmed this interaction. Therefore, two independent approaches to investigating protein-protein interactions reveal the direct association of synaptopodin with MAGI-1. We have provided additional evidence that this interaction is biologically relevant by showing that a full-length synaptopodin exogenously expressed in MDCK cells partially colocalized with endogenous MAGI-1 at tight junctions. Of the two WW domains present in MAGI-1, we found that the second, or C-terminal, WW domain is responsible for mediating the interaction with synaptopodin. Although both PY motifs of synaptopodin share the consensus PPXY sequence at their core, we have yet to establish to which PY motif of synaptopodin MAGI-1 preferentially binds or if both PY motifs can serve as binding sites for MAGI-1. Because the amino acids flanking the two core PPXY sequences of synaptopodin are quite different, they may contribute to the specificity of these PY motifs in binding to different WW domains. Initially, synaptopodin was found only in the neurons of the telencephalon-derived regions of the brain and glomerular podocytes of the kidney; but more recently, it has been found in other tissues as well (24). MAGI-1 has been found in most tissues examined (19), making it likely that synaptopodin is a common binding partner for MAGI-1 in those tissues expressing both proteins. Although this does not exclude the possibility that other proteins may bind to the second WW domain of MAGI-1 when synaptopodin is absent, to date, no other proteins have been reported to bind this protein interaction domain of MAGI-1. With synaptopodin as a binding partner for the second WW domain of MAGI-1, the first WW domain would be available to bind to a protein yet to be identified.

The recent discovery of mutations in the gene for α-actinin-4 that cause a hereditary form of autosomal dominant FSGS (8) provides a link between the actin cytoskeleton and disease in the kidney. Interestingly, the FSGS-associated mutations in α-actinin-4 occur between the actin-binding domain and the first rod domain of the protein and cause an increase in α-actinin-4 association with F-actin in co-sedimentation assays. These α-actinin-4 mutations would not be expected to affect MAGI-1 binding to α-actinin-4, but instead would presumably increase MAGI-1 association with the actin cytoskeleton. This increase in MAGI-1 association with the actin cytoskeleton could have an effect on the dynamics of actin cytoskeleton regulation by increasing the local concentration of potential MAGI-1-binding proteins that are known to have an effect on actin cytoskeleton dynamics (see below). We also have shown here that α-actinin-4 exogenously expressed in MDCK cells partially localized at the tight junction along with MAGI-1. In addition, we have observed endogenous α-actinin (α-actinin-1) in MDCK cells to be localized all along the lateral membrane border stretching up to and overlapping with the tight junction, as is seen with α-actinin-4. Of the 19 amino acids from α-actinin-4 that were used as a GST fusion protein in this study, 18 are identical to the other non-muscle isoform α-actinin-1, including the PDZ domain-binding motif. It was not surprising

FIG. 4. GST pull-down assays and co-immunoprecipitation of α-actinin-4. A, lysates from HER293 cells transiently transfected with a HA-α-actinin-4 construct were incubated with GST alone or GST-MAGI-1 PDZ5 and processed as described under “Materials and Methods.” The resulting blot was incubated with an anti-HA antibody and the appropriate horseradish peroxidase-conjugated secondary antibody. Input control lysate lanes represent 10% of the amount that was used in the pull-down assay. B, lysates from glomerulus-enriched preparations of mouse kidney were incubated with GST alone or GST-MAGI-1 PDZ5 and processed as described under “Materials and Methods.” The resulting blot was incubated with an anti-α-actinin-4 antibody (NCC-Lu-632) and the appropriate horseradish peroxidase-conjugated secondary antibody. Input control lysate lanes represent 10% of the amount that was used in the pull-down assay. Molecular mass markers (in kilodaltons) are indicated at the left of each panel. C, lysates of HER293 cells transfected with full-length Myc-MAGI-1, full-length HA-α-actinin-4, or both constructs were incubated with anti-Myc antibodies and protein A-Sepharose beads. Proteins bound to the beads were eluted off, separated on a 7% SDS-polyacrylamide gel, and blotted onto a nitrocellulose membrane. The blot was developed with anti-Myc or anti-HA antibodies and visualized with chemiluminescence reagents. Input control lysate lanes (lower two panels) represent 5% of the amount that was used in the immunoprecipitations (IP).

In the presence of doxycycline, the construct of interest is not expressed, thereby avoiding complications such as toxicity or instability observed in constitutive expressing systems. Tet-Off MDCK cells stably transfected with HA-synaptopodin and maintained in the absence of doxycycline expressed synaptopodin in a pattern very similar to that observed with α-actinin-4 (Fig. 5, E and F). Synaptopodin was seen to partially colocalize with MAGI-1 at the tight junction of cells. The overlapping expression of synaptopodin and α-actinin-4 with MAGI-1 at the tight junctions of MDCK cells supports the binding data that MAGI-1 can interact with these two actin-bundling proteins and that these interactions may have some biological significance.

