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Conformation and Glycosylation of a Megalin Fragment Correlate with Nephritogenicity in Heymann Nephritis

Alfonso Tramontano and Sudesh P. Makker

Active Heymann nephritis (AHN), a rat model of autoimmune glomerulonephritis, is induced by immunization with autologous megalin, a 600-kDa cell surface glycoprotein isolated from crude renal extracts. Recombinant proteins containing a 563-residue N-terminal sequence of megalin were obtained from Escherichia coli and baculovirus-insect cell expression systems. Rats immunized with the soluble, secreted protein encoded by a baculovirus construct elicited high titer anti-megalin autoantibodies and developed glomerular immune deposits and elevated proteinuria consistent with AHN. Rats treated with the bacterial or nonsecreted insect cell proteins produced a milder anti-megalin response and did not develop the disease. Nephritogenicity appeared to correlate with conformational or other structural features of native megalin. All three recombinant proteins were reactive in Western blots with rabbit anti-megalin antiserum, whereas the insect cell-derived proteins reacted preferentially in Western blot and ELISA with anti-megalin autoantibodies from rats with AHN induced by native megalin. Only the secreted insect cell product was stained in a lectin blot, suggesting its specific glycosylation. These observations provide evidence that a megalin N-terminal domain includes B and T cell epitopes sufficient for a pathogenic autoimmune response and that a native-like conformation and glycosylation are essential for the induction of disease. The importance of conformational B cell epitopes for pathogenic autoantibodies recapitulates observations made in other models of organ-specific autoimmune disease. Glycosidic modifications could influence the presentation of either B or T cell epitopes in AHN, consistent with emerging evidence of the role of post-translational modifications in pathogenic autoimmune responses.


Abbreviations used in this paper: AHN, active Heymann nephritis; B max, saturation binding fraction; ID, immune deposit; LBD, ligand binding domain; MDR, multiplicity of infection; nM60, recombinant N-terminal megalin polyepitope spanning residues 1–563 produced intracellularly in SF9 cells; nM60s, the same polyepitope produced and secreted from High Five cells; nM60GST, the corresponding polyepitope expressed as a GST fusion protein in bacteria; NFM, nonfat dry milk.

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The functional significance of megalin in renal physiology remains unclear, although it is underscored by the low m.w. tubular proteinuria observed in megalin-deficient mice and the severe pathology associated with AHN. It is known to bind >30 different small protein ligands and has been suggested to serve as an endocytic receptor for many of these (19, 20). A potential function of megalin in the kidney could be to provide a mechanism for the tubular clearance of proteins filtered in the glomeruli.

The primary sequence of megalin identifies it as a member of the LDL receptor family (7). The size, structural complexity, and extensive glycosylation of the intact molecule present serious challenges for an analysis of its immunogenic and nephritogenic determinants. Limited structural information is available to suggest immunologically relevant features of the megalin extracellular region. However, the modular architecture of four ligand binding domains (LBD), each comprised of epidermal growth factor repeats and separated by YWTD spacer regions, suggests the possible display of homologous determinants for Ab binding. In the

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passive model of Heymann nephritis, each of the LBD presents epitopes for Abs that induce ID (21). It has been suggested that the receptor-associated protein, with which megalin intimately associates, is also a target of pathogenic autoantibodies (22–24). Recombinant polypeptides representing megalin domains or smaller structural elements have been employed in studies aimed at characterizing these determinants (21, 25, 26). In some cases the recombinant peptides can bind to AHN autoantibodies and can also induce Abs in the rat that deposit in the kidney (25, 26). However, none of these derivatives has been shown to induce full-blown disease in the AHN model.

Recently, a fragment of ~60 kDa corresponding to a region from the megalin N terminus, obtained by limited proteolysis of native megalin, was shown to be as effective as the whole protein for inducing AHN (27). To verify these data and to further characterize the small megalin derivative, we have cloned the megalin N-terminal domain and expressed recombinant forms of the fragment in two expression systems. In this study we show that a protein expressed in insect cells from a secretory baculovirus construct induces a strong anti-megalin response leading to AHN, whereas the corresponding nonsecreted insect cell product or GST fusion protein expressed in bacteria does not produce disease. All three forms present an identical megalin primary sequence, but appear to differ in their disulfide structures, conformations, or post-translational modifications. These differences could suggest structural features of B or T cell determinants that prime the autoimmune response. The potential to drive a diversified anti-megalin response with a small fragment of the autoantigen has further implications for the mechanism of propagating the nephritogenic response.

