Megalin Mediates Selenoprotein P Uptake by Kidney Proximal Tubule Epithelial Cells*
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Selenoprotein P (Sepp1) contains most of the selenium in blood plasma and it is utilized by the kidney, brain and testis as a selenium source for selenoprotein synthesis. We recently demonstrated that Apolipoprotein E receptor-2 (ApoER2) is required for Sepp1 uptake by the testis and that deletion of ApoER2 reduces testis and brain, but not kidney, selenium levels. This study examined the kidney Sepp1 uptake pathway. Immunolocalization experiments demonstrated that Sepp1 passed into the glomerular filtrate and was specifically taken up by proximal tubule epithelial cells. Neither the C-terminus selenocysteine-rich domain of Sepp1 nor ApoER2 were required for Sepp1 uptake by proximal tubules. Tissue ligand-binding assays using cryosections of Sepp1⁻/⁻ kidneys revealed that the proximal tubule epithelium contained Sepp1-binding sites that were blocked by the receptor-associated protein, RAP, an inhibitor of lipoprotein receptor-ligand interactions. Ligand blotting assays of kidney membrane preparations fractionated by SDS-PAGE revealed that Sepp1 binds megalin, a lipoprotein receptor localized to the proximal tubule epithelium. Immunolocalization analyses confirmed the in vivo co-localization of Sepp1 and megalin in wild type kidneys and demonstrated the absence of proximal tubule Sepp1 uptake in megalin null mice. These results demonstrate that kidney selenium homeostasis is mediated by a megalin-dependent Sepp1 uptake pathway in the proximal tubule.

Selenium, an essential dietary micronutrient, is incorporated into selenoproteins as the amino acid selenocysteine. In humans 25 genes encoding selenoproteins have been identified (1). Most selenoproteins are intracellular enzymes with their activities dependent upon a single selenocysteine residue in their primary structures. However two family members, selenoprotein P (Sepp1) and glutathione peroxidase-3 (Gpx3) are extracellular proteins and they account for the bulk of selenium in blood plasma. Sepp1 is the only selenoprotein whose primary structure contains more than one selenocysteine residue, and human, mouse and rat Sepp1 each contain 10 residues (2). Sepp1 is expressed in most tissues. However, the liver is the predominant site of its production and secretion to the blood where it accounts for approximately 65% of the plasma selenium content. Gpx3 also is expressed at low levels in several tissues, but the kidneys represent its principal site of production and secretion to blood (3). Both Sepp1 and Gpx3 are proposed to function as extracellular antioxidants. However, recent studies have demonstrated that Sepp1 also performs a critical role in selenium transport throughout the body. Mice with deletion of the selenoprotein P gene (Sepp1⁻/⁻) have reduced selenium levels in the brain, testis and kidney (4,5). These animals also show abnormal neurological function, male infertility and reduced plasma Gpx3 activity, consistent with reduced brain, testis and kidney selenium levels. Furthermore mice with liver-specific inactivation of selenoprotein synthesis show reduced levels of plasma and kidney glutathione peroxidase activity, which is further evidence that liver Sepp1 provides selenium to the kidney (6); conversely Sepp1⁻/⁻ mice with transgenic expression of human selenoprotein P (SEPP1), driven by liver-specific promoter, show increased levels of selenium in the brain, testis and kidney supporting the crucial role.
of hepatic Sepp1 in selenium transport to target organs (7). Intriguingly, selenium levels in the brain and kidney, but not in the testis, of Sepp1−/− mice are nearly restored to levels found in Sepp1+/+ mice by feeding supplemental selenium as sodium selenite (4), indicating that these organs can also capture selenium from molecules other than Sepp1. These data raise the question of whether the organs that utilize Sepp1 as a selenium resource employ different mechanisms for its uptake and processing.

Gpx3 is synthesized by epithelial cells of the kidney proximal tubule (8,9) and secreted into the extracellular space from where it enters the blood (3). Sepp1 provides selenium that is utilized for kidney Gpx3 synthesis, but it is not known whether Sepp1 is internalized by a receptor-dependent pathway or by specific kidney cell types. The potential for receptor mediated Sepp1 uptake was suggested by early studies showing saturable binding of Sepp1 to membranes from a variety of organs including the kidney (10). We recently demonstrated that Apolipoprotein E receptor 2 (ApoER2/Lrp8) is required for Sepp1 uptake by the testis and that testes of ApoER2−/− males exhibited low selenium levels in comparison to testes of wild type animals (11). However while deletion of the ApoER2 gene also reduces brain selenium, it does not affect kidney selenium content (12). These data suggest that the kidney utilizes a distinct Sepp1 trafficking pathway to capture its selenium. The objectives of the present study were to define the cell types and potential receptor(s) that function in kidney Sepp1 uptake.

