H2, THE MINOR SUBUNIT OF THE HUMAN ASIALOGLYCOPROTEIN RECEPTOR, TRAFFICKS INTRACELLULARLY AND FORMS HOMO-OLIGOMERS, BUT DOES NOT BIND ASIALO-OROSOMUCOID*

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Running Title: The ASGP-R H2 subunit forms oligomers that do not bind ligand

*This research was supported by NIH grant GM49695 from the National Institute for General Medical Sciences.

Keywords: H2 subunit, asialoglycoprotein receptor, membrane protein, endocytosis, coated pits.
The functional human hepatic asialoglycoprotein receptor (ASGP-R) is a hetero-oligomer composed of two subunits, designated H1 and H2, which are highly homologous. Despite their extensive homology, the major H1 subunit is stably expressed by itself, whereas in the absence of H1 most of the H2 subunits are degraded in the ER. In this study, we were able to investigate the capability of the minor ASGP-R subunit, H2, to function independently of H1, because it was apparently stabilized by fusing its NH2-terminus with an epitope tag. We could thus create stable cell lines in hepatoma-derived SK-Hep-1 cells that expressed the H2 subunit alone. H2 was expressed on the cell surface and was internalized, predominantly through the clathrin-coated pit pathway. Since, the internal pool of H2 was also able to traffic to the cell surface, we conclude that H2 recycles between the surface and intracellular compartments, similar to the constitutive recycling of hetero-oligomeric ASGP-R complexes. However, the rate of H2 recycling and internalization was ~25-33% that of H1. Similar to H1, the H2 polypeptides were also able to self-associate to form homo-oligomers, including trimers and tetramers. However, unlike H1, which can bind the ligand asialo-orosomucoid (ASOR) when overexpressed in COS-7 cells, H2 failed to bind or endocytose ASOR. In summary, the H2 subunit of the human ASGP-R contains functional, although weak, signal(s) for endocytosis and recycling and has the ability to oligomerize. H2 homo-oligomers, however, do not create binding sites for desialylated glycoproteins, such as ASOR, that contain tri- and tetra-antennary N-linked oligosaccharides. Nonetheless, these results raise the intriguing possibility that naturally occurring H2 homo-oligomers may exist in human hepatocytes and have an as yet undiscovered function.
The ASGP-R is an endocytic recycling receptor localized to the sinusoidal face of the hepatocyte plasma membrane where it could be responsible for the removal and degradation of potentially deleterious circulating glycoconjugates. The ASGP-R is a hetero-oligomer containing multiple polypeptides derived from two different genes. In the human ASGP-R complex, the major H1 subunit, which is the predominant polypeptide, is expressed at a ~3-fold higher concentration than the minor H2 subunit. The mature and fully glycosylated forms of the H1 and H2 subunits are ~46 kDa and ~50 kDa, respectively. The H1 and H2 polypeptides are highly homologous to each other, as well as to analogous ASGP-R polypeptides from other mammals. The ASGP-R is a type-II membrane receptor; thus both subunits contain a short cytosolic NH2-terminus, a single transmembrane domain, a stalk segment and a Ca2+-dependent CRD.

In stably transfected murine NIH3T3 fibroblasts, H1 is expressed alone as a 40 kDa precursor containing high mannose oligosaccharides, which is then processed to a 46 kDa mature form found in native ASGP-Rs. When expressed alone in fibroblasts the kinetics of H1 biosynthesis and transport to the cell surface are similar to that of the native H1 subunit endogenously produced in the presence of the H2 subunit in HepG2 hepatoma cells. The mature H1 subunit on the plasma membrane is internalized through clathrin-coated pits. The Tyr5 residue in the cytoplasmic tail of H1 plays a significant role in its endocytosis. In addition, internalized H1 is also capable of recycling to the plasma membrane in transfected fibroblasts, and the efficiencies of the internalization and recycling processes are similar to those of endogenously expressed ASGP-Rs. Although wild-type H1-H2 receptor complexes binds ASOR with high affinity, the H1 subunit is unable to bind ASOR when expressed at low levels in stably transfected fibroblasts. In contrast, when overexpressed in COS-7 cells, H1 forms homo-oligomers that generate ASOR binding sites, although with a Kd four-times higher than
that of native ASGP-Rs, indicating a lower affinity of ASOR binding by H1 homo-oligomers (22).

Unlike H1, H2 is usually unstable when expressed by itself. In the absence of H1, newly translated H2 is glycosylated to a 43 kDa precursor form but then rapidly degraded in approximately 45-60 min (19). Co-expression of the H1 subunit prevents H2 from being degraded in the ER (23), indicating that oligomerization of the H2 subunit with H1 is necessary for H2 stability. Due to its unstable nature when expressed alone, studies of the H2 subunit thus far have been limited to transfected non-hepatic cell lines expressing sub-physiological levels of H2 polypeptides that escaped ER degradation. One such report, which described the different sorting information carried by the two human ASGP-R subunits when expressed alone (24), found that the rate of H2 internalization was about 25% that of H1 in transfected fibroblasts. However, in these experiments almost 90% of the expressed H2 polypeptide was degraded in the ER.

Here we expressed and analyzed the behavior of H2, in the absence of H1, in a cell line of hepatic origin, in order to provide an environment similar to that in which the H2 protein is normally expressed. We were able to create stably transfected hepatoma-derived SK-Hep-1 cells expressing the H2 subunit, which had apparently been stabilized by fusing its NH2-terminus with an epitope tag. Here we report that H2, independently of the H1 subunit, can internalize and recycle to the cell surface and form homo-oligomers, but these H2 homo-oligomers do not bind and mediate the internalization of ASOR.
EXPERIMENTAL PROCEDURES

Materials. ASOR was prepared by desialylation of human orosomucoid (Sigma) with neuraminidase as described previously (25). Na\(^{125}\)I (10-20 mCi/µg of iodine) was from Amersham Corp. 1,2,4,6-Tetrachloro-3\(\alpha\),6\(\alpha\)-diphenylglycouril (Iodo-gen), BSA protein standard and protein assay reagents were from Pierce Chemical Co. 125I-ASOR and 125I-IgG was prepared by the Iodo-gen method as described previously (25). ASOR conjugated to Alexa Fluor\textsuperscript{®} 488 (fl-ASOR) was prepared using the Alexa Fluor\textsuperscript{®} 488 Protein Labeling Kit from Molecular Probes according to the manufacturer's instruction. All other reagents were from Sigma unless otherwise noted.

