All Four Putative Ligand-Binding Domains in Megalin Contain Pathogenic Epitopes Capable of Inducing Passive Heymann Nephritis

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Abstract. Megalin (gp330) is the main target antigen involved in the induction of Heymann nephritis (HN), a rat model of human membranous nephropathy. Its large extracellular region contains four putative ligand-binding domains separated by spacer regions. Previously, it was reported that the second ligand-binding domain (LBD II) of megalin is involved in the pathogenesis of passive HN because it is capable of binding antibodies in vivo and initiating formation of immune deposits (ID). This study explores the possibility that pathogenic epitopes might also be present in the other putative ligand-binding domains. Recombinant fragments of ligand-binding domains (LBD) I through IV expressed in a baculovirus system were used to generate polyclonal domain-specific antibodies. Antibodies raised against each of the recombinant megalin fragments reacted preferentially with its respective antigen and with whole megalin by immunoblotting. Each of the antibodies also gave a characteristic brush-border staining for megalin by indirect immunofluorescence on rat kidney. When rats were injected with the domain-specific antibodies to test their ability to produce passive HN, glomerular ID were present in kidneys of all injected animals. The staining pattern in glomeruli of rats injected with LBD I, III, or IV was similar to that obtained with antibodies to LBD II. It is concluded that passive HN can be induced with antibodies against LBD I, III, and IV, as well as LBD II, and that each of the ligand-binding domains contains a pathogenic epitope. These findings provide further evidence for the multiple epitope model of HN.

Heymann nephritis (HN) is a well characterized experimental rat model of human membranous nephropathy (1). The glomerular lesions that represent the hallmark of this disease consist of subepithelial immune deposits (ID) formed mainly by shedding of antigen-antibody complexes from podocytes (2). The major pathogenic antigens of HN are megalin (gp330) and the receptor-associated protein (RAP) (2,3). Megalin is a multiligand-binding endocytic receptor expressed in clathrin-coated pits at the surface of a number of epithelia, including those of the kidney glomerulus (4), proximal tubule (5,6), yolk sac, type II cells of the lung, etc. (7). Sequence analysis of the cDNA for megalin (8) reveals that it is a large (>600 kD) transmembrane protein containing an extracellular region (4400 amino acids), a single transmembrane domain (22 amino acids), and a C-terminal cytoplasmic tail (213 amino acids). The extracellular domain of megalin contains structural motifs characteristic of members of the LDL receptor family: namely, four clusters of cysteine-rich LDL receptor ligand-binding repeats (LBD I through IV), growth factor repeats, an epidermal growth factor repeat, and YWTD spacer regions. Megalin binds RAP (9), a specialized chaperone that assists in the folding of megalin in the endoplasmic reticulum (10) and in its delivery to the cell surface (11). Together, megalin and RAP constitute what we have called the Heymann nephritis antigenic complex (HNAC) (2,9). HN can be induced by immunization with either megalin or RAP, and ID can be passively induced by injection of either antimegalin (2,4,12) or anti-RAP (12–16) antibodies. Thus, both megalin and RAP contain pathogenic epitopes capable of binding antibodies and inducing HN.

Previously we mapped the pathogenic epitope in RAP to a 14-amino acid sequence near its N terminus (16). We also mapped a major pathogenic epitope in megalin to 46 amino acids (amino acids 1160–1205) that correspond to the fifth ligand-binding repeat of the second ligand-binding domain (LBD II) (17). Similar findings were obtained independently by Raychowdhury et al. (18), who reported that amino acids 1114–1250 from the second LBD of megalin are involved in induction of HN. It has not yet been determined whether other LBD of megalin contain pathogenic epitopes. It is important to map the pathogenic epitopes in megalin to elucidate the molecular mechanisms of ID formation in HN.
For this study, we generated recombinant fragments representing the four clusters of ligand-binding repeats (LBD I through IV) in megalin, using a baculovirus expression system, and raised domain-specific polyclonal antibodies against these fragments. We determined that each of the antibodies is capable of causing ID formation (passive HN), indicating that pathogenic epitopes are present in all four LBD of megalin.

