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Mapping rat megalin: The second cluster of ligand binding repeats contains a 46-amino acid pathogenic epitope involved in the formation of immune deposits in Heymann nephritis

(membranous nephropathy/low density lipoprotein receptor gene family)

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ABSTRACT **Megalin (gp330), an epithelial endocytic receptor, is a major target antigen of Heymann nephritis (HN), an autoimmune disease in rats. To elucidate the mechanisms of HN, we have mapped a pathogenic epitope in megalin that binds anti-megalin antibodies. We focused our attention on four clusters of cysteine-rich, low density lipoprotein receptor (LDLR) ligand binding repeats in the extracellular domain of megalin because they represent putative ligand binding regions and therefore would be expected to be exposed *in vivo* and to be able to bind circulating antibodies. Rat megalin cDNA fragments I through IV encoding the first through fourth clusters of ligand-binding repeats, respectively, were expressed in a baculovirus system. All four expression products were detected by immunoblotting with two antisera capable of inducing passive HN (pHN). When antibodies eluted from glomeruli of rats with pHN were used for immunoblotting, only the expression product encoded by fragment II was detected. This indicates that the second cluster of LDLR ligand binding repeats is directly involved in binding anti-megalin antibodies and in the induction of pHN. To narrow the major epitope in this domain, fragment II was used to prepare proteins sequentially truncated from the C- and N-terminal ends by *in vitro* translation. Analysis of the truncated translation products by immunoprecipitation with anti-megalin IgG revealed that the fifth ligand-binding repeat (amino acids 1160–1205) contains the major epitope recognized. This suggests that a 46-amino acid sequence in the second cluster of LDLR ligand binding repeats contains a major pathogenic epitope that plays a key role in pHN. Identification of this epitope will facilitate studies on the pathogenesis of HN.**

Megalin (gp330) is a multiligand endocytic receptor expressed in clathrin-coated pits at the surface of a number of epithelia including those of the kidney glomerulus (1), proximal tubule (2, 3), yolk sac, type II cells of the lung, etc. (4). It was originally discovered (1, 3) as the target antigen of an autoimmune disease known as Heymann nephritis (HN), a rat model of human membranous nephropathy (5). Megalin binds a ~40-kDa protein called RAP (for receptor-associated protein) (6), which serves as a specialized chaperone during the biosynthesis of megalin (7) and in its delivery to the cell surface (8). Together megalin and RAP constitute what we have called the Heymann nephritis antigenic complex (5, 6). HN is induced when antibodies bind to HNAC and the resulting immune complexes are shed from the cell surface and become attached to the glomerular basement membrane to form subepithelial immune deposits (9), which are the hallmark of membranous nephropathy (5). HN can be induced by immunization with either megalin (1) or RAP (10), and it can be passively induced by injection of either anti-megalin (1, 11) or anti-RAP (10, 12–14) antibodies. This indicates that both megalin and RAP contain "pathogenic epitopes" capable of binding antibodies and inducing HN. We have recently mapped the pathogenic epitope in RAP to 14 amino acids near its N terminus (13). We also recently determined the complete primary structure of rat megalin (15), making it possible to pinpoint the pathogenic epitopes in megalin. As a member of the LDLR gene family (16), megalin contains three characteristic motifs—36 ligand-binding repeats arranged in four clusters, 16 growth factor repeats separated by YWTD spacer regions, and one C-terminal epidermal growth factor repeat (see Fig. 1). To date, the location of the pathogenic epitope(s) in megalin has not yet been determined. It is clear that there is no primary sequence homology between megalin and RAP, but it is not known whether there is any structural similarity between the pathogenic epitope identified in RAP and the epitope(s) in megalin. It is important to map the pathogenic epitopes in megalin to elucidate the molecular mechanisms of immune deposit formation in HN.

In this study we have used recombinant fragments of megalin produced in insect cells and antibodies eluted from rats with passive HN (pHN) induced with anti-megalin antibodies to locate a pathogenic epitope in megalin. We have pinpointed a 46-amino acid sequence in the second of four clusters of ligand binding repeats in megalin as containing an epitope that may play a key role in the induction of HN.