EXPERIMENTAL PROCEDURES

Animals- Care and use of animals conformed to National Institutes of Health guidelines for humane animal care and use in research. The Vanderbilt Institutional Animal Care and Use Committee approved all animal protocols. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) approved facility on a 10h:14h light:dark cycle, with food and water provided ad libitum. The diet provided was either Purina rat chow 5001 or pelleted semi-synthetic torula yeast diet supplemented with 0.25 or 1.0 mg selenium, as sodium selenite, per kg diet (Harlan-Teklad, Madison, WI). Sepp1+/− mice, ApoER2−/− mice and Sepp1A240−361 mice, that express a truncated Sepp1 lacking amino acids 240-361 which includes 9 selenocysteine residues, were produced and genotyped by PCR as described previously (4,11,13). Mice with deletion of the low density lipoprotein receptor-related protein 2 (megalin/Lrp2) gene (14) were the kind gift of Dr. J. Herz (University of Texas Southwestern Medical Center). Mice were sacrificed using CO2 and their kidneys and testes were removed and immediately utilized in the following protocols. Most megalin null mice die at birth, so adult megalin+/− males and females were paired overnight and the following morning females were examined for plug formation, designated as day 1 of pregnancy. Mice were sacrificed at day 19 of pregnancy, fetuses were genotyped by PCR and their kidneys were utilized in protocols described below.

Kidney and Testis Membrane Preparation- Kidney and testis membrane preparations were prepared from wild type or Sepp1−/− adult mice using slight modifications of an established protocol (15). Organs were placed in ice-cold 0.3 M sucrose, 2 mM CaCl2, 25 mM Tris-HCl, pH 7.5, containing a protease inhibitor cocktail (Cat. #P8340, Sigma, St. Louis MO) of 1 mM AEBSF, 0.8 μM aprotinin, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin A and 14 μM E64) and homogenized with a Brinkman Polytron. The homogenates were centrifuged 10 minutes at 1000 xg at 4°C and the supernatant fraction was then centrifuged at 100,000 xg for 45 minutes at 4°C in a Beckman TL100.3 rotor. The pellets were suspended in TBS (150 mM NaCl, 2 mM CaCl2, 25 mM Tris-HCl pH 7.5, containing protease inhibitors), homogenized and centrifuged at 100,000 xg to obtain a membrane-enriched pellet. Membranes were solubilized in TBS containing 0.5% Triton X100 for 2 hours at 4°C, and particulate material was removed by centrifugation at 14,000 xg for 15 minutes at 4°C.

SDS-PAGE, Western Blotting and Ligand Blotting- Protein levels of the membrane fractions were determined (16) and aliquots were solubilized in sample buffer, with or without 50 mM DTT as a reducing agent, at 75°C for 5 minutes. SDS-PAGE was performed on either 10% Bis-Tris or 3-8% Tris-Acetate NuPAGE Novex acrylamide gels (Invitrogen Inc., Carlsbad
Polypeptide bands were either visualized by staining with Coomassie Blue or transferred to nitrocellulose membranes for Western blot analysis (17). Blots were rinsed in PBS (150 mM NaCl, 10 mM sodium phosphate pH 7.4) and blocked in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE). Blots were then incubated in primary antibody diluted 1:2,500 to 1:5,000 in Odyssey blocking buffer containing 0.1% Tween 20; parallel control blots substituted equivalent levels of nonimmune IgG from the appropriate species. Primary antibodies included monoclonal rat anti-mouse Sepp1 which reacts with an epitope in its N-terminal region (provided by Dr. T. Naruse of Kaketsuken, The Chemoso-Therapeutic Research Institute, Kumamoto, Japan) and polyclonal affinity-purified rabbit anti-megalin (gift from Dr. D. Biemesderfer, Yale University) which reacts with the C-terminus region of megalin from several species (18). Blots were then washed at least 4 times with PBS containing 0.1% Tween (PBST) and then incubated in Alexa 680-conjugated goat anti-IgG or goat anti-rabbit IgG secondary antibody (Invitrogen Inc., Carlsbad CA) diluted 1:5,000 in PBST. Blots were then washed extensively in PBST and imaged using an Odyssey infrared imager.

