Selective interaction of megalin with postsynaptic density-95 (PSD-95)-like membrane-associated guanylate kinase (MAGUK) proteins

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Megalyn is an integral membrane receptor belonging to the low-density lipoprotein receptor family. In addition to its role as an endocytotic receptor, megalyn has also been proposed to have signalling functions. Using interaction cloning in yeast, we identified the membrane-associated guanylate kinase family member postsynaptic density-95 (PSD-95) as an interaction partner for megalyn. PSD-95 and a truncated version of megalyn were co-immunoprecipitated from HEK-293 cell lysates overexpressing the two proteins, which confirmed the interaction. The two proteins were found to be co-localized in these cells by confocal microscopy. Immunocytochemical studies showed that cells in the parathyroid, proximal tubuli of the kidney and placenta express both megalyn and PSD-95. We found that the interaction between the two proteins is mediated by the binding of the C-terminus of megalyn, which has a type I PSD-95/*Drosophila* discs-large/zona occludens 1 (PDZ)-binding motif, to the PDZ2 domain of PSD-95. The PSD-95-like membrane-associated guanylate kinase ('MAGUK') family contains three additional

members: PSD-93, synapse-associated protein 97 (SAP97) and SAP102. We detected these proteins, apart from SAP102, in parathyroid chief cells, a cell type having a marked expression of megalyn. The PDZ2 domains of PSD-93 and SAP102 were also shown to interact with megalyn, whereas no interaction was detected for SAP97. The SAP97 PDZ2 domain differed at four positions from the other members of the PSD-95 subfamily. One of these residues was Thr³⁸⁹, located in the α B-helix and part of the hydrophobic pocket of the PDZ2 domain. Surface plasmon resonance experiments revealed that mutation of SAP97 Thr³⁸⁹ to alanine, as with the other PSD-95-like membrane-associated guanylate kinases, induced binding to megalyn.

Key words: megalyn, membrane-associated guanylate kinase (MAGUK), postsynaptic density-95 (PSD-95), PSD-95/*Drosophila* discs-large/zona occludens 1 (PDZ) domain, synapse-associated protein 97 (SAP97).

INTRODUCTION

Megalyn is a cell-surface receptor belonging to the low-density lipoprotein receptor (LDLR) family [1]. The human receptor has a large extracellular portion composed of 4398 amino acid residues, a single transmembrane region and a cytoplasmic tail consisting of 209 amino acid residues. Megalyn is expressed in several cell types, including kidney proximal tubule cells, placental cytotrophoblasts, parathyroid chief cells, ependymal cells of the brain, type II pneumocytes of the lung, and epididymal and small intestinal cells [2–4]. Several ligands have been identified for megalyn. Among them are numerous plasma proteins such as retinol-binding protein, vitamin D-binding protein, β_2 -microglobulin, parathyroid hormone, serum albumin, plasminogen activator–plasminogen activator inhibitor type I complexes, apolipoprotein E, the cell-surface-associated protein cubilin, lipoprotein lipase and pharmaceuticals, including aminoglycosides [1].

Megalyn has been assigned different functions in different organs, including tubular resorption of low-molecular-mass

proteins filtered through the kidney glomerulus [5–7], endocytosis of lipophilic molecules in type II pneumocytes [8,9] and in the brain [7,10], and also signalling functions [11,12]. Megalyn has indeed been suggested to be involved in sonic hedgehog signalling in the brain [13].

The extracellular portions of LDLR family members share the following repeats: cysteine-rich complement-type/LDLR class A repeats present in cluster(s) separated by epidermal growth factor-like repeats and spacer regions containing Tyr-Trp-Thr-Asp (YWTD) motifs (for a recent review of megalyn, see [1]). In contrast, the cytoplasmic tail of megalyn displays low overall amino acid sequence similarity to those of other members of the LDLR family, apart from the conserved intracellular Phe-Xaa-Asn-Pro-Xaa-Tyr (where Xaa is any amino acid; FXNPXY) motifs, known to be necessary for clathrin-coated-pit-mediated endocytosis [14,15] and for downstream signalling events through phosphotyrosine-interaction domains ('PIDS') [16–18]. Several other potential signalling motifs have been described in megalyn [11], including one Src-homology 2 ('SH2')-binding motif, several Src-homology 3

Abbreviations used: Dlg, *Drosophila* discs-large; FBS, fetal bovine serum; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; GluR, glutamate receptor; GST, glutathione S-transferase; GUK, guanylate kinase-like; LDLR, low-density lipoprotein receptor; MAGUK, membrane-associated guanylate kinase; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; NHE3, Na⁺/H⁺ exchanger 3; NHERF, NHE regulatory factor; PSD, postsynaptic density; PDZ, PSD-95/Dlg/zona occludens 1; RT-PCR, reverse transcription-PCR; RU, resonance units; SAP, synapse-associated protein; SH3, Src-homology 3.

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(SH3)-binding sequences, three protein kinase C phosphorylation sites, seven casein kinase II sites, one potential cAMP/cGMP-dependent protein kinase phosphorylation site and the C-terminal (Ser/Thr)Xaa-Val sequence, known to bind postsynaptic density 95 (PSD-95)/Drosophila discs-large (Dlg)/zona occludens 1 (PDZ) domains [19].

Only limited information is available on signal transduction pathways mediating the putative intracellular signals evoked by megalin. Recently, a number of proteins binding to the intracellular domain of megalin have been identified by yeast interaction cloning, although some of these putative interactions still need to be confirmed *in vitro* [12,20–22]. In parallel with these studies, we initiated a screening for proteins binding to the C-terminal 55 amino acid residues of the receptor cytoplasmic tail. Our screening revealed interaction of megalin with PSD-95/synapse-associated protein 90 (SAP90), a protein belonging to the membrane-associated guanylate kinase (MAGUK) family, an observation that confirmed an earlier report [12]. The subfamily of PSD-95 contains three additional members: SAP97/human homologue of Dlg ('hDlg'), PSD-93/Chapsyn110 and SAP102/neuroendocrine-Dlg ('NE-Dlg') [23]. They all contain three N-terminal PDZ domains, followed by an SH3 domain and a guanylate kinase-like (GUK) domain (see Figure 1A). The recognized function of these proteins is to serve as scaffolds, binding different signal transduction components to the cell membrane [23]. The four MAGUKs are mainly expressed in neuronal tissue, but a more ubiquitous expression of SAP97 has been reported [24–26]. In the present study, we provide detailed information on the interaction of megalin with members of the PSD-95-like MAGUK family.