MATERIALS AND METHODS

Antibodies. Polyclonal anti-megalin (rat) antiserum was prepared as described (1). Rabbit antiserum was also raised to a rat renal microvillar fraction (17). IgG was purified on Protein A-Sepharose 4B. Both antisera are highly reactive toward megalin and induced pHN when injected to rats. Induction of pHN with anti-microvillar antisera and elution of antibodies from glomeruli of rats with pHN was carried out as described (1, 3, 9).

Construction of pSPH. pSPH (Fig. 2) was constructed by modifying pSPUTK (Stratagene) using DNA cloning techniques including PCR as described (18). Using a human integrin VLA-2 cDNA clone 2.72 (19) (kindly provided by Dr. Yoshikazu Takada, Department of Vascular Biology, Scripps Research Institute, La Jolla, CA), the nucleotide sequence for the signal peptide sequence and four amino acids of the N

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Abbreviations: HN, Heymann nephritis; pHN, passive Heymann nephritis; LDLR, low density lipoprotein receptor; RAP, receptor-associated protein; MCS, multiple cloning site.

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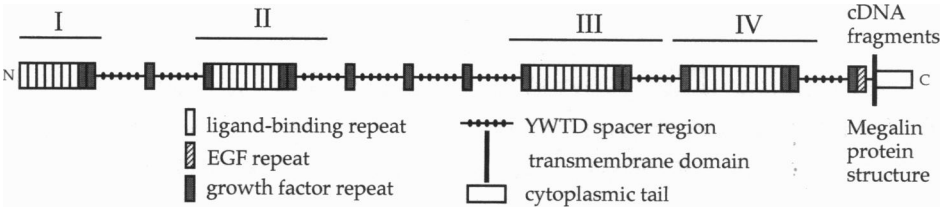

FIG. 1. Structure of rat megalin (15) showing the three characteristic motifs—36 ligand-binding repeats arranged in four clusters, 16 growth factor repeats separated by YWTD spacer regions, and one C-terminal epidermal growth factor (EGF) repeat. We expressed four cDNA fragments (I–IV), encoding the first, second, third, and fourth clusters of ligand binding repeats, respectively, for identification of pathogenic epitopes by immunoblotting and immunoprecipitation analysis (see Figs. 3–5).

terminus of VLA-2 was amplified by PCR with *Pfu* DNA polymerase (Stratagene) using the following primers: 5′-GGAAAGATCTGCGGCCGCTCGCGACCATGGGGCCAGAACGGACAGG-3′, TTGCGGCCGCTCAATGATGATGATGATGATGATATCCCGTCGACGCCCGGGCCCAGCTGGACCAACATTGTAGGCCAAA. The 186-bp PCR product was digested with *Bgl*II and inserted between *Bgl*II and *Hpa*I sites of pSPUTK to replace the 139-bp sequence encoding the multiple cloning site (MCS) (Fig. 2). The sequence was confirmed by the dideoxynucleotide chain-termination method.

Subcloning cDNA Fragments in pSPH. Rat megalin cDNA clones (15) in pBluescript SK− were used for restriction digestion to prepare the following four cDNA fragments, which encode the individual clusters of ligand binding repeats (Fig. 1). Clone 63 was used to prepare fragment I (nucleotides 106-1364, *Ava*I–*Acc*III restriction fragment, amino acids −25 to 389), clone 55 for fragment II (nucleotides 2886–4916, *Dra*I–*Bgl*II, amino acids 897-1573), clone 83 for fragment III (nucleotides 7834–10134, *Nhe*I–*Acc*I, amino acids 2547–3312), and clone 207 for fragment IV (nucleotides 10315–12405, *Bst*EII–*Bam*HI, amino acids 3374–4069). These restriction fragments were all blunt-ended with the Klenow fragment of DNA polymerase I. The cDNA fragments I through IV were cloned at the *Nru*I–*Sma*I, *Sma*I, *Eco*RV, and *Eco*RV sites of pSPH, and the resulting constructs were named pSPH-I, -II, -III, and -IV, respectively. The VLA-2 signal peptide sequence of pSPH was removed for pSPH-I, which encodes the probable signal peptide sequence of rat megalin.