Ligand-blotting analyses were performed to identify Sepp1-binding proteins in the kidney membrane preparations (19–21). Mouse Sepp1 was immunoaffinity purified from mouse plasma using monoclonal anti-Sepp1 conjugated to Aminolink Coupling Gel (Pierce Biotechnology, Rockford, IL) and its purity was assessed by SDS-PAGE. Western blots were blocked in Odyssey blocking buffer and then incubated overnight at 4°C with, or for controls, without 0.7 µg/ml mouse Sepp1 diluted in Odyssey blocking buffer containing 0.1% Tween 20 and 2 mM CaCl2. Blots were then washed in PBST containing 2 mM CaCl2 and fixed 10 minutes at RT with 4% formaldehyde in PBST. Blots were then rinsed in PBST containing 0.2% glycine and immunostained with monoclonal rat anti-Sepp1 as described above.

Immunofluorescence Microscopy- Mouse kidneys were fixed 1 hour at 4°C with 4% formaldehyde in 0.1 M sodium phosphate, pH 7.4, rinsed in phosphate buffer and infiltrated overnight at 4°C in phosphate buffer containing 20% sucrose. Tissue was then immersed in OCT (Optimal Cutting Temperature Compound, Fisher Scientific, Atlanta GA), frozen in liquid nitrogen and stored at −70°C. Cryosections were rinsed with TBST (20 mM Tris–HCl, pH 8.0, 150 mM sodium chloride, 0.05% Tween 20, 0.025% sodium azide and blocked with TBST containing 1% bovine serum albumin (BSA) and 0.1% glycine for 1 hour. Sections were then incubated in rat monoclonal anti-Sepp1 and/or rabbit anti-megalin primary antibodies in blocking solution for 1 hour at room temperature. Control sections were incubated with equivalent levels of nonimmune rat or rabbit IgG. Sections were then washed 3x 5 minutes in TBST and then incubated for 1 hour in affinity-purified secondary antibodies diluted in blocking solution. These included Cy3- or Cy5-conjugated mouse anti-rat IgG (Jackson Immunoresearch Laboratories, West Grove, PA) and Alexa 488-goat anti-rabbit IgG (Invitrogen Corporation, Carlsbad, CA). Hoechst 33258 and Alexa 488-phalloidin, to stain f-actin, (Invitrogen Corporation, Carlsbad, CA) were added to the secondary antibody solution. For double antibody staining, additional controls validated that the secondary antibodies only bound to the appropriate primary antibody. Slides were washed 3x 5 minutes with TBST and mounted in 50% glycerol in TBS. Specimens were examined by phase contrast and fluorescence microscopy and images of experimental and control specimens were obtained using identical photographic exposures. Each immunostaining experiment was repeated using at least three animals and the images shown are representative of all replicates.

Tissue ligand-binding assays were performed to localize Sepp1 binding-sites in kidneys from Sepp1−/− mice (22). Cryosections, 4–5µm in thickness, were blocked for 30 minutes in blocking solution of Hepes buffered saline (HBS = 20mM Hepes, pH 7.5, 150 mM sodium chloride, 2 mM calcium chloride, 0.25% sodium azide) containing 1% BSA and 0.1% glycine. Slides were then incubated one hour with 1-5 µg/ml of affinity-purified mouse Sepp1 in blocking solution. Two controls were performed: in the first, sections were preincubated for 1 hour in blocking solution containing 10 µg/ml GST-RAP (provided by Dr. E. Weeber, Vanderbilt University), an inhibitor of lipoprotein receptor-ligand interactions (21,23,24), prior to incubation with Sepp1; the second control omitted the Sepp1
from the incubation solution. Slides were washed 3 X with HBS and then fixed with 4% formaldehyde in HBS for 20 minutes. Slides were then rinsed with TBST and immunostained with rat anti-mouse Sepp1 and Alexa 488-phalloidin as detailed above.