MATERIALS AND METHODS

Materials

Tissue culture media, L-glutamine and penicillin/streptomycin were obtained from the National Veterinary Institute (Uppsala, Sweden). Fetal bovine serum (FBS) was purchased from Invitrogen (Groningen, The Netherlands). Oligonucleotides were from DNA Technology (Aarhus, Denmark), and restriction endonucleases were from MBI Fermentas (Vilnius, Lithuania).

Cell culture

HEK-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS, 50 µg/ml streptomycin sulphate, 60 µg/ml penicillin G and 2 mM L-glutamine in a 5% CO₂ atmosphere at 37 °C.

Antibodies

Anti-(PSD-95 N-terminal) and anti-(megalin MM6) antisera were produced by immunizing rabbits with glutathione S-transferase (GST) fusion proteins produced from pGEX-5X-2 (Amersham Biosciences, Uppsala, Sweden) incorporating residues 1–105 of PSD-95 or residues 4576–4630 of human megalin. A mouse anti-FLAG monoclonal antibody (clone M2; where FLAG is Asp-Tyr-Lys-Asp-Asp-Lys) was purchased from Sigma (St. Louis, MO, U.S.A.). A mouse anti-PSD-95 monoclonal antibody (MA1-046) against residues 1–246 of rat PSD-95 was obtained from Affinity BioReagents (Golden, CO, U.S.A.). An anti-mouse IgG-horseradish peroxidase conjugate was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Normal goat serum, and rat, rabbit and mouse IgGs were purchased from Sigma. Goat anti-rabbit IgG-fluorescein conjugate was

from Vector Laboratories (Burlingame, CA, U.S.A.). Goat anti-mouse IgG-Texas Red conjugate was purchased from Becton Dickinson (Mountain View, CA, U.S.A.). All antibodies used in immunofluorescent staining were diluted in PBS [0.13 M NaCl and 10 mM sodium phosphate (pH 7.4)] containing 0.1% BSA, 150 mM transemic acid, 20 µg/ml trasylol (Bayer AG, Leverkusen, Germany), 1.8 mM EDTA and 2 mM iodoacetic acid, and were used at optimal concentrations determined after serial dilution. All incubations were performed at room temperature.

Peptides

Meg-SEV (last 14 amino acid residues in the C-terminus of megalin with Ser-Glu-Val being the final three residues), Meg-VES (as with Meg-SEV, but with the final three amino acids reversed) and Kv1.4 (last 10 amino acid residues in the C-terminus of the voltage-gated potassium channel Kv1.4) peptides were synthesized by fluorenylmethoxycarbonyl ('Fmoc') chemistry using an ABI 431 peptide synthesizer (Applied Biosystems, Foster City, CA, U.S.A.). After deprotection, the peptides were purified by reversed-phase chromatography and analysed by matrix-assisted laser-desorption ionization-time-of-flight (MALDI-TOF)-MS using a Kompakt IV instrument (Kratos, Manchester, U.K.). The expected *m/z* of the peptides appeared as a dominant peak in the mass spectra.

Plasmids

Vector pMM6, used as bait in the yeast two-hybrid screening, was made by cloning a PCR fragment encoding amino acid residues 4576–4630 of human megalin [11] into the *EcoRI* and *BamHI* sites of pAS2-1 (Clontech, Palo Alto, CA, U.S.A.). Full-length PSD-95 (pPSD-95) including the complete N-terminus was amplified by PCR with a sense primer including the missing nucleotides of clone p4 (described in Results section), using clone p4 as the template. The product was subsequently cloned into pcDNA3 (Invitrogen). Full-length SAP97 (pSA30) was produced by ligating PCR-products of SAP97 from human parathyroid cDNA into pcDNA3 using *KpnI* and *XbaI* sites. For the interaction assay, two cytoplasmic constructs of megalin, pSEV and pNoSEV, were made. pSEV encoded amino acid residues 4421–4630 and pNoSEV residues 4421–4627, and both were produced by PCR using pGH3 (see below) as the template. The products were cloned into pAS2-1 using the *EcoRI* and *PstI* sites. DNA constructs encoding domains of PSD-95 (GenBank® accession number U83192) corresponding to amino acid residues 80–206 (PDZ1), 193–305 (PDZ2), 335–453 (PDZ3), 80–305 (PDZ1 + 2), 193–453 (PDZ2 + 3), 437–556 (SH3) and 555–766 (GUK) were obtained by PCR using clone p4 as the template and ligated into the *BamHI* (PDZ1, PDZ2, PDZ3, PDZ1 + 2, PDZ2 + 3 and SH3) or *NcoI* (GUK) and *EcoRI* sites of pACT2 (Clontech). PDZ2 was also cloned with *EcoRI* and *BamHI* sites into pGEX-5X-2. The N-terminal construct was obtained by PCR using the pPSD-95 clone as the template and ligated into the *EcoRI* and *XhoI* sites of pACT2. DNA constructs encoding SAP97 (GenBank® accession number U13897) domains corresponding to amino acid residues 308–421 (PDZ2), 204–421 (PDZ1 + 2), 308–553 (PDZ2 + 3), 204–553 (PDZ1 + 2 + 3), 563–686 (SH3) and 563–904 (SH3 + GUK) were produced by PCR using clone pSA30 as the template and ligated into *BamHI* and *EcoRI* (PDZ1 + 2, PDZ2 + 3 and PDZ1 + 2 + 3) or *EcoRI* and *XhoI* (SH3 and SH3 + GUK) sites of pACT2, or *BamHI* and *EcoRI* sites of pGEX-5X-1. Mutated SAP97 PDZ2 (Mut97) was created using a 'QuikChange' site-directed mutagenesis kit (Stratagene, La

