Receptor-associated Protein and Members of the Low Density Lipoprotein Receptor Family Share a Common Epitope

AN EXTENDED MODEL FOR THE DEVELOPMENT OF PASSIVE HEYMANN NEPHRITIS

(Received for publication, July 22, 1996, and in revised form, August 27, 1996)

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Heymann nephritis is an experimental rat model for human membranous glomerulonephritis. Two target antigens have been identified in the proximal tubule brush border of rat kidneys. One of them is megalin, a 600-kDa membrane protein that belongs to the family of low density lipoprotein receptor (LDLR)-related proteins. The other one is receptor-associated protein (RAP), a polypeptide of 40 kDa that associates with members of the LDLR family. Here we show that antibodies produced against recombinant human RAP strongly cross-react with the chicken oocyte receptor for very low density lipoprotein and vitelligenin (LR8), and with two other members of the LDLR family, LDLR-related protein and megalin. The interaction of this antibody with LR8 showed binding characteristics exactly as those demonstrated for the physiological ligands of this receptor, in that binding of the antibody: (i) is Ca"-dependent; (ii) is abolished by unfolding of the cysteine-rich binding domain by reduction; and (iii) interferes with the binding of very low density lipoprotein and vitelligenin. Immunopurification of the LR8-specific subpopulation of the polyclonal antiserum yielded an IgG fraction strongly reacting with LR8 as well as with RAP. Using recombinant fragments of RAP and peptide mapping, the cross-reacting epitope(s) could be narrowed down to three short sequences (5–7 residues) in the COOH-terminal part of the protein. After immunization with RAP, anti-LR8 antibodies and anti-RAP antibodies arise simultaneously, indicating that the receptor-specific activity is not due to anti-idiotypic antibodies. These findings suggest the existence of a common epitope(s) on RAP and members of the LDL receptor family. Based on these results, we present an extended molecular model for the development of passive Heymann nephritis.

Receptor-associated-protein (RAP)† was initially described as a 39–40-kDa protein copurifying with LDL receptor (LDLR)-related protein (LRP) (1, 2). At the same time, a putative target antigen in Heymann nephritis (HN), an experimental rat model of human membranous nephropathy, was identified and cloned (3). On molecular characterization of RAP, it became evident that the protein identified as the target antigen in HN was identical to RAP and not, as previously assumed, a fragment of glycoprotein 330/megalin (4). RAP subsequently was shown to bind not only to LRP but also to other members of the LDLR family, including megalin (5), the VLDL receptor (6), the chicken oocyte receptor for VLDL and vitelligenin (LR8) (7, 8) and, to a lesser extent, to the LDLR itself (8, 9). The ability to interfere with ligand binding to these receptors has made RAP a perfect tool to study ligand-receptor interaction both in vitro (8, 10–14) and in vivo (15). The latter experiments especially have established the role of LRP as a chyomicron remnant receptor in the liver. Despite the successful use of RAP to study the physiological function(s) of members of the LDLR family and of LRP in particular, clues about the function of RAP in vivo emerged only recently. The tetrapeptide sequence HNEL at the carboxyl terminus of RAP was shown to be necessary for the retention of RAP in the endoplasmic reticulum; thus, RAP associates in vivo with LRP early in the secretory pathway and dissociates from the receptor before reaching the cell surface (16). These results suggested a specialized role of RAP as a chaperon for LRP, possibly regulating the interaction of the receptor with ligands along the secretory pathway. This was confirmed by elegant studies by Herz and colleagues in RAP knockout mice (17) and in cultured cells in experiments relating the biosynthesis and functional expression of LRP and megalin with that of RAP (18). These experiments show that at least one of the physiological functions of RAP can be defined as that of a specialized escort protein, which protects certain receptors from ligand-induced aggregation along their intracellular itinerary.

We have previously shown that LR8 can serve as a model system to study ligand binding and structural aspects of LDLR family members (8, 19, 20). Here we have used LR8 to demonstrate that all receptors that strongly bind RAP share a common immunological epitope with the escort protein. Based on this observation, we propose a model for the development of passive Heymann nephritis induced by anti-RAP antibodies, in which megalin present in the brush borders of the proximal kidney tubule in rats constitutes an additional target antigen.