Materials and Methods

Abs and proteins

Native megalin was purified from rat kidney as previously described (5). Antisera containing autoantibodies to megalin were collected from Lewis rats immunized with purified megalin according to the reported procedure for induction of AHN (27). Rat anti-megalin autoantibodies in glomerular ID were obtained from AHN rat kidney by acid elution as described previously (13). Rabbit anti-megalin antiserum was obtained by standard immunization as previously described (28). HRP-conjugated rabbit anti-rat and goat anti-rabbit Abs and lectin (Con A) were obtained from Sigma–Aldrich (St. Louis, MO). FITC-labeled goat anti-rat IgG and FITC-labeled goat anti-rat C3 complement Abs (Cappel Laboratories, Cochranville, PA) were used for direct immunofluorescence microscopy. HRP-conjugated goat anti-GST and anti-His6 mAb were obtained from Amersham Pharmacia Biotech (Piscataway, NJ) and BD Biosciences (Palo Alto, CA), respectively. Protein concentrations were determined by modified Bradford assay (Bio-Rad, Hercules, CA).

Cloning and expression of a 563-residue polypeptide from the megalin N terminus

A 1692-bp megalin sequence was obtained by PCR amplification of a rat kidney cDNA library (Clontech Laboratories, Palo Alto, CA) using primers CGGATCCGCGCAAGAATGCGGCAGTGGGAATTTTC and CCTTCTGCAATTCCGTGCTATGCTGAG and sequenced to the 5′ end and the internal site, respectively, of the megalin gene. The fragment was purified, digested with BamHI and EcoRI, and cloned in pGEX-3X (Amersham Pharmacia Biotech) to provide the in-frame construct pGEX-nM60 for expression of the recombinant protein was induced by adding isopropyl-β-D-thiogalactoside (1 mM) to a culture in log phase growth. After 5 h cells were collected and lysed by sonication in 10 mM Tris-HCl, pH 8.5, containing 1 mg/ml lysozyme. The insoluble fraction was collected by centrifugation, washed with PBS, and lyzed by sonication. The insoluble fraction was collected by centrifugation, washed with PBS, and resuspended in PBS/0.05% Tween. The material was used as such for immunizations.

A second baculovirus construct for expression of a secreted form of the 60-kDa fragment was obtained by cloning in the transfer plasmid pBAC-3 (Novagen, Madison, WI) providing a gp64 signal sequence and His6 affinity tag. A DNA sequence obtained by amplification of pGEX-NM60 with 5′ primer TCCCCGCGGACACAGAATGCGGCAGTGGGAA and 3′ primer TCCCCGCGGACACAGAATGCGGCAGTGGGAATTTTC resulted in a 60-kDa fragment was obtained by cloning in the transfer plasmid pBAC-3. Recombinant baculovirus Bac-nM60i was prepared by cotransfecting SF9 cells with linearized BacPAK6 and isolation of viral plasmid as before. Viral stock was used to infect SF9 cells at an MOI of 10, and after 4 days the cells were collected, washed with PBS, and lysed by sonication. The soluble fraction was collected by centrifugation, washed with PBS, and resuspended in PBS/0.05% Tween. The material was used as such for immunizations.

