Megalin mediates renal uptake of heavy metal metallothionein complexes

R. Bryan Klassen, Kimberly Crenshaw, Renata Kozyraki, Pierre J. Verroust, Laura Tio, Silvia Atrian, Patricia L. Allen, and Timothy G. Hammond. Megalin mediates renal uptake of heavy metal metallothionein complexes. Am J Physiol Renal Physiol 287: F393–F403, 2004. First published May 4, 2004; 10.1152/ajprenal.00233.2003.—Although several heavy metal toxins are delivered to the kidney on the carrier protein metallothionein (MT), uncertainty as to how MT enters proximal tubular cells limits treatment strategies. Prompted by reports that MT-I interferes with renal uptake of the megalin ligand β₂-microglobulin in conscious rats, we tested the hypothesis that megalin binds MT and mediates its uptake. Three lines of evidence suggest that binding of MT to megalin is critical in renal proximal tubular uptake of MT-bound heavy metals. First, MT binds megalin, but not cubulin, in direct surface plasmon resonance studies. Binding of MT occurs at a single site with a K_d ~ 10^{-9} and, as with other megalin ligands, depends on divalent cations. Second, antisera and various known megalin ligands inhibit the uptake of fluorescently labeled MT in model cell systems. Anti-megalin antisera, but not control sera, displace >90% bound MT from rat renal brush-border membranes. Megalin ligands including β₂-microglobulin and also recombinant MT fragments compete for uptake by megalin-expressing rat yolk sac BN-16 cells. Third, megalin and fluorescently labeled MT colocalize in BN-16 cells, as shown by fluorescent microscopic techniques. Follow-up surface plasmon resonance and flow cytometry studies using overlapping MT peptides and recombinant MT fragments identify the hinge SCKKSCC region of MT as a critical site for megalin binding. These findings suggest that disruption of the SCKKSCC motif can inhibit proximal tubular MT uptake and thereby eliminate much of the renal accumulation and toxicity of heavy metals such as cadmium, gold, copper, and cisplatinum.

Although neither the apoprotein nor the zinc complex appears toxic, Cd-MT is a renal tubular toxin whose damage is marked by proteinuria, glucosuria, and aminoaciduria, or in more severe cases, acute tubular necrosis or chronic renal failure (37). Conflicting reports implicate different transporters or, more likely, receptor-mediated pathways in the cellular uptake of Cd-MT (4, 5, 15, 22, 28, 47). At least some of the uncertainty concerning uptake pathways arises from the use of in vivo and in vitro models that differ significantly in their behavior. For example, while CdCl₂ is more toxic than Cd-MT to cultured rat kidney proximal tubules and LLC-PK₁ cells, Cd-MT shows greater in vivo nephrotoxic effects (26, 27, 35). Furthermore, in vivo models indicate that free cadmium and Cd-MT target different subsegments of the proximal tubule (S3 and S1/S2, respectively) (38). The lack of consensus complicates the search for a therapy for renal heavy metal poisoning. Identifying the entry step, critical to the design of protective agents, was the main objective of the research reported here.

Understanding the process of heavy metal uptake is critical in both physiology and therapeutics because heavy metals have a narrow margin between their essential or useful and their toxic levels (45). Mutations of MT are associated with several copper-storage diseases (33, 45). MT also carries the commonly prescribed therapeutics gold and cisplatinum; both of these are limited in acute dose selection and duration of chronic therapy by nephrotoxicity based in the renal proximal tubule (7, 18, 36, 42, 43). Inhibition of renal uptake is desirable to enable the broadening of dose selection and treatment duration. Similarly, cadmium turnover in the body suggests that an appropriate therapy aimed at urinary excretion, secondary to high renal proximal tubular uptake of Cd-MT, could eliminate much of the accumulation.

We hypothesize that megalin or cubulin might be involved in the uptake of heavy metal MT complexes because these scavenger receptors mediate the proximal tubular uptake of many ligands with quite different properties. Megalin binds not only proteins like β₂-microglobulin, cytochrome C, and retinol-binding protein, but also polybasic antibiotics such as gentamicin (29, 41, 48, 49). Cubulin, the other abundant proximal tubular receptor, also has diverse ligands, including many of megalin’s ligands (6). Interestingly, proximal tubular uptake of MT and of β₂-microglobulin is mutually inhibitory in conscious rats (4). Taken together with the observation that megalin mediates uptake of β₂-microglobulin, this result provided indirect evidence implicating megalin in MT uptake. As all

HEAVY METALS COMPLEXED TO METALLOTHIONEIN (MT) class I disturb many functions within the proximal tubules, but the entry route of these complexes into epithelial cells remains unknown (10, 14, 16, 23, 24, 27, 31, 44). The best-studied heavy metal at present is cadmium. Environmental and occupational exposure to cadmium are widespread but mostly chronic and low level (1). Whether ingested or inhaled, the majority of absorbed cadmium eventually complexes with MT (9, 32), which is produced by several tissues and is largely intracellular but readily detectable at low levels in the circulation. The resulting heavy metal complex Cd-MT, containing seven cadmium ions (Fig. 1), is small enough (~7 kDa) to be freely filtered through the renal glomerulus into the proximal tubular fluid, before reuptake into proximal tubular cells (15).