Jolla, CA, U.S.A.) with the pGEX-5X-1 SAP97 PDZ2 clone as the template. The oligonucleotides used were 5'-ACTCATGAAGAAGCAGTAGCTGCCTTAAAGAACACAT and 5'-ATGTGTTCTTTAAGGCAGCTACTGCTTCTTCATGAGT (mutated nucleotides are underlined). To obtain the PSD-93 (GenBank® accession number U32376) PDZ domain constructs, PCR was performed on human parathyroid cDNA using primers corresponding to amino acid residues 3–509 (PDZ1 + 2 + 3), 85–191 (PDZ1), 186–293 (PDZ2) and 395–509 (PDZ3), and the products were cloned into pACT2 using the *Sma*I (PDZ1 + 2 + 3), *Bam*HI and *Xho*I (PDZ1) or *Eco*RI and *Xho*I (PDZ2 and PDZ3) sites. The PSD-93 PDZ2 construct for GST fusion was made by primers corresponding to amino acid residues 185–295 and ligated into pGEX-5X-2 using the *Bam*HI and *Eco*RI sites. The SAP102 (GenBank® accession number U49089) PDZ2 constructs were produced by PCR performed on a human kidney cDNA using primers corresponding to amino acid residues 225–312 and ligated into pACT2 and pGEX-5X-2 respectively, using the *Eco*RI sites. A DNA construct (pGH3) encoding a truncated version of megalin was produced using a 'three-step' strategy. First, complementary synthetic oligonucleotides encoding the N-terminal signal peptide of human megalin [11] were cloned into the *Kpn*I and *Bam*HI sites of pcDNA3. Thereafter, two oligonucleotides encoding a FLAG epitope were ligated into the *Bam*HI site of the first construct. This also destroyed the 5' *Bam*HI site in the new construct. Finally, a cDNA fragment encoding amino acid residues 4308–4630 of human megalin was obtained by PCR and subsequently cloned into the *Bam*HI and *Eco*RI sites of the second construct. This portion corresponded to the two most C-terminal extracellular cysteine-rich domains, the transmembrane part and the entire cytoplasmic region of the receptor.

Reverse transcription (RT)–PCR

PCR was performed on human parathyroid cDNA using primers 5'-CCGCTACCAAGATGAAGACA and 5'-CGAATCGGCTGTACTCTTC for PSD-95; 5'-GTCACGCCTCTCTTCAGACA and 5'-CCGTTGAGCCACGATGAAGAA for SAP97; 5'-GGCAGCTGCTGCACTAAAG and 5'-GCGCAGTAGCTAATTTATAAC for PSD-93; and 5'-ATGCACAAGCACCAGCACTG and 5'-GATTCAAAGCGACTGTATTCT or 5'-GTATTCTTCAGGTCTGTACTG for SAP102. The authenticity of the PCR products was confirmed by DNA sequencing.

DNA sequence analysis

DNA sequencing of all DNA constructs was performed using dRhodamine Terminator chemistry (Applied Biosystems) or BigDye Terminator chemistry version 2 (Applied Biosystems) using an ABI Model 310 DNA sequencer (Applied Biosystems). DNA sequences were analysed using the EditView (Applied Biosystems) and MacVector (Accelrys, Cambridge, U.K.) software packages.

Production and cleavage of GST fusion proteins

GST fusion proteins of recombinant PSD-95, SAP97, Mut97, SAP102 or PSD-93 PDZ2 domains were expressed in *Escherichia coli* BL21 Star (DE3)pLysS cells (Invitrogen). Cells were induced for 3 h with 2 mM isopropyl β -D-thiogalactoside and pelleted by centrifugation for 30 min at 1500 g. The pellet was resuspended in ice-cold PBS containing 1 % Triton X-100, aprotinin (2 μ g/ml), leupeptin (0.5 μ g/ml), pepstatin (0.7 μ g/ml) and pefabloc SC (100 μ g/ml). The suspension was sonicated and centrifuged for 30 min at 2000 g. The supernatant was passed through a

0.45 μ m filter and loaded on to a glutathione–Sephadex 4B column (Amersham Biosciences). Bound material was eluted by adding 5 mM glutathione in 50 mM Tris/HCl (pH 8.0). The single cysteine residue in SAP97 and Mut97 was thereafter reduced and alkylated with iodoacetamide. The size and purity of eluted material were verified by MALDI–TOF–MS and SDS/PAGE. Eluted material was then either used directly in surface plasmon resonance analysis (SAP97, Mut97 and SAP102) or dialysed overnight against Factor Xa cleavage buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl and 1 mM CaCl_2] in a Slide-a-lyzer with molecular mass cut-off of 3500 Da (Pierce, Rockford, IL, U.S.A.) (PSD-95, PSD-93 and SAP97). GST was removed by addition of 10 units of Factor Xa (Amersham Biosciences) per mg of fusion protein and digested for 6 h at 23 °C. Digested fusion proteins were directly loaded on to a Sephadex G-75 gel-chromatography column equilibrated and eluted with 150 mM NH_4HCO_3 (pH 9). Peaks corresponding to recombinant PSD-95, SAP97 or PSD-93 PDZ2 domains respectively, were collected, freeze-dried and dissolved in 200 μ l of 0.1 % trifluoroacetic acid. Proteins were then subjected to reversed-phase chromatography on a Brownlee Aquapore BU-300 column (Perkin Elmer, Norwalk, CT, U.S.A.) using a SMART system (Amersham Biosciences). Elution was made with a gradient of acetonitrile from 0 to 60 %. Peaks corresponding to recombinant PSD-95, SAP97 or PSD-93 PDZ2 domains were analysed on a Kompakt IV MALDI–TOF–MS system (Kratos) and, subsequently, the concentrations were determined by amino acid analysis.

Yeast two-hybrid screening

pMM6 was transformed into the Y190 yeast strain (Clontech) using the lithium acetate method [27] and grown in leucine-deficient medium. Yeast cells from an individual colony were subsequently transformed with a human fetal brain MatchMaker cDNA library (Clontech) and selected on leucine/tryptophan/histidine-deficient drop-out medium supplemented with 25 mM 3-amino-1,2,4-triazole for suppression of background growth. Robust colonies were picked, re-streaked on the same selection medium and later assayed for β -galactosidase activity. Plasmid preparation from yeast clones, positive also in the β -galactosidase assay, was performed according to a protocol provided by the manufacturer (Clontech). Plasmid preparations were electroporated into DH10b bacteria cells (Invitrogen) and subsequently purified with a QIAquick plasmid mini kit (Qiagen, Valencia, CA, U.S.A.). Positive library clones were re-transformed into Y190 yeast containing pMM6 and, as negative controls, also into yeast containing either an empty library vector, pACT2, or pVA3-1 (Clontech), which contains part of the murine p53 cDNA. Once again, the ability for growth under histidine-selection and performance in the β -galactosidase assay were evaluated. For mapping of the binding site in PSD-95 towards the megalin receptor tail, pSEV-containing yeast was transformed with plasmid constructs containing various parts of human PSD-95, SAP97, SAP102 or PSD-93 and plated on leucine/tryptophan-deficient medium for selection of the two plasmids. Three colonies from each transformation were picked and re-streaked on leucine/tryptophan-deficient agar plates for β -galactosidase assay and on leucine/tryptophan/histidine-deficient agar plates containing 30 mM 3-amino-1,2,4-triazole for evaluation of their ability to induce the *HIS3* reporter gene.

