Nested N-Terminal Megalin Fragments Induce High-Titer Autoantibody and Attenuated Heymann Nephritis

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It was shown previously that an N-terminal fragment (nM60) that encompasses amino acid residues 1 to 563 of megalin could induce active Heymann nephritis (AHN) as efficiently as the native protein. For delineation of a minimal structure within this fragment that is sufficient to induce AHN, smaller protein fragments that encompass residues 1 to 236 (L6), 1 to 195 (L5), 1 to 156 (L4), and 1 to 120 (L3), representing successive C-terminal truncations within ligand-binding repeats of nM60, were cloned and produced in a baculovirus insect cell expression system. Protein fragments L4, L5, and L6 clearly were glycosylated. All four fragments stimulated proliferation of megalin-sensitized lymph node cells and induced high-titer anti-megalin autoantibodies in Lewis rats. A full-blown disease, as assessed by severity of proteinuria, was observed in rats that were immunized with L6 and L5, whereas animals that were immunized with L4 and L3 developed only mild disease. The proteinuria levels correlated with staining for complement (C3, C5b-9) and IgG1 isotype in glomerular immune deposits. The results suggest that one or more molecular determinants on the region that comprises amino acid residues 157 to 236 contribute to the induction of a full-blown form of AHN. Study of the structure, conformation, and posttranslational modifications of these determinants could provide greater insight into the molecular correlates of immunopathogenesis in this disease model.

Megalin, a 600-kD membrane glycoprotein that is abundant on the renal proximal tubule and on podocytes, is the principal autoantigen of active Heymann nephritis (AHN) (1,2). The model reproduces important clinical features of human idiopathic membranous glomerulonephritis, including proteinuria and the accumulation of subepithelial immune deposits (ID) at the glomerular basement membrane. It also has historical significance in the development of the classical view of immune complex diseases (3–5). In AHN that is induced with crude renal extract (Fx1A), high-affinity anti-megalin autoantibodies are deposited in glomerular ID. However, the autoantibody titers in sera vary widely. In rats that were immunized with purified megalin, the serum titers were shown to correlate with the severity of proteinuria (6). Experiments in passive Heymann nephritis (PHN) generally reinforce the significance of anti-megalin autoantibodies, although antisera generated to Fx1A are more effective than antisera to purified native megalin for induction of PHN that includes onset of proteinuria (7,8).

Opportunities for investigating the immune responses in relation to pathogenesis of AHN are enhanced through cloning of megalin (9) and expression of recombinant fragments (10–13). The autoantigen presents a complex immunologic target on its large amino terminal, extracellular region that comprises four cysteine-rich ligand-binding domains (LBD I through IV).

It has been suggested that autoantibodies that are deposited in kidney of AHN rats are specific for certain determinants within these domains. Rats that were immunized with a recombinant fusion protein that contained residues 1114 to 1250 of megalin, mapping to a putative ligand-binding site (14), developed glomerular ID suggestive of incipient disease (11). In PHN, heterologous antisera that were produced against recombinant proteins that represented the isolated domains LBD I through IV all were capable of producing glomerular ID (15). Proteinuria was not reported in either of these models. We found that a 60-kD proteolytic fragment of megalin that was derived from the N-terminus could elicit AHN with attendant proteinuria (16) and also showed that a recombinant protein that represented this fragment could produce similar disease (13). Full activity in the model required expression of the fragment from insect cells as a secreted product, suggesting that posttranslational modifications or conformational determinants are essential for the pathogenic potential.

Although expressed in a number of organs (17), megalin that was obtained from tissues other than kidney has not been reported to induce AHN. Native megalin in renal tissue presents both N- and O-linked glycosides, including high mannose, sialic acid, and unique neuraminic acid residues (18,19). These differences lend support to the hypothesis that glycosylation is a distinguishing feature of the renal autoantigen.

