Primary Structure of $\alpha_2$-Macroglobulin Receptor-associated Protein

HUMAN HOMOLOGUE OF A HEYMANN NEPHRITIS ANTIGEN*

(Received for publication, January 22, 1991)

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The $\alpha_2$-macroglobulin ($\alpha_2$M) receptor complex as purified by affinity chromatography contains three polypeptides: a 515-kDa heavy chain, an 85-kDa light chain, and a 39-kDa associated protein. Previous studies have established that the 515/85-kDa components are derived from a 600-kDa precursor whose complete sequence has been determined by cDNA cloning (Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gassepohl, H., and Stanley, K. (1988) EMBO J. 7, 4119-4127). We have now determined the primary structure of the human 39-kDa polypeptide, termed $\alpha_2$M receptor-associated protein, by cDNA cloning. The deduced amino acid sequence contains a putative signal sequence that precedes the 323-residue mature protein. Comparative sequence analysis revealed that $\alpha_2$M receptor-associated protein has 73% identity with a rat protein reported to be a pathogenic domain of Heymann nephritis antigen gp 330 and 77% identity to a mouse heparin-binding protein termed HBP-44. The high overall identity suggests that these molecules are interspecies homologues and indicates that the pathogenic domain, previously thought to be a portion of gp 330, is in fact a distinct protein. Further, the 120-residue carboxyl-terminal region of $\alpha_2$M receptor-associated protein has 26% identity with a region of apolipoprotein E containing the low density lipoprotein receptor binding domain. Pulse-chase experiments revealed that the newly formed $\alpha_2$M receptor-associated protein remains cell-associated, while surface labeling experiments followed by immunoprecipitation suggest that this protein is present on the cell surface forming a complex with the $\alpha_2$M receptor heavy and light chains.

$\alpha_2$-Macroglobulin ($\alpha_2$M) is a plasma glycoprotein capable of inhibiting numerous proteinases. The inhibition is thought to occur via a trap mechanism (1), in which limited proteolysis at a specific region of the $\alpha_2$M subunit (2) is followed by conformational changes in the inhibitor (3-5). These conformational alterations result in reduced activity of the trapped protease toward large molecular weight substrates and also expose regions within the carboxyl-terminal portion of the inhibitor (6) that are recognized by $\alpha_2$M receptors.

The $\alpha_2$M receptor ($\alpha_2$MR) has been purified from detergent extracts of placenta (7, 8) and liver (9) and has been shown to be identical with LDL receptor-related protein (10, 11) whose complete sequence has been derived from cDNA sequencing (12). In addition to removal of $\alpha_2$M-protease complexes, this receptor system is also thought to bind and internalize large cholesterol-containing remnant lipoproteins, enriched with apolipoprotein E (13, 14). $\alpha_2$MR is synthesized as a 600-kDa precursor that is cleaved to give a 515-kDa heavy chain and an 85-kDa light chain (15). The 85-kDa light chain contains a cytoplasmic domain, a hydrophobic membrane-spanning region and an extracellular portion that binds non-covalently to the heavy chain (15). Associated with the 515/85-kDa receptor, we have found a 39-kDa polypeptide (7) that has been termed $\alpha_2$MR-associated protein ($\alpha_2$MRAP) This molecule binds with high affinity ($K_d = 18\,\text{nM}$) to the 515/85-kDa $\alpha_2$MR (10).

The objective of the present study was to learn more about the structure and function of $\alpha_2$MRAP. The results indicate that this molecule is complexed with the $\alpha_2$MR on the cell surface and that it shares considerable sequence identity with a rat protein thought to be a pathogenic domain of Heymann nephritis antigen gp 330 (16) and with a mouse heparin-binding protein, termed HBP-44 (17). The high degree of identity between these molecules suggests that they are interspecies homologues and indicates that the Heymann nephritis antigen previously thought to be a portion of gp 330 (16), is in fact a distinct protein. These studies raise questions regarding the role of $\alpha_2$MRAP and the $\alpha_2$MR complex in the pathogenesis of membranous glomerulonephritis.