Plasmid Constructs. pcDNA4/His/Max/lacZ vector (Invitrogen) was utilized to create pH2/HisMax, a plasmid with a NH\(_2\)-terminal fusion of the Xpress epitope and 6xHis tag to the cDNA encoding H2b. A BamHI-EcoRI H2 cDNA fragment, excised from the parental plasmid H2/pcDNA3.1 (26), was inserted into corresponding sites in pcDNA4/His/Max/lacZ version C plasmid to create pH2/HisMax. The pH2/HisMax plasmid was used to create H2 stable cell lines in SK-Hep-1 cells as well as for transient expression of H2/HisMax fusion protein in COS-7 cells. The BamHI-EcoRI H2 cDNA fragment was also cloned into pcDNA3.1/Zeo(+) (Invitrogen) and used to transfect SK-Hep-1 cells, previously transfected with the H1/pIRESneo plasmid, in order to generate stable cell lines expressing both H1 and H2 (26). The pcDNA4/TO/myc-His plasmid (Invitrogen) was used to create an H2 C-terminal fusion with the myc epitope and 6xHis tag (pH2/myc-His) by inserting the BamHI-PstI H2 fragment into the corresponding sites in pcDNA4/TO/myc-His version C plasmid. The H2 cDNA fragments were purified using the Wizard PCR prep kit (Promega) prior to ligation. To generate stable transfectants expressing H1, the H1 cDNA fragment was subcloned into pIRES/neo (Clontech)
as follows. H1 cDNA, inserted at HindIII-EcoRI sites in the parental plasmid H1/pcDNA3.1 (27) was digested with HindIII and blunt-ended using the Klenow fragment of DNA polymerase (Promega) and dideoxynucleotides (Pharmacia). EcoR1 linkers (New England Biolabs) were ligated to the blunt ends and subsequently digested with EcoR1. The EcoR1 fragment containing H1 cDNA was gel-purified and ligated into the EcoR1 site of pIRES/neo (from Clontech). The recombinant H1/pIRESneo plasmids in the ligation mix were transformed into E. coli (Top10 strain from Invitrogen) for screening and amplification. The correct orientation of inserted H1 cDNA in recombinant plasmids was checked by restriction digestion. The final H1/pIRESneo plasmid was sequenced to verify the complete open reading frame and then used to create stably transfected SK-Hep-1 cell lines.

Cell Culture and Transfection. SK-Hep-1 and COS-7 cells were obtained from ATCC and cultured in complete medium: DMEM (GIBCO-BRL), supplemented with 10% fetal bovine serum (Summit Biotechnology), 2 mM glutamine and 100 units/ml each of penicillin/streptomycin (GIBCO-BRL). Transfection of COS-7 cells for transient expression or of SK-Hep-1 cells for stable expression was performed using a Calcium Phosphate Transfection Kit (Invitrogen) according to the manufacturer’s protocol. Two µg plasmid DNA was used to transfect 60-70% confluent cells in each 35 mm dish. The resultant stably transfected SK-Hep-1 cell lines were selected 3 days post-transfection in complete medium containing either 400 µg/ml G418 (GIBCO-BRL) or 200 µg/ml Zeocin (Invitrogen). Cell lines expressing both H1 and H2 were selected in medium containing 200 µg/ml each of G418 and Zeocin antibiotics. The clonal cell lines were maintained in the same medium used for their selection.
Immunoblotting. Cells were solubilized in lysis buffer (1% Triton X-100, 0.1% SDS, 5 mM EDTA in PBS) for 15 min on ice and centrifuged at 14,000 rpm for 10 min. The supernatant was removed and the samples were prepared by boiling for 5 min in 1x Laemmli buffer (28) containing 2% (v/v) β-mercaptoethanol. The samples were subjected to SDS-PAGE and the proteins were electrotransferred to nitrocellulose membranes (Protran, 0.1 μm pore size from Schleicher and Schuell) by the method of Burnette (29). Nonspecific binding sites were first blocked by incubating for 30 min in 10 mM HEPES, pH 7.4, 150 mM NaCl, 6.7 mM KCl containing 1.5% (w/v) BSA (Buffer1/BSA). The membranes were then incubated for 1 h at 4 °C with the same Buffer1/BSA containing 1 μg/ml of affinity purified rabbit polyclonal anti-H1 or anti-H2 IgG (26). Membranes were washed with 10 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.5% Tween-20 and incubated with alkaline phosphatase conjugated to goat anti-rabbit secondary antibody (Sigma) in Buffer1/BSA for 1 h at room temperature. Proteins were visualized using the alkaline phosphatase substrates p-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (Bio-Rad) in 0.1 M Tris-HCl, pH 9.5, 0.5 mM MgCl₂ according to the manufacturer’s protocol.

¹²⁵I-ASOR and anti-H2 ¹²⁵I-IgG Binding to the Cell Surface. Transiently transfected COS-7 cells (36-48 h post-transfection) or stably transfected SK-Hep-1 cells were grown in 12-well culture dishes in complete medium. Cells were washed with ice-cold PBS and incubated at 4 °C for 1 h with 0.5 ml Buffer1/BSA per well containing 1 μg/ml ¹²⁵I-ASOR or affinity purified anti-H2 ¹²⁵I-IgG. For competition binding experiments, a 100-fold excess of unlabeled ASOR or anti-H2 IgG was added to the binding medium. The binding medium was aspirated and cells were washed three times with cold PBS. Cells were solubilized in 1N NaOH, radioactivity was determined
using a Packard COBRA™ II gamma counter and protein concentration in the cell lysate was estimated by the method of Bradford (30) using BSA as standard.