Co-immunoprecipitation and Western-blot analysis

HEK-293 cells were transfected at 75 % confluency with DNA constructs encoding the truncated megalin and PSD-95 using

the Superfect transfection reagent (Qiagen), according to the manufacturer's instructions. After 48 h, cells were washed in PBS and lysed for 40 min in 300 μ l of ice-cold lysis buffer [50 mM Hepes (pH 7.5), 250 mM NaCl, 0.2 % (v/v) Nonidet P40 and protease inhibitors (Complete, Roche Diagnostics Scandinavia AB, Bromma, Sweden)]. Anti-(PSD-95 N-terminal) antiserum (3 μ l) was added to the centrifuged lysate and incubated end-over-end for 2 h at 4 °C. Complexes were collected on Protein G-Sepharose (100 μ l; Amersham Biosciences) for 2 h at 4 °C, and subsequently washed four times with lysis buffer. The Sepharose beads were boiled in SDS/PAGE sample buffer and the supernatant retrieved by centrifugation. Protein samples were separated by SDS/PAGE and transferred on to a nitrocellulose membrane. For analysis, mouse anti-FLAG and goat anti-mouse IgG antibodies conjugated to horseradish peroxidase were used. Bound antibodies were detected by enhanced chemiluminescence (Amersham Biosciences).

Double immunofluorescent staining and confocal imaging

HEK-293 cells were transfected with DNA constructs encoding PSD-95 and/or truncated megalin as described above, and grown on bovine collagen type I-coated cover slips (20 μ g/ml) in 24-well plates in RPMI medium containing 20 % (v/v) FBS and antibiotics. Cells were washed twice in PBS and then fixed in fresh 4 % (v/v) paraformaldehyde for 10 min at room temperature. Cover slips were then rinsed in PBS, permeabilized in PBS containing 0.1 % Tween 20, rinsed and then blocked in PBS containing 0.1 % BSA and 0.1 M glycine. Cells were subsequently incubated for 1 h at room temperature with anti-(megalin MM6) antiserum and then incubated with fluorescein-conjugated goat anti-rabbit IgG for 30 min and then rinsed. Cells were then incubated with anti-PSD-95 monoclonal antibody, rinsed and incubated with Texas Red-conjugated goat anti-mouse IgG for 30 min, and finally rinsed five times in PBS and once with distilled water. Cells were mounted in fluoromount G and analysed using an MRC-1024 confocal microscope equipped with an argon/krypton laser (Bio-Rad, Hercules, CA, U.S.A.). A series of sequential optical sections were digitalized, filtered with edge definition and median filters. Exchange of primary and secondary antibodies for IgG or PBS was performed in all combinations necessary to establish the specificity of the observed staining.

Immunohistological localization in human tissues

The localization of megalin and PSD-95 was analysed immunohistochemically in tissues from human parathyroid, kidney and placenta. The tissues were obtained from anonymous routine cases at the Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden. The staining was performed with the Ventana System (Ventana, Tucson, AZ, U.S.A.). Briefly, 5 μ m cryosections were cut and air-dried overnight. The sections were fixed for 10 min in ice-cold acetone, dried and rinsed in 100 mM Tris/HCl (pH 7.5) containing 150 mM NaCl. The sections were applied to the Ventana system adapted for frozen sections using Ventana basic DAB detection kit, including endogenous biotin blocking and amplification kits. The antibodies were applied to the section for 30 min at a dilution of 1:400 for anti-(megalin MM6) antiserum and 1:800 for anti-(PSD-95 N-terminal) antiserum. After running the Ventana procedure, the sections were counterstained with haematoxylin, mounted and investigated under a microscope. Photographs were taken using a Leitz Axiophote microscope (Leica Microsystems, Wetzlar, Germany).

Surface plasmon resonance measurements

The interaction of PSD-95, SAP97, SAP102 and PSD-93 with megalin was investigated by the use of a Biacore 2000 biosensor (Biacore, Uppsala, Sweden) based on principles described previously [28]. Biotinylated peptides (Meg-SEV, Meg-VES or Kv1.4) were attached to an SA sensor chip using the manufacturer's instructions at a flow rate of 20 μ l/min, giving a gain of 410.6 resonance units (RU) or 43.7 RU for Meg-SEV, 416.7 RU for Meg-VES and 294.8 RU for Kv1.4. Binding experiments were performed with the PSD-95, SAP97 or PSD-93 recombinant PDZ2 proteins over a range of concentrations from 50–500 nM or GST-SAP97, GST-Mut97 and GST-SAP102 PDZ2 domains. The analyte was injected at a flow rate of 30 μ l/min over the Fc1 (control) and Fc2 (capture) in running buffer [10 mM Hepes (pH 7.4), 150 mM NaCl, 3 mM EDTA and 0.005 % surfactant P20]. Binding was measured as the difference between the Fc1 and Fc2 curves. The binding surface was regenerated with 120 s pulses of 1 M NaCl in 50 mM NaOH. Experiments were performed at 25 °C. Data were analysed using BIAevaluation program 3.1 (Biacore).

