The Minor Subunit Splice Variants, H2b and H2c, of the Human Asialoglycoprotein Receptor Are Present with the Major Subunit H1 in Different Hetero-oligomeric Receptor Complexes

Jasper H. N. Yik, Amit Saxena, and Paul H. Weigel†‡

From the Department of Biochemistry & Molecular Biology, and The Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

The hepatic asialoglycoprotein receptor (ASGP-R) is an endocytic receptor that mediates the internalization of desialylated glycoproteins and their delivery to lysosomes. The human ASGP-R is a hetero-oligomeric complex composed of H1 and H2 subunits. There are three naturally occurring H2 splice variants, designated H2a, H2b, and H2c, although the expression of the H2c protein has not been reported. Following deglycosylation of purified ASGP-R, we detected the H2b and H2c proteins in HepG2 and HuH-7 hepatoma cells, using an antibody directed against a COOH-terminal peptide common to all H2 isoforms (anti-H2-COOH) and another antibody against a 19-amino acid cytoplasmic insert found only in H2b (anti-H2-Cyto19). H1 and both H2b and H2c were co-purified by affinity chromatography, using asialoorosomucoid (ASOR), anti-H1, or anti-H2-COOH-Sepharose, whereas only H1 and H2b were immunoprecipitated with anti-H2-Cyto19. These results indicate that H2b and H2c are not present in the same ASGP-R complexes with H1. Similar to the H2b isoform, H2c was also palmitoylated, indicating that the 19-residue cytoplasmic insert does not regulate palmitoylation. Stably transfected SK-Hep1 cell lines expressing ASGP-R complexes containing H1 and either H2b or H2c had similar binding affinities for ASOR and endocytosed and degraded ASOR at similar rates. The pH dissociation profiles of ASOR-ASGP-R complexes were also identical for complexes containing either H2b or H2c. We conclude that the H2b and H2c isoforms are both functional but are not present with H1 in the same hetero-oligomeric ASGP-R complexes. This structural difference between two functional subpopulations of ASGP-Rs may provide a molecular basis for the existence of two different pathways, designated State 1 and State 2, by which several types of recycling receptors mediate endocytosis.

Although its function in vivo is unknown, the hepatic ASGP-R† can mediate the clearance of injected asialoglycoproteins from the circulation via the clathrin-coated pit pathway (1–6). The human ASGP-R is a hetero-oligomer composed of a major subunit (H1) and a minor subunit (H2) that are encoded by two different genes. The two subunits are glycoproteins that are highly homologous to each other, with the exception that an 18-aa cytoplasmic insert is found only in the 50-kDa H2 but not in the 46-kDa H1 (7). The two distinct proteins of ~50 and 60 kDa can be separated by SDS-PAGE due to differences in glycosylation, and they are, therefore, designated as RHL2 and RHL3, respectively (9). The mouse ASGP-R is also composed of a major subunit, MHL-1 (10), and a minor subunit, MHL-2 (11). In all species examined, each ASGP-R subunit contains a short 40- to 60-aa NH2-terminal cytoplasmic domain, a single-pass transmembrane domain, an ~80-aa extracellular stalk region, and a ~130-aa carbohydrate recognition domain that is capable of recognizing a terminal galactose or N-acetylgalactosamine residue found on the carbohydrate chains of naturally occurring desialylated glycoproteins or neoglycoproteins (12–14). High affinity ligand binding by the human ASGP-R, with Kd values in the nanomolar range, requires the formation of hetero-oligomeric ASGP-R complexes that are composed of both H1 and H2 subunits (15) as well as ligands containing carbohydrate chains with two or more branches (12).

The cDNAs of three naturally occurring H2 splice variants, designated H2a, H2b, and H2c (see Fig. 1), have been isolated from human liver and HepG2 hepatoma cells (7, 16). Relative to H2c, the H2a cDNA contains a 57-nt cytoplasmic insert and a 15-nt insert near the junction between the transmembrane domain and the ectodomain (7). H2a is not part of native ASGP-R complexes, because the 5-aa sequence, encoded by the 15-nt insert, serves as a cleavage signal that results in proteolysis and the secretion of the entire H2a ectodomain (17). H2b lacks the 5-aa insert and is, therefore, not proteolytically cleaved, but it oligomerizes with H1 to form native human ASGP-Rs. H2c (originally designated i-H2) lacks both the 19-aa and 5-aa inserts (16), and its function remains unknown. Although the native H2c protein was successfully expressed in transfected tissue culture cells (18), its expression in human liver or hepatoma cell lines and its role in ASGP-R function has not been determined.