RESULTS

Distribution of Sepp1 in the Kidney. Cryosections of adult wild type mouse kidneys immunostained with anti-Sepp1 revealed a discrete Sepp1 distribution pattern within the cortex, but no staining was detected in the medulla. Sepp1 displayed a punctate distribution at the luminal surface and within the apical cytoplasm of epithelial cells of the proximal tubule and of the urinary pole of the glomerulus (fig. 1A). At the cell surface Sepp1 localized just below the epithelial brush border, consistent with its endocytic uptake. Sepp1 staining was always detected in the proximal tubule as it emerged from the glomerulus, but not in its distal regions, suggesting an efficient mechanism for Sepp1 uptake from the glomerular filtrate. Sepp1 was not detected in any other kidney tubules. Specificity of the anti-Sepp1 staining was validated using two controls. First wild type kidney sections immunostained by substituting nonimmune IgG for anti-Sepp1 displayed no detectable specific staining (not shown). Second cryosections of kidneys from Sepp1^{−/−} mice immunostained with anti-Sepp1 displayed no detectable fluorescence (fig. 1B, C), this demonstrates that Sepp1, not other proteins, accounts for the positive staining. These data suggested that Sepp1 passes from blood plasma to the glomerular filtrate where it is removed by endocytic activity of the proximal tubule epithelium.

Truncated Sepp1 Binds to the Proximal Tubule Epithelium. Kidneys of mice expressing only truncated Sepp1^{Δ240-361}, that lacks the C-terminus amino acids 240-361 containing the selenocysteine-rich domain (13), were immunostained with anti-Sepp1. Like full-length Sepp1, Sepp1^{Δ240-361} was detected at the apical surface of the proximal tubule epithelium (fig. 1D, E). This demonstrates that the C-terminus domain is not required for Sepp1 binding and/or uptake by the proximal tubule epithelium.

Sepp1 Uptake by the Proximal Tubules Does Not Utilize Apolipoprotein E Receptor 2 (ApoER2). We recently demonstrated that Sepp1 uptake by the testis is mediated by ApoER2 and that testes of ApoER2^{−/−} adult males displayed sharply reduced selenium levels, similar to those of Sepp1^{−/−} males (11). To determine if the ApoER2 pathway functions in the kidney Sepp1 uptake, we examined Sepp1 distribution in kidneys of ApoER2 null animals. The Sepp1 distribution pattern in kidneys of ApoER2 null animals appeared identical to those of wild types, with Sepp1 binding to the apical surface of proximal tubule epithelial cells (fig. 1F, G). This supports the finding that kidney total selenium levels between wild type and ApoER2 null animals are similar (12). These data demonstrate that ApoER2 is not required for kidney Sepp1 uptake and indicates that the kidney and testis utilize distinct Sepp1 uptake mechanisms.

Proximal Tubule Epithelial Cells of Sepp1^{−/−} Mice Possess Sepp1-binding Sites. The above data demonstrate Sepp1 uptake by the proximal tubule epithelium but do not resolve whether it is a receptor-mediated process. To test if a specific receptor(s) mediates proximal tubule Sepp1 uptake, cryosections of kidneys from Sepp1^{−/−} mice were tested for Sepp1-binding activity. Sepp1 was immunoaffinity purified from mouse plasma and by SDS-PAGE contained a major Coomassie stained band of M, ≈50,000 (fig. 2, lane 1); Western blotting confirmed that anti-Sepp1 recognizes the M, ≈50,000 polypeptide (fig. 2, lane 2), whereas control non-immune primary IgG displays no detectable Sepp1 binding (fig. 2, lane 3). Control sections verified the absence of endogenous Sepp1 in proximal tubules (figs. 3 A-C). However cryosections incubated with purified Sepp1, revealed Sepp1 binding sites as punctate structures located below the brush border of proximal tubule epithelial cells (figs. 3 D-F) and indicated these cells possess receptors for Sepp1. Although ApoER2 does not function in proximal tubule Sepp1 uptake (fig. 1F, G), it is possible that other lipoprotein receptors play that role. The receptor-associated protein RAP inhibits the ligand binding activity of all members of the lipoprotein receptor family (23-25). Preincubation of kidney sections with RAP, prior to incubation...
with Sepp1, blocked Sepp1 binding (figs. 3 G-I). These data suggest that a lipoprotein receptor, other than ApoER2, participates in Sepp1 uptake by the proximal tubule epithelium.