**EXPERIMENTAL PROCEDURES**

Purification of $\alpha_2$MRAP—$\alpha_2$MRAP was purified from detergent extracts of human placenta (obtained from Montgomery General Hospital, Olney, MD) by affinity chromatography over immobilized methylamine-activated $\alpha_2$M followed by anion exchange chromatography over a Mono Q (Pharmacia LKB Biotechnology Inc.) column as previously described (7). $\alpha_2$MRAP was dialyzed overnight versus 50 mM HEPES, 50 mM NaCl, pH 7.4, and applied to a Mono S (Pharmacia LKB Biotechnology Inc.) column equilibrated with the same buffer. The column was eluted with a linear gradient of 50 mM NaCl in 50 mM HEPES, pH 9.6. The eluted $\alpha_2$MRAP was then applied to a Superdex 200 (Pharmacia LKB Biotechnology Inc.) column equilibrated with 50 mM sodium phosphate, $pH$ 7.4. $\alpha_2$MRAP derived from this column was homogeneous upon SDS-PAGE followed by silver staining. Deter-
minution of protein concentrations were based on absorption measurements at 280 nm. The absorption coefficient was calculated from the amino acid composition (based on the deduced measurements) as described (18) using the following equation: $E_1^1%  \text{cm}^{-1}$, $280 \text{ nm}$ = 0.97(569.0 + 1260.9 + 120.35), where $W$, $Y$, and $S$-type) were present. The mature molecule.

**Protein Sequencing**—-30 $\mu$g of aMRAP were purified by SDS-PAGE, transferred to nitrocellulose, and digested in situ with trypsin as described (21). The peptides were eluted from the nitrocellulose and sequenced on an Applied Biosystems Model 130 microbore HPLC and RP300 L8 cartridge. Major peaks were collected and used for sequencing on an Applied Biosystems Model 477A polypeptide sequenator with an on-line Applied Biosystems Model 130 phenylthiohydantoin analyzer.

**Circular Dichroism Measurements**—Circular dichroism measurements were performed on a JASCO J-50OC Spectropolarimeter at room temperature. Prior to use, the instrument was calibrated using ammonium d-10-camphorsulfonate (Katayama Chemical Co.) as described (25). Cell pathlength was 0.2 cm, and a baseline spectrum of 4°C. Following washing, the IgG was eluted with 0.1 M glycine, pH 1.9. The pH of the eluted fractions was immediately raised, and the IgG was precipitated. Antibodies against the aMRAP complex (i.e. 515/86-kDa and 38-kDa polypeptides) were prepared in a similar manner using the eluate from the a-M-methylamine affinity column as a source of antigen.

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**Isolation and Sequencing of aMRAP cDNAAs**—A human placental cDNA library (20) was immobilized by affinity-purified antibody against the aMRAP complex (i.e. 515/86-kDa and 39 kDa). Phage that expressed fusion protein reacting with the antibody were cloned to homogeneity, and their insert cDNAs were prepared by EcoRI restriction endonuclease digestion or polymerase chain reaction (22) and then subcloned into the phage vector M13mp19. Sequence was determined by the dideoxy-chain termination method (23) using modified T7 polymerase (United States Biochemicals) and synthetic oligonucleotide primers based on derived sequences. All of the sequences reported are derived from sequencing of both strands of the cDNA inserts. Since immunological screening failed to identify a polypeptide encoding the initiator methionine residue, a primer extension library was prepared using a synthetic oligonucleotide primer (nucleotides 393–409) from a region near the 5' end of the longest cDNA (clone 5). The resulting lambda library was screened with a ^32P-labeled oligonucleotide based on the 5' sequence of clone 5 (nucleotides 17 to 29). Of the 12 positive clones identified in multiple screenings, only a single clone was found having sequence extending 5' of that of clone 5. This cDNA encoded an in-frame methionine and a short 5'-untranslated segment.