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these ligands are freely filtered by the glomerulus, they are available in the proximal tubule for competition with MT to act as protective agents, if we can demonstrate that inhibition observed in direct molecular interactions matches the whole animal pathophysiology. Our results, reported here, indicate that megalin binds MT and implicates the highly conserved hinge or interdomain region of MT, centered on a lysine repeat, as a critical site for binding to megalin.

MATERIALS AND METHODS

Animals, reagents, and antibodies. Male Sprague-Dawley rats (200–250 g) were obtained from Sasco (Omaha, NE). All reagents were from Sigma (St. Louis, MO) unless otherwise stated. MT-I isolated from either rabbit liver or horse kidney was used as received. The supplier-reported metal assays of MT samples show ~7% metals by mass, which indicated complete occupation of all metal-binding sites by zinc and/or cadmium. Purified human megalin and cubilin receptors were obtained by detergent solubilization of renal cortex brush-border membranes followed by affinity chromatography using immobilized receptor-associated protein (29a). Polyclonal antibodies against cubilin, megalin, and transferrin were generated against recombinant proteins (8). Anti-giantin was kindly provided by Dr. Jacques Couraud (Gif-sur-Yvette, France) (8). Anti-giantin was monoclonal antibodies coupled to Sepharose 4B (19, 29a, 39, 40). Polyclonal antibodies against cubilin, megalin, and transferrin were generated against recombinant proteins (8). Anti-giantin was monoclonal antibodies coupled to Sepharose 4B (19, 29a, 39, 40). 

Protein yield and heavy metal content of recombinant mouse metallothionein proteins

<table>
<thead>
<tr>
<th>Clone</th>
<th>Protein Concentration/Total Yield by A280</th>
<th>Zinc Content as Predicted Ideal</th>
<th>ICP</th>
<th>Zinc/Protein Ratio</th>
<th>Predicted Ideal Zinc/Protein Ratio</th>
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</thead>
<tbody>
<tr>
<td>MT full-length</td>
<td>1.18 × 10^-4 M</td>
<td>6.73</td>
<td>7</td>
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<tr>
<td>Recombinant</td>
<td>2.14 mg total</td>
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<tr>
<td>MT α-subunit</td>
<td>1.37 × 10^-4 M</td>
<td>3.93</td>
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<tr>
<td>Recombinant</td>
<td>1.58 mg total</td>
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<td></td>
<td></td>
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<tr>
<td>MT β-subunit</td>
<td>1.87 × 10^-4 M</td>
<td>3.01</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recombinant</td>
<td>2.01 mg total</td>
<td></td>
<td></td>
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</tbody>
</table>

Table 1. Protein yield and heavy metal content of recombinant mouse metallothionein proteins

F394 MEGALIN MEDIATES METALLOTHIONEIN UPTAKE
typically harsh, solvent) was necessary. The “double-referencing” technique of Myszka (30) was used to eliminate additional instrumental artifacts; the results are reflected in Fig. 2. The blank injections used for this procedure were identical to sample solutions except for the omission of MT. Thermodynamic constants were calculated using Biacore’s BIAevaluation 3.1 software.

**SPR studies of peptide inhibition.** Six 16-amino acid peptides spanning the entire MT sequence (Biosource International) were used to inhibit the binding of MT to megalin. The peptide concentrations were ~5 μM, while rabbit liver MT was ~250 μg/ml, corresponding to the ligand K_d (estimated). The SCKKSCC peptide, representing the overlap sequence between two of these peptides, was also obtained from Biosource International. Megalin was immobilized as described above. An equal amount of transferrin (0.10 μg/ml in 10 mM acetate, pH 4.96) was immobilized in a second flow cell to provide real-time reference correction. Dose-dependent peptide binding was examined by injecting the peptide at concentrations ranging from 0 to 500 μg/ml. Inhibition of MT binding by peptide was examined by injecting rabbit liver MT as described above and comparing the results to samples that contained varying concentrations of peptide but were otherwise identical. No regeneration was necessary. Additional artifacts were eliminated before curve fitting by applying double-referencing techniques. The blank injections used for this procedure were identical to sample solutions except for the omission of MT and peptide. Peptides unrelated to MT but having a central KK motif, specifically a v-ATPase β-subunit peptide with a KK motif (CLQK-FEQKINQSYEREK) and an apolipoprotein A1 peptide with KK motif (ALEEYTKKLNQT; Biosource International), served as control peptides.