Ligand-Blotting Identification of Kidney Sepp1-Binding Proteins. Kidney and testis membrane lysates were fractionated by nonreducing SDS-PAGE and stained with Coomassie blue or transferred to nitrocellulose for ligand blotting analysis. Coomassie blue staining revealed a high molecular weight band of Mr > 460,000 in kidney (fig. 4, lane 1) but not testis (fig. 4, lane 2) membrane lysates. To identify Sepp1-binding proteins, Western blot ligand-blotting assays were performed using kidney and testis membranes prepared from Sepp1−/− mice (fig 4, lanes 3-8). Blots incubated with purified Sepp1 and then immunostained with anti-Sepp1 demonstrated that Sepp1 bound the high molecular weight polypeptide that was present in kidney, but not testis, membranes (fig. 4, lanes 3, 4). Two controls were performed to verify Sepp1-binding specificity. First, blots not incubated with Sepp1 showed no positively immunostained bands (fig. 4, lanes 5, 6) and, second, blots immunostained using nonimmune primary antibody showed no positive staining (fig. 4, lanes 7, 8). The kidney Sepp1-binding protein (fig. 4, lane 3) displayed an apparent molecular weight similar to that of megalin, a lipoprotein receptor family member that localizes to the apical membrane of proximal convoluted tubule epithelial cells (26-28). Western blots of membranes fractionated by nonreducing SDS-PAGE and immunostained with anti-megalin revealed a prominent megalin band in kidney membranes (fig. 4, lane 9) that co-migrates with the Sepp1-binding protein identified by ligand blotting (lane 3), but no megalin was detected in testis membranes (fig. 4, lane 10). Control blots immunostained with a non-immune primary antibody displayed no immunoreactive bands (fig. 4, lanes 11, 12). These data show that megalin, a multispecific receptor in the apical plasma membrane of proximal tubule epithelial cells, binds Sepp1.

Sepp1 Co-localizes with Megalin in Proximal Tubule Epithelial Cells. Cryosections of adult wild type mouse kidney double immunostained for Sepp1 and megalin reveal their overlapping distribution in a subset of proximal tubules (figs. 5A, B). However megalin, unlike Sepp1 which is only detected in tubule profiles near the glomerulus, is present throughout the length of the proximal tubules (fig. 5A, B). These results demonstrate that Sepp1 and megalin co-localize at sites of endocytic activity in the proximal tubule epithelium.

Megalin is Required for Sepp1 Uptake by the Proximal Tubule. The majority of megalin−/− mice die at birth (14). Therefore megalin−/− mice were mated and pregnant females were sacrificed at day 19 of pregnancy. Double immunostaining of wild type and megalin−/− fetal kidneys revealed megalin at the apical surface of the proximal tubule epithelium and Sepp1 within the epithelium of a subset of the proximal tubules (fig. 5C, D). Compared to adult kidneys, those of day 19 wild type or megalin−/− fetuses show a more prominent accumulation of Sepp1 within vesicle-like structures in the epithelial cell cytoplasm in addition to Sepp1 at the cell surface. In contrast neither megalin nor Sepp1 were detected in proximal tubules of megalin−/− fetuses (fig. 5E, F). These data indicate that Sepp1 uptake is megalin-dependent.

DISCUSSION

This study demonstrates that the lipoprotein receptor megalin mediates Sepp1 uptake by the kidney proximal tubule, a tissue requiring a high selenium supply to support synthesis of GPx3 (3). Megalin localizes to the apical plasma membrane of proximal tubule epithelial cells where it facilitates endocytic uptake of several proteins from the glomerular filtrate (26,27,29-31). This study shows that after Sepp1 passes into the glomerular filtrate, it is endocytosed by the initial portion of the proximal tubule. Kidneys of both Sepp1−/− mice, which only express truncated Sepp1 lacking the C-terminal selenocysteine-rich domain, and of wild type mice display the same pattern of Sepp1 immunostaining in the proximal tubule. These data indicate that the Sepp1 selenocysteine-rich C-terminus domain is not required for megalin-dependent uptake. This finding also raises the question of whether all Sepp1 isoforms identified in blood plasma (32) pass into the glomerular filtrate. Full-length mouse Sepp1 displays an apparent Mr of ~50,000 by SDS-PAGE, which is
near the size limit of the filtration barrier. In rats full-length Sepp1 and three C-terminus-truncated isoforms with 1, 2 or 6 selenocysteine residues respectively are present in blood plasma (32). However which Sepp1 isoforms are present in the mouse remain to be demonstrated. It is intriguing that when fed a selenium adequate diet, both Sepp1Δ240-361 and wild type mice display comparable kidney selenium levels (13), while kidney selenium content is reduced by 25%-35% in Sepp1Δ mice (4,5). It is possible that only the truncated isoform(s) of Sepp1 can pass into the glomerular filtrate and that they provide sufficient selenium to support proximal tubule selenoprotein synthesis.