**Deduced Amino Acid Sequence**—The aMRAP cDNA and deduced amino acid sequence of aMRAP is shown in Fig. 1. The deduced amino acid sequence was found to contain the amino-terminal sequence obtained from protein sequence analysis of aMRAP. In addition, two sequences derived from protein sequencing of tryptic fragments isolated from aMRAP were also found within the cDNA deduced sequence. These findings confirmed the identity of the cDNA clones as encoding aMRAP. The amino-terminal sequence of the mature protein is a sequence with properties of a signal peptide which include a core of hydrophobic residues preceded by charged residues (28) and an initiator methionine residue. The signal peptide cleavage site, however, does not fit the criteria described by Von Heijne (29) for eukaryotic signal cleavage sites in that position -1 consists of a lysine residue rather than the usual Ala, Ser, Gly, Cys, Thr, or Gin. The mature protein contains 323 residues having a molecular mass of 37,714 daltons, excluding the contribution of any carbohydrate. This value is in excellent agreement with the estimated 39–40 kDa as measured by SDS-PAGE and that of 42 kDa determined by gel filtration chromatography. The sequence contains one potential N-linked glycosylation site (N-X-S/T). A sequence fitting the consensus pattern of a leucine zipper (L-X$_1$–L-$X_n$–L-$X_L$) (30) at positions 28-49 is present. A tetrapeptide sequence, HNEL, was found at the carboxyl terminus of aMRAP and is similar to the endoplasmic reticulum retention consensus sequence of KDEL (31). In addition, sequence similarity was noted in the regions surrounding each of the 3 tryptophan residues contained in the mature molecule.

**Secondary Structure Analysis**—The secondary structure analysis (32) of the deduced sequence suggested a relatively high content of p helix. This was confirmed by circular dichroism measurements of the purified protein (data not shown) from which an a-helical content of

**RESULTS**

**Isolation of cDNA Clones for the aMRAP**—Agt11 phase plagues from a human placental cDNA library (20) were immunologically screened with a rabbit antisera against the aMRAP complex. Five clones were isolated that expressed a fusion protein which was reactive with the antisera. The fusion protein from these clones absorbed antibodies that reacted specifically with aMRAP upon Western blotting. Nucleotide sequencing revealed that the cDNA inserts, which ranged from 1.4 to 1.5 kilobases, encoded identical and overlapping sequences. DNA sequencing showed that the composite cDNA contains a single open reading frame of 1071 nucleotides that is followed by a 409-nucleotide 3'-untranslated region, terminating in a poly(A) tail. A potential polyadenylation signal, AAATAG, was detected 16 nucleotides upstream from the poly(A) tail.

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Structure of the α2-Macroglobulin Receptor-associated Protein

The sequence of the cDNA encoding α2MRAP and the deduced amino acid sequence. All of the sequence was determined from sequencing both strands of the cDNA inserts and was derived from overlapping clones: clone 1, 28 to 1495; clone 3, 56 to 1482; clone 5, 17 to 1528; and clone 9, 1 to 41. Underlined amino acid residues represent sequences determined by protein sequence analysis. A blank was obtained during protein sequence analysis at the putative N-linked glycosylation site, which is shown by a potential polyadenylation signal sequence is double underlined. The arrow designates the cleavage site that generates the amino terminus of the mature protein.

Fig. 2. Alignment of human α2MRAP (39 kDa) with mouse heparin-binding protein 44 (HBP-44) (17) and with rat Heymann nephritis antigen (HN antigen) (16) using FASTA (33). Identical residues are boxed. Numbering is based on that of α2MRAP.

38% was calculated (25). In addition, two regions (residues 189 to 206 and residues 303 to 320) of α2MRAP were found to have potential for forming amphipathic helices.