**Preparation of recombinant mouse MT and α- and β-subunit and SPR analysis of binding.** Atrian has produced recombinant fragments of mouse MT successfully and reproducibly. Her approach seems more practical than site-directed mutagenesis, as most attempts to produce recombinant MT have been characterized by very low yields or by mixtures of several abortive cleavage fragments of the MT molecule (2, 20). Atrian et al. (2) solved this problem by making recombinant MT subunits in *Escherichia coli* using a GST fusion vector followed by thrombin cleavage to release the free MT subunit. The thrombin cleavage leaves three amino acids, specifically SCM derived from the COOH terminus of the GST, on the NH2 terminus of the product. To understand the data, we must be aware that we postulate the critical binding site on MT to be the intradomain SCK-KSCC region, with SCK representing the COOH-terminal end of the β-subunit and KSCC the NH2-terminal start of the α-subunit. The recombinant α-subunit, therefore, has a conservative GST-derived SCM substitution for SCK on its NH2 terminus, leaving our postulated critical SCK-KSCC sequence essentially intact. The recombinant β-subunit starts with SCM- and ends in SCK, rendering the postulated critical SCK-KSCC disrupted. The full-length recombinant MT has an intact SCK-KSCC sequence as well as an additional NH2-terminal SCM. Atomic absorption (inductively coupled plasma) analysis of the zinc content of the recombinant subunits proved them to be at the predicted heavy metal content to within the error of the methods (see Table 1). Protein concentrations were assayed by the Bradford method (Pierce Biotechnology, Rockford, IL). Recombinant mouse MT proteins, and native mouse MT as a control, were dialyzed and excess metal was removed with resin. The proteins were used for SPR analysis or redialyzed into appropriate buffers for cell uptake studies.

**Preparation of fluorophore-conjugated MT.** MT was conjugated to Alexa Fluor 594, FluorX, or Cy3 (Molecular Probes) following the supplier’s protocols. Because MT is a very small protein, unreacted dye was removed by dialysis against PBS at pH 7.4 in Slide-A-Lyzer dialysis cassettes having 3,500-kDa molecular mass cutoff (catalog no. 66330, Pierce) rather than with the use of the columns provided in the manufacturer’s kit.

**Binding of MT to rat renal brush-border membrane vesicles: inhibition by anti-megalin, anti-cubulin, and control antibodies as well as MT peptides and other ligands.** Rat cortical brush-border membrane vesicles were isolated by magnesium precipitation techniques as described previously (3, 19, 39). The binding of MT was investigated in the presence of 100- to 3,300-fold dilutions of anti-cubulin or anti-megalin polyclonal antibodies that recognize the holoprotein (3, 19, 39, 40). Antibodies to the AT_1 receptor and anti-NK_1 peptide antibodies were chosen as negative controls for nonspecific interference by binding because they bind brush-border membrane vesicles at the same titer as the anti-megalin antiserum. Binding of FluorX (Amersham Biosciences, Piscataway, NJ)-conjugated MT was analyzed by flow cytometry using a FACStar Plus flow cytometer (Becton Dickinson Immunocytochemistry, San Jose, CA) to collect data files of 2,000 observations/sample. All antisera were used at 1:1,000 dilutions, which represented peak binding on dilution curves. Synthetic peptides were used at concentrations of 400 μg/ml, which was enough to inhibit significantly the binding of MT when observed by SPR. For consistent comparison, the known megalin ligand β2-microglobulin was also used at 400 μg/ml.

**Cell culture studies.** Except as noted, experiments were conducted using immortalized yolk sac cells from the Brown Norway rat (BN-16) (25). An apical brush border and a specialized endosomal pathway

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**Fig. 2.** SPR analysis of the dose-dependent binding of MT to megalin. Rabbit kidney MT in HEPES-buffered saline (HBS) containing 2 mM Ca and Mg. A (from bottom to top): traces representing responses obtained with 75, 150, 300, 600, 1,200, and 2,400 μg/ml MT. B: fit of the maximum responses obtained after 2.5 min. The double-referencing method of Myszka (30) was used to eliminate artifacts in the data. RU, resonance units.
similar to the renal proximal tubule, including abundant expression of megalin and cubulin, characterize these cells. The cells were grown in DMEM (GIBCO/Invitrogen, Carlsbad, CA) supplemented with 10\% fetal calf serum and 50 \( \mu \)g/ml streptomycin or ciprofloxacin. Cells were passaged every 4 days with a split ratio of 10:1. Madin-Darby canine kidney (MDCK) cells were grown in a modified minimal essential medium as described in American Type Culture Collection (Manassas, VA) protocols.

**MT uptake by BN-16 cells analyzed by epifluorescence and confocal microscopy.** Uptake experiments were performed with confluent monolayers cultured in eight-chamber glass slides (Nalge Nunc, Naperville, IL). The BN-16 cells were cultured on chambered slides until confluent (~10–18 h). The monolayers were washed twice with cold PBS and allowed to equilibrate at 4\°\C in a cold room. The labeled MT in DMEM containing 0.01\% ovalbumin was added at concentrations ranging from 0.075 to 12 \( \mu \)M. After incubation at 37\°\C for 20 min, the medium was removed and the cells were washed successively with PBS/0.1\% ovalbumin (2\times) and PBS before being fixed and mounted. The slides were examined by use of a fluorescence microscope (Leica DMR, Basel, Switzerland) equipped with a color video camera (Sony 3CCD). The experiment was used to select a concentration of 1.0 \( \mu \)M for subsequent experiments involving the labeled ligand. In a time-dependent uptake experiment using labeled MT, cells were incubated as before but incubated with 1.0 \( \mu \)M ligand for intervals of 5, 15, 30, and 45 min. In receptor colonization experiments, the cells were permeabilized with Triton X-100 (0.05\% in PBS) and treated with the appropriate primary and secondary antibodies after fixation. The primary antibodies included anti-megalin, anti-cubulin, anti-TIR, and anti-giantin. To follow the internalization of MT, Alexa-labeled MT was added at concentrations of 1.0 or 6.0 \( \mu \)M and the cells were incubated in the cold for intervals ranging from 5 to 45 min before being fixed. Based on these experiments, confluent monolayers were washed with PBS and allowed to equilibrate in a cold room with labeled MT (2.5 \( \mu \)M) for 1 h at 4\°\C. After being washed with PBS, the cells were treated with warm DMEM containing 2.5 \( \mu \)M unlabeled MT and 0.01\% ovalbumin and immediately transferred to an incubator. Cells were fixed at intervals of 5, 15, and 45 min. Finally, the cells were permeabilized and incubated with the appropriate primary and secondary antibodies to localize megalin, cubulin, and TIR.