Immunoblot and lectin blot analysis of recombinant megalin fragments

Proteins expressed in E. coli or in insect cells were separated by SDS-PAGE using precast Tris-glycine gels with a 4–16% acrylamide gradient (Bio-Rad). Proteins were then transferred to Immobilon-P (Millipore, Billerica, MA) or nitrocellulose membranes for 3 h at 75 V at 4°C, and the membrane was blocked with TBS containing 3% nonfat dry milk (NFM) for 1 h. The gel or blot was incubated with rabbit anti-megalin or rat anti-megalin antiserum (diluted 1/2,000 and 1/200, respectively, in TBS containing 3% NFM/0.05% Tween) for 2 h at 4°C. The blot was then washed and developed using goat anti-rabbit or goat anti-rat-HRP conjugates (Southern Biotechnologies, Birmingham, AL) at 1/10,000 dilution in TBS/Tween and developed with ECL substrates (Amersham Pharmacia Biotech). Anti-tag blots were blocked as described above and incubated in the presence of anti-GST (1/2,000) and anti-His6 mAb HRP conjugates diluted 1/2,000 or 1/10,000 respectively, in TBS-Tween. For lectin blotting the membranes were incubated with Con A-HRP, diluted 1/10,000 in TBS/Tween, NFM, and 2 mM α-D-methylmannopyranoside for 4 h at room temperature. The blots were then washed and developed for ECL detection as described above.

Induction and assessment of AHN

Female Lewis rats were injected intradermally with 100 μg of the recombinant protein in 100 μl of PBS emulsified 1/1 in CFA. Two booster immunizations (50 μg) were given by the same route at 4-wk intervals. Blood and urine were collected from rats at 0, 4, 8, and 12 wk. Urine was collected in metabolic cages over a 24-h period during which the rats had access to water only. Total urinary protein was determined by the borsalicylic acid method (5). ELISA plates were coated with purified megalin (0.1 μg/well in Tris-HCl, pH 7.6), and antiserum was serially diluted in PBS/Tween. Rats were sacrificed at wk 12, and kidneys were collected for preparation of frozen sections. Unfixed, frozen sections (4 μm) were stained with FITC-labeled Abs for microscopy as previously described (28).

Immunassay and competition ELISA

Microtiter plate wells were coated with purified rat megalin as described above or with recombinant 60-kDa proteins (1 μg/ml in TBS, pH 7.5; 100 μl/well) for 2 h at 23°C and then blocked for 1 h with 200 μl of 1 mg/ml BSA in PBS. Rat antisera were serially diluted in PBS/0.02% Tween, and 100-μl aliquots were dispensed into the wells. Plates were incubated for 1 h at room temperature, then washed three times with the dilution buffer, blocked with 1/10,000 diluted goat anti-rabbit HRP conjugate, and developed with tetramethylbenzidine-stable peroxide substrate (Amersham Pharmacia Biotech). For determination of inhibition by ELISA, the antiserum was diluted in the range of their titers (1/1000 to 1/1500) and preincubated with serially diluted nM60s. The mixtures were allowed to equilibrate for 4 h and then applied to an ELISA plate coated with native megalin or nM60s. Plates were further developed by the standard ELISA procedure described above,
Competition was indicated by reduction in the ELISA absorbance with increasing concentration of competitor. Absorbance values were corrected for background and divided by mean absorbance in the absence of inhibitor to obtain percent inhibition values.

**Results**

**Recombinant N-terminal megalin fragments**

The N-terminal 60-kDa region of the megalin extracellular domain was selected for cloning based on previous findings that a corresponding proteolytic fragment of native megalin was sufficient for induction of AHN in Lewis rats (27). A 1.7-kb fragment encoding the first 563 residues of the mature megalin N terminus was obtained by PCR amplification of a rat kidney cDNA library with appropriate primers. The *E. coli* expression construct pGEX-nM60 encoded the N-terminal GST fusion protein nM60GST, which accumulated exclusively in bacterial inclusion bodies, as evidenced by SDS-PAGE and immunoblotting with anti-GST Ab. The protein was solubilized in 4 M urea and dialyzed against Tris buffer to obtain material of 70–90% purity with an apparent size of 72 kDa under nonreducing conditions and ~90 kDa under reducing conditions (Fig. 1A). The upward shift in electrophoretic mobility of the reduced form was indicative of a high cysteine content with extensive intramolecular disulfide bonding.

The megalin cDNA was further subcloned in baculovirus transfer vectors pBacPAK-His1 and pBAC-3 for expression in insect cells. The pBacPAK-nM60i construct encodes the 6×His tag and seven additional residues (VDVKLGSV) before the mature N terminus of the megalin sequence. The pBAC-nM60s construct provides in-frame fusion to the 20-aa gp64 signal peptide sequence for directing expressed protein into the secretory pathway and the reduced form was indicative of a high cysteine content with extensive intramolecular disulfide bonding.