Identity between α2MRAP, a Rat Protein Identified as Heymann Nephritis Antigen and a Mouse Heparin-binding Protein—When the sequence of mature α2MRAP was compared (33) with that of other molecules in protein databases, two rodent proteins were found that displayed a high degree of identity. A rat protein, identified as a major pathogenic domain of the Heymann nephritis antigen gp 330 (16), has 73% identity with α2MRAP. A mouse protein, termed heparin-binding protein-44 (HBP-44) (17), has 77% identity with α2MRAP. Fig. 2 shows an alignment of the three proteins. We conclude from the high degree of identity between α2MRAP and these molecules that they are interspecies homologues of one another.

Sequence Similarity with Apolipoprotein E—Database searching also revealed similarity between α2MRAP and several other proteins. Among these, apolipoprotein E was particularly interesting given that this molecule is thought to mediate lipoprotein binding to LDL receptor-related protein (13, 14), now known to be the α2MR (10). An alignment of the sequence of α2MRAP with apolipoprotein E revealed 26% identity between the carboxyl terminal region of α2MRAP (residues 203–321) and a region of apolipoprotein E (residues 47–154) that contains the LDL receptor binding domain (34, 35). The similarity between these two molecules seems confined not only to their primary structure. The homology also includes regions with the potential to form amphipathic helices (Fig. 3). A similar amphipathic region in apolipoprotein E (residues 132–149) is known to be directly involved with lipoprotein-receptor interactions (34). Whether or not the corresponding region in α2MRAP mediates its interaction with α2MR remains to be established. In addition to serving a possible role in receptor-ligand interactions, amphipathic
helices are thought to serve as lipid-associating domains in apolipoproteins (36).

**RNA Hybridization Analysis**—RNA hybridization analysis was performed using an α₂MRAP-specific cDNA fragment. Two transcripts, of approximately 1.6 and 3.2 kilobases, were detected in total RNA prepared from gingival fibroblasts (Fig. 4). Similar profiles (data not shown) were obtained with RNA isolated from placenta, a human placenta choriocarcinoma cell line (JAR), and a human nasal septum tumor cell line (RPMI 2650). The length of transcripts encoding α₂MRAP-associated protein corresponded closely to the 1.8- and 3.5-kilobase transcripts reported for mouse HBP-44 (17). The two transcripts of HBP-44 encode overlapping RNA sequences differing only in the length of their 3′-untranslated regions. In the case of HBP-44, the two messages were thought to arise from the presence of two sets of transcription-termina-
polyadenylation signals. We assume that a similar mechanism gives rise to the two transcripts hybridizing with the α₂MRAP cDNA probe.

**Localization of α₂MRAP on the Cell Surface**—The deduced amino acid sequence predicts that α₂MRAP could be a secretory protein because it possesses a putative signal peptide and lacks any obvious transmembrane domain. Pulse-chase experiments were performed to examine the time course of biosynthesis and potential secretion of α₂MRAP. Immunoprecipitation experiments were conducted with [³H]leucine, rather than [¹S]-labeled amino acids because the deduced sequence lacks cysteine and contains only 1 methionine residue. The results of pulse-chase immunoprecipitation (Fig. 5) show that α₂MRAP remains exclusively associated with the cellular fraction with none secreted into the medium, even after 120 min of chase. The high molecular weight polypeptide seen after 15 min of chase presumably corresponds to the heavy chain of the α₂MR suggesting that α₂MRAP interacts with the newly formed α₂MR at an early stage in its biosynthesis, evidently before the complex reaches the cell surface. Within the first minutes of chase, a 56-kDa immunoreactive polypeptide is also detected. The relationship between this polypeptide and α₂MRAP, if any, remains to be established.

To further evaluate whether or not α₂MRAP is secreted, conditioned medium was concentrated 5-fold and subjected to Western blot analysis using affinity-purified antibody to α₂MRAP. These experiments failed to detect any media-derived immunoreactive material, but α₂MRAP was readily detected in detergent extracts of the cells. In earlier studies, we found that α₂MRAP, like HBP-44 (17), binds to heparin-Sepharose. We utilized this affinity matrix to concentrate any α₂MRAP in serum, plasma, and conditioned media. Immuno blot analysis of initial and eluted fractions failed to detect any α₂MRAP in these fractions. Thus, α₂MRAP does not appear to accumulate in cell culture media, nor does it appear to be a component of normal human plasma or serum.

