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# Sequence of a second human asialoglycoprotein receptor: Conservation of two receptor genes during evolution

**(cDNA/protein sequence/differential splicing)**

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ABSTRACT **The asialoglycoprotein (ASGP) receptor isolated from human liver and from the human hepatoma cell line HepG2 migrates on $NaDodSO_4$ gel electrophoresis as a single species of 45,000 daltons. Recently, we isolated a cDNA clone encoding this receptor (H1) from a HepG2 λgt11 library. From the same library, we have isolated and sequenced a clone encoding a second ASGP receptor, H2, with a protein sequence homology of 58% to H1. There are two subspecies of H2 that differ only by the presence of a five-amino acid insertion in the COOH-terminal extracytoplasmic domain. Comparison with the available sequences of the two rat ASGP receptors R1 and R2 indicates that H1 is more homologous to R1 than to H2, and H2 is more similar to R2 than to H1. Thus, the two receptor genes evolved before the separation of rat and man. As judged by RNA blot hybridization of HepG2 RNA using RNA transcribed *in vitro* from cDNA clones of the human receptors as standards, H1 and H2 mRNA are present in equimolar amounts, each 0.005–0.01% of the total mRNA. This finding raises the question of whether the three ASGP receptor proteins are functional as heterodimers or whether they might serve different functions in the cell.**

The asialoglycoprotein (ASGP) receptor is localized to mammalian parenchymal hepatocytes and is specific for desialylated (galactosyl-terminal) glycoproteins (1, 2). Internalization of the receptor–ligand complex, its transport to an acidic sorting compartment, recycling of the receptor to the cell surface, and degradation of the ligand in lysosomes have been characterized in rat hepatocytes and in the human hepatoma cell line HepG2 (3–5). As judged by analysis on $NaDodSO_4$/polyacrylamide gels, the isolated rabbit receptor consists of two molecular species (40,000 and 48,000 Da; see refs. 6 and 7) and the rat liver receptor consists of three (8). One rat receptor (R1, 41,500 Da) and its cDNA have been sequenced (9, 10) as has been a part of the two larger rat receptor proteins (R2, 49,000 Da; R3, 54,000 Da). Since the sequences of R2 and R3 are identical, but different from that of the corresponding part of R1, it was assumed that R2 and R3 differ only in the extent of glycosylation and/or other posttranslational modifications.

The human ASGP receptor, in contrast, migrates on $NaDodSO_4$/polyacrylamide gels as a single protein species of 46,000 Da (11). It is synthesized as a polypeptide of 34,000 Da, is cotranslationally glycosylated by the addition of two *N*-linked oligosaccharides with a high concentration of mannose, and then converted by oligosaccharide modification to the mature species. We recently reported the amino acid sequence of the human ASGP receptor, as derived from the sequence of a cDNA clone, and the identification of the two glycosylation sites (12).

In cloning the human ASGP receptor, we discovered mRNAs coding for a second receptor protein, H2, which is very homologous to H1. We identified two versions of H2 cDNA, differing only by the presence or absence of a segment of 15 base pairs (bp) within the coding region. This is probably due to differential splicing of an intron. Here we present the cDNA sequences of these receptors and the mRNA quantification of H1 and H2. Comparison of all the available DNA and protein sequences of the human and rat ASGP receptors shows that H1 and H2 are more homologous to R1 and R2/3, respectively, than to each other. Although all ASGP receptors clearly have originated from a common ancestor gene, the expression and conservation of more than one ASGP receptor protein in humans and rats raises the question of the function of the different receptor polypeptides.

## MATERIALS AND METHODS

**Materials.** Restriction and modifying enzymes were purchased from New England Biolabs and Bethesda Research Laboratories. DNA sequencing reagents were purchased from P-L Biochemicals; sodium [$^{125}$I]iodide, and deoxycytidine 5′-[$\alpha$-$^{32}$P]triphosphate, and adenosine 5′-[[$\alpha$-$^{35}$S]-thio]triphosphate were from Amersham. The riboprobe *in vitro* transcription system (SP6 polymerase, RNasin, pSP64 DNA) was from Promega Biotec (Madison, WI) and RNase-free DNase was from Cooper Biomedicals (Malvern, PA).