**Internalization Assays.** Internalization rates were determined as In/Sur ratios as described by Wiley and Cunningham (31). Following binding and removal of excess unbound radiiodinated antibody or ASOR as described above, internalization was induced by the addition of 1 ml prewarmed DMEM to each well and placing culture dishes in a 37 °C water bath. After the desired internalization periods, the medium was aspirated and cells were washed three times with cold PBS on ice. The rest of the procedure was performed on ice. PBS was aspirated and 1 ml 10 mM sodium phosphate buffer (pH 6.8) was added to cells for 5 min. Phosphate buffer was removed by aspiration and uninternalized ASOR or IgG was removed from the cell surface by incubation with 0.5 ml 100 mM glycine, pH 2.5 for 15 min. This Glycine wash was removed, saved and the cells were solubilized in 1.0 ml 1N NaOH. The radioactivity in the glycine wash and solubilized cells were measured and the ratio of internalized (cell-associated) to surface counts (glycine wash) was calculated and plotted versus time. The slope of this line indicates the receptor internalization rate. To monitor the effect of hyperosmolarity on receptor internalization, cells were pretreated with DMEM containing 0.4 M sucrose for 15 min, and the binding and internalization steps were performed as described above in the continuous presence of 0.4 M sucrose.

**Recycling of Receptor Subunits.** SK-Hep-1 cells expressing H1 or H2/HisMax subunits were grown to confluence in 35 mm dishes and washed with PBS. Prewarmed DMEM containing 1 mg/ml proteinase-K (Roche Biochemical) was added to the cells, which were then incubated at 37 °C for various times. Detached cells were collected, pelleted at 4 °C and washed three times
with ice-cold PBS containing 100 µM phenylmethylsulfonyl fluoride. Cells were then solubilized in lysis buffer containing 100 µM phenylmethylsulfonyl fluoride and the lysates were subjected to reducing SDS-PAGE. The H1 and H2 subunits were detected by Western analysis with the subunit-specific polyclonal antibodies as described above. Images of the Western blots were captured digitally and the density of the H1 and H2 protein bands was quantified using a Fluorchem™ 8000 Imaging System (Alpha Innotech Corp.) The percentages of H1 and H2 remaining at each time-point, compared to the amount recovered at time zero, were plotted.

Co-immunoprecipitation of H2/HisMax and H2/myc-His. COS-7 cells in 60 mm dishes were co-transfected with 4 µg each of pH2/HisMax and H2/myc-His plasmid DNA and were lysed 48 h post-transfection as described above. All steps were performed at 4 °C unless otherwise noted. The volume of cell lysate was adjusted to 450 µl with lysis buffer and an equal volume of Buffer 1/BSA was then added. The lysate was pre-cleared with 50 µl protein-G Sepharose (1:1 slurry) that was previously treated with Buffer 1/BSA for 1 h. The lysate was then centrifuged and the supernatant was incubated with 2 µg of mouse IgG or monoclonal antibody against either the myc (H2/myc-His) or the Xpress (H2/HisMax) epitopes (Invitrogen). After overnight immunoadsorption on a rotating platform, 50 µl protein-G Sepharose (1:1 slurry) was added and the samples were incubated for an additional 1 h. The antigen-antibody-protein-G Sepharose complexes were washed three times with lysis buffer and once with PBS. The immuno-adsorbed proteins were dissociated from protein-G Sepharose by boiling for 5 min in 1x Laemmli buffer (28). The immunoprecipitates were subjected to 10%-SDS-PAGE, followed by detection of H2 fusion protein in parallel Western blots using anti-Xpress or anti-myc monoclonal antibodies as described above.
Chemical Cross-linking. COS-7 cells in 60 mm dishes were transfected with pH2/HisMax, washed and scraped 48 h post-transfection into 200 µl cold PBS. Scrapped cells (50 µl) were incubated in microfuge tubes on ice with 2 mM disuccinimidyl suberate (Peirce Chemicals), freshly prepared as a 100 mM stock solution in dimethyl sulfoxide. The cross-linking reaction was carried out for various times on ice and then quenched by the addition of Tris, pH 7.5 to 10 mM. Cells were pelleted and then solubilized in lysis buffer as described above. Samples were boiled in Laemmli buffer (28) for 5 min and subjected to non-reducing SDS-PAGE using a 10% (w/v) gel. H2 was detected as described above using affinity-purified anti-H2 IgG and the Mr values of reactive bands were estimated using a Fluorchem™ 8000 imaging system (Alpha Innotech Corp).

Confocal Fluorescence Microscopy. Stably transfected SK-Hep-1 cells were grown in Lab-Tek® II chamber slides (Nalge Nunc Int.) to approximately 50-75% confluence and washed in PBS. The cells were fixed with 4% formaldehyde in PBS for 15 min on ice and then washed in PBS. The rest of the protocol was performed on ice. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and non-specific binding sites were blocked with Buffer1/BSA. Clathrin was detected using a monoclonal antibody against clathrin heavy chain (Transduction Laboratories) diluted 1:500 in Buffer1/BSA and Orange-green conjugated to goat anti-mouse IgG (Molecular Probes), while H2 was detected with polyclonal rabbit anti-H2 IgG (1 µg/ml) and Cy3-labeled sheep anti-rabbit IgG. Buffer1/BSA was also used for appropriate secondary antibody dilutions as suggested by the supplier. Cell samples were incubated with secondary antibody conjugates for 15 min, washed in PBS, mounted in FluorSave (Calbiochem) and fluorescence was detected using a Leica TCS NT laser confocal microscope. Digital images were recorded using Leica TCS software. In the experiments to detect internalized ASOR, cells
were incubated with 1 µg/ml fl-ASOR for 1 h at 37 °C in DMEM. Following a PBS wash, the cells were fixed with 4% formaldehyde, mounted, and examined as described above.