Human parathyroid megalin cDNA sequence

Eight overlapping PCR fragments were amplified covering the full-length coding sequence using synthetic oligonucleotides designed from a human kidney megalin cDNA sequence [11]. The primer pairs used were: F1s 5'-GCAGACCTAAAGGAGCGTT and G7as 5'-CCCGACCATTTGGAGAAGATA; G20s 5'-GCCA-GTACCAGTGCCATGA and G29as 5'-GCTCATGACACTGAT-ACTCTT; G26s 5'-GGCTGTGAGCAGGTCTGT and G16as 5'-CGACCACTAATTGAATCAAAATC; G16s 5'-CGGTGC-TCGTGTGATACAG and E2as 5'-ATCCACATCCACATGCAG; E4s 5'-CCTCAAATGGCTGTAGCAACAA and B9as 5'-CTGC-TGCTGCACGTGTGA; B5s 5'-CCAGTCTGGATACACAAA-ATGT and 23.5 5'-GGCGCACTGCCATTC; G19s 5'-CTCAGAT-GGCTCTGATGAACT and G36as 5'-GCGTTTTCTCTTTC-TTTCCTT; and G35s 5'-GAGAGTCATTGCAAAGGAAGCA and G31as 5'-AATATATGTGCAAAAGTGTGTTT. First-strand cDNA synthesis on total RNA from human parathyroid was done in four separate reactions using either oligonucleotides G29as, E2as, 23.5 or G31as as the primer. Oligonucleotide G29as-primed cDNA was used as template for primer pairs F1s/G7as and G20s/G29as, oligonucleotide E2as-primed cDNA for primer pairs G26s/G16as and G16s/E2as, oligonucleotide 23.5-primed cDNA for primer pairs E4s/B9as and B5s/23.5, and oligonucleotide G31as-primed cDNA for primer pairs G19s/G36as and G35s/G31as. PCR products of expected size were purified after separation by agarose-gel electrophoresis and thereafter used for direct sequencing.

RESULTS

PSD-95 interacts with megalin in the two-hybrid system

To identify intracellular interacting partners for megalin, a human fetal brain cDNA library was screened in the yeast two-hybrid system using part of the human megalin intracellular domain as bait. A small number of clones showed a positive response in both reporter-gene assays and failed to cause a response using the negative control vectors. As judged by agarose-gel electrophoresis of the library cDNA inserts, all yeast colonies contained the same plasmid clone. The 3 kb insert of one of these clones, p4, was sequenced. A BLAST search in the National Center for Biotechnology Information databases with the amino acid

Figure 1 Schematic structure of PSD-95 and co-immunoprecipitation of megalin with PSD-95

(A) Schematic structure of the PSD-95 protein displaying the three N-terminal PDZ domains, the SH3 domain and the C-terminal GUK domain. The protein encoded by the p4-clone, identified in the yeast two-hybrid screen, is aligned underneath. (B) Co-immunoprecipitation of PSD-95 and truncated megalin in overexpressed HEK-293 cells. Immunoprecipitation (IP) was done as described in the Materials and methods section with antiserum against the N-terminus of PSD-95 (α -PSD-95) or with preimmune serum from the rabbit (control serum). The membrane was probed with an antibody against the FLAG epitope of truncated megalin. Molecular-mass markers (in kDa) are indicated on the right.

sequence deduced from this clone revealed a 100% identity with the human PSD-95/SAP90 (GenBank[®] accession number U83192) [29,30]. However, our human p4 clone lacked the first 53 N-terminal amino acid residues found in the published human PSD-95 sequence. Instead, the first seven amino acid residues of p4 were found to be identical with residues 4–10 of the rat PSD-95 (GenBank[®] accession number M96853) [31], residues not found in the published human PSD-95 sequence. This indicated that clone p4 encoded a full-length novel human splice variant of PSD-95 apart from the N-terminal three amino acid residues. We therefore produced a full-length construct of PSD-95 containing the three lacking N-terminal residues.

PSD-95 consists of three PDZ domains, one SH3 domain and one GUK domain (Figure 1A). PDZ domains are known to bind different types of sequences, and one of them is the C-terminal class I sequence (Thr/Ser)Xaa-Val (where Xaa is any amino acid) [19]. Megalin has the C-terminal sequence Ser-Glu-Val, consistent with a class I motif. Recently, a more thorough description of the requirements for binding to the PDZ2 domain of PSD-95 was reported [32]. The consensus sequence (Glu/Gln)(Ser/Thr)Xaa-Val was defined, which fits quite well with the C-terminal sequence of megalin, although position –3 contains an aspartate residue instead of a glutamate or glycine residue.

Co-immunoprecipitation of megalin and PSD-95

To verify further the binding of PSD-95 to megalin, co-immunoprecipitation experiments were performed using cell lysates from HEK-293 cells co-transfected with full-length PSD-95 and a truncated variant of megalin from a plasmid denoted pGH3. The truncated receptor contained the megalin signal peptide, a FLAG epitope, the two juxtamembrane epidermal growth factor repeats, the membrane-spanning segment and the entire cytoplasmic tail. Cell lysates were subjected to immunoprecipitation with a polyclonal antiserum against the

Table 1 Yeast two-hybrid interaction assay using *HIS3* and β -gal

–, Denotes no growth for the *HIS3* marker, and indicates no, or low blue, background colour in the β -gal assay. +++++, robust yeast growth for the *HIS3* selection, and strong blue colour in the β -gal assay.

Bait	Prey	<i>HIS3</i>	β -gal
pSEV	PSD-95 N-terminus	–	–
pSEV	PSD-95 PDZ1	–	–
pSEV	PSD-95 PDZ2	+++++	+++++
pSEV	PSD-95 PDZ3	–	–
pSEV	PSD-95 PDZ1 + 2	+++++	+++++
pSEV	PSD-95 PDZ2 + 3	+++++	+++++
pSEV	PSD-95 SH3	–	–
pSEV	PSD-95 GUK	–	–
pSEV	PSD-93 PDZ1 + 2 + 3	+++	+++++
pSEV	PSD-93 PDZ1	–	–
pSEV	PSD-93 PDZ2	++	++
pSEV	PSD-93 PDZ3	–	–
pSEV	SAP102 PDZ2	++	+++
pSEV	SAP97 PDZ2	–	–
pSEV	SAP97 PDZ1 + 2	–	–
pSEV	SAP97 PDZ2 + 3	–	–
pSEV	SAP97 PDZ1 + 2 + 3	–	–
pSEV	SAP97 SH3	–	–
pSEV	SAP97 SH3 + GUK	–	–
pNoSEV	PSD-95 PDZ2	–	–

N-terminus of PSD-95 or with preimmune rabbit serum as a control. The immune complexes were separated by SDS/PAGE, transferred on to nitrocellulose membranes and then probed with an antibody against the FLAG epitope of the truncated megalin protein. As shown in Figure 1(B), immunoblotting with the anti-FLAG antibody gave rise to a strong band of the expected size for the truncated megalin (39 kDa), consistent with a complex formation between PSD-95 and megalin. The control serum did not precipitate megalin, nor was megalin detected in the untransfected HEK-293 cells.