EXPERIMENTAL PROCEDURES

Preparation and Radiolabeling of Ligands—VLDL was prepared from plasma of estrogen-treated roosters by sequential ultracentrifugation according to the method of George et al. (21). VLDL was labeled with 125I to a specific activity of 250–400 cpm/ng using the iodine

This paper is available on line at http://www-jbc.stanford.edu/jbc/
monochloride method as described previously (22). Vitellogenin was purified from plasma of estrogen-treated roosters by ion exchange chromatography (DEAE-cellulose) as described (23). Recombinant RAP was produced as a glutathione S-transferase (GST) fusion protein using a pGEX 2T-derived (Pharmacia Biotech Inc.) expression plasmid in DH5α bacteria (13). Purified RAP-GST was cleaved by incubating 10 mg of the fusion protein in 20 ml HEPES, 150 mM NaCl, and 2 mM CaCl₂, pH 7.5, for 12 h at 37 °C with 10 units of thrombin in a final volume of 5 ml. For immunization, the cleaved RAP was purified by preparative, nonreducing SDS-polyacrylamide gel electrophoresis and eluted from the gel by electroelution using the Elutrap system (Schleicher & Schuell). Bovine lactoferrin was obtained from Serva (Vienna, Austria). Purified human RAP was a gift from D. Strickland (Biochemistry Laboratory, American Red Cross, Rockville, MD).

Preparation of Membrane Extracts from Various Tissues and Cells—
Oocyte membranes were prepared from previtellogenic follicles (4–6 mm diameter) excised from mature laying hens and extracted with 1% Triton X-100 as described previously (21). Membrane extracts from rat (male Lewis) kidney microvilli were prepared as described (24). LDLa-7 cells carrying an expression plasmid (pHLR82) for the human VLDL receptor (25) and control cells were grown in Ham’s F-12 medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 5% fetal calf serum, and 0.7 mg/ml G418 for 48 h. For Western blotting experiments, cell monolayers were processed as described (26).

Preparation of Antibodies—
Polyclonal antibodies against LR8 were described previously (27). Polyclonal antibodies against recombinant human RAP (GST and RAP) were obtained by immunization of New Zealand White rabbits with the purified proteins as described (28). IgG fractions were purified from sera on protein A-Sepharose CL-4B matrix (Pharmacia) according to the method of Beisiegel et al. (29).

Immunopurification of Antibodies—RAP-GST was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer’s instructions (5 mg of RAP-GST/ml column material). Ten mg of protein A-purified anti-RAP-GST IgG in 5 ml of buffer A (90 mM NaCl, 20 mM Tris, and 2 mM CaCl₂, pH 7.4) was applied onto the column, and the flow-through was recycled twice. The column was washed with 15 ml of buffer A, and bound antibodies were eluted in two batches using 1 ml of 0.1 M glycine, pH 2.5, in each step. Eluted IgG was pooled and dialyzed against buffer A for 12 h.

To immunopurify anti-RAP-GST antibodies on LR8, a partially purified receptor preparation (30) was electrophoretically separated on 4.5–18% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose. The region of the membrane containing LR8 was cut out, and the membrane was blocked for 2 h in a 3% nonfat dry milk, pH 7.2. The membrane was incubated with protein-A-purified anti-GST-RAP GST fusion protein (protein A purified, 5 μg/ml) for 1 h and then washed three times for 10 min and two times for 30 min with ligand blot buffer. Elution of bound antibodies was performed by incubating the membranes twice for 1 min each in 1 ml of 0.1 M glycine, pH 2.5. Samples obtained from both elution steps were pooled and dialyzed against buffer A for 12 h.

Electrophoresis and Transfer to Nitrocellulose—One-dimensional SDS-polyacrylamide gel electrophoresis under nonreducing conditions was performed according to the method of Laemmli (31) on 4.5–18% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose. Western blotting experiments, cell monolayers were processed as described (26).