**Screening.** The preparation of a cDNA library from HepG2 cell poly(A)$^+$ RNA in λgt11 (13) has been described (12). Antibody screening of this library was performed by using an affinity-purified polyclonal rabbit anti-human ASGP receptor antibody (11) and [$^{125}$I]iodinated protein A as described (14). For screenings by DNA hybridization, we essentially followed the plaque-lift procedure described in ref. 15. After baking the nitrocellulose filters for 2 hr, they were prehybridized for 6–16 hr at 65°C in 0.6 M NaCl/60 mM sodium citrate/5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/20 mM sodium phosphate, pH 7/0.1% $NaDodSO_4$/25 $\mu$g of poly(A) per ml/100 $\mu$g of denatured calf thymus DNA per ml, then hybridized for ≈20 hr at 65°C in the same solution containing ≈10,000 cpm of the nick-translated probe per ml. Using a cDNA clone for one or the other human ASGP receptors as a probe, plaques containing cDNA for either of the two ASGP receptor mRNAs produced a signal (although usually not with equal intensity) after washing for 2 hr in 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$ at 50°C. After washing in the same solution for another 2 hr at 70°C, only clones corresponding to the homologous probe could be detected. Positive clones were subcloned into pUC13 (16) to facilitate characterization.

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Abbreviations: ASGP, asialoglycoprotein; bp, base pair(s).

**Sequence Analysis.** The sequences of cDNA clones A22 and A34 were determined by subcloning random sonication fragments of the isolated and self-ligated cDNA inserts into M13 (17) and sequencing by a modified Sanger "dideoxy" procedure (18, 19). The sequence data were assembled and analyzed by using the computer-assisted methods of Staden (20).

**RNA Blot Analysis.** RNA was isolated by the guanidinium isothiocyanate/CsCl gradient method (21) and by chromatography twice on oligo(dT)-cellulose (15). Up to 10 μg of RNA from HepG2 cells and, as a control, from HeLa cells, was denatured in 50% (vol/vol) formamide/6% (vol/vol) formaldehyde/phosphate buffer (18 mM $Na_2HPO_4$/2 mM $NaH_2PO_4$), and fractionated on a 1.5% agarose gel in 6% formaldehyde and phosphate buffer at ≈0.6 V/cm for at least 24 hr. The gel was then soaked in 3 M NaCl/0.3 M Na citrate and the RNA was transferred to nitrocellulose (15). After baking at 80°C for 2 hr, the filter was prehybridized in 50% formamide/5× Denhardt's solution/0.1% $NaDodSO_4$/0.9 M NaCl/50 mM sodium phosphate, pH 7.4/5 mM EDTA/100 μg of denatured calf thymus DNA per ml/100 μg of poly(A) per ml at 45°C for 6 hr to overnight, and then hybridized in the same solution containing ≈10,000 cpm of nick-translated DNA probe per ml at 45°C for 40 hr. The filter was washed in 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$ for 2 hr at 50°C. Under these conditions, only RNA and cDNA probes of identical type (H1 or H2) hybridize to a significant extent. To probe the same filter a second time, at least 95% of the first probe was removed by washing the filter in 70% formamide/90 mM NaCl/5 mM sodium phosphate, pH 7.4/0.5 mM EDTA at 70°C for 2 hr. The filter was kept wet at all times. Autoradiographs were scanned by using an LKB 2202 laser densitometer with an integrator.

Table 1. Extent of homology between the human and rat ASGP receptors

| Compared sequences | % homology: DNA coding sequence | % homology: Protein sequence (COOH terminus) |
|---|---|---|
| H1 and H2 | 72 | 58 (58) |
| R1 and R2 | | (51) |
| H1 and R1 | 81 | 80 (87) |
| H2 and R2 | | (67) |
| H1 and R2 | | (51) |
| H2 and R1 | 67 | 56 (60) |

The homology is expressed as the number of identities divided by the number of residues of the shorter sequence. In parentheses, the homologies in the segment covered by the known COOH-terminal part of the sequence of R2 are given.