Identification of interacting sequences in PSD-95 and megalin

To elucidate which part of PSD-95 bound megalin, the different domains of PSD-95 were cloned into the bait vector of the two-hybrid system and an interaction assay was performed. This showed that the cytoplasmic tail of the receptor bound to PSD-95 constructs containing the PDZ2 domain, but not to constructs lacking the PDZ2 domain (Table 1). To find out if megalin bound via the C-terminal Ser-Glu-Val sequence, a construct denoted pNoSEV was made. pNoSEV contained the megalin C-terminal 55 amino acid residues, except for the very last three residues Ser-Glu-Val. In the two-hybrid interaction assay, no binding of pNoSEV to the PDZ2 domain of PSD-95 was detected (Table 1).

Intracellular co-localization of megalin and PSD-95

To see if megalin and PSD-95 co-localized within the cell, HEK-293 cells were transfected with PSD-95 and/or the truncated version of megalin and double-stained using antibodies specific for the two proteins. Megalin was localized to the plasma membrane or to vesicles located close to the membrane (Figure 2B). Some faint cytosolic staining of megalin was also seen (Figure 2B), but this was mainly due to invagination of the cell membrane, which could be clearly seen in different sequential

Figure 2 Co-localization of megalin and PSD-95 in HEK-293 cells

HEK-293 cells were transfected with PSD-95 and megalin (A–C), PSD-95 alone (D–F) or megalin alone (G–I), sown on to collagen type I-coated cover slips, fixed in paraformaldehyde and double-stained using antibodies specific for PSD-95 (A, D and G; red) and megalin (B, E and H; green). Merged images (C, F and I; yellow) are also shown. In cells transfected with both PSD-95 and megalin (A–C), co-localization of PSD-95 and megalin in the cell membrane (arrows) is shown. No expression of endogenous megalin (E) was found in cells transfected with PSD-95 alone. In cells transfected with megalin alone, megalin co-localized with endogenous expression of PSD-95 (I). Scale bars, 10 μ m.

sections of the cells (results not shown). PSD-95 was mainly found co-localized with megalin, as seen by the merged image (Figure 2C). However, some PSD-95 was also found in the cytosol (Figure 2A). This staining might either correspond to free PSD-95 or PSD-95 bound to proteins other than megalin. A small part of the cytosolic PSD-95 staining was also due to invagination of the cell membrane, as seen in the different sequential sections of the cells (results not shown). No endogenous expression of megalin could be detected in the HEK-293 cells (Figure 2D–2F), but there was endogenous expression of PSD-95 which co-localized with megalin, the latter expressed from the pGH3 plasmid (Figure 2G–2I).

Localization of megalin and PSD-95 in tissues

Megalín is expressed by a number of specific cell types in different tissues, whereas PSD-95 has mainly been reported to be expressed in neuronal cells [31]. For the interaction between megalín and PSD-95 described above to be physiologically relevant, the two

Figure 3 Immunohistological localization of megalin and PSD-95 in human tissues

Parathyroid (A and B), kidney (C and D) and placental (E and F) tissues were immunohistologically stained as described in the Materials and Methods section. Antisera were used against megalin (A, C and E) or PSD-95 (B, D and F). Scale bar, 50 μ m.

proteins must be expressed in the same cell. Since megalín has been found in such diverse cells as the parathyroid chief cells, kidney proximal tubule cells and placental cytotrophoblasts, sections from relevant tissues were stained with antisera against megalín and PSD-95. As shown in Figures 3(A) and 3(B), parathyroid chief cells were markedly stained with both antisera, providing strong evidence for the co-occurrence of the two proteins in these cells. Also, kidney tubule cells and placental cytotrophoblasts were stained with the anti-megalín and anti-PSD-95 antisera (Figures 3C–3F). Preimmune sera derived from the same rabbits prior to immunization did not react with the tissue sections (results not shown). This demonstrates that megalín and PSD-95 are expressed in the same cell types in different tissues.

Expression of MAGUK family members in the parathyroid

Since the parathyroid chief cells were strongly stained with an anti-PSD-95 antiserum, we investigated whether PSD-95-like MAGUK proteins were also expressed in human parathyroid tissue. Therefore a number of RT-PCRs were carried out. PCR fragments of the expected sizes were obtained for primer pairs specific for PSD-95 and PSD-93 (Figure 4, lanes 1 and 2

Figure 4 Expression of PSD-95-like members of the MAGUK family in the human parathyroid as determined by RT-PCR

DNA-fragments of PSD-95 (lane 1), PSD-93 (lane 2) and SAP97 (lane 3) were produced by RT-PCR. The expected sizes were 1167 bp, 1183 bp and 1540 bp respectively. MM, molecular-size marker.

respectively), as well as SAP97 (Figure 4, lane 3). The identity of the fragments was verified by DNA sequencing. These results show that, in addition to PSD-95, PSD-93 and SAP97 are, most likely, expressed in the parathyroid. We failed to detect SAP102, which was somewhat surprising, since SAP102 has been reported to be expressed in endocrine cells in addition to neuronal tissue [33]. However, we do not exclude the possibility that SAP102 is expressed in parathyroid chief cells, since there are many technical reasons for the failure to amplify a given DNA sequence. Indeed, a splice variant of SAP102 was encoded by an expressed sequence tag from a parathyroid tumour (GenBank® accession number AA772095). Different splice variants of SAP97 have been described previously [24–26]. The parathyroid-encoded SAP97 identified in the present study lacked insertions I1A, I3 and I4, but contained insertions I1B, I2 and I5 [24–26]. It also contained two polymorphisms, Gln²⁷⁸ → Arg and Gly⁸⁰¹ → Glu, compared with the published sequence (GenBank® accession number U13897). The parathyroid-encoded PSD-93 was a novel splice variant containing a deletion of positions 342–393 and two polymorphisms, Ala¹⁷⁷ → Val and Asn²⁷⁸ → Lys (corresponding to SAP97 position 404 in Figure 6), compared with the published sequence (GenBank® accession number U32376).

To investigate if the other members of the PSD-95-like MAGUK family could also bind megalin, PSD-93, SAP102 and SAP97 constructs were used in a two-hybrid interaction assay. Table 1 shows that constructs of PSD-93 and SAP102 containing the PDZ2 domain bound the cytoplasmic domain of megalin; however, no part of SAP97 bound to megalin.