Preparation of Immobilized Overlapping Synthetic Peptides (SPOTs Assays)—SPOTs membranes (Imperial Chemical Industries, Cambridge, United Kingdom) were obtained with 96 preformed spots in which a single amino acid was conjugated to the cellulose matrix as an anchoring site for peptides. NH₂-terminally acetylated peptides (157 11-mer peptides with a 2-amino acid offset covering the entire sequence of RAP) were synthesized as described (35). The membranes were incubated with anti-RAP IgG (0.1 mg/ml) overnight and stained with anti-rabbit IgG conjugated to β-galactosidase (1:200, Genosys) and the staining reagents provided with the kit (Genosys).

**RESULTS**

Panels of polyclonal antibodies against recombinant and purified human RAP obtained from different sources and laboratories have been used in our laboratory to study the expression of RAP in chicken tissues. Surprisingly, in addition to recognizing chicken RAP, most of these antibodies persistently recognized recombinant human RAP obtained from different sources and laboratories, indicating that chicken RAP is highly conserved and that RAP is an attractive target for vaccine development.

**Induction and Analysis of Passive Heymann Nephritis—**Lewis rats were injected in the tail vein with 10 mg of anti-RAP IgG in 1 ml of phosphate-buffered saline. Animals were sacrificed on day 3, and kidneys were flushed blood-free with ice-cold phosphate-buffered saline, followed by perfusion fixation with paraformaldehyde-lysine-periodate solution, as described (33). Immunofluorescence was performed on 1-μm frozen sections of paraformaldehyde-lysine-periodate-fixed kidneys cut on a Reichert Ultracut ultramicrotome equipped with an F4 cryostage, as described (34). Immune deposits were detected by direct immunofluorescence with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200) and an enhanced chemiluminescence system.

**FIG. 1. Western blot analysis of anti-RAP IgG.** Purified human RAP (lane 1, 0.5 μg), thrombin-cleaved recombinant RAP-GST (lane 2, 1 μg), and uncleaved recombinant RAPGST fusion protein (lane 3, 1 μg) were subjected to electrophoresis under reducing conditions on a 4.5–18% SDS-polyacrylamide gradient gel and transferred to nitrocellulose. The membrane was incubated with an antibody raised in rabbits against the GST-RAP fusion protein (protein A purified, 5 μg/ml). Bound IgG was visualized with protein A-HRP and a chemiluminescence system.
2, this antibody also strongly reacts with chicken LR8. In Western blots using crude oocyte membrane extracts, both the 95-kDa protein and RAP are dominantly labeled (Fig. 2A, lane 1). After long exposure of the blot, another high molecular mass band became visible (see Fig. 2B). Control blots with crude extracts using an anti-LR8 antibody that cross-reacts with oocyte-specific LRP (8) (Fig. 2A, lane 2) and with purified LR8 using the anti-RAP antibody (Fig. 2A, lane 3) clearly show that the 95-kDa protein is chicken LR8. The cross-reactivity with LR8 is not species-restricted, since the human VLDLR (25), which is the mammalian homologue of chicken LR8, also reacts with the antibody. This is shown in Fig. 2A, lane 7, in which an extract of LdlA-7 cells stably transformed with an expression plasmid for the human VLDLR was blotted with the anti-RAP antibody. In Fig. 2A, lane 4, the same band is visualized with an anti-human VLDLR antibody. As a control for both antibodies, mock-transfected cells were used (Fig. 2A, lanes 5 and 6). The nature of the additional distinct band above the human VLDLR seen in control and transfected LdlA-7 cells (Fig. 2A, lanes 6 and 7) with the anti-RAP antibody is not clear yet. Since these cells lack LDL receptor expression, the cross-reacting protein might be a hitherto unknown member of the LDL receptor family.