In this study we confirmed the natural expression of the H2c splice variant protein and verified that H2c is part of native hetero-oligomeric ASGP-R complexes in HepG2 and HuH-7 hepatoma cell lines. We also studied the effect of deleting the 19-aa cytoplasmic insertion on the palmitoylation of H2c, the ligand-binding affinity of ASGP-R complexes containing H2c, as well as continuous uptake and degradation of ligand mediated by these ASGP-Rs. A preliminary report of these results has been presented (19).
EXPERIMENTAL PROCEDURES

Materials—[9,10-3H]Palmitate (30–60 Ci/mmol) was purchased from PerkinElmer Life Sciences. ASOR was prepared by desialylation of human orosomucoid (Sigma Chemical Co.) with neuraminidase and iodinated as described previously (20). ASOR-Sepharose 4B was prepared as described previously (21). Na218O (10–20 μCi of iodine) was from Amersham Biosciences, Inc. 1,2,4,6-Tetrachloro-3,3,5,5-tetraethylbenzene (TBB) Triton X-100, and protein assay reagents were from Pierce Chemical Co. BSA was from Interco Gen. Buffer 1 contains 10 mM HEPES (Research Organics, Inc.), pH 7.4, 150 mM NaCl, and 6.7 mM KC1. Hanks’ balanced salt solution contains 1.26 mM CaCl2, 5.36 mM KC1, 0.3 mM KH2PO4, 0.5 mM MgCl2, 0.4 mM MgSO4, 137 mM NaCl, 0.33 mM Na2HPO4, 5.5 μM glucose, 4.2 mM NaHCO3, and 0.001% (v/v) phenol red, pH 7.4. Digitonin was from Acros Organics. All chemicals for electrophoresis were obtained from Bio-Rad laboratories. All other reagents were from Sigma unless otherwise noted.

Oligonucleotides—The oligonucleotides used as RT-PCR primers for the analysis of mRNA encoding H2 isoforms were: 5′-CCCCACGCTT-CAGAGCAAC (H2F) and 5′-GACGCGTGGCTGGAGGAG (H2R), whose 5′-ends correspond to nucleotide positions 71 and 499 in the H2b cDNA sequence, respectively. Primers used for the analogous rat subunits RHL2/3 were: 5′-GGCTCCAGCCTAGGGCCCATC (RHL2F) and 5′-GCCCCTTGGCTCAGGAAGAAC (RHL2R), whose 5′-ends correspond to nucleotide positions 20 and 497 in the RHL2/3 cDNA sequence, respectively. All oligonucleotides were custom made by Midland Certified Reagent Co.

Plasmid Constructs and Cell Lines—H2c cDNA, inserted into the pVos plasmid, was generously provided by Dr. Elizabeth Paietta (University of Basel, Switzerland). H1 cDNA was subcloned into pcDNA3.1(zeo) using HindIII and EcoRI sites. H2b and H2c cDNA sequence, respectively. Primers used for the analogous rat subunits RHL2/3 were: 5′-GGCTCCAGCCTAGGGCCCATC (RHL2F) and 5′-GCCCCTTGGCTCAGGAAGAAC (RHL2R), whose 5′-ends correspond to nucleotide positions 20 and 497 in the RHL2/3 cDNA sequence, respectively. All oligonucleotides were custom made by Midland Certified Reagent Co.

Antibodies—Polyclonal rabbit anti-H1 antibody (anti-H1-COOH) against the peptide corresponding to the COOH-terminal sequence CETELDKASQEPPLL was custom generated by Alpha Diagnostics, Inc. polyclonal rabbit anti-H2 antibody (anti-H2-COOH) against the peptide corresponding to the COOH-terminal sequence CEKRNRAT-CHCQGLDRKQPRNPRGNNFPLKG was made by Bethyl Laboratories, Inc. All antibodies used in this study were affinity-purified from the serum using Sepharose beads conjugated to the respective peptide used for immunization. The preimmune sera from the animals used to generate the three subunit-specific antibodies did not react in Western analyses with ASGP-Rs isolated from HepG2 cells. Anti-H1-, anti-H2-COOH, and anti-H2-Cyto19 specifically recognized the respective H1 or H2 subunits but did not cross-react with the other subunits, as determined by Western analyses using H1, H2b, and H2c proteins isolated from individually transfected COS-7 cells.