Immunoprecipitation of cell extracts prepared from surface- labeled fibroblasts was performed to establish whether or not α₂MRAP is present on the cell surface. The results showed that indeed a radiolabeled band with mobility corresponding to α₂MRAP was precipitated with affinity-purified α₂MRAP antibodies (Fig. 6). Co-precipitating with the α₂MRAP were the 515/85-kDa heavy and light chains of the α₂MR suggesting that α₂MRAP and the α₂MR form a complex, thus supporting previous data documenting their interaction (7, 10). Overall, the results from these experiments suggest that α₂MRAP is located on the cell surface and associates with the α₂MR.

**DISCUSSION**

This paper reports the complete primary structure of α₂MRAP, a molecule that co-purifies with the α₂MR during affinity chromatography (7). The prominent structural features of α₂MRAP include a putative signal sequence, a leucine

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**Fig. 3.** Shiffer-Edmundson helical wheel diagram (49) for Thr<sup>399</sup> to His<sup>428</sup> of α₂MRAP. The drawing shown represents a vertical view down the axis of one of α₂MRAP’s predicted helix with Thr<sup>399</sup> at the top of the helix.

**Fig. 4.** RNA hybridization analysis of α₂MRAP transcript(s). Total RNA from gingival fibroblasts (10 µg) was denatured and separated by agarose gel electrophoresis. After transferring to nitrocellulose membranes, RNA was hybridized to <sup>32</sup>P-labeled 5′-apolipoprotein (36).

**Fig. 5.** Pulse-chase labeling and immunoprecipitation of α₂MRAP. Gingival fibroblasts were pulse-labeled with leucine-free medium containing [³H]leucine and chased with medium containing unlabeled leucine. At the designated chase time intervals, cell extracts (A) and culture media (B) were subjected to immunoprecipitation using 10 µg of affinity-purified anti-α₂MRAP IgG. Immunoprecipitates were analyzed by SDS-PAGE on gradient gels using a discontinuous pH gel system (Laemmli) with a 4% polyacrylamide stacking gel and a 4–12.5% separating gel. Following electrophoresis, the gels were dried and exposed to X-ray film.
was confirmed by circular dichroism measurements.

An unexpected finding in the present study is that α₂MRAP appears to be the human homologue of a rat polypeptide identified as a Heymann nephritis antigen (16). Heymann nephritis is a rat model of human membranous glomerulonephritis that is characterized by the accumulation of immune deposits within the glomerular basement membrane. One target of autoimmunity in this disease is gp 330 (37), a large glycoprotein that is isolated from renal proximal tubule brush borders. Using antibodies eluted from glomeruli of rats with Heymann nephritis, Pietromonaco et al., (16) isolated a partial cDNA encoding for a protein of approximately 35 kDa. They demonstrated that antibodies prepared against this molecule, when injected into rats, induced formation of subepithelial immune deposits characteristic of Heymann nephritis. The authors concluded that the protein encoded by their cDNA represents the carboxyl terminal portion of gp 330. The results of the present investigation suggest that α₂MRAP, mouse HBP-44 (17), and the rat protein identified by Pietromonaco et al. (16) are interspecies homologues and are distinct from gp 330.

An examination of the available amino acid sequence derived from sequencing partial cDNAs encoding gp 330 (38) reveals that gp 330 has considerable homology with both LDL receptor and α₂MR, clearly indicating that these molecules are members of a receptor family. The homology between these proteins includes the overall organization of structural and functional motifs, such as repeated cysteine-rich regions, a transmembrane domain, and one or more consensus sequences (NPXY) within the cytoplasmic domain known to be involved in internalization of the LDL receptor (39). The interaction of α₂MRAP with the α₂MR is now well documented (7, 10), and it is possible that α₂MRAP interacts with additional members of the LDL receptor family given the similarities between these molecules.