We wanted to know whether the cross-reactivity of the anti-RAP antibody is restricted to VLDLR receptors or applies to further members of this receptor family. Thus, we tested a fraction of the chicken oocyte membrane extract that had been enriched by RAP-Sepharose chromatography for LDL receptor relatives. In such a fraction, a high molecular mass band with the same electrophoretic mobility as the band seen in Fig. 2A, lane 1, after long exposure became clearly visible (Fig. 2B, lane 1). This band is somatic LRP, previously shown to be present in whole follicles (36), as demonstrated by comparison with purified chicken LRP-515 (Fig. 2B, lane 2). Rat megalin, an additional member of the LDLR family, also is recognized by the anti-RAP antibody, as demonstrated in Fig. 2C, using crude extracts from rat kidney microvilli (lane 1) as well as the purified protein (lane 2). Surprisingly, the LDLR itself does not react with the anti-RAP antibody. This was analyzed with human embryo fibroblasts grown either in the presence of fetal calf serum to suppress the LDL receptor or with the addition of lipoprotein-deficient serum to maximally induce the expression of the receptor (26), as well as with membrane extracts from bovine adrenal cortex (37; data not shown).

The observation that not only LR8 but also megalin, which is involved in the development of passive and active HN, cross-reacts with an antibody against RAP, which is associated with megalin and also a target antigen for the development of passive HN, is intriguing. Since chicken LR8 is highly abundant, easily obtained, and well characterized, we used this protein as a model system to further study the interaction of anti-RAP antibodies with members of the LDLR family. Fig. 3 demonstrates that the electrophoretic mobility of LR8 decreases on reduction (lanes 1 and 2). This is due to unfolding of the ligand binding domain held in a rigid conformation by intramolecular disulfide bonds. The interaction of the anti-RAP antibody with LR8 is completely abolished on reduction of the receptor (Fig. 3, lanes 3 and 4), whereas the recognition of RAP is independent of disulfide-mediated folding of the protein. This result suggests that the cross-reacting epitope on the receptor is not a linear epitope but, rather, a structural one. Results presented in Fig. 4 further support this notion. In sharp contrast to the interaction with RAP, binding of the antibody to the receptor is competed for by an excess of physiological ligands for the receptor, such as vitellogenin (Fig. 4, lane 2), VLDL (Fig. 4, lane 3), and lactoferrin (Fig. 4, lane 4). In addition, antibody binding to the receptor and, to a weaker extent, to RAP, is Ca2+-dependent, since the addition of 20 mM EDTA completely inhibits the reaction with the receptor and significantly reduces that with RAP (Fig. 4, lane 5). These results strongly suggest that the antibody reacts like a ligand for the receptor. To demonstrate that this observation is not produced by an experimental artifact, we performed the reverse inhibition experiment using radiolabeled VLDL with and without the addition of an excess of the antibody. As shown in Fig. 4 (lanes 6 and 7), a 1000-fold molar excess of anti-RAP antibody completely inhibits the binding of VLDL to its receptor. Taken together, these experiments strongly suggest that the anti-RAP-antibody reacts with the ligand binding domain of the receptor and that the binding characteristics of the antibody are similar to those of the physiological ligands for the receptor.

It has been described that, sporadically, mice immunized with ligands or their respective receptors spontaneously de-
Epitope Sharing in Heymann Nephritis

Fig. 4. Competitive Western and Ligand blot analysis for chicken LR8. Crude membrane extracts (Triton X-100, 5 μg/lane) of chicken follicles were subjected to electrophoresis under nonreducing conditions on a 4.5–18% SDS-polyacrylamide gradient gel and transferred to nitrocellulose. Western blots with anti-RAP-antibody (protein A purified, 10 μg/ml) were performed with the following additions: lane 1, none; lane 2, vitellogenin (0.3 mg/ml); lane 3, VLDL (1.5 mg/ml); lane 4, lactoferrin (0.2 mg/ml); and lane 5, EDTA (20 mM). Ligand blots with 125I-labeled VLDL (5 μg/ml, 300 cpm/ng) were performed without (lane 6) and with (lane 7) the addition of 2.5 mg/ml anti-RAP IgG.

Fig. 5. Western blot analysis with immunopurified fractions of the anti-RAP antiserum. Partially purified RAP (200 ng), prepared by thrombin cleavage of RAP-GST fusion protein (A, lanes 1 and 3; B, lanes 2 and 4) and 50 ng of purified LR8 (A, lanes 2 and 4; B, lanes 1 and 3) were subjected to electrophoresis on a 10% polyacrylamide gel, and proteins were transferred to nitrocellulose. Lanes 3 and 4 were incubated either with an anti-RAP antibody fraction that had been affinity-purified on RAP-Sepharose as described under “Experimental Procedures” (A) or with an anti-RAP antibody fraction that had been affinity-purified on nitrocellulose-bound LR8 (B). As a control, lanes 1 and 2 were incubated with the unfractionated anti-RAP-IgG preparation. Bound IgG was visualized with protein A-HRP (1 μg/ml) and a chemiluminescence system.