**MT uptake by MDCK cells analyzed by confocal microscopy.** MDCK cells were cultured on chambered slides until confluent (~2 days). The monolayers were washed twice with PBS and treated with labeled MT in DMEM containing 0.01\% ovalbumin. The labeled MT was added at a concentration of 1.0 \( \mu \)M. After incubation at 37\°\C for 30 min, the medium was removed and the cells were washed successively with PBS/0.1\% ovalbumin (2\times) and PBS before being fixed and mounted. To assist in visualization of the cells, some samples were permeabilized with Triton X-100 (0.05\% in PBS) and stained with DAPI. After a preliminary examination with a fluorescence microscope as described above, confocal microscopy was carried out with a Leica TCS equipped with a DMR inverted microscope and a 63/1.4 objective. Image processing was performed with the use of the Leica’s online Scanware software. Numeric images were processed with the use of Scion Image and Photoshop 5.0 software.

**MT uptake by BN-16 cells analyzed by flow cytometry.** Uptake experiments used FluorX- or Cy3-labeled MT and were performed with confluent monolayers cultured in 96-well plates. In preliminary experiments, we determined that MT uptake was linear for at least 3 h and exhibited dose-dependent saturation. The concentration producing half-maximal uptake was ~5 \( \mu \)M. Uptake experiments were performed as follows. The confluent monolayers were washed with serum-free DMEM and allowed to equilibrate for 2 h at 37\°\C. The cells were then incubated with 5 \( \mu \)M labeled MT and any inhibitor for 1–2.5 h at 37\°\C. Incubations were performed in DMEM containing 0.1\% ovalbumin to reduce nonspecific binding. The cells were washed several times with PBS, acid-washed to release membrane-bound proteins, released with trypsin, and washed several more times with PBS. In this state they could be analyzed immediately, without fixing, by flow cytometry analysis. The positive control was labeled MT without added inhibitor; the negative control was unlabeled MT. Inhibitor concentrations were generally 10–100\times greater than the concentration of labeled MT.

**RESULTS**

We present several lines of evidence indicating that megalin is the receptor responsible for the uptake of Cd-MT in the proximal convoluted tubules.

**Molecular studies of receptor-MT binding using SPR.** We studied cubulin and megalin separately by using SPR, immobilizing purified membrane-free samples of each receptor, and studying its interaction with rabbit liver MT. The dose-dependent binding to megalin is shown in Fig. 2A. The responses uniformly increased with dose over a 32-fold increase in concentration, 75–2,400 \( \mu \)g/ml. The observed variations and noise are normal for the very low signal levels used to optimize a study of binding kinetics. Even at high MT concentrations, >90\% saturation was not achieved, and therefore some errors occurred in the fit. An approximate fit using the maximum (but nonequilibrium) responses obtained at each concentration yielded an estimated dissociation constant of 9.8 \( \times 10^{-5} \) M (Fig. 2B). Repeated experiments consistently indicate the binding of ~0.7–0.9 mol of MT/mol of megalin, consistent with one binding site. In contrast, no binding of MT to cubulin was observed.

The binding shown in Fig. 2 was specific for megalin and depended on metal ions but not on the MT source. Omitting either Ca or Mg from the sample buffers abolished the binding (data not shown); both appeared to be required. Samples of MT from horse kidney and from rabbit liver provided nearly identical results (data not shown).

Interestingly, oligomerized MT bound more effectively to megalin than did the monomer. Nondenaturing gel electrophoresis showed that over time, MT forms trimers, tetramers, and even much larger oligomers (data not shown). The binding of such molecules to megalin was significantly stronger. Owing to difficulties in purifying these oligomers, the actual binding constants for oligomers could not be determined with any precision. Qualitatively, compared with monomeric MT, oligomeric MT dissociated much more slowly, and harsher conditions were required to dislodge it from immobilized megalin. Using the tetramer as a basis for calculations, one may estimate a 100-fold change in \( K_d \) (7 \( \times 10^{-7} \) M).