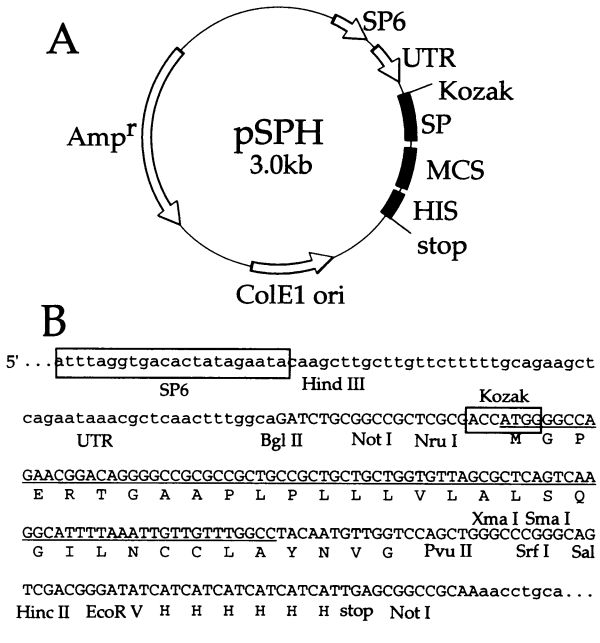

FIG. 2. Structure of the pSPH vector. (*A*) Following the SP6 promoter and the *Xenopus* β-globin 5′-untranslated region (UTR) sequence derived from pSPUTK, pSPH encodes the Kozak sequence, human VLA-2 signal peptide (SP) sequence, a MCS, and a hexa-His tag sequence (HIS) terminated by a stop codon. (*B*) The sequence replaced to construct pSPH from pSPUTK is capitalized. The sequence was introduced between the *Bgl*II (at nucleotide 52) and *Hpa*I sites (at nucleotide 191) of pSPUTK. The VLA-2 SP sequence (underlined) is followed by the sequence encoding its four N-terminal amino acids.

Baculovirus Expression. pSPH-I was digested with *Not*I to release the fragment I insert with the hexa-His tag sequence and blunt-ended with the Klenow fragment of DNA polymerase I. The released fragment was subcloned at the *Stu*I site of pAcSB2 (PharMingen); the resulting construct was named pAc-I. pSPH-II was digested with *Pvu*II, and the released 1.3-kb fragment was subcloned at the *Sma*I site of pAcGP67B (PharMingen). The recombinant plasmid was then double-digested with *Sma*I and *Not*I, and the released 0.1-kb fragment was replaced by a 0.9-kb *Sma*I–*Not*I fragment from pSPH-II to construct pAc-II. pSPH-III and pSPH-IV were double-digested with *Sma*I and *Not*I, and *Srf*I and *Not*I, respectively. The released inserts, with the hexa-His tag sequence, were subcloned between the *Sma*I and *Not*I sites of pAcGP67A (PharMingen) to make the pAc-III and pAc-IV constructs, respectively. In pAc-II, -III, and -IV, the cDNAs were inserted downstream of the gp67 signal peptide sequence. The constructs pAc-I through pAc-IV were transfected into Sf9 cells to generate recombinant baculoviruses. The viruses were amplified, and cells were infected and induced to express recombinant proteins following the protocol of the Baculovirus Expression Vector system (PharMingen).

Preparation of Megalin Fragments and Immunoblotting. Sf9 cells were infected with the amplified recombinant viruses derived from pAc-I, -II, -III, and -IV and cultured for 72 hr, after which they were lysed in guanidinium buffer (6 M guanidine HCl, 20 mM sodium phosphate, 500 mM NaCl (pH 7.8), for 30 min at room temperature followed by sonication (3 × 5 sec). Lysates were cleared by centrifugation (10,000 × *g* for 15 min), and the supernatants were incubated for 15 min with nickel-Sepharose (Invitrogen). The resin was washed step-wise with 8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride at successive pH of 7.8, 6.0, 5.3, and 4.0. Bound proteins were eluted with a 10–320 mM step gradient of imidazole in the urea containing buffer (pH 4.0). Baculovirus-derived megalin fragments were identified by silver staining following 7% SDS/PAGE. The individual fragments were then pooled, dialyzed against 10 mM Tris (pH 7.4)/50 mM NaCl, lyophilized, and resuspended in dH_20 to ~1 mg/ml.

For immunoblotting, proteins were boiled for 5 min in Laemmli sample buffer supplemented with 5% 2-mercaptoethanol and separated by SDS/PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Millipore) and probed with IgG prepared from both antisera (2 μg/ml) and the glomerular eluted antibodies. The protein–antibody complexes were detected with secondary antibodies conjugated to horseradish peroxidase using chemiluminescence (Pierce).

