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Commentary

Molecular Analysis of the Pathological Autoimmune Antigens of Heymann Nephritis

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In this issue, Raychowdhury et al[1] report the induction of Heymann nephritis (HN) with a 137-amino-acid (137-aa) fragment of gp330/megalin made as a fusion protein in bacteria. This paper represents another landmark in the molecular characterization of the target antigens of HN because it represents the first time that HN has been induced with a fragment of gp330/megalin, and at the same time it clearly pinpoints a pathogenic epitope in this large molecule.

Identification of gp330/Megalin as a Target Antigen

HN, a valid model for membranous nephropathy, has provided valuable insights into the molecular pathogenesis of this disease.[2–4] HN was originally induced with crude homogenates of kidney cortex,[5] but it was recognized a number of years ago that the antigen is present in the brush border of the proximal tubule because circulating autoantibodies stained the brush border,[6,7] and the active disease could be produced by immunizing rats with microvillar fractions.[8] gp330/Megalin was identified as the main antigen in brush border fractions by taking advantage of the selected population of autoantibodies bound to glomeruli of rats with active HN. When these antibodies were eluted and used for immunoprecipitation, a single protein with an estimated molecular mass of 330 kd was precipitated from solubilized microvillar proteins.[9,10] That gp330/megalin is a target antigen in HN was validated by showing that rats immunized with gp330/megalin developed glomerular immune deposits characteristic of the active disease, and heterologous antibodies raised against gp330/megalin bound to glomeruli when injected into normal rats (passive disease).[9,10] Immunocytochemical localization of gp330/megalin revealed that it is found in clathrin-coated pits located on the sides and base of the foot processes of podocytes and at the base of the proximal tubule microvilli,[10–12] and immunocytochemical localization of bound antibodies revealed that the coated pits at the base of the foot processes were the site where the immune deposits initially form[10,12] (see Figure 1). The localization of endogenous gp330/megalin in coated pits provided the first clue that this protein might function as an endocytic receptor.

Structure of gp330/Megalin and Location of the Pathogenic Epitope

In 1989, Raychowdhury et al[13] obtained a partial cDNA sequence of gp330/megalin that revealed it contains motifs characteristic of the low density lipoprotein (LDL) receptor gene family. The complete cDNA sequence and predicted protein sequence of gp330/megalin was obtained only recently,[14] and its structure is shown in Figure 2. Its predicted molecular weight, M_r 516,715 (exclusive of oligosaccharides), is considerably larger than originally estimated from its mobility on sodium dodecyl sulfate polyacrylamide gel electrophoresis, which led to the renaming of gp330 to megalin (from the Greek mega, meaning large). Given the large size of megalin, the pinpointing by Raychowdhury et al[1] of a tiny, 137-aa fragment of this huge molecule as a pathogenic epitope represents a remarkable feat. It was accomplished,

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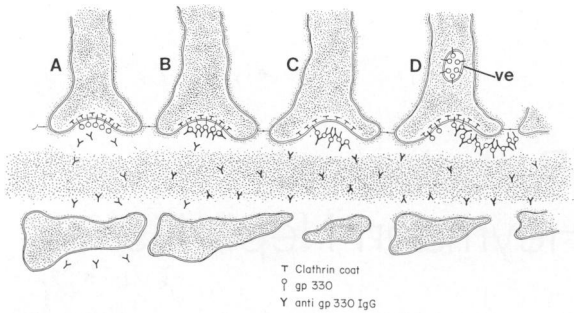