**Inhibition of MT binding by megalin ligands and small peptides derived from MT.** When using SPR, we observed no reproducible inhibition of MT binding to megalin by known megalin ligands but did observe inhibition by some synthetic peptides corresponding to sequences within MT. Commercial sources of \( \beta_2 \)-microglobulin dissociated only with difficulty from the immobilized megalin, leading to erratic, nonreproducible binding, and loss of binding of control ligands after the harsh regeneration modalities necessary. For this reason, SPR
Table 2. Binding to megalin by peptides derived from MT and interference with binding of the native protein using SPR techniques

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Binding to Megalin</th>
<th>Competition With MT for Megalin Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KMDPNCSCATGNSCTCA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>GNSGCGSSCKCKECKC</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>CCKCKKEKCTSCCKSCC</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>SCKKSCCSCPAGCTK</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>CPAGCTKCAEGCGICCKG</td>
<td>Insoluble; no data</td>
<td>Insoluble; no data</td>
</tr>
<tr>
<td>6</td>
<td>CAQGCGICGKASDKSCCA</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Results obtained by injecting synthetic peptides in HEPES-buffered saline (HBS) containing 2 mM Ca and Mg are shown. To locate a peptide sequence within native MT, see Fig. 1.

Assessment of competitive β2-microglobulin binding with MT was impractical.

We prepared a series of peptides spanning the sequence of rabbit liver MT and used SPR to study their effect on the binding of MT to megalin (Fig. 1). Six 16-amino acid peptides, each overlapping its neighbors by 7 amino acids, were prepared as shown in Fig. 1 (21). The results of these initial qualitative studies are summarized in Table 2. Interestingly, peptides 3 and 4 bound quite tightly to megalin and also disrupted the binding of MT. Although peptides 1, 2, and 6 contain cysteines, they did not bind megalin, suggesting that the binding of peptides 3 and 4 is a specific interaction, rather than a nonspecific disulfide interaction between the peptides and megalin. Technical issues prevented direct confirmation; reduction with DTT denatured megalin and abolished the binding of all ligands. Because the behavior of peptides 3 and 4 differed significantly from that of the other soluble peptides, we turned our attention to the overlap sequence these peptides have in common.

A peptide representing this overlap sequence, SCKKSCC, bound to megalin and also disrupted the binding of native MT (Table 3; Fig. 3). The dose-dependent binding of this peptide to megalin is shown in Fig. 3A and Table 3. The ability of the peptide to affect the binding of MT to megalin is apparent in Fig. 3B, in which the binding of MT decreased when coinjected with peptide. In contrast, peptides containing a lysine repeat but derived from unrelated ATPase or apolipoprotein A-I sequences had no apparent effect, producing instead responses that were essentially additive (Table 3). The polybasic megalin ligand gentamicin bound megalin with an affinity much lower than MT and showed no interference with MT binding (Table 3).

 SPR analysis of binding to megalin shows that, when corrected for the molecular mass of the protein fragments (n = 2 for each analysis), the recombinant full-length MT clone bound 95% as well as the native protein, the α-subunit with an intact conservatively substituted SCKKSCC region, also bound ~94% as well as the native MT (see Table 4). However, the β-subunit in which the SCKKSCC region is divided at KK has binding reduced to 30% of the predicted value (see Table 4).

Table 3. Binding to megalin by polybasic peptides and gentamicin and interference with binding of native MT using surface plasmon resonance techniques

<table>
<thead>
<tr>
<th>Peptide or Reagent</th>
<th>Sequence</th>
<th>Binding to Megalin Compared With MT</th>
<th>Effect on MT Binding to Megalin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT sequence overlap of peptides 3 and 4*</td>
<td>SCKKSCC</td>
<td>Affinity ~ MT</td>
<td>Competitive with MT binding</td>
</tr>
<tr>
<td>v-ATPase β-subunit peptide with KK motif</td>
<td>CLQKFEK11QSPYKRR</td>
<td>Affinity &lt;&lt; MT</td>
<td>Additive to MT binding</td>
</tr>
<tr>
<td>Apolipoprotein-A-1 peptide with KK motif</td>
<td>ALEETYKXLNTQ</td>
<td>Affinity &lt;&lt; MT</td>
<td>Additive to MT binding</td>
</tr>
<tr>
<td>Gentamicin</td>
<td></td>
<td>Affinity &lt;&lt;&lt;&lt; MT</td>
<td>Additive to MT binding</td>
</tr>
</tbody>
</table>

Results obtained by injecting synthetic peptides in HBS containing 2 mM Ca and Mg are shown. *See Table 2.
Table 4. Binding to megalin by recombinant proteins derived from mouse MT and interference with binding of the native protein using surface plasmon resonance techniques

<table>
<thead>
<tr>
<th>Clone</th>
<th>% Predicted Binding by SPR</th>
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<tr>
<td>Native full-length MT</td>
<td>100 (Positive control)</td>
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<tr>
<td>MT full-length recombinant</td>
<td>95</td>
</tr>
<tr>
<td>MT α-subunit recombinant</td>
<td>94</td>
</tr>
<tr>
<td>MT β-subunit recombinant</td>
<td>30</td>
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</table>

Results obtained by injecting recombinant proteins in HBS containing 2 mM Ca and Mg are shown. SPR, surface plasmon resonance. To locate a protein sequence within native MT, see Fig. 1.

Protein-receptor binding in membrane vesicles and displacement by antibodies studied by flow cytometry. The binding of fluorescent MT to vesicles was readily detected (Fig. 4). The addition of anti-megalin antibodies was able to displace nearly all bound MT (no antibody, MT binding 161 ± 4 fluorescence units, n = 5; anti-megalin antibodies 14 ± 5, n = 5, P < 0.01 by ANOVA and Scheffé, as well as on each individual run by Kolgomorov-Smirnov) (43). Antibodies to cubilin had a small but significant effect on binding (146 ± 5, n = 5, P < 0.05 by Kolgomorov-Smirnov compared with no antibody). However, antisera to the unrelated NK1 receptor (a negative control) had little or no effect (150 ± 21, n = 5, P > 0.05 compared with no antibody).

