Megalin (gp330) Possesses an Antigenic Epitope Capable of Inducing Passive Heymann Nephritis Independent of the Nephritogenic Epitope in Receptor-Associated Protein

Robert A. Orlando, Dentscho Kerjaschki, and Marilyn Gist Farquhar

R.A. Orlando, M.G. Farquhar, Division of Cellular and Molecular Medicine and Center for Molecular Genetics, University of California, San Diego, La Jolla, CA
D. Kerjaschki, Section of Ultrastructural Pathology and Cell Biology, Institute of Clinical Pathology, University of Vienna, Vienna, Austria


ABSTRACT

The Heymann nephritis antigenic complex (HNAC) consists of two glycoproteins, megalin (gp330), and the receptor-associated protein (RAP). HNAC is expressed on the surface of the glomerular epithelium where it plays a primary role in the pathogenesis of Heymann nephritis (HN). Several models were previously proposed describing how antibody binding epitopes in HNAC may contribute to the initiation and progression of HN. Although these models suggest that nephritogenic epitopes capable of initiating HN are present in both megalin and RAP, the structural relationship between these epitopes has not been established. Previously a nephritogenic epitope was identified and characterized in RAP that initiates immune complex formation in HN. In this report, the immunologic relationship between nephritogenic epitopes in megalin and RAP were examined to determine whether these epitopes are immunologically distinct or antigenically related. To this end, a polyclonal antibody to megalin was generated that does not recognize RAP by immunoblotting or immunoprecipitation and whether this antibody is capable of inducing passive HN was determined. It was found that antimegalin antibodies devoid of RAP cross-reactivity induced the formation of subepithelial immune deposits (passive HN) when injected into rats. Antibodies eluted from glomeruli of the injected rats recognized only megalin by immunoblotting a cortisol extract and did not recognize a RAP fusion protein or any other renal protein. In addition, the eluted antibodies immunoprecipitated two proteolytic fragments of megalin (140 and 75 kd) identifying a nephritogenic epitope within a smaller fragment of megalin. These results indicate that at least one nephritogenic epitope is present in megalin that can bind circulating antibodies and initiate immune complex formation independent of the pathogenic epitope in RAP. The data presented here support the multiple epitope model, proposing that both megalin and RAP possess nephritogenic epitopes that are not antigenically related and can independently induce HN.

Key Words: Megalin, gp330, RAP, HNAC, membranous glomerulonephritis

The Heymann nephritis antigenic complex (HNAC) is a heterodimer consisting of a large cell surface receptor named megalin (1) (previously called gp330) (2,3) and a smaller polypeptide referred to as the receptor-associated protein (RAP) (4). Biochemical studies have shown that RAP and megalin associate shortly after biosynthesis and form a stable oligomeric complex (4–6). In the rat kidney, megalin is primarily localized to clathrin-coated pits in the proximal tubule brush border and at the base and at the sides of podocyte foot processes (3,7,8). RAP, however, is most abundant in the endoplasmic reticulum, and only limited amounts are present on the cell surface (4,6,9).

Both megalin and RAP have been implicated as antigens in Heymann nephritis (HN), the experimental rat model of human membranous glomerulonephritis. We (2,3,8) and others (10,11) have provided evidence for megalin's role in HN by showing that (1) bound antibodies eluted from the glomeruli of HN rats react specifically with megalin, and (2) HN can be induced by injecting rats with antimegalin antibodies (passive HN, pHN). However, recent data indicate that the antimegalin antisera used previously by us (9) as well as others to induce pHN contain antibodies that also recognize RAP. Because RAP contain a well-characterized nephritogenic epitope that binds antibodies and initiates immune complex formation (12,13), the question arises, is an immunologically similar epitope also expressed in megalin or does megalin present epitopes capable of forming immune complexes in the absence of RAP?
Megalin and RAP Have Unique HN Epitopes

We have previously proposed three models describing how pathogenic epitopes may be presented on the surface of podocytes in HN (4,13). In one model (Model I), nephritogenic epitopes are expressed within megalin and RAP that are immunologically distinct. Another model (Model II) suggests that the nephritogenic epitopes are structurally and immunologically related. To determine which of these two models best describes the relationship between pathogenic epitopes in megalin and RAP, we have generated a polyclonal antibody to megalin that demonstrates no cross-reactivity with RAP or any other renal antigen and tested if these antibodies are capable of inducing pHN. We present evidence that this highly specific antimegalin antibody preparation binds to podocyte foot processes and induces the formation of large immune deposits. These results, suggesting that megalin can initiate the pathogenic events of HN in the absence of RAP, supports Model I, indicating that megalin and RAP express immunologically distinct nephritogenic epitopes capable of independently binding antibodies leading to immune complex formation. Based on these findings, we propose a modified model that reflects our current understanding of the contributions by megalin and RAP to HN at a molecular level.