**Immunochemical characterization of recombinant megalin fragments**

The presence of megalin sequences in insect cell-derived proteins nM60s and nM60i and in the bacterial fusion protein nM60GST was confirmed by their reaction with anti-megalin antiserum obtained in rabbit or AHN rats. In Western blot analysis all three proteins resolved under reducing conditions reacted with rabbit antiserum (Fig. 1C). Anti-megalin Abs from AHN rats reacted with both baculovirus products nM60s and nM60i under nonreducing conditions, but failed to react with any of the three under reducing conditions. Reactivity was equivalent with rat anti-megalin Abs obtained from sera or eluted from glomeruli of AHN rats. As previously observed, native megalin reduced with 2-ME was similarly unreactive with these autoantibodies (13). The bacterial protein nM60GST also reacted with rabbit antisera against the megalin fragment 7F-GST (26), although this could be attributed in part to Abs directed against the common GST fusion partner. In contrast, it did not stain with autoantibodies from AHN rats in either the reduced or unreduced state (data not shown).

The relative immunoreactivities of unreduced recombinant proteins nM60GST and nM60s were compared by ELISA with anti-megalin autoantibodies generated in AHN rats. The proteins were adsorbed on 96-well plates, and wells were incubated with serially diluted rat antisera induced with purified native megalin. Titers were weakest against the bacterial product nM60GST, whereas the secreted baculovirus product nM60s provided titers that were 30–50% of the titer determined on plates coated with purified native megalin (Fig. 2). The intracellular product nM60i, available only as a crude cell extract, was not tested.

**Immune responses and induction of AHN**

Lewis rats were immunized using a standard protocol for induction of AHN, consisting of intradermal injection of the Ags emulsified in CFA, followed by booster immunizations at 4 and 8 wk from the initial treatment. Rats were monitored for assessment of disease by collection of blood and urine after each treatment and analysis of anti-megalin immune responses and total proteinuria. Each recombinant protein elicited serum Abs that cross-reacted with purified megalin, as detected by ELISA. Animals immunized with nM60s elicited a strong anti-megalin response with titers that peaked at 8 wk and subsequently diminished by 12 wk, corresponding to those of rats treated with native megalin. In contrast, rats immunized with nM60GST or nM60i showed weaker anti-megalin responses and no appreciable diminution in the titer over the course of the experiment (Fig. 3).

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**FIGURE 1.** Immunoblot characterization of megalin-derived recombinant fragments. A, Coomassie Blue-stained SDS-PAGE showing molecular mass standards (lane 1), nM60GST (lane 2), and nM60s (lane 3). B, Western blot of nM60s (lane 1) and nM60i (lane 2) developed with anti-His6-HP and of nM60GST developed with anti-GST-HP (lane 3). C, Immunoblots of nM60s, nM60i, and nM60GST (lanes 1, 2, and 3, respectively) stained with rabbit anti-megalin antiserum.

**FIGURE 2.** Comparison of recombinant megalin fragments by anti-megalin autoantibody reactivity. AHN antiserum induced with native megalin was serially diluted from 1/100 to 1/6400, incubated on 96-well plates coated with nM60s, nM60GST, and megalin (adsorbed at 8.0, 15, and 0.1 μg/well, respectively), and the plate was developed as described in Materials and Methods. The relative binding to megalin and the recombinant proteins was expressed by their titers (dilutions for half-maximal binding) after correcting for background absorbance.
Only animals treated with the secreted product nM60s developed pronounced proteinuria, as estimated from urine collected over 24 h (Fig. 4). This was comparable to that seen in animals immunized with Fx1A (3), native megalin, or its proteolytic fragment (27). Rats that received either nM60i or the bacterial product nM60GST produced no abnormal proteinuria, similar to controls immunized with cell culture extract in CFA.

Immuno-fluorescence microscopy staining for IgG and complement component C3 was performed on unfixed sections prepared from renal cortex of rats sacrificed at 12 wk. All animals immunized with the recombinant nM60s showed pronounced staining of ID in the glomerular basement membrane, equivalent to that seen in AHN rats induced with native megalin (Fig. 5). Kidney sections of rats immunized with nM60i or nM60GST showed no appreciable staining, similar to control rats treated with Sf9 cell extracts in CFA (data not shown).