Materials and Methods

Baculovirus Expression and Purification of Megalin Fragments

Recombinant baculoviruses expressing cDNA fragments encoding the individual clusters of ligand-binding repeats (LBD I through IV) (Figure 1) were prepared and used to infect Sf9 cells. The proteins expressed in Sf-9 cells were affinity-purified by Ni²⁺ chromatography as described (17). In brief, fragment I (nucleotides 106–1364, amino acids 25–389), fragment II (nucleotides 2886–4916, amino acids 897–1573), fragment III (nucleotides 7834–10134, amino acids 2547–3312), and fragment IV (nucleotides 10315–12405, amino acids 3374–4069) were cloned into pSPH vector. Fragment I and fragment II inserts with a hexa-histidine tag sequence were subcloned into pAcSB2 and pAcGP67B (PharMingen, San Diego, CA), respectively. Fragment III and fragment IV inserts with the hexa-histidine tag sequence were subcloned into pAcGP67A (PharMingen). Sf9 cells were transfected with the constructs using the BaculoGold transfection kit (PharMingen) to generate recombinant baculoviruses. The viruses were amplified, and cells were infected and cultured for 72 h, after which they were lysed in 6 M guanidine HCl, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8, for 30 min at 23°C followed by sonication (three times, for 5 s each). Lysates were cleared by centrifugation (10,000 × g for 15 min), and the supernatants were incubated for 15 min with nickel-Sepharose (Invitrogen, Carlsbad, CA). The resin was washed stepwise using 8 M urea, 20 mM sodium phosphate, 500 mM NaCl at decreasing pH values of 7.8, 6.0, 5.3, and 4.0. Bound proteins were eluted with a 10 to 320 mM step gradient of imidazole in the urea-containing buffer, pH 4.0. Baculovirus-derived megalin fragments were visualized by silver staining after 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Individual fragments were then pooled, dialyzed against 10 mM Tris, pH 7.7, 150 mM NaCl, 0.1% Triton X-100, and concentrated using Ultrafree-15 (Millipore, Bedford, MA).

Antibodies

Antimegalin LBD domain-specific antisera were raised in rabbits by immunization with 50 μg of each purified recombinant megalin fragment (LBD I through IV) in complete Freund’s adjuvant, followed by two boosts with 50 μg of protein in incomplete Freund’s adjuvant at 3 and 6 wk. Polyclonal antisera against full-length megalin were prepared by immunization with megalin purified from the rat renal cortex by gel filtration and lentil lectin chromatography (4). All procedures on animals were conducted in accord with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Immunoblot Analysis

Rat kidney cortex proteins and the purified recombinant megalin fragments were boiled for 5 min in Laemmli sample buffer supplemented with 5% 2-mercaptoethanol and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with phosphate-buffered saline, 0.2% Triton X-100, 5% calf serum for 1 h at 23°C, and incubated with primary antibodies for 2 h at 23°C. They were then washed three times (10 min), incubated with goat anti-rabbit horseradish peroxidase conjugate (BioRad, Hercules, CA), and bound primary antibodies were detected by SuperSignal chemiluminescence (Pierce, Rockford, IL).

Induction of Passive HN and Immunofluorescence Analysis

Female Sprague Dawley rats (200 to 250 g) were injected intravenously with 5 to 10 mg of IgG specific for megalin LBD I, II, III, or IV, purified on protein A agarose (BioRad). Kidneys of normal and 3-d passive HN rats were perfused with paraformaldehyde-lysine-periodate fixative. Samples of renal cortex were cryoprotected and frozen in liquid nitrogen for cryosectioning as described (12). Semi-thin (0.5 μm) sections of normal rat kidneys were incubated with antimegalin LBD I–IV antisera, followed by FITC-conjugated goat anti-rabbit IgG (DAKO, Carpinteria, CA). Sections of kidneys from rats with passive HN were incubated directly in FITC-conjugated goat anti-rabbit IgG, and examined and photographed using a Zeiss Axiosphot microscope equipped for epifluorescence.

Figure 1. Structure of rat megalin indicating the regions of megalin against which antibodies were generated. Four cDNA fragments (ligand-binding domains I through IV [LBD I–IV]), encoding the first, second, third, and fourth LBD, respectively, were expressed in a baculovirus system, purified to homogeneity, and used to raise polyclonal, domain-specific antibodies against LBD I through IV, respectively.
Urinalysis

Rats were housed overnight in metabolic cages, and 16-h urine specimens were collected and analyzed for protein content by the biuret method.

Homology Search

Amino acid homology searches were performed using MacVector (version 6.0, International Biotechnologies, Inc., New Haven, CT).