Figure 1. *Drawing summarizing the early events in the formation of an immune deposit in passive HN.* **A:** *Circulating anti-megalin IgG (anti-gp330) penetrates the GBM and approaches megalin/gp330, a resident membrane glycoprotein of clathrin-coated pits located at the base of the epithelial foot processes.* **B:** *Anti-megalin IgG binds to its antigen in coated pits forming an immune complex.* **C:** *The initial immune complex is shed from the cell surface and becomes attached to the GBM as early as 15 minutes after injection of antibody.* **D:** *This immune deposit grows in size by repeated cycles of* in situ *immune complex formation and shedding into the lamina rara externa of the GBM until it eventually encroaches on the area of the slit diaphragm. The continued growth of the immune deposit appears to require the* de novo *synthesis by the glomerular epithelium of megalin/gp330, which, like other membrane glycoproteins, is assumed to be delivered via vesicles (ve) that eventually fuse with the cell membrane at the base of the foot processes. From Kerjaschki et al.*[12]

once again, by taking advantage of the selected population of antibodies bound to the glomeruli of rats with active HN. The fact that β-galactosidase fusion proteins containing this insert were recognized by the eluted antibodies in their earlier paper[13] led the authors to focus on this fragment. Analysis of the location of the 137-aa peptide in the megalin/gp330 molecule reveals that it is present in the second of four clusters of cysteine-rich, LDL-receptor-ligand-binding repeats where it spans the fourth to the sixth ligand-binding repeats (see Figure 2). Interestingly, Saito et al[15,16] have also independently identified the second cluster of ligand-binding repeats as the site of a pathogenic epitope based on its recognition by antibodies eluted from glomeruli of rats with passive HN. Using a different approach involving immunoprecipitation and deletion analysis of megalin fragments translated *in vitro*, Saito et al[15] have narrowed the epitope to the fifth ligand-binding repeat consisting of 46 aa (aa 1160 to 1205). It remains to be seen whether this 46-aa fragment is capable of inducing active HN. As the 137-aa megalin fragment identified by Raychowdhury et al[1] has all of the properties necessary for stimulating helper T cells, triggering the immune system, and inducing immune deposits, additional analysis of its structural properties and additional attempts to narrow the pathogenic epitope and determine its participation in the active *versus* the passive disease should be fruitful. The finding of Raychowdhury et al[1] that the immune deposits contained portions of the ectodomain of gp330/megalin other than those of the fusion protein and that they were not stained by antibodies against the cytoplasmic tail of megalin is in keeping with the scheme presented in Figure 1, according to which binding of antibodies triggers cleavage of endogenous megalin and its shedding and attachment to the glomerular basement membrane (GBM).

Raychowdhury et al[1] have also noted that the immune deposits induced by immunization of rats with the 137-aa fragment of gp330/megalin, like those induced by immunization with recombinant receptor-associated protein (RAP),[17] are somewhat smaller than those found in glomeruli of rats immunized with intact megalin. This may indicate that additional epitopes present in the megalin molecule can contribute to the generation and enlargement of immune deposits.