Total RNA Isolation—Total RNA was isolated from HepG2 human hepatoma cells or hepatocytes obtained from Sprague-Dawley rats (Charles River Laboratories) using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, 100 mg of HepG2 cells or rat hepatocytes were lysed in 1 ml of TRIzol reagent at 22 °C for 5 min. The rest of the procedure was performed on ice. Chloroform (200 μl) was added to the cell lysates, and the samples were mixed by vortexing. The samples were centrifuged at 12,000 × g for 15 min at 4 °C, and the aqueous phase was then transferred to an Eppendorf tube containing 0.5 ml of isopropyl alcohol. After a 10-min incubation, the precipitated RNA was pelleted by centrifugation at 12,000 × g for 10 min at 4 °C. The RNA pellet was resuspended and washed in 1 ml of 75% ethanol and then pelleted. The pellet was air-dried and dissolved in 300 μl of water.

RT-PCR—Partial cDNAs of H2 and RHL2/3 were synthesized from the corresponding total RNAs using the ThermoScript RT-PCR system (Invitrogen) according to the manufacturer’s instructions. The gene-specific primers H2R and RHL2R were used in a single round of reverse transcriptase reactions for the synthesis of H2 and RHL2/3 cDNAs, respectively. The cDNAs were then amplified by the PCR protocols using gene-specific primer pairs: H2F and H2R for H2 cDNA, or RHL2F and RHL2R for RHL2/3 cDNA. The PCR products were resolved in a 3.5% agarose gel and visualized by ethidium bromide staining.

Transient and Stable Transformations—All transfections were performed using a Calcium Phosphate Transfection Kit (Invitrogen) according to the manufacturer’s instructions. pcDNA3.1(+)/zeo plasmid containing H1 cDNA and 5 μg of pcDNA3.1(+)/zeo containing either H2b or H2c cDNAs were co-transfected into ~60–70% confluent COS-7 cells grown in 60-mm dishes. Transfected COS-7 cells were washed ~16 h later with PBS and cultured in complete medium for ~24 h before being harvested. The generation of SK-Hep-1 cell lines stably expressing ASOR-Rs containing H1 and H2b will be described elsewhere.2 For the generation of stable cell lines co-expressing H1 and H2c, pcDNA3.1(+)/zeo containing H2c cDNA was transfected into SK-Hep-1 cells that had been previously transfected with pIRES/neo plasmid containing H1 cDNA and selected with 400 μg/ml G418. Stable doubly transfected cell lines were selected, cloned, and maintained in complete medium supplemented with 400 μg/ml G418 (Meditech) and 290 μg/ml Zeocin (Invitrogen).

Purification of ASOR-Rs—Hetero-oligomeric ASOR-R complexes containing H1, H2b, and/or H2c from HepG2, HuH-7, Trf1, and transfected COS-7 cells were purified by ASOR-Sepharose affinity chromatography as described previously (21). Briefly, cells from 60-mm dishes were solubilized on ice in buffer 1 containing 1.5% Triton X-100 and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin-A). The cell lysates were cleared by centrifugation at 10,000 × g for 10 min, and the supernatant fluids were transferred to Eppendorf tubes containing an equal volume of 2 mg/ml BSA in buffer 1 and 20 μl of packed ASOR-Sepharose 4B. After incubation for at least 1 h at 4 °C, the ASOR-Sepharose beads were washed three times with 0.05% Triton X-100 in buffer 1, and the isolated ASOR-Rs were eluted with buffer containing 0.05% Triton X-100, 150 mM NaCl, 40 mM acetic acid, pH 5, and 10 mM EGTA. For immunoprecipitation of ASOR-Rs, cells were solubilized as described above and the supernatant from the cell lysate was transferred to an Eppendorf tube containing an equal volume of 2 mg/ml BSA, 10 mM EGTA in buffer 1, and 1 μg of affinity-purified anti-H1 or anti-H2 IgG. After 30 min on ice, 20 μl of packed protein A-Sepharose was added, followed by an additional 1-h incubation. The samples were then washed four times with 0.05% Triton X-100, 150 mM NaCl, 40 mM acetic acid, pH 5, and 10 mM EGTA. The proteins were resolved by SDS-PAGE in a 15% gel, followed by electro-transfer to nitrocellulose paper. H2 isoforms were detected by Western blotting using anti-H2-Cyto19 or anti-H2-COOH.