***In Vitro* Transcription.** Linearized plasmid DNAs and PCR products that contain the SP6 promoter were used as templates to synthesize capped RNA with mMESSAGE mMACHINE (Ambion). The DNA fragments (0.5–1 μg) were mixed with 10 μl of 2 × ribonucleotide mix (10 mM ATP, CTP, and UTP/2 mM GTP/12 mM m7G(5′)ppp(5′)G cap analogue), 2 μl of 10 × Transcription buffer, 1 μl of RNase inhibitor (Promega), and 2 μl of enzyme mix containing SP6 RNA polymerase in a final volume of 20 μl and incubated at 37°C for 2 hr. The RNA was precipitated using lithium chloride and dissolved in 30–50 μl of TE [10 mM Tris·HCl (pH 7.5) at 20°C/1 mM EDTA).

***In Vitro* Translation.** The capped RNA was translated *in vitro* using the Flexi rabbit reticulocyte lysate system (Promega). The RNA (~1 μg) in TE was mixed in a total volume of 25 μl with the following: 16.5 μl of Flexi rabbit reticulocyte lysate, 0.5 μl of amino acid mixture excluding Met, 1.0 μl of ^{35}S-labeled Met (Premium Stabilized Translation Grade, DuPont/NEN), 0.7 μl of KCl (2.5 M), 0.5 μl of RNase inhibitor (Promega), and RNase-free H_2O and incubated at 30°C for 90 min.

Preparation of Fragments Truncated from the C Terminus. Proteins encoded by cDNA fragment II were truncated from the C terminus in two ways. Truncation fragments a, b, c, g, and h (Fig. 4*A*) were prepared by digesting pSPH-II with *Eco*RV, *Pvu*II, *Sma*I, *Ban*I, and *Afl*III, respectively. Truncation fragments d, e and f were prepared by digesting pSPH-II with *Sap*I, which only cuts the vector, and were used as templates for PCR to amplify fragments containing the SP6 promoter and 5′ portions of the inserts. The *Sap*I-digested pSPH-II was used for PCR with a sense primer (SPH-S, CCTTATGTATCATACACATACG) and antisense primers (II-D, GGTCTTAGGAACACAGCCAG; II-E, CATGCCGGGAGGCCTGGTGG; II-F, GTTTAACACACAGCCCGCCT), respectively. The PCR products were purified with the Prep-A-Gene DNA purification system (Bio-Rad). The linearized pSPH-II DNAs and PCR products were used for *in vitro* transcription and translation. The translation products were mixed with Laemmli sample buffer supplemented with 5% β-mercaptoethanol, boiled for 5 min, and separated by SDS/PAGE. Gels were stained with 0.05% Coomassie R in 40% MeOH/10% acetic acid, destained, incubated for 20 min in Amplify (Amersham), dried, and exposed to Kodak X-Omat AR radiographic film. Peak volume quantitation of radioactive signals was performed on a Molecular Dynamics PhosphorImager (Molecular Dynamics). Gels were exposed to a phosphor screen for 1 day, and data were quantitated using IMAGEQUANT version 3.3 software after subtracting background values. The translation products were equalized based on radioactivity and separated by SDS/PAGE. Autoradiograms were scanned using an OmniMedia XRS 12cx scanner and processed using Adobe Photoshop on a MacIntosh 8500/66 PowerPC. Images were produced on a Kodak ColorEase PS printer.

Preparation of Fragments Truncated from the N Terminus. To prepare fragments a′, b′, c′, and d′, truncated from the N-terminus (Fig. 5A), pSPH-II digested with *Sap*I was used for PCR with an antisense primer (II-ENDA, TCCCCTCGAGATCTTTTCCTGGACAATGA) and sense primers (II-A′, CCCACACATGCCACGTCGTC; II-B′, CAAGCTTCAGGGACCTGCCA; II-C′, GTGTTAAACTGTACGAGTGC; II-D′, CCCACCAGGCCTCCCGGCAT), respectively. The PCR products were digested with *Xho*I whose site was introduced at the 3′ end of II-ENDA. The digested products were purified and subcloned between the *Sma*I and *Sal*I sites of pSPH. The resulting constructs were linearized with *Sap*I and were used for *in vitro* transcription and translation. The radioactivities of the specific translation products were quantitated and equalized as described above.