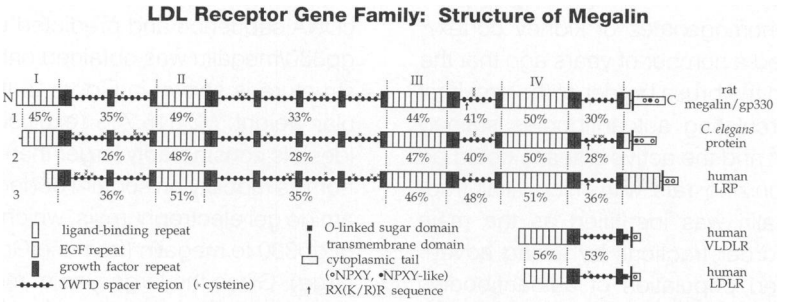

Figure 2. *Comparative protein structure of megalin and several other members of the LDL receptor gene family. Megalin consists of 4660 amino acids and has a predicted molecular weight of 516,715. It contains all of the motifs characteristic of this family, ie, the ligand binding, growth factor and EGF cysteine-rich repeats, and the YWTD spacer regions in the ectodomain and two NPXY motifs and one very similar motif, NQXY; (presumptive coated pit internalization signals) in the cytoplasmic tail. Four putative ligand-binding domains of megalin are indicated by I to IV. Megalin's overall structure is very similar to that of the LDL receptor-related protein/α2-macroglobulin receptor (LRP) and a protein identified in* C. elegans. *Megalin also possesses an RXRR sequence, which constitutes a presumptive proteolytic processing site for precursor processing endoproteases of the furin family.*[14] *Such processing could be responsible for generation of the soluble form of megalin identified in the proximal tubule*[45] *and in L2 yolk sac cells.*[40] *It could also be responsible for the cleavage and shedding of megalin that occurs after antibody binding (see Figure 1). Megalin also has unique features compared with other members of the LDL receptor gene family in that there are differences in the number of the ligand-binding and EGF repeats and in the structure of the cytoplasmic tail (this figure). The cytoplasmic tail of megalin has no sequence homology to those of other members of the LDL receptor gene family except for the NPXY internalization sequences. From Saito et al.*[14]

Amino Acid Sequence of RAP

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1    Y S R E K N E P E M A A K R E S G E E F R M E K L N Q
28   L W E K A K R L H L S P V R L A E L H S D L K I Q E R
55   D E L N W K K L K V E G L D G D G E K E A K L V H N L
82   N V I L A R Y G L D G R K D T Q T V H S N A L N E D T
109  Q D E L G D P R L E K L W H K A K T S G K F S S E E L
136  D K L W R E F L H Y K E K I H E Y N V L L D T L S R A
163  E E G Y E N L L S P S D M T H I K S D T L A S K H S E
190  L K D R L R S I N Q G L D R L R K V S H Q G Y G P A T
217  E F E E P R V I D L W D L A Q S A N F T E K E L E S F
244  R E E L K H F E A K I E K H N H Y Q K Q L E I S H Q K
271  L K H V E S I G D P E H I S R N K E K Y V L L E E K T
298  K E L G Y K V K K H L Q D L S S R V S R A R H N E L
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Figure 3. *Amino acid sequence of rat RAP. RAP is a ~ 40-kd soluble protein that has a potential N-linked glycoprotein site* (asterisk). *A pathogenic epitope has been mapped to aa 39 to 53*[22] *and a heparin-binding site to aa 287 to 306. Modified from Pietromonaco et al.*[17]

A curious, unanswered question is why other members of the LDL receptor gene family, especially megalin's close cousin (see Figure 2), the LDL-receptor-related protein/α2-macroglobulin receptor (LRP/α2MR), fails to induce HN as evidenced by the fact that immunization of rats with liver membranes[8] that are enriched in LRP/α2MR[18] fail to induce HN whereas immunization with kidney membranes and those of yolk sac that are enriched in megalin can induce the disease.

RAP Also Serves as a Target Antigen in HN

In 1990 another protein, now called RAP, was identified, sequenced (see Figure 3), and shown to be a target antigen of HN.[17] This protein was also identified using antibodies eluted from glomeruli of rats with passive HN, this time to screen a λGT-11 rat kidney expression library. Originally thought to be a fragment of gp330/megalin, it soon became clear that RAP is a distinct protein that binds tightly to megalin in a calcium-dependent fashion and co-precipitates with megalin (see Figure 4).[19,20] That RAP can serve as a target antigen was clearly demonstrated by the finding that immune deposits could be produced by immunizing rats with a RAP fusion protein (active HN), and antibodies raised against the fusion protein in rabbits produced passive HN.[17] The pathogenic epitope was initially mapped to the amino-terminal 86 aa of RAP,[21] and more recently has been narrowed to 15 aa (aa 39 to 53; see Figure 3).[22] RAP has two megalin-binding sites as well as a binding site for heparin that were also recently mapped[23] (Figure 5). The fact that RAP has a heparin-binding site is of special interest because it suggests that RAP could play a role in the binding and subsequent cross-linking of the HN antigenic complex to the GBM, which contains abundant