Palmitate Labeling—At 40 h post-transfection, COS-7 cells transfected with cDNAs encoding H2b or H2c were serum-starved for 1 h and then metabolically labeled with 400 Ci/ml [3H]palmitic acid at 37 °C for 4 h in serum-free DMEM containing 1 mg/ml delipidated BSA. Cells were washed with buffer 1, then solubilized, and the cell lysates were subjected to immunoprecipitation using anti-H2-COOH antibodies and protein A-Sepharose as described above. The eluted samples containing ASOR-Rs were used for the generation of the cross-reaction peptide pairs involving 4× Lasmilki buffer, and heated at 55 °C for 5 min, and the proteins were resolved by non-reducing SDS-PAGE, followed by fluorography of the dried gel as described previously (21).

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**Fig. 1.** Splice variants of H2. A, the diagram shows the three H2 splice variants, H2a, H2b, and H2c. The 57-nt insert is located in the H2 cytoplasmic domain, whereas the 15-nt insert is at the junction of the transmembrane domain (TMD) and the ectodomain. The locations of two primers, H2F and H2R, used in RT-PCR are indicated (small arrows) with their 5'-ends corresponding to nucleotide positions 71 and 499 in the H2b sequence. The regions are indicated for the two anti-H2 antibodies that recognize either the 19-aa peptide encoded by the 57-nt sequence (anti-H2-Cyto19) or the COOH-terminal peptide of H2 that is common to all H2 isoforms (anti-H2-COOH). Because the NH2-terminal Gly residue of the 19-aa insert is also present at the junction site in H2c, the actual novel peptide in H2b is 18-aa. B, the nucleotide sequence alignment of H2b (7, 16), RHL2/3 (9), and MHL2 (11) show the boundaries of the alternatively spliced 57-nt region in H2b and the consensus splice donor/acceptor sites (underlined). The amino acid translation is for the 19-aa insert in H2b.

**Equilibrium Binding and Scatchard Analyses—SK-Hep-1 cells stably transfected with cDNAs encoding H1 and either H2b or H2c were grown to confluency in 6-well plates and serum-starved for 30–60 min. Cells were then incubated on ice with 10 ng/ml 125I-ASOR (~330 cpm/fmol) and 0.055% (w/v) digitonin in Hanks’ balanced salt solution, in the presence of increasing amounts of unlabeled ASOR ranging from 0 to 5 μg/ml. After 1-h incubation, the binding medium was removed and washed. Cells were then washed three times with ice-cold Hanks’ balanced salt solution and then lysed with 0.3 M NaOH. The radioactivity in the lysed cells (bound 125I-ASOR) was measured using a Packard COBRAII gamma counter. Protein content in the cell lysates was determined by the method of Bradford (24) using BSA as the standard. The amount of radioactivity recovered from the pH 7.4 (HEPES was used as the buffer for pH below 6.3 and buffer 1 for pH above 6.3) was washed as set at 100%. The second washing was performed with 0.3 M NaOH. The radioactivity in the supernatant was then measured. The cells were washed three times with ice-cold Hanks’ balanced salt solution and then lysed with 0.3 M NaOH, and cell-associated radioactivity was then determined. Nonspecific uptake and degradation were determined by incubating cells with 125I-ASOR in the presence of a 10-fold excess of unlabeled ASOR.

**RESULTS**

**Detection of H2b and H2c Splice Variant Transcripts in Huh-7 Cells**—The cDNAs of three H2 splice variants, H2a, H2b, and H2c (Fig. 1A), have been isolated from human liver or HepG2 cells (7, 16). However, the expression of the H2c protein in human hepatocytes or hepatoma cell lines had not been reported. We, therefore, sought to confirm the natural expression of the H2c protein in hepatoma cells and assess the role of H2c in ASGP-R function.