To quantify the amount of H1 and H2 mRNA, known amounts of synthetic RNA, prepared by using the cDNAs for H1 and H2 as templates, were analyzed in parallel. The *Hin*dIII/*Eco*RI fragment of H1 clone A21 (12) was subcloned into pSP64 cut with *Hin*dIII and *Eco*RI. H2 clone A34 had been subcloned into *Sma* I-cut and phosphatase-treated pUC13 after filling in the terminal *Eco*RI sites using the Klenow fragment of DNA polymerase I. It was further subcloned as the *Bam*HI/*Eco*RI fragment (i.e., the entire cDNA insert plus part of the polylinker of the plasmid) into *Bam*HI- and *Eco*RI-cut pSP64. The two resulting plasmids, pSA1 and pSA2, were linearized at the 3′ end of the inserts

FIG. 1. cDNA and protein sequences of the two human ASGP receptors. The hydrophobic membrane-spanning segments are indicated by lines, the two potential polyadenylylation sites are indicated by boxes, and the potential sites for *N*-linked glycosylation are indicated by dots. The cDNA sequence of H2 was obtained from the two clones A34 (nucleotides −120 to 1031) and A22 (nucleotides 640–1247). The overlapping sequences were identical except for nucleotide 699, which was missing in A22. At the 3′ end, A22 contains a stretch of 56 As, only a few of which are shown. The sequence of H1 was described in ref. 12.

by *Eco*RI digestion. *In vitro* transcription was carried out using the riboprobe system following the manufacturer's protocol. Two micrograms of linearized pSA1 or pSA2 was incubated in a total volume of 50 μl containing 20 units of SP6 polymerase, 25 units of RNasin per μl, 10 mM dithiothreitol, 40 mM Tris·HCl (pH 7.5), 6 mM $MaCl_2$, 2 mM spermidine, and ATP, CTP, GTP, and UTP (0.5 mM each) at 40°C for 1 hr. To allow quantitation of the RNA produced, a small amount of adenosine 5'-[[$\alpha$-$^{35}$S]thio]triphosphate (≈20,000 cpm per nmol of ATP) was added to the reaction mixture. The DNA template was removed by incubation with RNase-free DNase (20 μg/ml) and an additional 1 unit of RNasin per μl at 37°C for 10 min. The RNA was then extracted with phenol and chloroform and precipitated with ethanol and ammonium acetate. Between 3 and 6 μg of RNA was usually obtained from these preparations. The length of the transcripts both from pSA1 and pSA2 is 1210 bases. To obtain similar backgrounds and efficiencies of transfer during RNA blot analysis as for total cell RNA, the synthetic RNAs were mixed with 8 μg of HeLa polyadenylylated RNA.

## RESULTS

We have previously reported the isolation of a cDNA coding for the human ASGP receptor, H1, from a λgt11 library prepared by using mRNA from HepG2 cells (12). In the initial screening of this library, using specific antibodies directed against the human ASGP receptor, we isolated and characterized six clones, of which five contained all or part of the sequence of H1. One clone, A10, hybridized to the other clones and to the same band on RNA blots of HepG2 mRNA, but it had a different restriction map. Its partial sequence was different from but homologous to H1. By rescreening the cDNA library by hybridization to clone A10, we isolated a set of clones that together contained the entire coding sequence of the mRNA for a second ASGP receptor, H2.

The total sequence of 1336 nucleotides contains a single large open reading frame of 1050 bp (nucleotides −120 to 933 in Fig. 1). Starting with the first ATG initiation codon (position 1), the mRNA could encode a polypeptide of 311 amino acids. We do not know how many bases are missing at the 5' end of the cDNA. The 3' end of the cDNA contains part of the poly(A) sequence; there are conceivable polyadenylylation site(s), AACAAA and AAGAAA starting 18 and 22 bp upstream of the poly(A) tail, although there is no "standard" AATAAA polyadenylylation sequence.

The noncoding regions of H1 and H2 are not homologous to one another except for 20–30 bp preceding the initiation codons. Similarly, the noncoding sequences of H1 and H2 are not homologous to those of R1, except for a short stretch of homology between the sequences immediately following the stop codons of H1 and R1. Within the coding region, however, there is considerable homology between H1 and H2. Alignment of the homologous regions (Fig. 1) reveals two insertions of 54 and 15 nucleotides (positions 67–120 and 244–258) in H2 relative to H1. These are located in the amino-terminal domain and immediately following the membrane-spanning region, respectively. Except for these interruptions, the DNA and protein homology is equally pronounced in all three domains of the protein: the amino-terminal cytoplasmic segment, the membrane-spanning segment, and the carboxyl-terminal extracellular domain. The two sites of *N*-linked glycosylation in H1 are conserved in H2, and there is a third potential glycosylation site at the very COOH terminus of H2.