RESULTS

Stabilization of the H2 polypeptide. The H2 polypeptide is unstable when expressed alone using the mammalian expression vector pcDNA3.1. However, we were able to study the characteristics of the H2 subunit in the absence of the H1 subunit, because masking its NH2-terminus with an epitope tag apparently stabilized the expression of H2. We generated an H2 fusion protein, designated H2/HisMax (Mr ~52 kDa), by the addition of both the 6xHis tag and Xpress epitopes to the NH2-terminus of the H2 protein using the pcDNA4/His/Max/lacZ expression vector. The native H2 polypeptides derived from pcDNA3.1 and the H2/HisMax fusion proteins derived from pcDNA4/His/Max/lacZ were expressed in COS-7 cells and the H2 proteins were detected by immunoblotting. The expressed H2/HisMax fusion protein was stable (Fig. 1, lane 2), whereas the H2 protein expressed from the pcDNA3.1 vector was present as a partially degraded ~35 kDa species (Fig. 1, lane 3). Since this 35 kDa band is not present in transiently transfected cells expressing H2/HisMax, it is unlikely to be a precursor of the mature H2 protein. In addition, a 35 kDa H2 precursor protein during the biosynthesis of H2 has not been described. In the HepG2 hepatoma cell line, H2 is synthesized as a 43 kDa high mannose-containing precursor, which is then processed to a 50 kDa mature form (9). The 35 kDa protein is likely a ER degradation product of H2, which has been reported before by others (23,32,33). Although the precise mechanism for the H2 stabilization we observed is unknown, the fusion of the H2 NH2-terminus with Xpress epitope and 6xHis tag apparently rescued the H2 protein from degradation in the ER.
**H2/HisMax is localized at the plasma membrane in the absence of H1.** The H2/HisMax fusion protein was then transfected into SK-Hep-1 cells, to create cell lines that stably expressed H2 at levels similar to native hepatoma cell lines. We determined the expression of H2/HisMax at the plasma membrane in these cell lines by quantifying anti-H2 $^{125}$I-IgG binding at 4 °C (Fig. 2). The anti-H2 $^{125}$I-IgG specifically bound to H2 in SK-Hep-1 cells expressing this protein, since excess unlabeled anti-H2 IgG markedly reduced the radioactivity associated with those cells and IgG binding to cells transfected with the plasmid alone was minimal (Fig. 2, "Vector"). We conclude, that the H2 subunit contains the necessary information required for its translocation to the plasma membrane in the absence of the H1 subunit.

**H2/HisMax is internalized in SK-Hep-1 cells.** Since H2 was present on the cell surface in transfected SK-Hep-1 cells, we tested its ability to be internalized by following the uptake of anti-H2 $^{125}$I-IgG that had been allowed to bind on ice to cell surface H2/HisMax. Cells with $^{125}$I-IgG bound to the cell surface were then warmed to 37 °C to induce internalization and the apparent internalization rate was determined from the ratio of IgG internalized to IgG remaining on the cell surface (*i.e.* the In/Sur ratio). The increase in the In/Sur ratio with increasing time showed that the H2/HisMax proteins were internalized in SK-Hep-1 cells (Fig. 3A). Initially, H2/HisMax was internalized more rapidly, with almost 20% and 31% of surface-bound IgG localized internally after 2 and 5 min, respectively (In/Sur ratios of 0.28 and 0.45). However, after the first 5 min, it took another ~30 min for half of the initial surface-bound anti-H2 IgG to be internalized (In/Sur ratio of 1.04), indicating a reduction in H2/HisMax internalization rate. Thus, after a period of initial rapid uptake, the H2 internalization rate decreased by ~80%. Nonetheless, the internalization of H2/HisMax was continuous, and almost 70% of the $^{125}$I-IgG bound to H2/HisMax on the cell surface was internalized after 3 h (Fig. 3B).
H2/HisMax is internalized at a slower rate than H1. We compared the rates of H1 and H2/HisMax internalization in stably transfected SK-Hep-1 cells expressing either H1 or H2/HisMax. H1 or H2/HisMax on the cell surface was allowed to bind subunit-specific, affinity-purified \(^{125}\text{I}\)-IgG. The rates of anti-H1 and anti-H2 IgG uptake were then assessed in parallel. The H1 subunits were internalized faster than H2/HisMax subunits (Fig. 4A). Thus, after 5 min ~50% of the H1 was internalized (In/Sur ratio of 1.1) compared to ~33% for H2/HisMax (In/Sur ratio of 0.5). The difference in their internalization rates was even more apparent at longer time; after 12 min ~67% of H1 was internalized compared to ~40% of H2/HisMax. A difference in H1 and H2/HisMax internalization rates was also seen in transiently transfected COS-7 cells (Fig. 4B). In COS-7 cells however, H1 internalization was slower than in SK-Hep-1 cells, whereas the H2/HisMax internalization rate was not significantly different in these two cell lines.

H2 is internalized through clathrin-coated pits. Since the hetero-oligomeric ASGP-R is internalized via the clathrin-coated pit pathway (34), we determined if H2 alone is also internalized via coated pits. The internalization rate of surface-bound anti-H2 \(^{125}\text{I}\)-IgG was determined in H2/HisMax stable transfectants after their ability to form clathrin-coated pits was impaired by hyperosmolar treatment (34,35). As shown in Fig. 5, sucrose treatment reduced anti-H2 \(^{125}\text{I}\)-IgG uptake. However, inhibition was not complete, since ~28% of surface H2 was still internalized after 30 min in sucrose-treated cells, compared to ~51% internalization in untreated cells. In contrast, similar treatment more substantially reduced ASOR uptake by ASGP-Rs in HepG2 cells (Fig. 5 inset). Almost 80% of the surface-bound ASOR was internalized in untreated HepG2 cells in 10 min, compared to ~20% in sucrose-treated cells. Thus, sucrose treatment led to a 75% reduction in ASOR internalization by ASGP-Rs in HepG2 cells, compared to a ~45% decrease observed for H2/HisMax in SK-Hep-1 cells.
Uptake of H2 via clathrin-coated pits was corroborated by the colocalization of H2/HisMax with clathrin heavy chains (Fig. 6). The orange/yellow color (Fig. 6F) in the overlay of pictures of clathrin (Fig. 6D, green) and H2/HisMax (Fig. 6E, red) staining suggests substantial colocalization of these molecules. Similar results were also obtained in HepG2 cells, in which a distinct overlapping clathrin and H2 population was evident (Fig. 6C). However, the overlap of green and red stain in SK-Hep-1 cells expressing H2/HisMax was not complete, since distinct red and green staining was still observed in these cells. Hence, when expressed alone, H2 was at least partially internalized via clathrin-coated pits. It is likely that the initial more rapid uptake of H2 (described above) occurs through clathrin-coated pits, whereas the slower continuous internalization of H2 occurs through a clathrin-independent pathway.