In further development of this model, we have considered the production of smaller, glycosylated protein fragments from the N-terminal region to delimit the determinants for the immune response. We compared four fragments of megalin, representing C-terminal truncations of the N-terminal 563-residue fragment that induced a full-blown disease. Molecular and immunochromic analysis of the recombinant protein fragments and their re-
spective immune responses and potential for disease induction in rats were investigated to derive further insight to the molecular correlates for pathogenic autoimmunity.

Materials and Methods
Reagents, Proteins, and Animals
Native megalin was purified from Lewis rat kidney as described previously (3,20). Antisera containing autoantibodies to megalin were collected from Lewis rats that were immunized with purified megalin according to the reported procedure for induction of AHN (16). Horse-radish peroxidase (HRP)-conjugated rabbit anti-rat and goat anti-rabbit antibodies and Con A were obtained from Sigma Chemical Co. (St. Louis, MO). FITC-labeled goat anti-rat IgG, goat anti-mouse IgG, goat anti-rabbit IgG, and goat anti-rat C3 complement antibodies that were used for immunofluorescence microscopy were purchased from Cappell Laboratories (Coehranville, PA). mAb to rat IgG1, IgG2a, IgG2b, and IgG2c were acquired from Southern Biotechnology Associates (Birmingham, AL). A rabbit anti-C5b9 antisum was described previously (21). HRP-conjugated anti-His6 mAb was obtained from BD Biosciences (Palo Alto, CA). Protein concentrations were determined by modified Bradford assay (Bio-Rad, Hercules, CA). Lewis rats at 6 wk of age were obtained from Charles River Laboratories (Wilmington, MA). Rats were killed by CO2 asphyxiation. Protocols were reviewed and approved by the institutional animal care and use committee.

Cloning and Expression of Recombinant Megalin Fragments
The previously described plasmid pGEX-nM60 encoding residues 1 to 563 of the megalin N-terminus served as template for PCR amplification of truncated fragments that encompassed residues 1 to 236 (L6), 1 to 195 (L5), 1 to 156 (L4), and 1 to 120 (L3) using the common forward primer TCCCGCGGAACAGATGCGGATGGAATTTTC and reverse primers ACCTAGTTAATCTTGTTACCTCACATCC, ACCTAGTTATATATTGCATGTGGCTC, ACCTAGTTAGTTAGTAGTGGCAGTTGCTC, and ACCTAGTTAGTTAATGTGGCAGTTTC, respectively. Agarose gel–purified fragments were digested with SaeI and Acr II and cloned in the baculovirus transfer vector pBAC-3 (Novagen, Madison, WI) using the respective sites, providing modified Bradford assay (Bio-Rad). Briefly, the membrane was treated in the dark with 10 mM NaIO4 in 100 mM sodium acetate and 5 mM EDTA (pH 5.5). After washing with PBS, the membrane was incubated in sodium acetate/EDTA and 0.025 μM biotin hydrazide (pH 5.5), washed as before, and finally detected with streptavidin–HRP (1:10,000 diluted in PBS-Tween), using ECL reagents for development as described above.}

Immunization of Rats and Assessment of AHN
Female Lewis rats were immunized by intradermal injection at several dorsal sites with 100 μg of recombinant protein in 100 μl of PBS emulsified 1:1 in complete Freund’s adjuvant (CFA) supplemented with 7 mg/ml Mycobacterium tuberculosis H37 RA (Difco Laboratories, Detroit, MI). A booster immunization (50 μg per rat) was administered in CFA by the same route at 4 wk. As previously established, the use of supplemented CFA for both primary and booster doses was shown to be advantageous to the model. For sensitization of rats that were used in lymph node cells (LNC) proliferation assays, a single 100-μg dose of megalin or nM60 (13) in CFA was distributed at four dorsal sites and one rear footpad.

Urines were collected at 4-wk intervals after immunization by placing animals individually in metabolic cages for 24 h. Total urinary protein was determined with 5% sulfosalicylic acid using BSA as standard (3). Blood samples were obtained via the tail artery from each rat at 0, 4, 8, and 12 wk after immunization. Rats were killed at week 12, and kidneys were collected for preparation of frozen sections.