**Lectin blot analysis of recombinant megalin fragments**

The recombinant proteins nM60i (50 μg), nM60s (10 μg), and nM60GST (150 μg) were resolved by SDS-PAGE and analyzed by lectin blotting on nitrocellulose membranes for evidence of glycosylation (29). The blot was incubated with Con A-HRP in the presence of 2 mM α-methylmannopyranoside, conditions known to detect high mannose on rat megalin (30). Only nM60s, the protein secreted from High Five cells, was strongly stained (Fig. 6). The prominent band was at approximately the same position as the band identified in immunoblots. Weak staining was observed for
nM60i at the position of the immunochemically stained band, which was comparable to the nonspecific staining of molecular mass standards, and no staining was seen with the bacterial product (Fig. 6, lanes 2, 1, and 4, respectively). Overloading of the bacterial and intracellular products nM60GST and nM60i relative to the secreted product nM60s diminishes the possibility that differences in staining were due to significantly greater purity of the latter sample.

**Specificity of anti-megalin autoantibodies**

Rats immunized with recombinant nM60s produced a strong autoantibody response against native megalin. To characterize the specificity of the anti-megalin response, the antisera were analyzed for inhibition of megalin binding by solution phase competition for nM60s. Specific inhibition of megalin binding would suggest that autoantibodies are directed primarily against determinants on the N-terminal region provided by the immunogen, whereas the lack of inhibition could indirectly suggest evidence of autoantibodies to other regions of megalin. Antisera from rats immunized with nM60s collected at 8 wk, when anti-megalin titers were greatest, were shown to bind soluble nM60s at concentrations in the range of 0.1–10 μg/ml when nM60s was also used as the capture Ag on the solid phase. More than 60% of the signal was inhibited, and the data showed good fit to a theoretical binding hyperbola with a saturation binding fraction (B_{max}) of 100. In contrast, when rat megalin was used for solid phase capture, the inhibition data were better represented by a curve with a B_{max} of 28, suggesting that the N-terminal fragment competes for binding with only ~28% of the available megalin-binding autoantibodies (Fig. 7A). Corresponding inhibition assays with sera collected at 4 wk showed no significant differences between nM60 fragment inhibition of anti-megalin and anti-nM60 Abs (Fig. 7B). These data provide an indication that the immune response may have diversified over time to include autoantibodies that bind to a number of epitopes of native megalin distinct from those on the N-terminal domain.

**Discussion**

Heymann nephritis provides an attractive model for investigating the role of autoantibodies in autoimmune disease pathogenesis. Megalin constitutes a major membrane glycoprotein autoantigen at the glomerular basement membrane where autoantibodies accumulate in ID, which could be a cause of tissue damage (4–6, 31). Specific autoantibodies or their B cells could also play a fundamental role in early mechanisms for induction of pathogenic autoimmunity. Potential B cell epitopes within a large array on the 600-kDa autoantigen can participate in either or both processes. A strategy to isolate and identify nephritogenic determinants of the autoantigen employs recombinant proteins representing fragments of the megalin polypeptide (21, 25, 26, 32–34). In particular, Yamazaki et al. (21) have shown that heterologous Abs generated against recombinant forms of each of the individual megalin domains LBD I–IV have the capacity to deposit in glomeruli in the passive model. However, no recombinant fragments have been reported to induce AHN. We recently described an N-terminal fragment encompassing the first LBD, produced by limited proteolysis of native megalin, which was able to induce full-blown AHN with attendant heavy proteinuria and nephrotic syndrome (27). This established the first successful effort to delimit the immunogenic determinants that can initiate the disease. In the present study we describe a set of recombinant proteins corresponding to this proteolytic fragment, their immunoochemical characteristics, and nephritogenicity in the AHN model.