*Endogenous Recycling Rates of H1 and H2 Subunits.* We next compared the rates of receptor subunit movement from intracellular compartments to the cell surface in SK-Hep-1 cells expressing either the wild-type H1 or the H2/HisMax fusion protein. Receptor subunits that translocate to the cell surface, including H2 proteins that recycled after their internalization, were removed by proteinase-K treatment at 37 °C for various times. The residual amounts of H1 and H2/HisMax proteins were detected by Western analysis of the cell lysates using subunit-specific antibodies (Fig. 7A). The wild-type H1 subunits were destroyed considerably faster than the H2/HisMax subunits. Almost 75% of the H1 subunits trafficked or recycled to the plasma membrane after 15 min, compared to only 15% of the H2/HisMax proteins (Fig. 7B). After 30 min, almost all of the H1 subunits were degraded by the proteinase-K, whereas only 25% of the H2/HisMax proteins had been degraded. These results indicate that in SK-Hep-1 cells H1 subunits recycle at a rate approximately 4-fold faster than that of H2/HisMax.

*Oligomerization of H2 Subunits.* Since H1 oligomers occur in hepatocytes or hepatoma cells (11) and when H1 is overexpressed in COS-7 cells (19), we used a co-immunoprecipitation
technique to test the ability of two different populations of H2 fusion proteins, containing either the Xpress (H2/HisMax) or myc (H2/myc-His) epitopes, to associate with each other. COS-7 cells were co-transfected with plasmids containing H2/HisMax or H2/myc-His cDNAs. The cells were lysed and subjected to immunoprecipitation with monoclonal antibodies against either the myc or Xpress epitope. The presence of H2/HisMax fusion protein (Mr ~ 52 kDa) in anti-myc immuno-precipitates (Fig. 8A, lane 4) and H2/myc-His fusion protein (Mr ~49 kDa) in anti-Xpress immunoprecipitates (Fig. 8B, lane 4) was detected by anti-Xpress and anti-myc antibodies, respectively (indicated by arrows). The presence of both H2 isoforms in each independent immunoprecipitate shows that the two different H2 fusion proteins interact strongly in vivo even after detergent solubilization. The interaction was specific, because mouse IgG or protein-G Sepharose alone did not precipitate H2/HisMax (Fig. 8A, lanes 2 and 3), although a minor amount of H2-myc-His was detected in mouse IgG/protein-G Sepharose immunoprecipitates (Fig. 8B, lanes 2 and 3). Co-immunoprecipitation of both H2 fusion proteins with either epitope-specific antibody demonstrates that the two different H2 fusion proteins oligomerize in vivo.

**Cross-linking of H2/HisMax.** The occurrence of RHL1 and RHL2/3 homo-dimers and homo-trimers in rat liver microsomes (16) led us to investigate the extent of H2 oligomerization in cultured cells ex vivo. COS-7 cells expressing the H2/HisMax fusion protein were treated with the non-cleavable homo-bifunctional cross-linker DSS on ice for different times and the cells were lysed. H2 was then detected by immuno-blotting using polyclonal anti-H2 IgG (Fig. 9). At least three additional H2-containing bands of larger mass (marked by arrows) were generated after cross-linking (Fig. 9, lanes 6-8). The masses estimated for the three larger bands (~100, 154 and 220 kDa), are consistent with the predicted sizes of H2/HisMax dimers, trimers and tetramers (based on a monomeric mass of 52 kDa for H2/HisMax). The formation of H2 homo-
oligomers was evident as early as 90 s (Fig. 9, lane 6). Samples from COS-7 cells transfected with vector alone did not contain any proteins cross-reactive with the H2-specific IgG (Fig. 9, lanes 1-4). These results showed the ability of H2 to oligomerize and form at least homotetramers.

**Inability of H2/HisMax to mediate ASOR uptake in SK-Hep-1 cells.** ASOR binding and uptake was studied in SK-Hep-1 cells expressing either H2/HisMax alone, or both H1 and H2 wild-type subunits. The cells were incubated with fl-ASOR for 1 h at 37 °C and internalized fl-ASOR was assessed by confocal microscopy. Whereas SK-Hep-1 cells expressing both H1 and H2 internalized fl-ASOR, detected as punctate green staining (Fig. 10A), cells expressing H2/HisMax alone did not accumulate fl-ASOR (Fig. 10C). Similarly, stably transfected cells expressing H2 did not internalize 125I-ASOR (not shown). Results from these experiments indicate that H2/HisMax is incapable of mediating the endocytosis of ASOR.

Since H1 specifically binds ASOR when overexpressed without H2 in COS-7 cells (22), we assessed the ability of H2 to bind ASOR under similar conditions. COS-7 cells expressing H2/HisMax protein or transfected with the backbone plasmid (HisMax), as well as HepG2 cells, were allowed to bind either anti-H2 125I-IgG (Fig. 11A) or 125I-ASOR (Fig. 11B) on ice. COS-7 cells expressing H2/HisMax did not bind ASOR (Fig. 11B), although in a parallel experiment these cells bound anti-H2 125I-IgG (Fig. 11A). HepG2 cells expressing native ASGP-Rs bound both ASOR and anti-H2 IgG, whereas COS-7 cells transfected with the empty vector (HisMax) did not bind either ASOR or anti-H2 IgG. These results demonstrate the inability of oligomerized H2 to present functional binding sites for a high affinity interaction with ASOR. This result is in contrast to findings about the H1 subunit, which can bind ASOR when overexpressed in COS-7 cells (22), although at a somewhat lower affinity than the heterooligomeric ASGP-R complex.
DISCUSSION

The present study was undertaken to address the ability of the minor H2 subunit of the human ASGP-R to function independently of the major subunit H1. Understanding the properties of H2 in the absence of H1 could ultimately elucidate its role in the function of native hetero-oligomeric ASGP-Rs. Previous studies were limited in their ability to monitor H2 by itself, since the protein could only be expressed at sub-physiological levels (24). Hence we generated stably transfected cell lines, derived from hepatic adenocarcinoma-derived SK-Hep-1 cells, that express stabilized H2 subunits. Our most significant findings are that H2 contains the necessary structural information to assemble into homo-oligomers, but these complexes are not able to bind an asialoglycoprotein ligand such as ASOR.