***In vitro* studies of the binding of MAGUK PDZ2 domains to the megalin cytoplasmic tail**

To investigate further the binding of the PSD-95-like MAGUK family members to megalin, the binding of different MAGUK PDZ2 domains, produced as recombinant proteins, to peptides corresponding to the megalin C-terminus was studied by surface plasmon resonance. Figure 5(A) shows that the PDZ2 domain of PSD-95 bound a peptide corresponding to the last 14 amino acids of megalin (Meg-SEV). When the last three amino acids in Meg-SEV were reversed from Ser-Glu-Val to Val-Glu-Ser (Meg-VES), no binding of the PSD-95 PDZ2 occurred. Figure 5(B) shows that the PSD-93 PDZ2 was also able to bind Meg-SEV, but not Meg-VES. SAP102 PDZ2 expressed as a GST fusion protein was shown to bind Meg-SEV with high affinity (Figure 5C). A GST fusion protein of SAP97 PDZ2 was unable to bind Meg-SEV (Figure 5C), which was consistent with the results obtained in the yeast two-hybrid interaction assay (Table 1). As a control experiment for the intactness of the SAP97 PDZ2 domain,

Figure 5 Superimposed sensorgrams from surface plasmon resonance experiments

Biotinylated peptides Meg-SEV, Meg-VES or Kv1.4 were attached to SA sensor chips as described in the Materials and methods section. Analyte in the form of recombinant PSD-95 PDZ2 protein (A), recombinant PSD-93 PDZ2 protein (B) and GST-SAP97 PDZ2, GST-Mut97 PDZ2, GST-SAP102 PDZ2 or GST (C) were injected on to Meg-SEV as described. Recombinant PSD-95 PDZ2 protein (A) and recombinant PSD-93 PDZ2 protein (B) were also injected on to Meg-VES. As a control experiment, recombinant SAP97 PDZ2 protein was injected on to the Kv1.4 peptide (D).

Figure 6 Sequence alignment of PDZ2 domains of the PSD-95-like MAGUK family

Asterisks indicate identity with PSD-95 sequence. Light grey boxes refer to the α -helical or β -strand regions of the PDZ domain, as inferred from X-ray determination of PDZ domains. Residues in the SAP97 (GenBank® accession number U13897) PDZ2 domain differing from those of the PSD-95 (GenBank® accession number U83192), SAP102 (GenBank® accession number U49089) and PSD-93 (GenBank® accession number) PDZ2 domains are boxed in dark grey. Dots below alignment indicate residues contributing to the hydrophobic-binding pocket of PDZ domains [36]. Arrow refers to amino acid polymorphism Asn $\rightarrow$ Lys in the parathyroid PSD-93 sequence compared with the published sequence. Numbering refers to the published sequence of the SAP97 protein [24].

Figure 7 Ribbon diagram showing the structure of the SAP97 PDZ2 domain modelled on the three-dimensional fold of the PDZ3 domain of rat PSD-95, corresponding to amino acids 312–391 (GenBank® accession number M96853)

A peptide with the sequence Gln-Thr-Ser-Val (QTSV) is inserted with ball-and-stick model in the hydrophobic-binding pocket of the PDZ domain. Lys³⁶⁴, Cys³⁷⁸ and Phe³⁹⁷ are shown as balls, and Thr³⁸⁹ of SAP97 PDZ2 as a ball-and-stick model. The Figure was generated by MOLSCRIPT [48] and rendered using POVray (<http://www.povray.org>), by using the atomic coordinates and X-ray structure factors deposited in the Protein Data Bank under accession number 1BE9.

the ability of this domain to bind a peptide consisting of the last 10 amino acid residues of the voltage-gated potassium channel Kv1.4 was investigated. As shown in Figure 5(D), the PDZ2

domain of SAP97 bound this peptide, as reported previously [34]. No dissociation constants of the interactions of the PDZ2 domain peptides were calculated due to mass transport limitations, but the shape of the surface plasmon resonance sensorgrams of the PSD-93 and SAP102 PDZ2 domains suggested a higher affinity for those domains than for the PSD-95 PDZ2 domain (Figures 5A–5C). Larger GST fusion proteins did not cause mass transportation limitations, but dissociation constants calculated from sensorgrams for these are not reliable due to dimerization of GST.

The PDZ2 domains of PSD-95, SAP102, PSD-93 and SAP97 are highly similar to each other in sequence, but the PDZ2 domain of SAP97 did not bind megalin, in contrast with those of PSD-95, SAP102 and PSD-93. A sequence alignment of the PDZ2 domains showed that SAP97 differs only at four positions from SAP102, PSD-93 and PSD-95 (Figure 6). In the alignment, these differences correspond to amino acid residues Lys³⁶⁴, Cys³⁷⁸, Thr³⁸⁹ and Phe³⁹⁷ of the PDZ2 domain of SAP97 (all marked with dark grey boxes in Figure 6). Modelling of the SAP97 PDZ2 domain on the three-dimensional structure of the PDZ3 domain of PSD-95 with a peptide in the pocket [35], showed that Lys³⁶⁴ is located between the α A-helix and the β D-strand, Cys³⁷⁸ in the β E-strand, Thr³⁸⁹ in the α B-helix and Phe³⁹⁷ in the β F-strand (Figure 7). The α B-helix, the β B-strand and the conserved Gly-Leu-Gly-Phe (GLGF) loop, which connects the β A- and β B-strands, contain most of the amino acids that build up the hydrophobic peptide-binding pocket in the PDZ domain. The dots underneath the alignment in Figure 6 show peptide-binding amino acids in the binding pocket of other crystallized PDZ domains [36]. Therefore, out of these residues, only Thr³⁸⁹ in the α B-helix seemed likely to be able to influence the structure of the binding pocket, since Lys³⁶⁴, Cys³⁷⁸ and Phe³⁹⁷ are located on the surface of the PDZ domain and not close to the hydrophobic-binding pocket. We mutated Thr³⁸⁹ of the SAP97 PDZ2 domain to an alanine residue to conform to the other members of the PSD-95-like MAGUK family. The corresponding mutated SAP97 PDZ2 domain was produced as a fusion protein with GST (GST-Mut97 PDZ2) and tested for its ability to bind the Meg-SEV peptide by surface plasmon resonance analysis. Figure 5(C) shows that the fusion protein bound the C-terminal sequence of megalin. GST itself did not bind the Meg-SEV peptide.