As demonstrated above, it is possible to immunopurify small amounts of an IgG fraction on purified LR8 spotted onto a nitrocellulose membrane. The amount of IgG in the eluted fraction was sufficient to determine its binding affinity to LR8 and to compare it with the affinity of the total (protein A-purified) IgG fraction toward the original antigen (RAP). Analysis was performed by plasmon surface resonance on the BIAcore system, as described under “Experimental Procedures.” The immunopurified fraction bound to purified LR8 with an extremely high affinity constant ($K_{aff} = 2.5 \times 10^{10}$ M$^{-1}$). In comparison, total IgG bound to RAP with a $K_{aff}$ of $1.3 \times 10^{5}$. This value is well within the range of affinities of polyclonal antibodies toward their antigens.

As a control we used the total IgG fraction in vivo to evaluate the actual immune deposits induced by our antibody. Three days after intravenous injection of anti-RAP IgG into rats, numerous distinct granular immune deposits typical for Heymann nephritis were detected in the peripheral capillary walls of the glomeruli (Fig. 6). In addition, some mesangial binding was observed. Unfortunately this experiment cannot be performed with an IgG fraction immunopurified on pure LR8. This is because at least 5 mg of the purified IgG would be required but it is only possible to obtain μg amounts of this fraction.

Finally, we initiated attempts to localize the epitope on RAP responsible for eliciting antibodies that cross-react with members of the LDLR family. In a first step, we analyzed the immunoreactivity of a fusion protein containing 27% of the amino-terminal region (residues 1–86) and compared it with antibodies toward their antigens. As expected, both recombinant fusion proteins reacted with the unfractionated anti-RAP IgG (Fig. 7, lanes 1 and 2). However, the IgG fraction immunopurified on LR8, which still reacted with complete RAP, did not recognize the amino-terminal fragment, suggesting that the cross-reactive epitope is located in the carboxyl-terminal moiety of RAP. To further identify the relevant epitopes on RAP we performed epitope mapping using 157 peptides (11 mer) covering the entire RAP sequence with...
an offset of 2 amino acids. These peptides were spotted on a membrane and immunoblotted with total anti-RAP IgG. A strong and specific reaction was observed toward five sets of three or four overlapping peptides corresponding to the following sequences: (i) PVRLAEL (amino acids 39–45), (ii) LD-EDGEK (amino acids 67–73), (iii) SGEELD (amino acids 131–136), (iv) VIDLWDL (amino acids 223–229), and (v) DKELE EDGEK (amino acids 67–73) (not shown). Considering the fact that the LR8-immunopurified fraction of the antibody (Fig. 7) did not recognize the 86-residue amino-terminal part of RAP, epitopes iii–v likely represent potential cross-reactive epitopes with the LDL receptor family.

**Discussion**

Here we report that RAP, an intracellular protein that interacts with most members of the LDLR family, shares an epitope(s) with these receptors. We used chicken LR8, which is abundantly expressed in growing oocytes, to study this intriguing and surprising observation in close detail. Antibodies against recombinant RAP produced in rabbits strongly react against recombinant RAP produced in rabbits strongly react with the LDL receptor family. This observation later was confirmed by demonstrating that mice immunized with bovine insulin spontaneously developed anti-insulin receptor antibodies that exerted an insulin-like effect (40, 41).

Our finding that the cross-reactivity is caused by a common epitope(s) on RAP and megalin has important implications for our understanding of the development of passive HN. Since a complex of megalin and RAP has been discovered as the antigen in HN, three molecular models for the presentation of the HN antigenic complex have been put forward (5). The first model proposes that multiple pathogenic epitopes are present on both megalin and RAP; these might bind specific antibodies independently and induce the formation of immune deposits. The second model states that a single cross-reacting epitope is present on both proteins, and the third proposes that a shared epitope is generated when both proteins form a complex. Our current results for the first time lend strong direct support, on a molecular basis, to the concept of the second model, as they demonstrate cross-reactive epitopes on both proteins.