H1 self-associates as homo-dimers and homo-trimers in HepG2 cells expressing native ASGP-Rs (12) and homo-trimers of both RHL1 and RHL2/3, (i.e. the rat homologues of H1 and H2), are present in isolated rat liver microsomes (16). We found that H2 fusion proteins, tagged with two different epitopes, were also able to associate with each other to form H2 homo-oligomers. Furthermore, the ability to cross-link H2/HisMax expressed in transfected COS-7 cells indicated that the protein was oligomerized to produce at least homo-tetralmers. These results thus raise the intriguing possibility that naturally occurring H2 homo-oligomers may exist in human hepatocytes, as observed in rat liver.

In cell expressing H2/HisMax, H2 was transported to the cell surface and internalized in the absence of H1. The lack of tyrosine- (24) or dileucine-based (37,38) internalization signals in the fused region of the H2/HisMax protein, indicates that the internalization of H2/HisMax via coated pits is due to the presence of at least one endogeneous endocytic signal in the H2 cytoplasmic segment. H2-IgG complexes on the cell surface were internalized rapidly at first,
followed by a slower rate of H2 uptake. Almost 20% and 31% of cell surface H2 complexes were internalized after 2 and 5 min, respectively. Bider and Spiess (39) observed that only 8% and 20% of surface H2 was internalized after 2 and 5 min, respectively, in the absence of ASOR. The endocytic rates of individual ASGP-R subunits in HepG2 cells cannot be determined because both receptor subunits are also present in native hetero-oligomeric ASGP-R complexes. Since the endocytic rate of H1 in ASGP-R hetero-oligomers affects the observed H2 internalization rate, the value for H2 determined in HepG2 cells likely does not reflect the true internalization rate of individual H2 subunits.

Fuhrer et al. (24) found that NIH/3T3 fibroblasts expressing the H2 subunit alone only internalized 10-15% of the cell surface H2 after 40-50 min. The majority of H2 internalization occurred in the initial 10 min, after which there was no significant change in the intracellular accumulation of H2. In SK-Hep-1 cells stably expressing H2, its uptake was faster, with almost 36% of the surface-bound H2 internalized in 10 min, compared to only 10-15% in transfected fibroblasts. Also, H2 clearance from the plasma membrane continued for hours in SK-Hep-1 cells, although at a reduced rate, in contrast to the saturation of H2 uptake in NIH/3T3 cells after 10 min. The initial phase of rapid H2 uptake followed by a subsequent slower rate suggests the existence of two different pathways for H2 internalization in SK-Hep-1 cells. Thus, it is possible that the initial rapid uptake of H2 from the plasma membrane occurs through clathrin-coated pits, whereas the following slow but continuous internalization occurs via another route.

H1 was internalized at a faster rate than H2 in both SK-Hep-1 and COS-7 cells. In SK-Hep-1 cells, the H1 subunits were internalized about 1.5-times faster than H2. The different endocytic rates observed for the two ASGP-R subunits could be due to the efficiency of different endocytic signals residing in their cytoplasmic tails. Recognition of the H1 and H2 endocytic signals in SK-Hep-1 appeared to be somewhat different than in COS-7 cells. Whereas H1 was
internalized at a slower rate in COS-7 cells than in SK-Hep-1 cells, H2/HisMax was internalized at similar rates in both cell lines. Nevertheless, the individually expressed H1 subunits were internalized at a faster rate than the H2 subunits in both cell lines. Tyr^5 in the H1 subunit is part of a tyrosine-based internalization signal (21) that is more effective for internalization than the endocytic signal present in the H2 cytoplasmic tail, which remains to be defined. In H2 a Phe residue is at the analogous site of Tyr^5 in H1. Internalization of the mutant H1(Tyr5Phe) is decreased but still retains significant endocytic activity (21,40,41). Hence, if the Phe^5 in H2 is responsible for its internalization, it constitutes a weaker signal for endocytosis than the Tyr^5 in H1. In H2 the mutation of Phe^5 to Ala did not reduce the H2 internalization rate any further in transfected fibroblasts (24). However, since fibroblasts do not normally express ASGP-R, they may lack some of the accessory endocytic factors necessary for the recognition of a putative Phe^5 internalization signal in H2, which could also explain the slower internalization rate of H2 in fibroblasts compared to SK-Hep-1 cells.

H1 is internalized via the clathrin-coated pit pathway in the absence of H2 in transfected fibroblasts (20). The observation that hyperosmolarity partially reduced H2 uptake in transfected SK-Hep-1 cells suggests that a fraction of the H2 is also targeted to clathrin-coated pits. This result was corroborated by the partial colocalization of H2 and clathrin. However, not all H2 in SK-Hep-1 cells was taken up via clathrin-coated pits, ~28% of surface-expressed H2 was still internalized within 30 min despite sucrose treatment. In support of this finding, not all H2 was co-localized with the clathrin heavy chain (Fig. 6). Thus, H2 could be internalized via clathrin-coated pits as well as another, unknown pathway. Other ongoing studies have indicated that similar stably transfected SK-Hep-1 cell lines have an active transcytosis pathway^2. Consistent with this finding, Heffelfinger et al. have suggested that the SK-Hep-1 cell line may actually be of endothelial rather than parenchymal origin (42).
Since intracellular H1 is capable of recycling to the cell surface in transfected fibroblasts (20), we tested the ability of H2/HisMax to recycle. The rates of H1 and H2 recycling were significantly different in stably transfected SK-Hep-1 cell lines. H1 recycled at a 4-fold faster rate than H2/HisMax. Since H1 homo-oligomers internalize and recycle at a rate comparable to that of H1-H2 hetero-oligomeric ASGP-R complexes, the signals in H1 subunits in the mature ASGP-R complex override any potential weak signals present in the H2 subunit. Thus the H2 subunits in hetero-oligomeric ASGP-Rs internalizes and recycles at a rate that is similar to H1, as noted above.