MATERIALS AND METHODS

Antibodies

Polyclonal antisera to megalin (antimegalin, 070) (14), RAP-glutathione-S-transferase (anti-RAP-GST, 072) (14), and monoclonal antibody 20B (15) were prepared as described.

Induction of pHN and Elution of Glomerular Bound Antibodies

Male Lewis rats (250 to 330 g) were injected intravenously with 10 to 15 mg of antimegalin immunoglobulin G (IgG) purified on Protein A-Sepharose 4B (Pharmacia, Alameda, CA). After 3 days, the kidneys were perfused with phosphate-buffered saline (PBS) at 4°C, kidney cortex was excised, and boiled for 5 min, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16). The blots were then washed and processed using ECL chemiluminescence (Amersham, Arlington Heights, IL) detection according to the manufacturer’s instructions.

Immunocytochemistry

Kidneys of normal and 3-day pHN rats were perfused with paraformaldehyde-lysine-periodate fixative. Some pieces of renal cortex were processed for routine electron microscopy, and others were cryoprotected and frozen in liquid nitrogen for cryosectioning. Semithin (0.5 μm) and ultrathin cryosections were prepared for immunofluorescence and immunogold labeling, respectively, on an Ultracut ultramicrotome, equipped with a F4 cryo-attachment (Reichert, Cambridge Instruments, Nussloch, Germany), as described (6,17). Semithin sections of normal rat kidneys were incubated with antimegalin IgG (10 μg/mL) or eluted glomerular IgG, followed by fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (DAKO, Carpinteria, CA). Sections of kidneys from rats with pHN were incubated directly in fluorescein isothiocyanate–conjugated goat anti-rabbit IgG. Sections were examined in a Zeiss Axiophot (Zeiss, Oberkochen, Germany) microscope equipped for fluorescence. Ultrathin frozen sections of PLP-fixed cortex of pHN rats were incubated with goat anti-rabbit IgG, conjugated to 10-nm gold particles (1:50, Amersham), and examined in a JEOL 1010 electron microscope (JEOL, Tokyo, Japan).

Purification of the RAP-GST Fusion Protein

The cDNA for rat RAP (9) was subcloned into the pGEX prokaryotic expression vector (Pharmacia, Alameda, CA). Purification of RAP-GST was performed by glutathione-agarose (Sigma, St. Louis, MO) affinity chromatography as described (18).

Protein iodinations

Iodinations were performed using the IODO-GEN (Pierce, Rockford, IL) method according to the manufacturer’s instructions. Specific activities for all iodinations were routinely 20,000 to 50,000 cpm/ng protein.

Immunoprecipitation

[125I] Megalin (5 × 10⁶ cpm/mL) or [125I] RAP-GST (2 × 10⁶ cpm/mL) was diluted into 1 mL of 20 mM N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.4), 150 mM NaCl, 2 mM CaCl₂, 1% Triton X-100, and 1% bovine serum albumin. Antisera or antibodies eluted from isolated glomeruli were added as indicated, followed by protein A-agarose (BioRad, Hercules, CA), and incubated for 16 h at 4°C. The immunoprecipitates were washed twice with RIPA buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% Tween-20, 0.1% SDS) and twice with PT (PBS, 0.5% deoxycholate). The beads were then resuspended in Laemmli sample buffer supplemented with 2% β-mercaptoethanol, boiled for 5 min, and resolved by SDS-PAGE on 10% gels (16).

Purification of Megalin

Microvillar membranes were prepared by Ca²⁺ precipitation as described (2). Megalin was purified from solubilized microvillar membranes by affinity chromatography on monoclonal antibody 20B in the presence of 5 mM EDTA as previously described (4).

Proteolytic Digestion of [125I] Megalin

[125I] Megalin (4 × 10⁶ cpm) was incubated with 1.0 μg of chymotrypsin (Sigma) in 50 mM NH₄OAc [pH 8.0], 2 mM CaCl₂ for 1 h at 37°C. The reaction was stopped by the addition of 2 mM phenylmethylsulfonyl fluoride and boiling.
RESULTS

Antibodies Generated Against Megalin Are Highly Specific

We raised a polyclonal antibody against a monoclonal antibody affinity-purified preparation of megalin from rat kidney microvilli. Because RAP binding to megalin is Ca\(^{2+}\) dependent (5,14,19), we isolated megalin in the presence of EDTA to prevent the copurification of RAP. Immunoblot analysis with anti-RAP antibodies demonstrated that the original antigen preparation was devoid of RAP. To confirm the specificity of the antisera for megalin, we performed immunoblotting studies on a renal cortical extract. By immunoblotting, the antimegalin antibody recognized only megalin and no other renal antigens including RAP (Figure 1A). As a second criterion to verify the specificity of the antimegalin antibody, we performed immunolocalization studies on the rat kidney. Incubation of semithin cryosections with antimegalin resulted in a staining pattern (Figure 1B) indistinguishable from that obtained previously with both polyclonal and monoclonal antibodies to megalin (3,8,15). The antibodies specifically bound to clathrin-coated pits along the proximal tubule brush border, which is the major site of megalin expression. In addition, there was no endoplasmic reticulum staining, further corroborating that the antibodies are devoid of RAP immunoreactivity. Collectively, these results demonstrate that the polyclonal antibody generated against megalin is specific for this large glycoprotein receptor and shows no cross-reactivity toward any other renal proteins including RAP.