Comparing the protein sequences of the human and rat ASGP receptors, H1 and R1 align perfectly, except for the deletion of residue 15 in R1. The extent of homology between all four sequences is summarized in Table 1. Only the COOH-terminal segment of R2 has been sequenced. Both in this segment and in the protein as a whole, H1 is more closely related to R1 than to H2. Likewise, H2 and R2 are more homologous to each other than to H1 or R1. This is further substantiated by a position-by-position analysis of the COOH-terminal segment for which the sequence of R2 is available (Fig. 2). In this segment, H1 and H2 differ in 44 positions. In 30 of these, the amino acid of H1 is the same as that in R1, and in 21 the residue of H2 is the same as that in R2. Of these 44 positions H1 and R2, and H2 and R1 have the same residue at only 5 and 4 positions, respectively. We conclude that two homologous ASGP receptor genes must have existed before the separation of the ancestors of rats and humans. Gene 1, however, has been more conserved than gene 2.

Subsequently, we have sequenced part of three more cDNA clones for H2—A33, A35, and A36—in the region between their 5' ends (positions 77, 122, and 24 in Fig. 1, respectively) and the *Hin*dIII site at position 293. In this segment, the sequences are identical to the one shown in Fig. 1 except for the 15-nucleotide insertion relative to H1 (positions 244–258), which is absent in all three of them (Fig. 3). It seems extremely unlikely that this difference is the product of a cloning artifact (insertion of a five-codon segment, or an identical deletion in three clones). It is conceivable, however, that the insertion is the result of the differential splicing of an intron, in particular since the segment is framed by the splice. The other insertion in H2

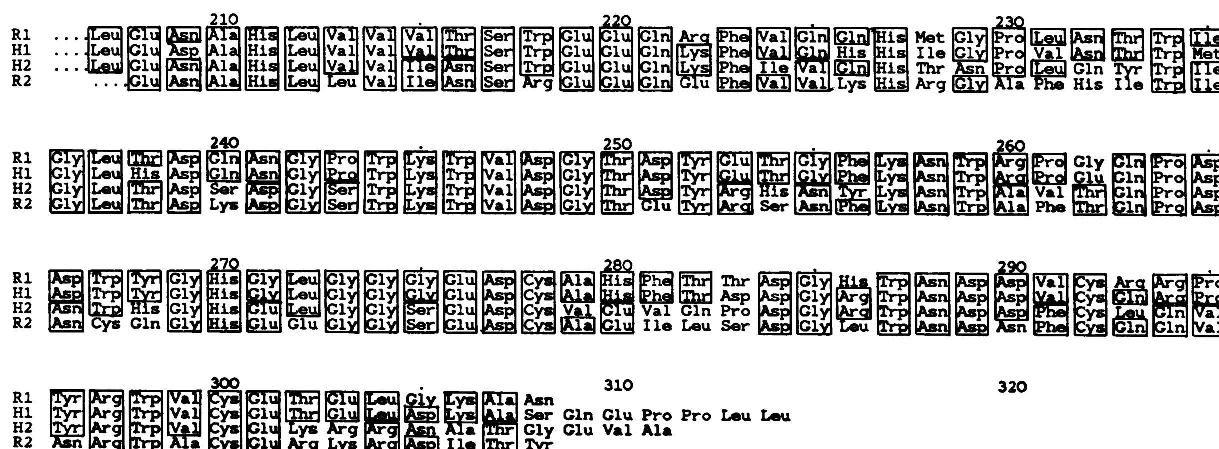

FIG. 2. Comparison of the human and rat ASGP receptor sequences near the carboxyl termini. The segment is shown for which all four protein sequences are known. Identical residues are boxed. R1 and R2 have been published in ref. 9 and H1 has been published in ref. 12.

FIG. 3. Deletion of 15 nucleotides in some H2 cDNA clones. The sequence of clone A34 (i.e., the sequence presented in Fig. 1) is shown in the region immediately downstream from the membrane-spanning domain (underlined) in comparison to the sequence of two other clones, A33 and A35.

(positions 67–120) is also present in clone A35 and A36. Thus, there are two variants of H2, called H2A and H2B (Fig. 3).