The CRDs of individual H1 and H2 subunits can bind to galactose-Sepharose in a Ca\(^{2+}\)-dependent manner (16,18,19). Zeng et al. (43) demonstrated that renatured RHL1 specifically binds to ASOR, which contains multiple tri- and tetra-antennary N-linked glycans with terminal galactose residues. Similarly, homo-oligomeric H1 complexes in COS-7 cells also bind ASOR, although at lower affinity than that of the native H1-H2 hetero-oligomeric ASGP-R (22). However, the homo-oligomeric H2 species observed here, which included trimers and tetramers, did not bind ASOR. Thus homo-oligomeric H1 presents binding sites for ASOR, whereas homo-oligomerized H2/HisMax failed to produce similar sites for stable interaction with ASOR. The requirement for the presence of H2 in native ASGP-R hetero-oligomeric complexes in order to achieve high affinity ASOR binding indicates a critical role of H2 in the proper assembly of the multiple CRDs in order for an ASGP-R to bind ligand effectively. Therefore, the presence of H2 in a hetero-oligomeric H1-H2 ASGP-R complex likely creates a special spacial organization of CRDs that leads to the generation of specific recognition sites for N-linked glycans with terminal galactose residues. This idea was first proposed for the rat ASGP-R by Hardy et al. (44), who suggested that the CRD arrangement of the minor and major subunits needed to achieve high affinity ligand binding could be described by a triangle whose sides were 15, 22, and 25 Å.
The present findings indicate that H2 homo-oligomers could exist in native human hepatic cells, although presumably at lower concentrations than hetero-oligomeric ASGP-R complexes. If H2 homo-oligomers exist naturally, they might have a different organization of CRDs and recognize a different set of endogenous ligands than the typical glycoproteins known to bind to hetero-oligomeric ASGP-Rs, i.e. tri- and tetra-antennary oligosaccharides containing gal-termini. H2 homo-oligomers could thus have an as yet undiscovered function, in a similar manner to the unexpected finding that homo-oligomeric RHL1 is the rat liver receptor for lactoferrin (45).
ACKNOWLEDGEMENTS

We thank Janet A. Weigel for iodination of ASOR and IgG preparations. We also thank Jim Henthorn for his valuable help in obtaining immunofluorescence images at the Confocal Imaging facility supported by the Warren Medical Research Institute.

FOOTNOTES

1. The abbreviations used are: ASGP-R, asialoglycoprotein-receptor; ASOR, asialoorosomucoid; BSA, bovine serum albumin; CRD, carbohydrate recognition domain; DMEM, Dulbecco's minimum essential medium; fl-ASOR, fluorescent ASOR; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

2. J. H. N. Yik, A. Saxena, J. A. Weigel and P. H. Weigel (manuscript submitted)
FIGURE LEGENDS

Figure 1. Stable production of H2 in COS-7 cells. COS-7 cells transfected with pcDNA4/HisMax (lane 1), H2/pcDNA4/HisMax (lane 2), or H2/pcDNA3.1 (lane 3), were lysed 40 h post-transfection and proteins in the cell lysates were separated by SDS-PAGE and transferred to nitrocellulose. H2 proteins were detected by immunoblotting with affinity-purified anti-H2 polyclonal IgG as described in “Experimental Procedures”.

Figure 2. Binding of anti-H2 125I-IgG to H2/HisMax expressed on the surface of SK-Hep-1 cells. SK-Hep-1 cells stably transfected with pcDNA4/HisMax (Vector) or H2/pcDNA4/HisMax (H2/Vector) were grown in 24-well dishes and incubated for 1 h at 4°C with 1 µg/ml anti-H2 125I-IgG with (+) or without (-) a 100-fold excess of anti-H2 IgG. Cells were lysed and cell-associated radioactivity was determined. Data presented are the mean ± S.D. of the results from three separate experiments each performed in duplicate (n = 6).

Figure 3. Internalization of H2/HisMax in SK-Hep-1 cells. SK-Hep-1 cells stably expressing the H2/HisMax fusion protein were grown in 12-well dishes. Affinity purified anti-H2 125I-IgG was bound at 4°C to cells and after washing, internalization was induced by warming the cells to 37°C. The internalization rate was determined by measuring the In/Sur ratio versus time as described in “Experimental Procedures”. The slope of the line indicates the internalization rate of H2/HisMax, which is shown at shorter (A) and longer duration (B). The data presented are the mean ± S.D. of the results obtained in duplicate from at least two experiments.
Figure 4. **H1 and H2/HisMax internalization rates in SK-Hep-1 and COS-7 cells.** SK-Hep-1 (A) or COS-7 (B) cells expressing either H1 (●) or H2/HisMax (○) were grown in duplicate in 12-well plates. The cells were chilled and allowed to bind $^{125}$I-IgG at 4°C and internalization was induced at 37°C for the indicated times. Internalization rates were assessed by determining the In/Sur ratio versus time. The data presented are the mean ± S.D. from three experiments.

Figure 5. **Effect of hyperosmolarity on the internalization rate of H2/HisMax.** SK-Hep-1 cells stably producing H2/HisMax were grown in 12-well dishes and then incubated in DMEM without serum at 37°C for 30 min either with (○) or without (●) 0.4 M sucrose. The cells were then allowed to bind anti-H2 $^{125}$I-IgG at 4°C, washed and incubated at 37°C. Internalization rates were determined from the In/Sur ratio plots as described in "Experimental Procedures". The data presented are the mean ± S.D. from three experiments. The effect of a 30 min sucrose treatment on $^{125}$I-ASOR uptake by wild-type ASGP-R in HepG2 cells is shown in the inset.