Protein-receptor binding in membrane vesicles and displacement by peptides and ligands studied by flow cytometry. The binding of fluorescent MT to freshly prepared rat brush-border membrane vesicles was readily detected [3 ± 1 arbitrary fluorescence units in unstained control vesicles, compared with 534 ± 180 when vesicles were labeled with fluorescent MT, n = 6, means ± SD] (Fig. 5). The addition of peptide 2, being amino acids 10–28, at equimolar concentrations to the fluorescent MT reduced MT binding to 434 ± 156 (P < 0.05 by ANOVA and Scheffé, as well as on each individual run by Kolgomorov-Smirnov) (50), with further reduction to 336 ± 97 when fluorescent MT competed with equimolar overlap peptide SCKKSCC, being amino acids 28–34 (P < 0.01). The known megalin ligand β2-microglobulin, which has been demonstrated to compete with MT in live rat studies (4), competed with fluorescent MT at equimolar concentrations (394 ± 128, n = 6, P < 0.05), whereas antibodies to the unrelated AT1 receptor (a nonspecific control) had little or no effect (513 ± 157). Fluorescently conjugated MT competes directly with equimolar unlabeled MT (398 ± 114, n = 6, P < 0.05 compared with MT; no competition). Solubility limitations prevented study of higher competing concentrations of peptides.

Cell culture studies by fluorescence microscopy. Confluent monolayers of BN-16 cells were incubated with fluorescently labeled MT at 37°C. After 30 min, fluorescence microscopy revealed that much MT could be found in the cells in a granular form, consistent with MT uptake into endosomes. To follow cellular uptake more closely, we incubated BN-16 cells with labeled MT at 4°C and chased with unlabeled MT for variable intervals. At 4°C, MT bound to the surface but did not enter the cells, whereas incubation at 37°C afterward led to uptake. Colocalization with antibodies to the transferrin receptor, an early endosomal marker, indicated that MT entered the early endosomes within 15 min but passed beyond them in <45 min.

Colocalization of MT with both megalin and cubilin was demonstrated by using receptor antibodies in conjunction with fluorescent secondary antibody. At 4°C, megalin, cubilin, and MT were colocalized on the surface, whereas after 15 min at 37°C they had all migrated to the early endosomes (Fig. 6, A–F). After 45 min, little evidence for colocalization remained (data not shown). No colocalization was observed with antibodies to giantin, an unrelated protein found in the Golgi apparatus and used as a negative control (data not shown).

Using confocal microscopy, results with this higher resolution method for colocalization confirmed that MT and megalin...
or cubilin were colocalized. In Fig. 6, G–I, one can see similar patterns of distribution for fluorescent MT and antibody. As a negative control, MDCK cells were examined for evidence of MT uptake. These cells do not express cubilin or megalin, and in fact we found that they did not import MT at all, demonstrating that ordinary membrane diffusion (of free dye or of conjugated MT) cannot explain our results with BN-16 cells.

MT uptake in cultured cells and inhibition by antibodies, ligands, and peptides studied by flow cytometry. Consistent with a receptor-mediated process, MT uptake may be saturated, inhibited by receptor ligands and by MT model compounds, and inhibited by receptor antibodies.

To determine the cellular uptake of fluorescently labeled MT and inhibition by known megalin ligands, we began with dose-and time-dependent uptake studies. Incubation of BN-16 cells with 0–80 μM fluorescently labeled MT for 3 h, followed by flow cytometry analysis, demonstrates that MT uptake is saturable and that MT concentrations of 4–5 μM produce half-maximal uptake. Under these conditions, the uptake of labeled MT was easily distinguished against background signal (Fig. 7A). Uptake of MT by BN-16 cells was then demonstrated to be linearly time dependent at doses above and below the half-maximal binding concentration (Fig. 7B). The addition of β2-microglobulin reduced MT uptake in a dose-dependent manner across a broad range of concentrations of both MT and β2-microglobulin (Fig. 7C).

Incubating BN-16 cells with anti-megalin antibodies before adding fluorescent MT greatly reduced the uptake of MT in a dose-dependent manner (Fig. 8A) (unstained cells geometric means 5 ± 0, increases to 132 ± 18, n = 5, P < 0.05, means ± SD, with antimegalin antisera at 1:100 dilution 17 ± 2, at 1:330 dilution 40 ± 3, at 1:1,000 dilution 90 ± 2, n = 5, P < 0.05). At the same titer, anti-cubilin antiserum had a smaller although significant effect (at 1:100 dilution 48 ± 7, at 1:330 dilution 96 ± 6, at 1:1,000 dilution 116 ± 12, n = 5, P < 0.05). AT1 antiserum, in contrast, had no effect (at 1:100 dilution 161 ± 14, at 1:330 dilution 149 ± 9, at 1:1,000 dilution 111 ± 19, n = 5, P > 0.05). Antibodies against megalin and cubilin had an additive effect (at 1:100 dilution 7 ± 4, at 1:330 dilution 23 ± 1, at 1:1,000 dilution 62 ± 5, n = 5, P < 0.05 against both anti-megalin and anti-cubilin alone). The effect of antibodies on MT uptake was not observable unless ovalbumin was used to reduce nonspecific effects.