Immunoblot and Glycoprotein Detection by Western Blot
Recombinant proteins were analyzed by SDS-PAGE on precast tris-glycine gels, 4 to 16% acrylamide gradient (Bio-Rad). Gels were stained with Coomassie blue or transferred to Immobilon-P membranes (Millipore) for 3 h at 75 V at 4°C. After blocking with TBS that contained 3% nonfat dry milk for 1 h, blots were incubated with rat anti-megalin antiserum (diluted 1:2000 in TBS, 3% nonfat dry milk, and 0.05% tween) for 2 h at 4°C. The blots were washed, incubated with goat anti-rat-HRP conjugate (Southern Biotechnologies, Birmingham, AL) at 1:10,000 dilution in TBS-Tween and developed with ECL substrates (Amersham Biosciences, Piscataway, NJ). For His-tag detection, the membranes were blocked as above and incubated in the presence anti-His6 mAb-HRP conjugate diluted 1:10,000 in TBS-Tween. Glycoprotein blotting was performed using a commercial kit according the manufacturer’s instructions (Bio-Rad). Briefly, the membrane was treated in the dark with 10 mM NaIO4 in 100 mM sodium acetate and 5 mM EDTA (pH 5.5). After washing with PBS, the membrane was incubated in sodium acetate/EDTA and 0.025 μM biotin hydrazide (pH 5.5), washed as before, and finally detected with streptavidin–HRP (1:10,000 diluted in PBS-Tween), using ECL reagents for development as described above.

Immunofluorescence Microscopy
Unfixed frozen sections (4 μm) of kidney cortex were cut on a cryostat and stained with FITC-conjugated goat anti-rat IgG (1:32 diluted) or anti-C3 complement (1:16 diluted) as described previously (22). Similarly, staining for IgG isotypes was done by incubating sections with IgG1-, 2a-, 2b-, or 2c-specific mAb (1:50 diluted) for 20 min, washing twice with PBS, and then incubating with anti-mouse FITC conjugate (1:32 diluted) for 20 min. Staining for C5b-9 was performed by incubating with rabbit anti-C5b-9 antisum (1:50 diluted), washing as above, and labeling with goat anti-rabbit FITC conjugate (1:32 diluted). Slides were viewed and photographed on a Nikon inverted-lens microscope equipped for epifluorescence. The intensity of ID was
scored on a semiquantitative scale from 0 (no fluorescence) to 4+ (intense fluorescence with granular appearance) by two independent observers who were unaware of sample identities. Indirect immunofluorescence was done on sections that were prepared from kidney of normal Lewis rats. Sections were soaked with antisera from rats that were immunized with fragments L3 through L6 diluted 1:50 in PBS and developed with goat anti-rat IgG-FITC conjugate, and fluorescence was recorded as above.

**LNC Proliferation Assay**

Rats that were immunized with nM60 fragment (50 μg), native syngeneic megalin (100 μg), or ovalbumin (100 μg) in CFA as described above were killed 12 d later, and lymph nodes (popliteal and axillary) were collected. Single-cell suspensions that were prepared in RPMI-1640 supplemented with 2% normal Lewis rat serum, 50 μM 2-mercaptoethanol, 100 U of penicillin, and 100 μg/ml streptomycin were dispensed in 96-well flat-bottom cell culture plates at 5 × 10⁶ cells/well. Proteins or Con A (5 μg/ml) in 20 μl of sterile PBS were added in triplicate wells, and plates were kept at 37°C in a CO₂ incubator for 72 h. PBS alone (20 μl) was added to control wells that were used for determining background proliferation. Cells then were pulsed with 0.5 μCi/well ³H-thymidine (Amersham Biosciences) and incubated for an additional 18 h. Cells were harvested on filter plates and washed, and counts were read on a TopCount plate reader (Perkin Elmer Instruments, Shelton, CT). Proliferation was expressed as the stimulation index (total counts - background counts from unstimulated cells)/background counts. Values represent mean of triplicate determinations ± SEM.