Immunoprecipitation. Protein A-agarose beads (Bio-Rad) were washed twice with 1 ml of RIPA buffer (10 mM Tris·HCl (pH 7.2)/150 mM NaCl/1% deoxycholate/1% Triton X-100/0.1% SDS) and incubated with equal amounts of radiolabeled *in vitro* translation products in 1 ml of RIPA buffer at 4°C for 4 hr to eliminate nonspecific binding. The supernatants were recovered, mixed with newly washed Protein A-agarose beads and 3 μl of anti-megalin IgG (4 mg/ml), and incubated at 4°C for 16 hr. The beads were then washed with 1 ml of RIPA buffer three times, resuspended in Laemmli sample buffer with 5% β-mercaptoethanol, boiled for 5 min, and proteins were separated by SDS/PAGE.

RESULTS

Structure of pSPH. As shown in Fig. 2, pSPH has an SP6 promoter and the 5′-untranslated sequence of the *Xenopus* β-globin gene—both derived from pSPUTK. The latter sequence is known to increase the efficiency of *in vitro* translation (20). In addition, pSPH encodes the signal peptide sequence and the N-terminal four-amino acid sequence of human integrin VLA-2. The signal peptide sequence is necessary for translocating the *in vitro* translation products to microsomes. pSPH has a unique MCS that allows any blunt-end cDNA fragments to be cloned in frame using the blunt-end restriction sites (*Pvu*II, *Sma*I, *Srf*I, *Hin*cII, or *Eco*RV) or 5′-overhanging *Xma*I or *Sal*I site after blunt-ending with Klenow fragment of DNA polymerase I. The MCS is followed by a hexa-His tag sequence to purify the expression products on Ni^{2+} resins (21).

Immunoblotting of the Baculovirus Expression Products. Rat megalin cDNA fragments I–IV encoding the first through fourth clusters of ligand binding repeats were used to generate recombinant baculoviruses. Sf9 cells were infected with recombinant baculoviruses, and the expressed proteins were affinity-purified by Ni^{2+} chromatography. Purified proteins representing fragments I through IV were subjected to immunoblot analysis using two polyclonal antibodies that recognize megalin and antibodies eluted from glomeruli of rats with pHN induced with one of the antisera. As shown in Fig. 3*A*, all of

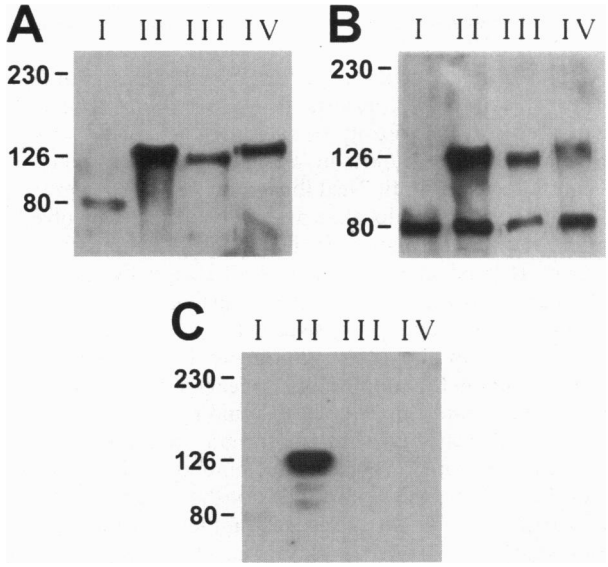

FIG. 3. Immunoblotting of baculovirus expression products of fragments I–IV. Antibodies to purified rat megalin (*A*) and antibodies to renal microvillar antigens (*B*) that contain megalin as the major component both strongly detected fragment II product (M_r ~ 126 kDa) and to a lesser extent products of fragment I (M_r ~ 80 kDa), fragment III (M_r ~ 124 kDa), and fragment IV (M_r ~ 130 kDa). The additional proteins in *B* (II–IV), M_r ~ 80–85 kDa, that are recognized by the anti-microvillar antibodies are most likely degradation products formed during purification of fragments II-IV products from the baculovirus-infected insect cells. (*C*) The fragment II product was selectively detected with antibodies eluted from glomeruli of rats with pHN, indicating that the second cluster of ligand binding repeats is directly involved in pHN.