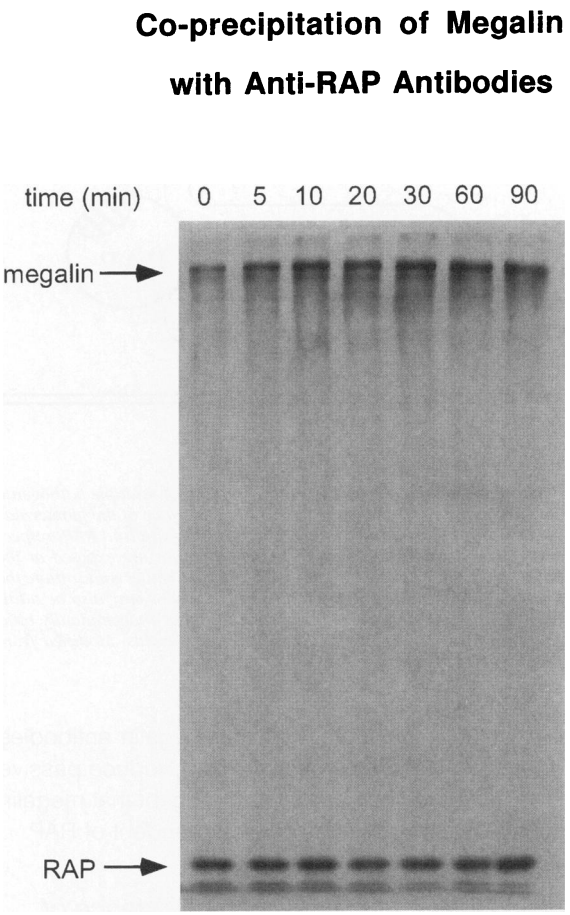

Figure 4. *Co-precipitation of newly synthesized RAP with megalin in L2 rat yolk sac carcinoma cells. Anti-RAP co-precipitates radiolabeled megalin at the end of a 15-minute pulse (0 minutes) and up to 90 minutes of chase. Each sample was immunoprecipitated with anti-RAP; the precipitates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (7.5% gels) and examined by fluorography. From Orlando and Farquhar.*[12]

heparan sulfate proteoglycans (perlecan) in the lamina rara interna and externa.[24] Due to the tight binding of RAP to megalin, many of the early anti-megalin/gp330 antibodies also recognized RAP, which originally created some uncertainties in the localization of pathogenic epitopes. The present findings by Raychowdhury et al,[1] together with the demonstra-

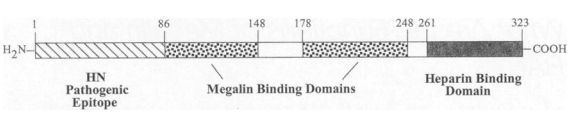

Figure 5. *Domain map of RAP. A pathogenic epitope capable of inducing HN has been identified between aa 1 and 86 based on direct binding of megalin and heparin to truncated recombinant fusion proteins. Two primary binding sites on RAP for megalin (aa 85 to 148 and 178 to 248) and a heparin-binding site (aa 261 to 323) were identified. Within this heparin-binding domain, RAP contains a putative heparin-binding motif (aa 287 to 306) structurally homologous to those in other heparin-binding proteins. From Orlando and Farquhar.*[23]