Receptor-mediated Sepp1 uptake by the proximal tubule epithelium represents a mechanism to provide the selenium required for Gpx3 synthesis (3,8,33). Ligand blotting identified megalin as the only Sepp1-binding protein in kidney membranes, but no megalin was detected in testis membranes which agrees with published immunocytochemical data (26). Megalin is a large ~600kDa glycoprotein whose extracellular domain, which possesses four large clusters of disufide bond-stabilized ligand-binding repeats (31,34,35), retains ligand-binding activity after fractionation by non-reducing SDS-PAGE (19-21). In contrast, ApoER2, the testis Sepp1 receptor, did not retain Sepp1-binding activity by ligand blotting; the extracellular domain of ApoER2 possesses only a single cluster of ligand-binding repeats (36,37), which in the testis isoform is reduced to four repeats (11). These data raised the possibility that ligand blotting also failed to identify other Sepp1-binding proteins. However the use of megalin null mice directly demonstrates that megalin is required for proximal tubule Sepp1 uptake. MegalinΔ mice display perinatal mortality, therefore day 19 fetuses were utilized since both glomerular filtration (38) and liver expression of Sepp1 (39) is established by this embryonic stage. Sepp1 was detected in both wild type and megalinΔ/Δ kidneys of day 19 fetuses and its absence from the proximal tubule epithelium of the megalinΔ mice reflects the failure of its uptake from the glomerular filtrate. The accumulation of Sepp1 in endosomes and/or lysosomes of proximal tubules of day 19 fetuses differed from adult kidneys. This could reflect an immature lysosomal system in the fetal kidney since lysosome biogenesis in the proximal tubule epithelium is dependent upon endocytic uptake of hydrolases from the glomerular filtrate (40), and the developmental timing of formation of mature lysosomes has not been established.

A critical question has been to elucidate mechanisms by which selenium is selectively accumulated by key target organs. Sepp1 is utilized for selenium delivery to the brain, testis and kidney (4,5,11). The testis, which requires ApoER2 but does not express megalin, and the kidney, which utilizes megalin but not ApoER2, employ distinct lipoprotein receptors for Sepp1 uptake. It will be of interest to define the Sepp1 trafficking pathway in the brain, since it expresses ApoER2 and megalin as well as other lipoprotein receptors (36,41,42). Collectively these data also raise the possibility that other members of the lipoprotein receptor family expressed in a variety of organs (43,44), may also function in Sepp1 utilization and selenium homeostasis. An important unresolved question is whether the kidney, testis and brain utilize distinct Sepp1 isoforms to supply their selenium requirements or whether they compete for the same Sepp1 isoform. Future studies will examine both the binding of the Sepp1 isoforms to both megalin and ApoER2, to assess whether these lipoprotein receptors recognize distinct domains of Sepp1, and define the mechanisms generating the different Sepp1 isoforms present in blood plasma.

REFERENCES


**FOOTNOTES**

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The abbreviations used are: Sepp1, selenoprotein P; ApoER2, Apolipoprotein E receptor-2; and Gpx3, glutathione peroxidase-3.

**FIGURE LEGENDS**

**Fig. 1.** (A): Merged fluorescence images of a cryosection of wild type kidney cortex showing α-Sepp1 immunostaining (red), f-actin (green), and nuclei (blue). Sepp1 is detected at the apical surface of epithelial cells of both the proximal tubule (pt) and the urinary pole (arrow) of the glomerulus (g). No Sepp1 is detected in epithelial cells of the distal tubule (dt). Epithelial cells of the proximal tubule (pt), but not the distal tubule (dt) possess a prominent f-actin containing brush border. Sepp1 is always detected in the segments of the proximal tubules near the glomerulus, but it is not detected in all profiles of the proximal tubule (pt*). Inset shows merged phase contrast and anti-Sepp1 immunofluorescence images of glomerulus and proximal tubule. Note positive Sepp1 staining of epithelium at urinary pole (arrow) of the glomerulus (g) and proximal tubule (pt).