**DISCUSSION**

The complex morphology observed for the renal podocyte is crucial for its function in helping to establish the glomerular filtration barrier. Although this morphology is well characterized at the light and electron microscope level, its establishment and regulation at the molecular level are only slowly being realized (23). We have recently shown that the MAGUK
therefore to find that a GST fusion protein containing only the fifth PDZ domain of MAGI-1 was able to pull-down endogenous H9251-actinin-1 from lysates of HEK293 cells (data not shown). MAGI-1 appears to be capable of binding to both non-muscle isoforms of H9251-actinin. Our previous results showing MAGI-1 in the Triton X-100-insoluble fraction of membranes from mouse glomerular preparations suggests a cytoskeletal association of MAGI-1 (11). Alternatively, this observed insolubility could also be due to the association of MAGI-1 with detergent-resistant microdomains, or lipid rafts. However, our finding of MAGI-1 interaction with the two actin-binding proteins H9251-actinin and synaptopodin suggests an association with the actin cytoskeleton as the reason for its resistance to extraction with Triton X-100.

Although the distinct subcellular localization of MAGI proteins in tissues is somewhat limited at this time, MAGI-1 was found localized at the tight junctions in intestinal epithelium using immunoelectron microscopy (22). It was also shown that the localization of endogenous MAGI-1 in MDCK cells overlaps perfectly with the localization of the tight junction protein ZO-1, a finding that we confirmed in this study. Our data provide additional evidence that MAGI-1 provides a link from membrane-associated protein complexes to the actin cytoskeleton as the reason for its resistance to extraction with Triton X-100.

It is apparent that the morphological changes observed in the foot processes of podocytes in the nephrotic syndrome are dependent on the reorganization of the actin-based cytoskeleton. Understanding the regulation of the actin cytoskeleton in podocytes is therefore crucial. The finding of MAGI-1 at membrane-associated complexes in epithelial cells suggests a model in which MAGI-1 would be localized at the membrane of foot processes and tethered to the actin cytoskeleton by way of its interaction with synaptopodin and H9251-actinin-4. Although the localization of MAGI-1 in podocytes at the ultrastructural level is not currently available, we feel that MAGI-1 could be localized in a manner that would at least partially overlap with synaptopodin and H9251-actinin-4. In addition to actin and H9251-actinin, the microfilament contractile apparatus of podocyte foot processes is also composed of myosin II, talin, and vinculin, which extends down to and is linked with the glucose transport system by an α/β integrin- and α/β-dystroglycan-based electron-dense protein complex called the sole plate (3, 28, 29). Like the localization of H9251-actinin at integrin-based protein complexes in cultured cells, H9251-actinin-4 is observed to be partially localized at the sole plate (3, 30). Although H9251-actinin-4 itself can provide a link from the sole plate protein complex to the actin cytoskeleton, its interaction with MAGI-1

![Fig. 5. Colocalization of exogenously expressed α-actinin-4 and synaptopodin with endogenous MAGI-1 in MDCK cells.](image)
would provide an additional link to the actin microfilament array via synaptopodin. Alternatively, it is plausible that MAGI-1 is providing a platform for regulators of actin dynamics in the foot process instead of merely playing an additional passive structural link between the actin cytoskeleton and the membrane. It is well established that integrin-based focal contacts in cell culture provide a signaling link from the substrate-contacting plasma membrane to the actin cytoskeleton and are regulated by the RHQ family of small GTPases. Interestingly, the guanine nucleotide exchange factor mouse NET1 has recently been identified as a binding partner for the first PDZ domain of MAGI-1 (31). Mouse NET1 activates RhoA and the stress-activated protein kinase/c-Jun N-terminal kinase signaling pathways (32). RhoA activation stimulates actomyosin-based contractility, which contributes to the assembly of stress fibers and focal contacts (33, 34). Additionally, the tumor suppressor PTEN has been shown to bind to the second PDZ domain of all three MAGI proteins (35, 36) and is implicated in focal contact assembly by antagonizing the phosphatidylinositol 3′-kinase signaling pathway. However, the precise localization of mouse NET1 and PTEN within the kidney is not known at this time.

The precise mechanism by which MAGI-1 associates with distinct plasma membrane subdomains or what function it may serve there is speculative at this time. Expression of the last PDZ domain of MAGI-1 (31) is solely responsible for the plasma membrane localization of mouse NET1 and PTEN within the kidney once it is at the membrane. We are able to detect neither tight junction nor plasma membrane localization of MAGI-1 in MDCK cells, but it is not clear if the fifth PDZ domain is required for proper localization of MAGI-1 throughout the cell (data not shown). This confirms that the fifth PDZ domain is required for proper localization of MAGI-1 to the plasma membrane, but it is not clear if the fifth PDZ domain alone is sufficient to properly target MAGI-1 to the tight junction of MDCK cells once it is at the membrane. We are not sure at this time whether the interaction of MAGI-1 with α-actinin-4 (or other proteins known to bind to the fifth PDZ domain of MAGI-1) is solely responsible for the plasma membrane localization of MAGI-1 in MDCK cells. We are currently investigating the potential involvement of the other protein interaction domains of MAGI-1 regarding their role in proper membrane localization.

The identification of the proteins in renal podocytes and other cells that bind to MAGI-1, whether they are integral transmembrane proteins or peripheral proteins, will undoubtedly help reveal the function of MAGI-1. This proteomic approach combined with transgenic technology will help provide an understanding of the role that MAGUK proteins play in the function and regulation of podocyte dynamics.