To quantitate the amounts of mRNA for H1 and H2 in HepG2 cells, we analyzed poly(A)-selected HepG2 RNA on RNA blots probed consecutively with labeled H1 and H2 under conditions in which the two clones do not cross-hybridize. As standards, we used synthetic mRNA transcribed *in vitro* from H1 and H2 cDNA by using the riboprobe SP6 transcription system (22, 23). Both *in vitro* transcripts had the same length of ≈1210 bases (see *Materials and Methods*). To ensure comparable electrophoretic mobility and transfer efficiency of the synthetic RNAs, they were diluted with the same amount of poly(A)-selected RNA from HeLa cells (which do not express the ASGP receptors) used in the HepG2 samples. After probing the RNA blot with nick-translated H2 cDNA, a single RNA of ≈1500 bases, was detected in HepG2 RNA (Fig. 4*A*) and was absent in HeLa RNA. Of the synthetic RNAs, only the one transcribed from H2 hybridized to H2 cDNA. Hybridization was linearly proportional to the amount of HepG2 RNA and to the amount of synthetic H2 RNA loaded on the gel. By comparison of the extent of hybridization to the synthetic and HepG2 RNA, we calculate that the concentration of H2 mRNA in HepG2 RNA preparation is 56 pg/μg. After stripping the filter of at least 95% of the hybridized H2 cDNA probe, it was reprobed with radioactive H1 cDNA. Now only the synthetic RNA transcribed from H1 hybridized, as well as an RNA from HepG2 cells with a mobility indistinguishable from that detected by the H2 cDNA (Fig. 4*B*). Using the synthetic H1 RNA as standard, we determined the concentration of H1 RNA to be 54 pg/μg, essentially the same as that of H2 RNA. Considering that poly(A)-selected RNA is usually contaminated with considerable amounts of ribosomal RNA, both ASGP receptor mRNAs together might comprise as much as 0.02% of the mRNA in HepG2 cells.

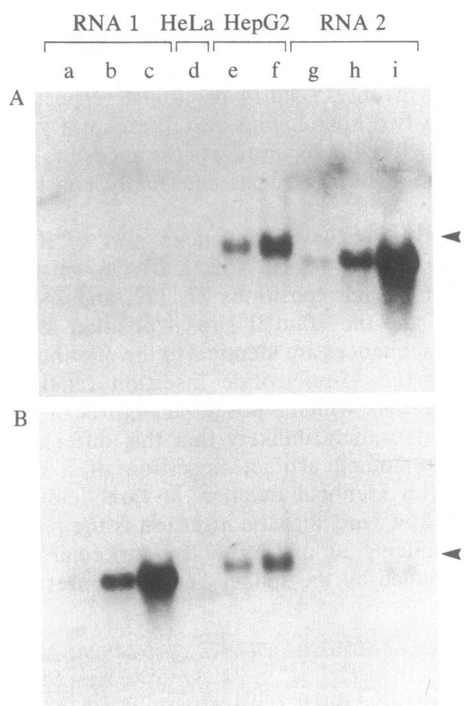


FIG. 4. RNA blot analysis for H1 and H2 in HepG2 cells. Poly(A)-selected RNA from HepG2 cells and HeLa cells, and synthetic RNA transcribed from cDNA for H1 and H2 were fractionated by electrophoresis and blotted onto nitrocellulose. The filters were probed first with radioactive cDNA for H2 (*A*) and then, after removal of the first probe, with cDNA for H1 (*B*). The mRNA concentrations of H1 and H2 were determined by comparison of the extent of hybridization of the HepG2 RNA to that of standard RNA analyzed and hybridized in parallel. Lanes a–c, 0.1, 0.5, and 2.5 ng, respectively, of synthetic RNA to H1 mixed with 8 μg of HeLa cell RNA; lane d, 8 μg of HeLa cell RNA; lanes e and f, 4 and 8 μg of HepG2 cell RNA, respectively; lanes g–i, 0.1, 0.5, and 2.5 ng, respectively, of synthetic RNA to H2 mixed with 8 μg of HeLa cell RNA. The size of the synthetic RNAs is 1210 bases. The position of α-tubulin mRNA (≈1800 bases) as determined by a third hybridization using a genomic rat α-tubulin clone (24) is indicated by arrowheads.