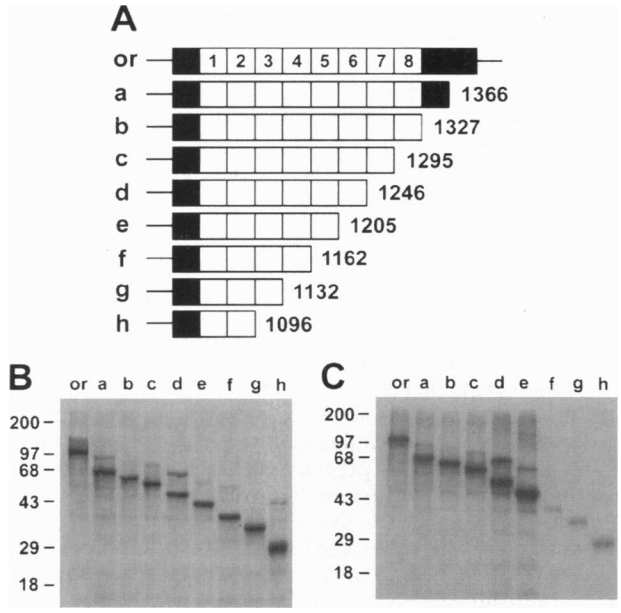

FIG. 4. Immunoprecipitation analysis of fragment II *in vitro* translation products truncated from the C terminus. (*A*) Schematic structures of the products of original fragment II (or) and the truncated fragments (a–h). The open boxes represent ligand-binding repeats numbered 1–8, and the shadowed boxes represent growth factor repeats. The numbers to the right indicate the positions of the terminal amino acids. (*B*) Equal amounts (based on radioactivity) of the *in vitro* translation products were separated by 12% SDS/PAGE to verify their predicted molecular weights. (*C*) Equal amounts of the products were used for immunoprecipitation with anti-megalin IgG and separated by 12% SDS/PAGE. The truncated translation products a–e containing the first through fifth ligand-binding repeats were efficiently precipitated, whereas products f–h were not. The small amount of binding seen with translation products f–h was equivalent to nonspecific adsorption to Protein A-agarose in the absence of antibodies (not shown).

the expression products were detected with both antibodies, indicating that all four clusters of ligand binding repeats are immunogenic. The intensity of each product varied between the two IgG preparations, but the fragment II product was strongly detected by both. Next the eluted antibodies were used for immunoblotting to localize an epitope directly involved in immune deposit formation in HN. Fig. 3*B* shows that the fragment II product was selectively detected by the eluted antibodies, indicating that the second cluster of ligand-binding repeats contains an epitope involved in the pathogenesis of pHN.

Localization of the Major Antigenic Epitope within the Second Cluster of Ligand Binding Repeats. Fragment II, which encodes the second cluster of ligand binding repeats, was used to prepare truncated *in vitro* translation products to further pinpoint the epitope responsible for binding pathogenic antibodies. Fig. 4*A* shows the structures of the *in vitro* translation products of fragment II truncated from the C terminus. Equal amounts of the *in vitro* translation products were used for immunoprecipitation with the nephritogenic antibody to further localize the major epitope (Fig. 4 *B* and *C*). The original fragment II translation product and the truncation products a–e were efficiently precipitated, but products f–h were not. Truncation product e contains the fifth ligand-binding repeat, but product f does not. To confirm these results, truncation was carried out from the N terminus of fragment II. The truncation products a′–d′ were efficiently precipitated but not e′, which lacks the fifth ligand-binding repeat (Fig. 5 *A–C*). Together, these results indicate that a 46-amino acid sequence (amino acids 1160–1205) of megalin representing the fifth ligand-binding repeat in the second cluster contains a major epitope of HN.