Megalin Antibodies Devoid of RAP Cross-Reactivity Induce pHN

To determine if this newly generated antibody binds to epitopes on megalin capable of inducing pHN, Lewis...
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Figure 3. Subepithelial immune deposits are seen by electron microscopy after the injection of antimegalin antisera. (A and B) Immune deposits (arrowheads) are present under the slit diaphragms (arrows) and between the epithelium and glomerular basement membrane (GBM). Rats were treated as in Figure 2, and their kidneys were processed for routine electron microscopy. Bar, 1 µm.

Figure 4. Detection of bound antimegalin antibodies by immunogold labeling. (A and B) Ultrathin cryosections from kidneys of rats injected with antimegalin antisera were labeled with goat anti-rabbit IgG coupled to 10-nm gold. Gold particles indicating the presence of immune deposits (ID) containing bound antimegalin antibodies are seen under the slit diaphragms in the epithelial filtration slits. Cap, capillary. Bar, 1 µm.

Figure 5. Antibodies bound to glomeruli of rats with pHN specifically recognize megalin. Antibodies were eluted from isolated glomeruli of rats with pHN induced with antimegalin antibodies as described in Materials and Methods and used for immunoblotting and immunoprecipitation assays. (A) Immunoblots prepared from kidney cortical proteins with the eluted antibodies and anti-RAP antibodies. The eluted antibodies demonstrate reactivity specifically toward megalin and no other renal proteins (Lane 1). RAP is recognized by anti-RAP-GST antibodies (Lane 2). (B) Affinity-purified RAP-GST fusion protein was radioiodinated, and immunoprecipitates were prepared with the indicated antibodies. Although anti-RAP-GST efficiently precipitated the labeled fusion protein (Lane 1), neither the antimegalin sera (Lane 2) nor the eluted antibody (Lane 3) recognized RAP. Ab, antibody.

Antibodies Eluted From Glomeruli of pHN Rats Specifically Recognize Megalin

By definition, antibodies bound to glomeruli of rats with pHN are enriched for specific IgG that bind to HNAC in vivo and initiate the formation of immune deposits. To determine whether the bound antibodies were specific for megalin or also recognized epitopes on RAP or other proteins, we eluted the antibodies...
from glomeruli of pHN rats injected with antimegalin antibodies and used them for immunoblotting and immunoprecipitation. As shown in Figure 5A, the eluted antibodies bound only to megalin in an immunoblot of rat cortical proteins. No cross-reactivity toward RAP or any other renal protein was detected.

As a further test to verify that the eluted antibody did not recognize RAP, we carried out immunoprecipitations with purified RAP-GST fusion protein. Because the RAP-GST is radioiodinated, this serves as a highly sensitive assay for the detection of trace amounts of antibodies toward RAP in the eluted antibody preparation. Figure 5B shows that although anti-RAP antibodies efficiently precipitated radioiodinated RAP-GST (Lane 1), no reactivity was detected with either the eluted antibody (Lane 3) or the original antisera (Lane 2) used for injection. Collectively, these results indicate that megalin antibodies are capable of inducing pHN in the absence of antibodies specific for RAP. In addition, the fact that no other renal antigens were recognized by the eluted IgG preparation indicates that megalin alone can induce HN.

The Eluted Antibodies Recognize Two Proteolytic Fragments of Megalin

Using the eluted antibodies that are enriched for IgG reactive toward epitopes involved in the initiation and progression of HN, we attempted to identify a proteolytic fragment(s) within megalin that binds these antibodies. Toward this end, we radioiodinated affinity-purified megalin and digested it with chymotrypsin. The digestion products were then subjected to immunoprecipitation analysis with antimegalin antisera and the eluted antibody preparation (Figure 6). Two major fragments with relative molecular masses of ~140 and 75 kd were identified by both the antimegalin antisera (Lane 2) and the eluted antibody (Lane 4). These fragments could represent two separate antibody-binding sites within megalin. Alternatively, the 140-kd fragment could represent an incomplete digestion product and the 75-kd fragment might be contained within the 140-kd polypeptide. A minor band was also detected at ~200 kd. Recognition of these proteolytic fragments of megalin by both the eluted IgG and the whole antisera indicates that these digestion products contain a nephritogenic epitope(s). Because our laboratory has recently isolated the cDNA for megalin (1), the identification of this fragment(s) will facilitate the characterization of megalin's pathogenic epitope(s).