These results were confirmed by screening the λgt11 library with cDNA for H2 as a probe, first under hybridization and washing conditions that permit cross-hybridization between DNAs coding for H1 and H2, and then by washing under more stringent conditions that do not (see *Materials and Methods*). Of ≈600,000 recombinant phage, a total of 108 clones were detected under low stringency. Of these, 51 hybridized to H1 cDNA and 57 hybridized to H2 cDNA under high stringency. Thus, H1 and H2 mRNAs are indeed present in equimolar amounts and together comprise ≈0.018% of HepG2 mRNA. This percentage is approximate, because only cDNAs longer than 600 bp were used to construct the library.

## DISCUSSION

We have shown that humans have at least two genes encoding related ASGP receptor proteins, H1 and H2, which correspond to the rat receptors R1 and R2, respectively. Among the cDNA clones for H2 we found two different sequences; H2A, which has a 15-bp segment at positions 223–272 immediately downstream of the region coding for the membrane-spanning portion of the polypeptide, and H2B, which lacks this segment. Since both sequences are otherwise identical, they seem to be the product of differential splicing of an intron. By analogy, the rat receptors R2 and R3, whose partial sequences at the COOH terminus are identical (9), might also differ by alternate splicing of the primary transcription product and not, or not only, by different modification of the polypeptide as was suggested (9). Thus, H2A might correspond to R3 and H2B might correspond to R2.

The predicted molecular size of the H2 polypeptide is larger than that of H1 by ≈2000 Da. The amounts of mRNA for H1 and H2 in HepG2 cells, as judged both by RNA blot analysis and by the frequencies of corresponding cDNA clones in the λgt11 library, are nearly identical. If we assume that H1 and H2 mRNAs are translated at equal efficiencies, then a problem is apparent, because receptor preparations from human liver and from pulse-labeled and long-term labeled HepG2 cells result in a single band on $NaDodSO_4$/polyacrylamide gels. However, we note that the receptor was isolated either by a complex purification procedure, which might enrich for one of the receptor species (25), or by immunoprecipitation using an antibody that might not bind to both receptors with the same efficiency. The same problems exist for the receptor preparation from rat and rabbit: The ratios of 2:1 for the two rabbit receptors (7) and of 4:1 for the R1 and R2 rat receptors (10) were based on protein staining of $NaDodSO_4$ gels and might not reflect the molar ratio of the two polypeptides in the cell. Autoradiographs of iodinated rat ASGP receptor by Schwartz *et al.* (26) indicated rather similar amounts of the R1 and R2 species. To solve this question, we need to raise antibodies specific for each of the two human receptors and test for H1 and H2 in HepG2 cells and in human liver.

Radiation inactivation experiments (27, 28) indicated that the functional ligand-binding unit of the ASGP receptor is a dimer in human and rat liver and possibly a higher oligomer in HepG2 cells. Until now, both the rat and human ASGP receptors were assumed to be homodimers—in the human because the receptor appeared to be a single protein, and in rat because the different species did not appear to be present in equimolar amounts. Because of our finding of two human ASGP receptors in equal amounts (at least at the mRNA level), it is necessary to consider the possibility that the functional receptor is a heterodimer.

It is also possible that H1 and H2 form homodimers (or homotetramers) that differ in some aspect of their function. First, both H1 and H2 (or R1 and R2) could be present at the cell surface but have different ligand-binding properties. The ASGP receptors on the surface of rabbit hepatocytes exhibit two affinities for single ligands (29), but it is not known how many ASGP receptors exist in rabbit hepatocytes. Second, only one of the two ASGP receptors could be present on the cell surface. Cell-surface iodination of rat hepatocytes resulted in labeling the two receptor species of 49,000 and 54,000 Da to a considerably larger extent than the smaller protein of 41,500 Da (26). Our original interpretation of this result was that the 41,500-Da species is a degradation product of the two larger species. Given the sequence data on R1 and R2 (9), this conclusion is obviously wrong, but the data do suggest that only R2 is found on the hepatocyte cell surface. Since only 35% of the ASGP receptor in rat hepatocytes is localized to the plasma membrane (4, 30), the receptor exposed on the cell surface to mediate endocytosis of ASGPs might be predominantly the product of gene 2, while the protein product of gene 1 might serve a different function in intracellular membranes. To answer these questions, we intend to transfect the two human cDNAs, both separately and in combination, into cells originally not expressing theASGP receptor and to study the receptors' location in the cell and their ability to bind and internalize ligand.

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