Results

Characterization of Polyclonal Antibodies Against Recombinant Megalin Fragments

Rat megalin cDNA fragments I through IV encoding the first through fourth clusters of ligand-binding repeats (LBD I–IV) were used to generate recombinant baculoviruses, and Sf9 cells were infected with these recombinant baculoviruses. The expressed proteins were affinity-purified by Ni^{2+} chromatography and used to raise four domain-specific polyclonal antibodies against LBD I, II, III, and IV. The specificity of these antibodies for each cluster of ligand-binding repeats was determined by immunoblot analysis. Each of the domain-specific antisera reacted preferentially with its respective recombinant protein used as immunogen, whereas all four domains were recognized by a polyclonal antibody raised against whole megalin (Figure 2).

To confirm the specificity of the anti-LBD I–IV antisera for megalin, we performed immunoblotting on rat renal microvillar extract and immunolocalization on sections of rat kidney. Each of the domain-specific antisera raised against recombinant megalin fragments reacted with native megalin in the microvillar extract (Figure 3). By immunofluorescence, megalin was detected at the base of the proximal tubule microvilli with all four antibodies (Figure 4, A through D). Thus, each of the domain-specific antibodies recognizes intact megalin in situ as well as by immunoblotting.

Polyclonal Antibodies Specific for Megalin LBD I Through IV Induce Passive Heymann Nephritis

To determine whether the domain-specific antibodies are capable of inducing passive HN, Sprague Dawley rats were injected intravenously with antimegalin LBD I–IV IgG, and 3 d later the kidneys were examined by direct immunofluorescence. Granular subepithelial ID were observed in glomeruli of rats injected with all four domain-specific IgG (Figure 5). The ID induced by anti-LBD I, II, and III IgG were similar in size, but those induced by anti-LBD IV IgG were smaller. This may reflect the somewhat lower titer of this anti-LBD IV antibody. An additional finding was that staining of proximal tubule brush border was seen in the specimen with anti-LBD I IgG, but not the others. This suggests that anti-LBD I IgG may pass through the glomerulus in greater amounts. Rats injected with all four of the domain-specific IgG had urinary protein excretion within the normal range (data not shown). We conclude

Figure 2. Immunoblot analysis demonstrating the specificity of anti-LBD I, II, III, or IV antibodies. Each of the four clusters of ligand-binding repeats, LBD I through IV, was separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF), and immunoblotted with polyclonal antibodies raised against whole megalin (antimegalin) or recombinant megalin fragments, (anti-LBD I–IV). The antibody raised against whole megalin recognized all four ligand-binding domains (LBD I–IV) migrating with relative electrophoretic mobilities of 80, 126, 124, and 130 kD, respectively. The antisera raised against megalin fragments LBD I through IV reacted preferentially with the recombinant fragment used as immunogen, indicating that each antibody is domain-specific.

Figure 3. Immunoblot demonstrating that domain-specific antibodies against LBD I through IV recognize megalin in a renal microvillar extract. Rat kidney microvillar proteins were separated by 5% SDS-PAGE, transferred to PVDF membranes, and immunoblotted using anti-whole megalin polyclonal antibody (lane M), anti-LBD I (lane 1), anti-LBD II (lane 2), anti-LBD III (lane 3), and anti-LBD IV (lane 4). All antibodies react strongly with megalin (arrow).
that antibodies against all four LBD are capable of producing passive HN.

Sequence Homology Between the Fifth Repeat of LBD II and Repeats in LBD I, III, and IV

Previously, we defined the fifth ligand-binding repeat in LBD II as containing a major pathogenic epitope of megalin (17). To investigate whether there are structurally similar regions in other LBD, we compared the amino acid sequence of LBD I, III, and IV with that of the fifth ligand-binding repeat of LBD II. Amino acid identity percentages are 30 to 47% among the ligand-binding repeats in LBD I, III, and IV. As shown in Figure 6, the third ligand-binding repeat of LBD I (amino acids 80–126), the third ligand-binding repeat of LBD III (amino acids 2753–2802), and the tenth ligand-binding repeat of LBD IV (amino acids 3857–3906) showed the highest homology, with 45 to 47% identity and 13 to 15% similarity.

Discussion

The extracellular domain of megalin contains four clusters of cysteine-rich regions, LBD I through IV, composed of 7, 8, 10, and 11 ligand-binding repeats, respectively, with adjacent growth factor repeats. To date only the second cluster, LBD II, of ligand-binding repeats has been shown to contain a pathogenic epitope involved in HN (17,18). In this study, we generated domain-specific polyclonal antibodies against recombinant fragments of all four LBD of megalin to determine whether additional pathogenic epitopes are present in megalin. We found that passive HN can be induced by injecting rats with each of the domain-specific antibodies, indicating that all four putative LBD contain pathogenic epitopes.