(B, C): Matched phase contrast (B) and fluorescence (C) images of a cryosection of the kidney cortex of a *Sepp1−/−* mouse immunostained with α-Sepp1. Note the absence of fluorescence in proximal tubules (pt) and glomerulus (g).

(D, E): Matched phase contrast (D) and fluorescence (E) images of a cryosection of the kidney cortex from a homozygous mouse expressing *Sepp1Δ240-361*. Note that, like full-length Sepp1, truncated Sepp1 also binds to the apical surface of the proximal tubule epithelium (pt).

(F, G): Matched phase contrast (F) and fluorescence (G) images of a cryosection of kidney cortex of an ApoER2−/− mouse immunostained with α-Sepp1. Note Sepp1 is localized at the apical surface of the proximal tubule (pt) epithelium, just as seen in wild type animals.
Fig. 2. SDS-PAGE and Western blot analyses of immuno-affinity purified mouse Sepp1. Lane 1 shows Coomassie blue stained gel lane showing that the purified Sepp1 fraction contains a single major polypeptide band of Mr ~50,000. Lane 2 shows Western analysis of purified Sepp1 stained with monoclonal α-Sepp1. Lane 3 shows a control lane of Sepp1 immunostained with nonimmune primary antibody. It shows no detectable band.

Fig. 3. Tissue overlay experiments identify Sepp1-binding sites in kidney cryosections of Sepp1<sup>−/−</sup> mice. Panels (A, D, G) phase contrast, (B, E, H) anti-Sepp1 immunostained (red) and (C, F, I) phalloidin stained (green), showing f-actin brush border, in images of proximal tubules. L= tubule lumen.

A-C show control overlay experiment in which cryosections were stained with α-Sepp1. No Sepp1 staining is detected in Sepp1 null animals, (B) but the proximal tubules still display a prominent f-actin brush border (C).

D-F show views of a proximal tubule from overlay experiment in which cryosections were incubated with purified Sepp1 before immunostaining. Note the intense punctate staining, representing bound Sepp1, in the proximal tubules (E, F), which localizes below the f-actin of the brush border, shown in the merged image (F).

G-I show views of a proximal tubule in which the specimens were sequentially incubated with RAP followed by Sepp1 before immunostaining. Note that pre-incubation in RAP blocks Sepp1 binding to proximal tubule epithelial cells (H).

Fig. 4. Non-reducing SDS-PAGE and blot overlay and immunoblot identification of Sepp1-binding proteins in kidney (K; lanes 1, 3, 5, 7, 9 and 11) and testis (T; lanes 2, 4, 6, 8, 10, 12) membranes isolated from Sepp1<sup>−/−</sup> mice. Coomassie blue staining reveals a high molecular weight band in kidney (lane 1) but not testis (lane 2) membrane preparations. Blot overlay analyses of lanes incubated with Sepp1 (lanes 3, 4) before immunostaining with α-Sepp1 show staining of the high molecular weight band in kidney (arrowhead) but not testis membranes. Control blot overlay (lanes 5, 6) lanes that were not incubated with Sepp1 prior to anti-Sepp1 immunostaining show no staining of the high molecular weight band. Control lanes (7, 8) immunostained using nonimmune antibody also show no immunoreactivity of the high molecular weight band. Western blots (WB) immunostained with affinity-purified rabbit anti-megalin (lanes 9, 10) or as a control, non-immune rabbit IgG (lanes 11, 12) show that the high molecular weight Sepp1-binding protein of kidney membranes reacts specifically with anti-megalin.

Fig. 5. (A, B): Phase contrast (A) and fluorescence (B) images of a cryosection of adult wild type kidney immunostained for megalin (green) and Sepp1 (red). Note that when Sepp1 is present in a proximal tubule it co-localizes with megalin at the apical surface of the epithelium.

(C, D) Phase contrast (C) and fluorescence (D) images of a cryosection of a day19 megalin<sup>+/−</sup> fetal kidney immunostained for megalin (green) and Sepp1 (red). Both megalin and Spp1 co-localize at the apical surface but an intracellular accumulation of Sepp1 within vesicular elements is also noted.

(E, F) Phase contrast (E) and fluorescence (F) images of a cryosection of a day19 megalin<sup>+/−</sup> fetal kidney immunostained for megalin (green) and Sepp1 (red). Neither megalin nor Sepp1 was detected.
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