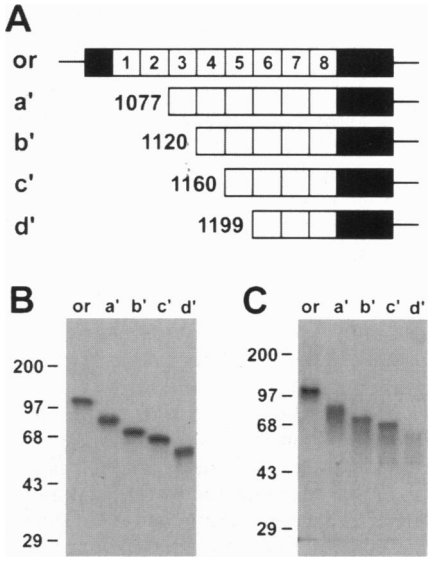

FIG. 5. Immunoprecipitation analysis of the *in vitro* translation products of fragment II truncated from the N terminus. (*A*) Structures of the original (or) and truncated forms (a′ through d′). The *in vitro* translation products (*B*) and the immunoprecipitates (*C*) were separated by 9% SDS/PAGE. All except the smallest fragment (d′), missing the fifth ligand-binding repeat, were efficiently precipitated. This, along with the C-terminal truncation analysis shown in Fig. 4, indicates that the major epitope is contained within the 5th ligand binding repeat.

DISCUSSION

The extracellular domain of megalin has four clusters of cysteine-rich regions that are composed of 7, 8, 10 and 11 ligand-binding repeats, respectively, with adjacent growth factor repeats. We have shown here that all four of these clusters are immunogenic since they are recognized by an antibody raised against purified rat megalin and another raised against rat renal microvillar antigens. pHN was induced in rats, and the antibodies that bound to glomeruli were eluted and used as tools to map the pathogenic epitopes involved in pHN. By immunoblotting, the eluted antibodies predominantly recognized the product containing the second cluster of ligand binding repeats, indicating that this region is directly involved in formation of immune deposits in pHN. Our results suggest that the second cluster of ligand-binding repeats is exposed on the surface of the glomerular epithelium, which binds circulating antibodies and presents a major epitope in HN. The epitopes in the other clusters of ligand binding repeats may be cryptic or occupied by other molecules masking the epitopes *in vivo*. To gain further insight into these processes, the baculovirus expression products are being used to prepare specific antibodies that will be used for immunohistochemical analysis and tested for their ability to induce pHN.

The second cluster of ligand binding repeats was then systematically analyzed to pinpoint the epitope using truncated products prepared by *in vitro* translation for immunoprecipitation. The *in vitro* translation products were not efficiently detected by the antibodies eluted from glomeruli of rats with pHN, perhaps because the titer of the eluted antibodies is too low to detect such a small amount of the products of *in vitro* translation. Alternatively, the epitope recognized by the eluted antibodies may be conformation-dependent, and the translation products may not be correctly folded. Therefore immunoprecipitations were carried out using high titer IgG from antiserum specifically raised against rat megalin. Using translation products truncated from both the C- and N-terminal ends for immunoprecipitation, the fifth ligand binding repeat (amino acids 1160–1205) was found to contain an epitope that

appears to be responsible for the antigenicity of the entire domain. We conclude that the fifth ligand binding repeat contains a key epitope involved in the formation of immune deposits in pHN. Megalin appears to bind the antibodies, and the resulting immune complexes are shed, presumably by cleavage of its ectodomain (9, 22), and become attached to the GBM (9). Antibodies to the baculovirus expression products will be useful for identification of other epitopes involved in active and passive HN (5).

Previously we identified a 14-amino acid sequence in RAP that contains a pathogenic epitope for pHN (12, 13). The sequence has no significant similarity with the fifth repeat in the second cluster of ligand-binding repeats of rat megalin. Identification of the pathogenic epitope on megalin will be helpful to investigate more precisely how megalin and RAP are associated to initiate HN.

We have recently shown that the second cluster of ligand binding repeats in megalin contains a binding site for both RAP and several specific ligands (23). Further studies in progress are designed to precisely map the ligand binding sites as well as the pathogenic epitopes in megalin.

In this study we showed that the second cluster of ligand binding repeats is involved in immune deposit formation in pHN. Furthermore, the fifth ligand-binding repeat in this domain was found to present the major epitope that appears to play a key role in the event. These findings will facilitate studies to clarify the mechanisms of pHN and hopefully will allow development of a novel strategy to treat membranous nephropathy, the human counterpart of HN.

Note Added in Proof. This research was presented at The American Society of Nephrology meeting in San Diego, November, 1995 (24).

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