**Statistical Analyses**

Proteinuria data are expressed as the mean ± SEM. Statistical differences between groups were evaluated by the t test and ANOVA using Prizm 4 software program (GraphPad, San Diego, CA).

**Results**

**Characterization of Recombinant N-Terminal Megalin Fragments**

Proteins that were expressed in baculovirus-infected High Five insect cells were characterized by SDS-PAGE and by immunoblot analysis, using megalin-specific antisera. Under reducing conditions, the major protein bands for L6, L5, L4, and L3 migrated at approximately 36, 31, 27, and 22 kD relative to size standards, representing a 9- to 10-kD shift from their theoretical masses of 26, 21, 17, and 13 kD, respectively (Figure 1). The discrepancy between apparent and theoretical masses of the polypeptide chains was attributed to glycosylation, as supported by carbohydrate-specific biotin-labeling chemiblot, where staining of fragments L6, L5, and L4 was evident (Figure 2A). The similar displacement of each fragment relative to its theoretical size suggested that all fragments were glycosylated to a similar degree. The lack of staining of L3 could be attributed to resistance of its carbohydrate residues to the oxidation procedure. Characteristically diffuse bands in these blots reflected the appearance of the corresponding Coomassie-stained bands in the gels. A duplicate blot that stained for the Hisx6 tag detected single bands (Figure 2B), consistent with those that were identified by Coomassie staining.

**LNC Proliferation Assay**

A proliferation assay was used to assess the relevance of the N-terminal regions that were investigated in T cell responses to native megalin that were elicited. LNC from rats that were sensitized with whole megalin or recombinant nM60 were stimulated in vitro with each of the purified fragments in the deletion series. Potent stimulation of the nM60-sensitized LNC was apparent with all four of the deletion fragments. Significant proliferation of the megalin-sensitized LNC required a five-fold higher dose of the proteins for detection (Figure 3). Neverthe-
less, the responses were two- to three-fold above the background stimulation of control LNC from ovalbumin-sensitized rats that were incubated with the same dosage of fragments. In agreement with our previous experience, LNC from either megalin-sensitized or nM60-sensitized rats failed to proliferate above background in the presence of native megalin.

Immunization and Autoantibody Characterization

Each of the secreted recombinant fragments L6, L5, L4, and L3 was used to immunize a group of Lewis rats according to a standard protocol. The anti-megalin titers of antisera that were collected at 4, 8, and 12 wk from each group were compared by ELISA. All four groups elicited high-titer anti-megalin autoantibodies at 4 wk, with no further increases at 8 or 12 wk despite the booster immunization (Figure 4); rather, titers decreased slightly during this time in all groups. These changes recapitulate similar but more marked reductions in anti-megalin titers that were noted previously in AHN rats immunized with native megalin (6) or with recombinant nM60 (13) (Figure 4).

The 12-wk antisera from each group of animals strongly stained proximal tubules in normal rat kidney sections as represented by a section stained with antiserum to L6 (Figure 5). The pattern was similar to that seen using serum of AHN rats induced with native megalin. Moreover, discernible glomerular staining, typical of very high-titer anti-megalin antibodies, also was observed.

Assessment of Disease

At 8 wk, all four groups of rats had slightly elevated proteinuria compared with controls. At 12 wk, the group that was immunized with L6 developed abnormal proteinuria, approaching levels that are typical of the standard AHN model. Rats that were treated with L5 also had pronounced proteinuria, although the levels were reduced relative to those of the L6-treated group. The differences were deemed to be statistically significant. However, proteinuria in groups that were treated with L3 or L4 remained stable but above the normal range (Figure 6). Potential increases or decrease in proteinuria at time points beyond 12 wk were not assessed.