Figure 6. **Co-localization of H2/HisMax and clathrin in stable SK-Hep-1 cells.** HepG2 or SK-Hep-1 cells stably expressing H2/HisMax were co-immunostained with antibodies against the clathrin heavy chain and H2 subunit. Clathrin was detected by Orange-green conjugated to anti-mouse IgG (green), whereas H2 was detected with Cy3-conjugated to anti-rabbit IgG (red). Staining of HepG2 cells expressing wild-type ASGP-Rs is shown in Panels A and B. A similar staining pattern in SK-Hep-1 cells expressing H2/HisMax is shown in Panels D and E. The overlay of red and green fluorescence is shown in Panels C (HepG2) and F (SK-Hep-1 expressing H2/HisMax). Staining of SK-Hep-1 cells expressing H2/HisMax with nonimmune
mouse and rabbit IgGs are shown in Panels G and H, respectively. Staining of SK-Hep-1 cells stably transfected with the empty vector using anti-H2 IgG is shown in Panel I. The bar is 20 µm.

**Figure 7. Recycling rates of H1 and H2/HisMax in SK-Hep-1 cells.** A. Analysis of the reduction in the amounts of H1 and H2 proteins following proteinase-K digestion: Mock transfected SK-Hep-1 cells (lane 1) or SK-Hep-1 cells stably expressing H1 (upper panel) or H2/HisMax (lower panel) were either untreated (lane 2) or treated (lanes 3-7) with 1 mg/ml proteinase-K at 37 °C for the indicated times to remove cell surface proteins. The remaining total cellular H1 or H2/HisMax was detected by Western blotting using either anti-H1 or anti-H2 IgG. B. Recycling rates of H1 and H2/HisMax: The density of the H1 (●) and H2/HisMax (○) bands in panel A at each time-point (lanes 4-7) relative to the amount recovered at time-zero (lane 3) were quantified as described in "Experimental Procedures". Data, presented as the percentage remaining compared to time zero, are the mean ± S.D. from three independent experiments.

**Figure 8. Homo-oligomerization of H2/HisMax and H2/myc-His in COS-7 cells.** Lysates prepared from COS-7 cells co-expressing H2/HisMax and H2/myc-His were subjected to immunoprecipitation in parallel with protein-G Sepharose and non-immune mouse IgG (lanes 2, A & B), no antibodies (lanes 3, A & B) or monoclonal antibodies against the myc epitope (lane 4, A) or the Xpress epitope (lane 4, B). The immuno-precipitated proteins were subjected to SDS-PAGE and immunoblotting following transfer of proteins to nitrocellulose. H2/HisMax protein in the anti-myc immunoprecipitate (lane 4, A) and H2/myc-His in the anti-Xpress immunoprecipitate (lane 4, B) are indicated by arrows. Lanes 1 show immuno-blots of
immunoprecipitates using anti-\textit{myc} (A) or anti-Xpress (B) from a lysate of COS-7 cells transfected with the backbone plasmids.

**Figure 9. Cross-linking of H2/HisMax in COS-7 cells.** COS-7 cells transfected with either pcDNA4/HisMax (lanes 1-4) or H2/pcDNA4/HisMax (lanes 5-8) were treated with disuccinimidylsulinate for the indicated times at 4 °C and lysed as described in Experimental Procedures. Samples were analyzed by SDS-PAGE, transferred to nitrocellulose and bands containing H2 were detected using affinity-purified anti-H2 IgG. The arrows indicate the predicted positions of the H2/HisMax monomer, dimer, trimer and tetramer bands.

**Figure 10.** Stably transfected SK-Hep-1 cells expressing H2/HisMax proteins do not internalize ASOR. SK-Hep-1 cells expressing both wild-type H1 and H2 subunits (A) or the H2/HisMax fusion protein (C), were incubated in DMEM with fl-ASOR for 1 h at 37 °C. Internalized fl-ASOR was visualized by confocal microscopy. The bar is 20 µm. Confocal image (A and C); phase contrast image (B and D).

**Figure 11.** Binding of $^{125}$I-ASOR and anti-H2 $^{125}$I-IgG to surface-expressed H2/HisMax in COS-7 cells and H2 in HepG2 cells. HepG2 and COS-7 cells transfected with the HisMax plasmid alone or H2/HisMax were allowed to bind either anti-H2 $^{125}$I-IgG (A) or $^{125}$I-ASOR (B). Following a 1 h incubation at 4 °C, the cells were washed in PBS, solubilized and cell-associated radioactivity, normalized per µg of cell protein, was determined in duplicate. Data are the mean ± S.D. from two separate experiments.
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Fig. 2

**Diagram:**

- **Y-axis:** ANTI-H2 $^{125}$I-IgG BOUND (cpm/µg)
- **X-axis:** Anti-H2 IgG: - - + +

- **Comparison:**
  - **Vector**
  - **H2/Vector**

The graph shows a significant increase in the bound $^{125}$I-IgG when Anti-H2 IgG is present with the H2/Vector, compared to the Vector control.
Fig. 3

**A**

![Graph A](image)

**B**

![Graph B](image)
Fig. 4

A

SK-Hep-1

B

COS-7
A

Proteinase-K: + - + + + + + +
Time (min): 0 0 0 5 15 30 60

H1

H2

1 2 3 4 5 6 7
Fig. 8

A

|       | 1 | 2 | 3 | 4 |
|-------|---|---|---|---|
| Mock  |   |   |   |   |
| H2/HisMax + H2/MycHis |   |   |   |   |

Anti-Xpress Blot

- Anti-Myc: + - - +
- Mouse IgG: - + - +
- ProtG-Seph: + + + +

B

|       | 1 | 2 | 3 | 4 |
|-------|---|---|---|---|
| Mock  |   |   |   |   |
| H2/HisMax + H2/MycHis |   |   |   |   |

Anti-Myc Blot

- Anti-Xpress: + - - +
- Mouse IgG: - + - +
- ProtG-Seph: + + + +
Fig. 9

| TIME (min): | 0 | 1.5 | 3 | 5 |
|-------------|---|-----|---|---|
| Vector alone |   |     |   |   |
| Vector with H2 |   |     |   |   |

kDa

- Tetra
- Tri
- Di
- Mono
**Fig. 11**

**A**

![Graph A](image1)

**B**

![Graph B](image2)
H2, the minor subunit of the human asialoglycoprotein receptor, trafficks intracellularly and forms homo-oligomers, but does not bind asialo-orosomucoid

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J. Biol. Chem. published online June 27, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M205653200

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