First, we verified the existence of both H2b and H2c transcripts in another human hepatoma cell line Huh-7, which also expresses native ASGP-Rs (26). Partial cDNAs derived from H2 transcripts were generated from Huh-7 total RNA by reverse transcriptase reaction using gene-specific primer H2R. The H2 cDNAs were then amplified by PCR using primers H2F and H2R, flanking the 57-nt insert at nucleotide positions 71 and 499, respectively, in the H2b sequence (Fig. 1A). Because there is a difference of only 57 base pairs between H2b and H2c transcripts, the positions at which the above primers hybridize were designed to generate relatively small PCR fragments, thus allowing separation of the two predicted PCR products from the H2b (570-bp) and H2c (513-bp) transcripts. As shown in Fig. 2A, two PCR fragments were generated (indicated by arrows) with molecular sizes matching that of the predicted PCR products. The two DNA bands were then excised from the gel and sequenced, which verified that the two bands were indeed derived from H2b and H2c transcripts (not shown).
The two ASGP-R subunits are highly homologous among all mammalian species, which prompted us to examine if splicing events similar to those observed in H2 also occur in the rat homologue RHL2/3 (9). Partial RHL2/3 cDNAs were generated from rat hepatocyte total RNA using primer RHL2R. The cDNAs were then amplified by PCR using primer pairs RHL2F and RHL2R, which hybridize to the rat sequence at analogous positions to those of the H2 primers H2F and H2R. Unlike the alternatively spliced H2 transcripts, only a single RHL2/3 transcript was detected in the region flanking the analogous 57-nt insertion (Fig. 2B), indicating that no rat transcripts were present that lack this domain. Inspection of the sequences bordering the 57-nt region in cDNAs encoding RHL2/3 and the mouse homologue MHL2 (Fig. 1B) did not reveal the typical AG/CT 5′-consensus splice donor and acceptor sites (27) that are present at both the 5′- and 3′-ends of the H2b sequences bordering the 57-nt insertion (16). Therefore, alternative splicing of the 57-nt cytoplasmic insert may be unique to the human H2 species.

Deglycosylation of ASGP-Rs Reveals Two H2 Species in HepG2 Cells—The H2 open reading frame contains three consensus N-glycosylation sites (7), and the heterogeneity of the carbohydrate chains causes the fully glycosylated H2 proteins to migrate as a broad and diffuse band at ~50 kDa in SDS-PAGE (28). Adding to the complexity of this band pattern is the similarity of the predicted molecular masses of the H2b (34.6 kDa) and H2c (32.5 kDa) polypeptides. As a result, these two H2 species are not readily resolvable by electrophoresis and thus cannot be correctly identified. ASGP-Rs were isolated from HepG2 cells by ASOR-Sepharose affinity chromatography, and the H2 subunits were detected by Western analysis using anti-H2-COOH antibody. The native glycosylated form of H2 can only be detected as a broad and diffuse band, as expected (Fig. 3, lane 1). However, following deglycosylation with N-glycosidase F, the H2 proteins separated into two distinct bands, which migrated at positions similar to the predicted molecular weights of H2b and H2c (Fig. 3, lane 2). These two H2-specific bands also migrated at positions identical to those of the corresponding H2b and H2c proteins isolated from individually transfected COS-7 cells (Fig. 3, lanes 3 and 4).

Expression of H2b and H2c Proteins in Various Hepatoma Cell Lines—To determine if the two H2-specific bands detected in HepG2 cells are indeed derived from the H2b and H2c proteins, two different anti-H2 antibodies were used in a parallel Western analysis of deglycosylated ASGP-Rs. The anti-H2-Cyto19 IgG, which is directed against the 19-aa cytoplasmic insert, only recognizes H2b. On the other hand, the anti-H2-COOH IgG recognizes both H2b and H2c, because it is generated against a COOH-terminal peptide epitope that is common to all H2 isoforms (see Fig. 1). Western blotting with anti-H2-Cyto19 IgG detected only one H2-specific protein in ASGP-Rs isolated from HepG2, Trf1 and HuH-7 cells (Fig. 4, lanes 2–4, top row). However, in the parallel Western blot, two H2-specific bands were detected with anti-H2-COOH IgG (lanes 2–4, bottom row). These results indicate that both the upper (larger) and lower (smaller) H2-specific bands detected with anti-H2-COOH IgG contain the COOH-terminal peptide epitope of H2 as expected but that only the upper band contains the 19-aa cytoplasmic peptide found exclusively in H2b.

These data above strongly indicate that the upper and lower bands correspond to the deglycosylated H2b and H2c proteins, respectively. Because an antibody specific for H2c alone is not available, we cannot directly prove that the lower H2-specific band detected by the anti-H2-COOH antibody is the H2c protein. However, in the control experiment, both anti-H2 IgGs detected a single H2b band in COS-7 cells expressing the H2b protein (Fig. 4, lane 1), whereas in the parallel Western blot, H2c protein expressed by transfected COS-7 cells was only detected with the anti-H2-COOH IgG (Fig. 4, lane 5). Furthermore, the upper and lower H2-specific bands detected in various hepatoma cells (Fig. 4, lanes 2–4) migrated at positions corresponding identically to those of the deglycosylated H2b and H2c proteins isolated from individually transfected COS-7 cells (Fig. 4, lanes 1 and 5). We conclude from the above results that H2b and H2c proteins are naturally co-expressed in HepTr1, Trf1, and HuH-7 hepatoma cell lines and that both the H2b and H2c splice variants are present in the human ASGP-R preparations isolated by ligand affinity chromatography.