The effect of the synthetic MT-derived peptides (Fig. 1) on MT uptake could be observed when nonspecific binding was...
carefully excluded. The addition of ovalbumin, which reduces the nonspecific binding of proteins to BN-16 cells (48), unmasked the differential effects of these peptides on the uptake of MT. The greatest effect was produced by peptides containing the KK sequence of the interdomain region of MT: peptides 2 and 4 and the SCKKSCC overlap peptide reduced binding

Fig. 7. Flow cytometry analysis of the dose dependence, time dependence, and competitive uptake of fluorescently labeled MT into BN-16 cells. A: concentration of MT producing half-maximal uptake is 4–8 μM. In view of this data, all later experiments used at least 4 μM MT (10 μM preferred when reagents were not limiting). B: nearly linear uptake of MT-FLUORX was observed over 3 + h at 2 doses. As a result, uptake experiments with antibodies and MT-Cy3 were performed for 1–2 h. C: known megalin ligand β2-microglobulin displayed dose-dependent interference with MT uptake over a broad range of concentrations of both β2-microglobulin and MT.

Fig. 8. Flow cytometry analysis of antibody, peptide, and recombinant (recomb) protein inhibition of uptake of fluorescently labeled MT into BN-16 cells. A: anti-cubilin antisera inhibited MT uptake into BN-16 cells in a concentration-dependent manner. The effect of anti-megalin (meg) antiserum was far greater than anti-cubilin (cub) antiserum; the 2 sera produced an additive effect. Anti-AT1R antiserum, which also binds BN-16 cells, was used as a nonspecific binding control but had no effect on MT uptake. B: peptide 4, containing the overlap sequence SCKKSCC, inhibited MT uptake, as did the overlap sequence itself; in contrast, peptide 2, distant from the overlap sequence but with heavy cysteine content, did not affect MT uptake. Concentrations of all peptides were 100 μM. C: recombinant (recomb) full-length mouse MT inhibited the uptake of fluorescently labeled MT by BN-16 cells, as did the α-subunit carrying the intact SCKKSCC motif; the β-subunit, in which this motif is disrupted, was far less effective at inhibiting MT uptake.
MEGALIN MEDIATES METALLOTHIONEIN UPTAKE

DISCUSSION

This study provides three lines of evidence that megalin binds MT and that megalin is by far the most quantitatively important mechanism of MT uptake into the renal proximal tubule. First, SPR directly demonstrates binding of the purified proteins in a dose-, ion-, and pH-dependent manner. Second, antibody interference experiments show that >90% of the MT binding on brush-border membrane vesicles, and cellular uptake into BN-16 cells, can be displaced or inhibited specifically with anti-megalin, but not control, antisera. Finally, megalin and MT colocalize at the cellular level in fluorescent microscopy studies. Megalin and MT colocalize and internalize concomitantly before separating in the late endosomal pathway.

These studies used commercially available MT-I, a highly conserved mammalian isoform. All known class I sequences contain 61 or 62 amino acids with 20 conserved cysteine residues and are able to bind up to 7 equivalents of divalent metal ions (20), commonly a mixture of zinc and cadmium. Although Zn-MT and Cd-MT differ dramatically in their toxic effects, they produce virtually identical profiles in their binding and uptake (13). In solution, MT tends to form oligomers (46). Therefore, like other investigators (38) we used MT as received, rather than saturated with cadmium in an extra step, to maximize the structural integrity of the metalloprotein during our analyses. The inclusion of recombinantly expressed MT samples in our analysis is therefore an important control for any impurities in the commercial reagents.

The new results are consistent with the binding properties and physiological observations of megalin and its ligands in other systems. The dissociation constant estimated at 9.8 × 10⁻⁵ M may appear small for a receptor-ligand interaction, but it is similar to values obtained for other known megalin ligands (17). The calcium dependence of MT binding is also consistent with similar ion requirements of other megalin ligands (6, 29a), but the dependence on magnesium is unusual. We report competition between β₂-microglobulin and MT binding to megalin, which confirms earlier observations that MT and β₂-microglobulin compete directly for renal uptake in live animals and now explains such competition in terms of megalin binding.

The molecular, physical, and chemical properties of MT were important drivers in our approach to this analysis. MT is so cysteine rich that it is easily oxidized (21). Many investigators use a reducing agent such as DTT in solution with MT to overcome this problem (2, 13, 21). In preliminary experiments, we found that DTT potently denatures megalin, abolishing the binding of known megalin ligands. This finding necessitated the continual use of an internal control, having cysteine content similar to active peptides, to correct for the nonspecific effects of sulfhydryl binding. The ability of peptide 2 partially to inhibit binding of MT to brush-border membranes, but not uptake in BN-16 cells, may fall into this category.