In a previous attempt to identify the antigenic epitope(s) on RAP responsible for induction of passive HN, antibodies against a series of fusion peptides derived from cDNA deletion clones have been obtained (42). Antibodies able to induce passive HN, antibodies against megalin (43) or intact RAP (Fig. 6). These results indicate that, in addition to the binding of specific antibodies to the pathogenic epitope on RAP, further antibodies—against megalin—might be required to induce fully developed immune deposits. This interpretation is in full accordance with our data obtained from the epitope mapping using the complete polyclonal anti-RAP IgG. The antibody strongly reacts with five distinct epitopes on RAP, one of which is identical to the pathogenic epitope (amino acids 39–53) described earlier (35). Three of the identified epitopes are indeed located in the carboxyl-terminal two-thirds of the protein, which was identified as the region that harbors the cross-reacting epitope (Fig. 7).

In summary, we believe that this conceptual paradox has now been solved by our demonstration of a further epitope(s) on RAP, located in the carboxyl-terminal part of the protein. This epitope(s) on RAP mimics a structural epitope in the ligand binding domain of megalin. Therefore we propose a conciliatory concept, which fully accounts for all aspects of the development of passive HN induced by antibodies against RAP. In such a scenario, antibodies against the pathogenic domain (amino acids 31–55) of RAP and, in combination, antibodies cross-reacting with the ligand binding domain of megalin bind to the RAP-megalin complex. Such combination fosters the formation of large immune deposits, which are the histological hallmark of the disease. Recently, in an effort to pinpoint disease-related epitopes on megalin, it was shown for the first time that a fusion protein carrying a small (137 amino acids) fragment of megalin alone was able to induce HN (44). This fragment corresponded to a sequence spanning from the forth to the sixth cysteine-rich ligand binding repeat in the second of four clusters of such repeats in megalin. Interestingly, this cluster is the only one in megalin containing eight repeats; VLDL receptors and LRP are the only other known receptors containing clusters of exactly eight repeats. Since all three proteins strongly cross-react with the anti-RAP antibody, the cross-reacting epitope(s) in the receptors might be located in the eight-ligand binding repeat clusters.

The finding that the carboxyl-terminal two-thirds of RAP contain a domain(s) that mimics certain structural epitopes in the ligand binding repeat domain of members of the LDLR family might also have physiological implications. In this context it is interesting that the LDL receptor does not contain this epitope. The LDL receptor harbors seven binding repeats, only weakly binds RAP, and most likely does not form an intracellular complex with RAP, in contrast to LRP (16), megalin (5), and possibly VLDL receptors. It is also the receptor with the most restricted ligand binding specificity, binding only apolipoprotein E and B with high affinity, whereas the others, including VLDL receptors with eight ligand binding repeats, bind a series of unrelated ligands (45–47). This is in accordance with the observation that functional expression of LRP, but not...
the LDL receptor, is impaired in the livers of RAP-deficient mice (17).

One key feature of the binding repeats in members of this receptor family is clusters of negatively charged residues exposed to the surface by a rigid scaffold held together by intramolecular disulfide bonds. RAP, which is a ligand for these receptors, also contains at least three similar clusters of negatively charged amino acids in its carboxyl-terminal part (amino acids 163–167, 215–220, and 237–241). Interestingly, one of these clusters (amino acids 237–241) is identical to one of the three possible cross-reacting epitopes identified by the peptide-mapping experiment. In a recent publication by Bu et al. (16) it was demonstrated that RAP associates with LRP early in the secretory pathway. This association is mediated by at least three independent domains of RAP containing clusters of mostly positively charged residues (14). Dissociation of RAP from LRP occurs later in the Golgi and is most likely induced by a lower pH in this compartment. Probably, the receptor-like domain in RAP plays an important role in this pathway. This domain in the chaperon may mask, in pH-dependent fashion, binding sites on RAP for LRP.

Acknowledgments—We appreciate the expert technical assistance of Robert Wandl and Harald Rumpler.

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