We have previously proposed a multiple pathogenic epitope model of HN in which both megalin and RAP contain at least one pathogenic epitope (12,19), and antibody binding to either epitope can trigger deposition of immune complexes. We subsequently mapped these two pathogenic epitopes to a 14-amino acid sequence in RAP (14,16) and 46 amino acids (amino acids 1160–1205) constituting the fifth ligand-binding repeat of LBD II in megalin (17). The present study indicates that all four putative LBD contain pathogenic epitopes involved in passive HN, thus providing further support for the multiple epitope model of HN. Raychowdhury et al. (18) reported that a 137-amino acid sequence (amino acids 1114–1250) within LBD II produces active HN but noted that the ID were smaller than those found in glomeruli of rats immunized with whole megalin. It seems likely that antibody binding to multiple pathogenic epitopes present in megalin or RAP are required for enlargement of ID.

The fact that the third ligand-binding repeat of LBD I, the third ligand-binding repeat of LBD III, and the tenth ligand-binding repeat of LBD IV showed the highest homology (45 to 47% identity and 13 to 15% similarity) to the fifth ligand-binding repeat of LBD II suggests that these ligand-binding repeats...
Figure 5. Injection of rats with each of the four antimegalin domain-specific IgG resulted in formation of glomerular immune deposits (ID). Rats were injected with antimegalin LBD I, II, III, or IV IgG, and 3 d later the kidneys were removed and processed for direct immunofluorescence. Subepithelial ID, the hallmark of passive Heymann nephritis (HN), are seen in rats injected with each of the antibodies. Staining patterns on semithin cryosections are similar among the four specimens, except that the ID induced by anti-LBD IV are somewhat smaller than the others. Also, specimens from rats injected with LBD I, but not anti-LBD II through IV, bound to the proximal tubule brush border. Magnification: ×400 and ×1000.

Figure 6. Comparative homology between ligand-binding repeats in LBD I, III, and IV and the fifth ligand-binding repeat of LBD II. The third ligand-binding repeat of LBD I (amino acids 80–126), the third ligand-binding repeat of LBD III (amino acids 2753–2802), and the tenth ligand-binding repeat of LBD IV (amino acids 3857–3906) showed the highest homology, i.e., 45 to 47 identity and 13 to 15% similarity. Gaps have been introduced to optimize the alignment. Identical residues are shaded, and conservative changes are boxed.

repeats could contribute to the immunogenicity and pathogenicity in passive HN. However, we cannot rule out that other ligand-binding repeats of lower sequence homology might have conformational homology and present pathogenic epitopes. Additional studies are needed to determine whether these homologous regions are involved in the pathogenesis of HN.

LBD II stands out as an important region in megalin from
both the structural and the functional standpoint. This region not only contains a pathogenic epitope for passive HN, but it also contains a binding site for several ligands, including apolipoprotein E-β very low density lipoprotein (ApoE-βVLDL), lipoprotein lipase, aprotinin, lactoferrin, and RAP (20). The fact that this region binds circulating antibodies indicates that this site is exposed in vivo on the basal surface of the glomerular epithelium where it is competent to bind multiple ligands (21), as well as pathogenic antibodies. This study demonstrates that not only LBD II, but also LBD I, III, and IV are exposed in vivo and can bind circulating antibodies. The finding that LBD I, III, and IV are exposed in vivo raises the possibility that LBD other than LBD II may contribute to ligand-binding by megalin. LBD II is also an important domain in the LDL receptor-related protein/α2-macroglobulin receptor (LRP), which is the closest relative to megalin and has four structurally similar LBD (8,22,23). LRP is also capable of binding multiple ligands (lipoprotein lipase, urokinase-type plasminogen activator: plasminogen activator inhibitor type 1 complex, pro-urokinase-type plasminogen activator, α2-macroglobulin and RAP), and all of these ligands have been shown to bind to LBD II (24–26), but not to LBD I, III, or IV. Only RAP has been shown to bind to other LBD (III and IV) (25,27). Thus, the functional contributions of LBD I, III, and IV in megalin and LRP are not yet clear.

In summary, in this study we show that all four clusters of ligand-binding repeats in megalin contain pathogenic epitopes capable of inducing passive HN. Additional studies are needed to determine the nature of the epitopes in LBD I, III, and IV. It is hoped that this information will provide insight into the mechanism of passive HN and human membranous nephropathy that can eventually lead to rational therapeutic interventions.

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References