Our initial analysis of the MT/megalin binding site used a peptide library, because the midregion of the MT molecule models to a linear peptide with little secondary or tertiary structure (2, 20). Comparison of protein isoforms from different species has, on occasion, provided clues to critical binding sites, but MT is so highly conserved across species and even phyla that this option was not open for the current analysis (2, 20). Even the naturally occurring isoforms of MT from Ia and Ib, through II, III, IV, V, and others, are sufficiently similar to be of little assistance in defining binding sites (2, 20). Furthermore, our enthusiasm for site-directed mutagenesis and expression of mutated MT isoforms was greatly diminished by numerous investigators who report extremely low yields and splice variants when attempting to express MT (2). After the peptide series implicated the interdomain of MT as a binding site, S. Atrian’s recombinant MT subunits, which disrupt the candidate binding motif, provided further evidence for this hypothesis. Recombinant production of MT fragments dividing MT at the lysine-lysine hinge yields intact α- and β-subunits that still bind heavy metals (2). The failure of non-MT-derived peptides with a central KK motif to inhibit megalin-MT interaction in direct SPR studies suggests that this interaction requires more than simply the charge cloud of the KK motif. Studies using site-directed mutagenesis have established the critical role of the conserved lysine repeat in the detoxification function of MT in yeast (11, 12). Replacement of one or both lysines in the hinge or interdomain region is inconsequential to the structure and function of MT unless both substituted residues are unchanged (11). However, our observations of charged peptides and the highly charged polybasic antibiotic gentamicin suggest more structural requirements than simply charge for a molecule to interfere with the megalin-MT interaction.

Our initial dissection of the site in the MT sequence critical for binding to megalin clearly implicates the hinge region. In lower species, MT exists as two separate molecules, binding three and four heavy metal moieties (21). However, in mammals and other higher organisms the two molecules have coalesced, joined by a hinge region centered on a highly charged lysine repeat. The hinge region sequence SCKKSCC is even more heavily conserved than the rest of the MT sequence, being identical in virtually all known mammalian species, and all the various MT isoforms in each species (20, 21). This fact would explain our own observations that diverse MTs bind megalin with the same kinetics and may be impor-
tant to ensure efficient reuptake of diverse isoforms in the proximal tubule.

While the data are consistent with megalin being the predominant uptake mechanism for MT, we cannot exclude a role for other pathways, especially a role for cubilin. The antibody binding data on both brush-border membrane vesicles and BN-16 cells shows an effect of anti-cubilin antiserum on MT binding and uptake. Megalin is a molecular chaperone for cubilin (48, 49), so the colocalization studies, not surprisingly, demonstrate colocalization of MT with both cubilin and megalin during the early steps of internalization and uptake. The only data we collected against a role for cubilin in MT binding and uptake are our direct studies of molecular interactions using SPR techniques. Although other known ligands of cubilin bound in control studies, we cannot be certain that partial denaturation of cubilin, which is inevitable during its purification, masks binding. It remains entirely possible that cubilin also plays a role in MT uptake in the proximal tubule of the kidney and other cubilin/megalin-expressing epithelia such as in the placenta.

The different roles of intracellular and circulating MT have created much confusion about the role of MT in cytotoxicity (26, 32, 36). Several lines of evidence suggest that increased intracellular MT is a scavenger for heavy metals, providing protection against the effects of free heavy metals (15, 16, 32). This is one basis for the practice of administering bismuth to induce tissue MT clinically, before administration of the heavy metal-based chemotherapeutic agent cisplatinum (16). In contrast, conjugating heavy metals such as copper and cadmium to MT not only changes the nephron sites of toxicity but also greatly enhances the nephrotoxic effect of these agents (33, 38). Based on our new observations, it may be possible to administer cisplatinum on a mutated MT, which does not bind megalin, and avoid some of the therapy-limiting nephrotoxicity of this group of anticancer agents. It remains to be seen whether such a reagent would still be taken up by cancer cells and maintain clinical efficacy, but there is some evidence for MT uptake in diverse tumor lines (1, 31) and no evidence for expression of megalin (48, 49).

Megaline has 4 binding sites, and cubilin at least 27 domains and 8 EGF repeats as binding sites, and yet these two proteins are thought to be largely responsible for the reabsorption of an immense volume of diverse ligands in the proximal tubule (41, 48, 49). Given the long list of ligands for megalin, and the abundance of these proteins in the glomerular filtrate, the effectiveness of the uptake likely relies on the very large content of megalin in the kidney (49). On simple SDS-PAGE, gels of renal proximal tubular brush borders, it is apparent that by far the two most abundant proteins are the distinctive 460- and 600-kDa molecular masses of cubilin and megalin (25, 48). When one combines the abundant expression of megalin with the large surface area created by brush-border formation, there is abundant megalin to facilitate reabsorption of all available ligands (48, 49).

In summary, this study provides three lines of evidence that megalin binds MT and that this is the predominant mechanism of uptake of MT and its conjugated heavy metals in the kidney. The hinge region of MT, based around the highly conserved lysine repeat, is one critical peptide sequence for the MT-megalin binding interaction. MT fragments and mutants truncating or altering the hinge region may prevent megalin-mediated renal uptake of conjugated heavy metals and secondarily diminish or abolish heavy metal renal tubular damage.

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MEGALIN MEDIATES METALLOTHIONEIN UPTAKE