A 1692-bp megalin sequence amplified from rat kidney cDNA was cloned for expression of the N-terminal fragment in bacterial and insect cell systems. This region encompasses the cysteine-rich LBD I of megalin, and recombinant forms could adopt a large number of unnatural disulfide structures. The protein expressed in E. coli was recovered from insoluble inclusion bodies. Nevertheless, the unreduced product was resolved as a monomer in SDSPAGE. A shift in mobility upon reduction indicated a high degree of intramolecular disulfide cross-linking. This disulfide structure was most likely unnatural, as the fragment was not recognized by anti-megalin autoantibodies from AHN rats. A baculovirus construct expressed in Sf9 insect cells also provided a monomeric polypeptide of low solubility, but better reactivity with anti-megalin autoantibodies. Finally, a secreted product obtained from High Five cells appeared to be both native-like and glycosylated, as shown by immunoblot with anti-megalin autoantibodies and lectin blot analysis. Its mobility in nonreducing gels suggested a heterogeneous multimeric composition. These observations are consistent with the idea that AHN autoantibodies recognize
conformational determinants of native megalin and that these determinants are maintained at least in part by native disulfide cross-links. Significantly, only the secreted insect cell derivative induced high titer autoantibodies and reproduced the nephritogenic effects of the native autoantigen. Kidney sections showed heavy glomerular ID of equal intensity to those seen in AHN rats injected with native megalin.

The immune response of rats immunized with the nephritogenic fragment nM60s had a remarkably high titer against the native autoantigen. The observed rise and subsequent decline of autoantibodies approximate the response kinetics induced with megalin itself and are consistent with previous studies of rats immunized with native megalin or crude renal extracts (8, 9). Reduction in titer in late sera could be attributed to renal filtration and sequestration of autoantibodies in ID or to the onset of regulatory mechanisms, which attenuate the response. In contrast, sustained titers in rats treated with the nonsecreted or bacterial fragments could reflect Ab responses against nonnative megalin that do not accumulate in glomerular ID and are not subject to autoimmune regulation.

An inhibition assay provided preliminary evidence for the diversity of autoantibody specificities against epitopes of megalin. Competitive binding to the nM60 fragment only partially inhibited circulating anti-megalin autoantibodies at 8 wk, consistent with a population of specificities extending beyond the megalin N-terminal domain. It is possible that Abs elicited against the N-terminal fragment cross-react with homologous structures on the highly repetitive sequences of megalin, but the fragment would be expected to inhibit this binding as well. Furthermore, this reactivity difference was not observed in the 4 wk sera. A more likely explanation is suggested by diversification of the autoantibody response, or epitope spreading, initiated on primary determinants of the N-terminal fragment and propagating to other epitopes of autologous megalin. Studies now in progress are designed to investigate the specificities and temporal appearance of anti-megalin autoantibodies elicited by the recombinant fragment.

Both inactive and nephritogenic insect cell products nM60i and nM60s reacted with anti-megalin autoantibodies from AHN rats; however, only the latter were specifically stained by lectin blotting. This could indicate that an essential determinant(s) for breaking of tolerance encompasses a glycosylated residue. Direct evidence implicating such a determinant in pathogenic autoimmunity remains to be demonstrated. Binding of AHN serum with a nonglycosylated fragment presumably reflects a variety of epitopes that may be the targets of nephritogenic autoantibodies, which could arise by the spreading mechanism proposed above. Con A reactivity of nM60s, indicative of high mannose, reproduces a feature of the native glycoprotein (35) that has been exploited in affinity purification (5) and for characterization of megalin in rat kidney (30). Various post-translational modifications, and glycosylation in particular, can introduce or mask immunoreactive determinants that can be associated with pathogenic autoimmune responses (36, 37).

The specific attribution of such a determinant to this model of pathogenic autoimmunity could lead to additional insight into the mechanism of disease progression. It could also explain the immunological distinction of megalin expressed in various tissues.

These studies also support a more general understanding of a class of organ-specific autoimmune diseases. The Heymann model demonstrates remarkable similarity to experimental autoimmune myasthenia gravis induced in mice or rats by acetylcholine receptor or its recombinant fragments (38, 39). In both models autoantibodies accumulate in the target organ where they appear to initiate tissue damage, the diseases can be passively transferred by autoantibodies, the pathogenic determinants appear to be localized toward the N terminus of the extracellular domain of a membrane autoantigen, and native-like conformations of peptides representing these determinants are critical to induction of the disease. The ability to dissect the domains of megalin into antigenic and nephritogenic fragments provides a novel approach for further investigation. In particular, it may be useful for assessment of epitope spreading in the mechanism of disease pathogenesis.

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