The fact that α₂MRAP appears to be a homologue of a rat protein shown to be an autoantigen in Heymann nephritis, suggests that this molecule, perhaps in complex with the α₂MR, may have some role in the pathogenesis of membranous glomerular nephritis. Given the role of the α₂MR in the uptake of α₂M-proteinase complexes, and probably certain lipoproteins, it is interesting to note that elevated levels of α₂M (40, 41) and hyperlipoproteinemia (42–44) are associated with the nephrotic syndrome. A characteristic feature of α₂MRAP deserves mention in view of the potential involvement of this molecule in membranous glomerular nephritis. Notably, the ability of this molecule to bind heparin is of interest since heparin sulfate proteoglycans are components of basement membranes (45) and elevated expression of proteoglycans, along with transforming growth factor-β and fibronectin, have been reported in an experimental model of glomerulonephritis (46). Furukawa et al. (17) found that message levels of the murine homologue, HBP-44, were considerably higher in kidneys than in other organs examined. It is apparent that an understanding of the distribution of this molecule, and the α₂MR, in normal and pathological kidneys is important for understanding the role of α₂MRAP in this disease process. The availability of specific monoclonal antibodies and cDNA probes should greatly facilitate these studies.

Comparative sequence analysis also revealed similarity between α₂MRAP and apolipoprotein E. These include the relatively high content of α-helix, the potential of several regions to form amphipathic helices, and sequence homology with a region of apolipoprotein E that is involved in receptor recognition. The similarity with apolipoprotein E is of interest since exogenously added apolipoprotein E appears to be required for the binding of large cholesterol-containing remnant lipoproteins to LDL receptor-related protein (13, 14), now known to be identical with the α₂MR. The structural similarity suggests that α₂MRAP may function in a similar manner and mediate the binding of large, cholesterol-containing remnant lipoproteins to α₂MR. We have been unable to detect α₂MRAP in conditioned media of metabolically labeled cells or in plasma. Further, pulse-chase experiments revealed that the newly formed α₂MRAP remains cell-associated, and surface-labeling experiments document that this molecule is labeled, suggesting that α₂MRAP is located on the cell surface. The detection of α₂MRAP on the cell surface is in agreement with the observations of Furukawa et al. (17) who noted a cell-surface staining pattern in indirect immunofluorescence studies when PYS-2 parietal endoderm cells were stained with antibodies prepared against the α₂MRAP murine homologue, HBP-44. All of the data obtained thus far support the proposal that α₂MRAP is located on the cell surface as a component of the α₂MR complex, perhaps representing a new ligand.

If not a new ligand, α₂MRAP may in some manner influence ligand binding by the 515/85-kDa polypeptides of the α₂MR. Another role of α₂MRAP may be in regulating the internalization of receptor-ligand complexes during the process of endocytosis.

The carboxyl terminal sequence of α₂MRAP, HNEL, was noted to be similar to the carboxyl-terminal sequence of KDEL that marks proteins for retention in the endoplasmic reticulum (31). Since several variations of the KDEL sequence direct intracellular retention of proteins (47), the HNEL sequence of α₂MRAP might serve a similar function. The fact that α₂MRAP can be detected on the cell surface is not entirely consistent with the observation that other proteins bearing an endoplasmic retention sequence are retained intracellularly. Given that α₂MRAP interacts with a receptor involved in endocytosis, it is possible that interactions involving
this carboxyl terminal sequence on α2MRAP play an impor-
tant role in α2MR trafficking.

In summary, the present investigation has determined the
primary structure of a component of the α2MR complex. The
results indicate that this molecule can be detected on the cell
surface and is a human homologue of a rat protein implicated
as an autoimmune antigen in Heymann nephritis. The function
of α2MRAP in the α2MR complex, its potential interac-
tion with this carboxyl terminal sequence on α2MRAP play an impor-
tant role in membranous glomerulonephritis remain to be established.

Acknowledgment—The assistance of Dr. Wilson Burgess in obtaining
protein amino acid sequences is greatly appreciated.

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