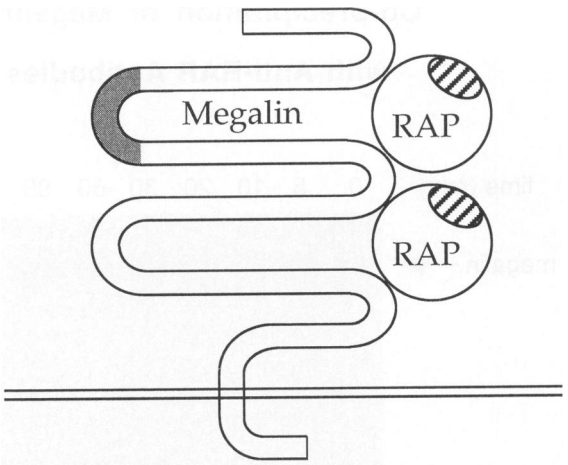

Figure 6. *Molecular model for the presentation of multiple pathogenic epitopes in the HN antigenic complex at the surface of the glomerular epithelium. Shaded and hatched regions in megalin and RAP, respectively, represent potential antigenic domains that are exposed at the basal surface of the podocyte and may bind antibodies and initiate the onset of immune complex formation in HN. There may also be additional exposed epitopes in megalin and RAP that independently bind antibody and induce formation of immune deposits. Modified from Orlando and Farquhar.*[23]

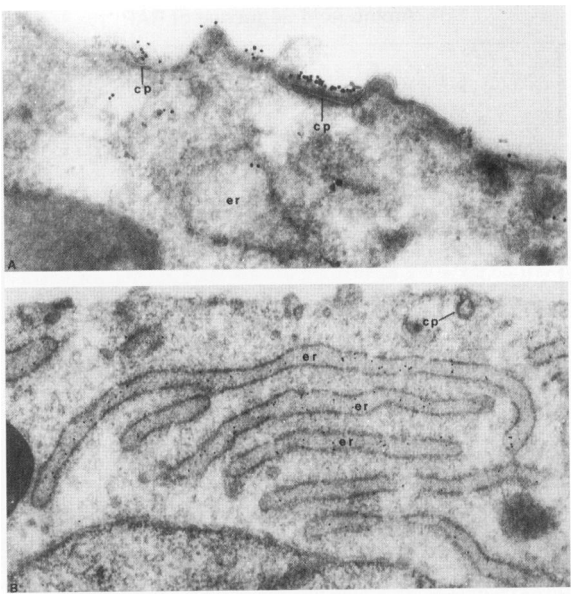

Figure 7. *Immunogold labeling for gp330* (**A**) *and RAP* (**B**) *in L2 yolk sac cells.* **A**: *Gold particles indicating the presence of gp330 are concentrated in coated pits* (*cp*) *at the cell surface. RAP is detected almost exclusively in the ER lumen* (*er*). *Magnification,* ×*50,000* (**A**) *and* ×*30,000* (**B**). *From Lundstrom et al.*[40]

tion by Orlando et al[25] that anti-megalin antibodies devoid of RAP cross-reactivity can produce passive HN, should remove any lingering doubt that megalin is capable of producing HN independent of RAP.

How Do We Account for the Existence of More Than One Antigen in HN?

Figure 6 presents the multiple epitope model indicating how pathogenic epitopes on megalin and RAP, the HN antigenic complex, may be displayed at the cell surface of the podocyte and serve as autoimmune antigens. Megalin is a membrane protein whereas RAP is a soluble protein that binds to megalin at the cell surface. In this model, both megalin and RAP contain at least one pathogenic epitope, and binding of antibodies to either epitope can trigger deposition of immune complexes, ie, cleavage of megalin, shedding of the complexes, and their rapid attachment to the glomerular basement membrane.

What Are the Functions of Megalin and RAP?

Megalin is a multiligand receptor that has been shown to bind a whole variety of ligands *in vitro* including apolipoprotein-E-enriched β-VLDL, lactoferrin, lipoprotein lipase,[26,27] aprotinin,[28] Ca^{2+},[29] plasminogen,[30,31] and plasminogen-activator/plasminogen-activator-inhibitor complexes.[26,27] In the kidney brush border it has been shown to bind Ca^{2+},[29] peptide hormones, and basic proteins[32] and to bind and internalize polybasic drugs.[33,34] Its function in the glomerulus is unknown, but it is intriguing to speculate that megalin may normally be responsible for clearance of filtration residues, including antibodies, by the podocyte. The podocyte has been shown to bind and take up proteins that cross the GBM by endocytosis in coated pits.[35,36] Thus, binding of anti-megalin or anti-RAP antibodies to the complex followed by its shedding and attachment to the GBM might be expected to paralyze normal removal mechanisms and lead to accumulation of ligands in immune deposits.