Sequence of parathyroid megalin

The sequence of human megalin has been reported previously from kidney and placenta cDNA and showed an overall identical sequence apart from a number of amino acid polymorphisms [11]. To look for splice variants and to ascertain that megalin expressed in the parathyroid possessed the Ser-Glu-Val (SEV) motif necessary for PSD-95 binding, human megalin cDNA fragments from parathyroid tissue were produced and sequenced (results not shown; GenBank® accession number AY265358). Ten single nucleotide polymorphisms (SNPs) were found. At two nucleotide positions, the parathyroid cDNA sequence could not be determined with certainty and were therefore treated as an N. In the present study, the four previously described polymorphic nucleotide sites giving rise to amino acid replacements encoded in the megalin sequence are Ala¹²⁶², Ala²⁸⁴⁷, Glu⁴⁰⁶⁹ and Leu⁴¹⁸⁵. No alternatively spliced forms of megalin were detected. In all tissues investigated, human megalin had the C-terminal Ser-Glu-Val sequence important for its binding to PSD-95.

DISCUSSION

Megalin has been suggested to mediate signals from the cell exterior to the cytoplasm through its intracellular domain. To elucidate signal transduction pathways coupled to this receptor, it is necessary to identify proteins interacting with specific motifs in the cytoplasmic segment. We therefore screened a fetal brain cDNA library with the C-terminal 55 amino acid residue segment of megalin as bait and were able to identify the binding of PSD-95, in accordance with a previous report [12]. The aims of the present study were to (i) define the binding domain in PSD-95, (ii) investigate if other PSD-95-like MAGUK proteins could bind megalin, (iii) identify the structural reason(s) for any differences in binding, and (iv) investigate further the potential physiological relevance of the reported megalin–PSD-95 interaction by co-localization of these proteins in cultured cells and human tissues.

Our present studies in yeast and by surface plasmon resonance analyses showed that the PDZ2 domains of PSD-95, SAP102 and PSD-93 bound megalin, but not the PDZ2 domain of SAP97 (Table 1). Alignment of the SAP97 PDZ2 domain sequence with those of the PDZ2 domains from PSD-95, PSD-93 and SAP102 identified four amino acid residues that distinguished the SAP97 PDZ2 domain from the homologous domain in the other MAGUK proteins. Modelling of the SAP97 PDZ2 domain on the three-dimensional structure of the PDZ3 domain of PSD-95 with a peptide in the binding pocket [35] showed that only Thr³⁸⁹ is located close to the binding pocket but pointing outwards (Figure 7). At the equivalent position in PSD-95, PSD-93 and SAP102 an alanine residue is found (Figure 6). We reasoned that this threonine residue might disrupt the regular α -helical hydrogen-bonding pattern, causing a distortion in the helix and, in turn, alter the peptide-binding pocket in a way that the megalin C-terminal Ser-Glu-Val would not fit. Our finding that replacing Thr³⁸⁹ with an alanine residue in the SAP97 PDZ2 domain allowed binding to the megalin C-terminal is consistent with this hypothesis.

An alternative explanation can be proposed from a recent report [37] showing that an upstream Ser-Ser-Gly (SSG) motif at positions –8 to –10 in the binding peptide was essential for binding of the ionotropic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid ('AMPA') receptor subunit glutamate receptor A (GluR-A) to SAP97 PDZ2. Also, changing the residues at position –9 from the hydrophilic serine residue to a basic

arginine residue, an acidic glutamate residue or the hydrophobic alanine or isoleucine residues abolished binding. It is possible that the bound C-terminal portion of GluR-A folded back in a U-turn on the outside of the PDZ2-binding pocket, allowing interaction of the hydrophilic serine residue at position –9 of the peptide with the hydrophilic Thr³⁸⁹. The hydrophobic alanine residue at the position in PSD-95, equivalent with Thr³⁸⁹ in SAP97, would then not favour binding of the peptide with the Ser-Ser-Gly sequence at positions –8 to –10, as was shown in the same report [37]. The same speculation may be true with megalin. The hydrophobic alanine residue at position –9 in the megalin cytoplasmic tail would not allow binding to the hydrophilic Thr³⁸⁹ of SAP97, but a hydrophobic alanine residue at the same position as in PSD-95 would allow binding.

The physiological role of the interaction between megalin and PSD-95 and other MAGUKs is far from clear. MAGUK proteins are able to bind numerous different proteins in different cell types, including neuronal nitric oxide synthase ('nNOS'), GluR subunits, kainate receptor subunits, potassium channels, neuro-ligins, guanylate kinase-associated proteins ('GKAPs'), plasma membrane calcium pumps ('PMCA's'), mint-1, syndecan, calmodulin, protein 4.1, neuroligins and many more [23,38], and it is therefore not possible at this time to draw any definite conclusions on the role(s) of megalin–MAGUK interactions from proteins binding to MAGUKs. However, in the renal proximal tubule brush border, the Na⁺/H⁺ exchanger 3 (NHE3) has been co-purified with megalin [39]. NHE3 appears in two forms, as determined by sedimentation centrifugation experiments, an active 9.6 S megalin-free form and an inactive 21 S megalin co-purifying form [39,40]. A direct physical interaction between megalin and NHE3 has not been demonstrated, but it is known that NHE3 can bind PDZ-containing proteins, such as NHE regulatory factor (NHERF)/ezrin–radixin–moesin-binding phosphoprotein 50 ('EBP50') and NHERF2/NHE kinase A regulating protein ('E3KARP') [41,42]. NHE3 does not have a perfect type I PDZ-binding consensus sequence; however, the PSD-95 PDZ2 domain can bind molecules in a different mode, such as the β -hairpin forming the 'pseudopeptide' structure in the PDZ domain of neuronal nitric oxide synthase [43]. Furthermore, the LDLR-like protein 1 ('LRP1'), which lacks a type I PDZ-binding sequence, has been reported to bind PSD-95 [12]. It has even been speculated that all PDZ domains can recognize internal sequence motifs and not just C-terminal sequences [44]. It may also be worthwhile investigating whether MAGUKs, such as PSD-95, and NHE3-binding PDZ-containing proteins, such as NHERF or NHERF2, can directly or indirectly interact, thus explaining the co-purification of megalin and NHE3 in a large complex [39,40]. A functional coupling between megalin and NHE3 in the proximal tubule would be plausible, since megalin has been demonstrated to mediate endocytosis of numerous proteins, hormones and vitamins in the proximal tubule [1] and NHE3 is involved in the acidification of endosomes [45–47].

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