Glomerular ID, which stained strongly for IgG, were detected in sections of renal cortex from all rats in each of the four groups that were immunized with secreted fragments L6 through L3 (Figure 7, left column). Staining on average was more intense in sections of rats that were treated with L6 (Figure 8). Staining for major IgG isotypes showed similarly intense staining for IgG2a and IgG2b, whereas IgG1-specific staining varied from strongest in sections of rats that were
treated with L6 to weak or trace staining in those that were immunized with L4 and L3 (Figure 7, middle column). Significant staining for complement C3 was evident only in ID of rats that received injections of L6 or L5 (Figure 7, right column). Sections that were stained for C5b-9 indicated a similar trend, with most intense staining in the groups that were immunized with L6 or L5 (Figure 9).

Discussion

Molecular trimming of the previously described fully pathogenic 60-kD N-terminal megalin fragment was expected to provide insight to the critical structural determinants in the fragment for induction of a pathogenic autoimmune response that leads to full-blown AHN. Truncations that terminate between the three C-terminal class A motifs of LBD I were designed to preserve intrachain disulfide linkages.

Each of the fragments stimulated megalin-sensitized LNC in vitro and induced a vigorous anti-megalin autoantibody response in vivo, suggesting that each contained a set of B and T cell epitopes that were sufficient for an autoimmune response relevant to this disease. However, quantitative differences were observed in glomerular ID and proteinuria levels among groups that were immunized with different fragments. Although all groups had abnormal proteinuria, only rats that were immunized with the larger fragments L6 and L5 had levels that approached those that were produced with whole megalin or its 60-kD N-terminal megalin fragment. We therefore can deduce that epitopes that are composed of residues within the sequence 157 to 236 are critical for expression of the full disease. The most severe disease was induced with L6, suggesting that this fragment provides a more complete set of epitopes or that its C-terminal sequence contributes to a critical conformational epitope that is shared with L5.

Anti-megalin autoantibody serum titers in all four groups were similar to or greater than those seen in rats with full-
blown AHN that was induced with nM60. Therefore, high titers alone cannot explain the variations in the levels of glomerular ID or proteinuria. Presumably, conventional peptide or carbohydrate determinants on these fragments account for qualitative differences in the immune response, including epitope specificity and isotype distribution. It is interesting to note that there was a positive correlation between disease severity and the level of staining of glomerular ID for IgG1 isotype and complement among rats that were immunized with different fragments. Autoantibodies to these epitopes could have unique properties with respect to deposition or effector functions such as complement activation.

Although both IgG1 and IgG2a can activate complement through the classic pathway (23), the results presented in this report suggest that complement deposition and, consequently, the pathogenic response correlate best with glomerular IgG1 deposition. A recently reported mouse model of membranous glomerulonephritis suggested a similar correlation of IgG1 deposition with the disease phenotype (24). Although it has been shown that AHN can be induced in C5 complement-deficient rats (25), a mechanism for glomerular injury that is caused by formation of a membrane attack complex cannot be ruled out (26,27). Results from this study are supportive of the role of complement involvement. Autoantibodies against complement regulatory protein Crry have been implicated in unregulated activation of complement in the AHN model that is induced with Fx1A, which is known to contain this autoantigen (28). This would be less plausible in a model that is induced with purified megalin or recombinant megalin fragments.

The requirement in this model for expression of the megalin fragments in insect cells suggests additional critical factors for investigation. Glycosidic modifications could influence folding of conformational B cell epitopes (29,30), the generation of relevant T cell epitopes, or activation of the innate immune system through carbohydrate-specific receptors. Differences in the complexity and pattern of megalin glycosylation during development (31,32) or in diverse tissues (17) could account for unique pathogenicity of the renal glycoprotein in the induction of AHN (33). Posttranslational modifications comprise a class of neodeterminants in several inducible and spontaneous autoimmune diseases (34,35). The size and the complexity of the relevant antigens often present obstacles to further study of these determinants. The characterization of the small, immunogenic fragments defined here should offer further insight into the molecular correlates for immunopathogenesis in AHN. These studies could prove relevant as well to the immunologic mechanisms of human idiopathic glomerulonephritis.

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References