The function of RAP is also unusual. RAP has the intriguing property that it prevents ligand binding to megalin as well as LRP,[26,37] the VLDL receptor,[38] and the LDL receptor.[39] That is, when RAP is bound to megalin, it is unable to bind any other ligand. At steady state, megalin is found primarily in coated pits, as already mentioned, but RAP is found primarily in the rough endoplasmic reticulum (ER)[19,40] (Figure 7). RAP binds to newly synthesized megalin in the ER shortly after its biosynthesis,[19,41] and the two proteins travel together as a complex to the cell surface.[40,42] Evidence has recently been obtained[43,44] that, upon reaching the cell surface, the megalin/RAP complex is internalized and transported to an endosomal compartment. Here the two proteins dissociate, and megalin is recycled to the cell surface where it is presumably competent to

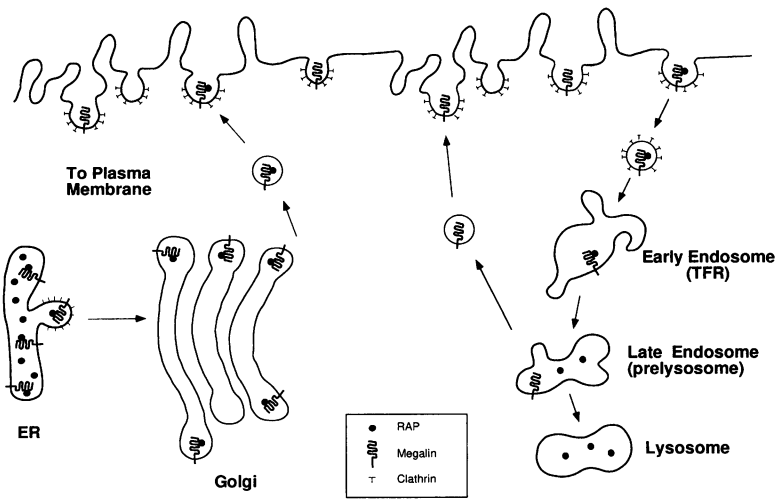

Figure 8. *Diagram indicating the proposed itinerary for the intracellular trafficking of megalin and RAP. Megalin and RAP are assembled into a hetero-oligomeric complex in the rough ER. Newly synthesized RAP and megalin leave the ER together, are transported to the Golgi and across the Golgi stack together, and from there traffic together to the cell surface. Megalin-RAP complexes are internalized in clathrin-coated pits and traffic to a late endosome compartment where they dissociate. RAP is delivered to lysosomes and megalin is recycled to the cell surface. From Farquhar et al.*[43]

bind ligands whereas RAP is delivered to lysosomes and degraded. This trafficking itinerary is shown in Figure 8. Thus, RAP appears to function as a traffic chaperone with the dual function of assisting in the folding of megalin and in preventing ligand binding to megalin in transit along the biosynthetic pathway.

Why Map Pathogenic Epitopes in HN?

Some may consider the molecular mapping of epitopes in megalin and RAP to be esoteric in view of the fact that neither molecule has been implicated as yet in the pathogenesis of membranous nephropathy in humans. This activity is driven by the striking similarity of the events in the pathogenesis of HN to its human counterpart, which suggests that at some level the human disease and the rat model share common underlying molecular mechanisms. The hope is that by further defining the molecular mechanisms involved in HN one will gain insights into the pathogenesis of human membranous nephropathy that can eventually lead to rational therapeutic intervention. Raychowdhury et al[1] have achieved the mapping of the first pathogenic epitope, but given the interest in the topic and its importance, one can expect rapid advancements in the further mapping not only of pathogenic epitopes on megalin but also of functional binding sites for megalin's ligands.

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