**DISCUSSION**

Previous studies have clearly shown that antisera raised against a highly enriched fraction of megalin will induce pHN (2,3,8,10,11). However, recent data indicate that RAP binds to and copurifies with megalin (14,20,21) and therefore is likely to be present in the antigen preparation used for generating antimegalin antisera unless specific measures are taken to avoid it (e.g., the addition of Ca²⁺ chelators). Therefore, antisera raised against megalin may also demonstrate reactivity against RAP (9). Because RAP expresses a nephritogenic epitope that is capable of independently inducing pHN (12,22), further data are necessary to provide conclusive evidence as to megalin's role in HN.

In this study, we have raised a polyclonal antibody to megalin that shows no cross-reactivity with RAP or any other renal antigens by immunobiochemical and immunocytochemical procedures. Injecting this antimegalin IgG into rats resulted in the formation of subepithelial immune deposits. Antibodies eluted from these immune deposits specifically recognized megalin and demonstrated no reactivity toward any other renal antigens including RAP.

We have previously proposed testable models describing how antibody-binding epitopes are presented on HNAC (4). These models suggest that nephritogenic epitopes presented by megalin and RAP located on the
basal surface of the glomerular epithelium are either (1) immunologically distinct or (2) antigenically related. The data presented here demonstrate that epitopes presented on megalin are capable of binding circulating antibodies and serving as a nucleation site for the formation of immune deposits in the absence of immunoreactivity toward RAP. We conclude that antibody-binding epitopes involved in the initial events of HN are expressed independently by megalin and RAP on the surface of the glomerular epithelium. Furthermore, this study affirms that at least two nephritogenic epitopes are present within HNAC that are capable of inducing pHN, thus providing new evidence in support of the multiple epitope model (Figure 7).

An important question in the pathogenesis of HN is whether one nephritogenic epitope can serve as a nucleation site for the formation of immune aggregates or if antibody binding to multiple sites is required. This question has been addressed in part through the use of monoclonal antibodies raised against megalin (23). When injected into rats, monoclonal antibodies are confined to coated pits of the glomerular epithelium. However, polyclonal antibodies are found in both coated pits and in electron-dense subepithelial deposits, suggesting that polyvalent antibodies are necessary for the initiation and growth of immune aggregates. The differences reported in the pathologic effects of monoclonal and polyclonal antibodies may be due in part to their rates of cell-mediated clearance, as recent studies demonstrated that the internalization of monoclonal antibodies by cultured glomerular epithelial cells is more rapid than that of polyclonal antibodies (24). Reduced clearance of polyvalent megalin antibodies may result in a greater amount of antibody binding to the surface of the glomerular epithelium and in turn increase the probability of immune complex deposition. Additionally, in HN, the binding of polyclonal antibodies to megalin also leads to receptor-antibody shedding from the podocyte cell surface and subsequent cross-linking to the basement membrane, forming insoluble immune deposits (8). The binding of polyvalent antibodies to multiple epitopes on megalin or RAP, which reduces the rate of receptor-mediated clearance, may provide the higher level of antibody binding to the surface of podocytes critical for promoting shedding and the later pathogenic events of HN.

To fully understand the pathologic events of membranous nephropathy, it is necessary to identify the mechanism of immune deposit formation at the molecular level. Developing effective therapies to prevent and/or reverse these pathologic events requires the identification of the amino acid sequence that binds circulating antibodies. We have previously shown that the N-terminal 86 amino acids of RAP contain an epitope that is responsible for inducing pHN after injection with anti-RAP antisera (12). Here, we report that antibodies eluted from the glomeruli of HN rats demonstrate reactivity toward two major proteolytic fragments of megalin with relative molecular masses of 140 and 75 kd. Whether these fragments represent two separate nephritogenic domains or whether the 75-kd fragment is contained within the 140-kd polypeptide is currently unknown. In addition, these same proteolytic fragments are also recognized by the anti-megalin antisera, suggesting that these domains are among the most immunogenic regions in megalin.

In this report, we have demonstrated that megalin alone can initiate the events of immune aggregate formation without contributions from the well-characterized nephritogenic epitope presented on RAP (12, 13). The results indicate that at least two pathogenic epitopes are present within the HNAC complex, and each is capable of independently inducing the formation of immune deposits. Using the cDNA for megalin (1), we are currently mapping the amino acid sequences that bind pathogenic antibodies. This information will allow us to determine the number of pathogenic epitopes that are expressed in HNAC and lead to a more complete understanding of the molecular nature of antibody interactions with the glomerular epithelium.

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