H2b and H2c Are Not Present with H1 in the Same ASGP-R Complexes—Because ASGP-R is a hetero-oligomeric complex...
composed of H1 and H2 subunits, we next examined whether H2b and H2c co-exist with H1 in the same ASGP-R complexes or whether they oligomerize with H1 in different ASGP-R complexes. ASGP-Rs from HepG2 cells were either immunoprecipitated using anti-H1-COOH (Fig. 5A, lane 1), anti-H2-COOH (lane 2), or anti-H2-Cyto19 (lane 3) IgG, or affinity-purified by ASOR-Sepharose chromatography (lane 4). The isolated ASGP-Rs were then deglycosylated, and the H2 proteins were detected by Western blotting using anti-H2-COOH antibody. Both H2b and H2c were co-purified by anti-H1- and anti-H2-COOH IgG (Fig. 5A, lanes 1 and 2), as well as ASOR-Sepharose (Fig. 5A, lane 4), indicating that both H2b and H2c were oligomerized with H1 in functional ASGP-R complexes. However, only H2b was immunoprecipitated with anti-H2-Cyto19 IgG (Fig. 5A, lane 3), indicating that H2b and H2c were not present in the same ASGP-R complexes. Similar results were obtained in experiments using ASGP-Rs isolated from HuH-7 cells (Fig. 5B). Western analysis using anti-H1-COOH antibody verified that H1 was also co-immunoprecipitated with both anti-H2-COOH and anti-H2-Cyto19 antibodies (not shown), indicating that H1 and H2 hetero-oligomeric complexes were intact following immunoprecipitation. Thus, we conclude that H2b and H2c oligomerize with H1 to form functional human ASGP-Rs, but that H2b and H2c are not present with H1 in the same ASGP-R hetero-oligomeric complexes.  

**Palmitoylation of H2b and H2c**—The presence of the 19-aa cytoplasmic insert is required for phosphorylation of Ser in H2b, although this insert itself does not contain any Ser residues (18). Because the location of this 19-aa cytoplasmic insertion is relatively close to the palmitoylated Cys residues in H2b, we examined the effect of deleting this region on the palmitoylation of H2. When COS-7 cells were transfected with either H2b or H2c cDNAs and metabolically labeled with [3H]palmitate, both the H2b and H2c splice variants were palmitoylated (Fig. 6, lanes 1 and 2). The H2 bands detected were specific, because mock transfection (i.e. vector alone) of COS-7 cells (lane 3) did not produce the same band. We conclude that the presence or absence of the 19-aa cytoplasmic insertion does not affect palmitoylation of H2.

**Ligand Binding Affinity of ASGP-R Complexes Containing Either H2b or H2c Isoforms**—High affinity ligand binding requires a hetero-oligomeric ASGP-R complex containing both H1 and H2 subunits (15). Although H2b and H2c are not present in the same hetero-oligomeric ASGP-R complex, our results indicate that they are both present in active ASGP-R complexes and able to bind ASOR-Sepharose. To measure the ligand binding affinity of ASGP-Rs containing either H1 and H2b, or H1 and H2c, equilibrium binding studies and Scatchard analyses were performed on two types of stable cell lines, generated in SK-Hep-1 cells, that co-express H1 with either H2b or H2c. These two stable cell lines showed a similar steady-state distribution of surface (i.e. ~one-fourth of total receptors) and intracellular ASGP-Rs as determined by ligand binding assays in the presence or absence of digitonin at 4 °C (not shown). As shown in Fig. 7 (insets), the saturation curves for ASOR binding were very similar between the two cell lines, with apparent K_d values for ASOR binding determined from Scatchard analyses for ASGP-Rs containing H1 and H2b, or H1 and H2c, were not substantially different at ~1.6 and 3.9 nM, respectively. We conclude that hetero-oligomeric ASGP-R complexes containing H1 and either H2b or H2c bind ASOR with similar affinities.

**Dissociation of 125I-ASOR-ASGP-R Complexes Containing H2b or H2c**—The rapid continuous uptake of ligand by a recycling receptor system requires an efficient process for dissociation of receptor-ligand complexes. We, therefore, examined the pH sensitivity of ASOR dissociation from the two different hetero-oligomeric ASGP-R complexes. Cells expressing H1-H2b or H1-H2c were allowed to internalize a pulse of 125I-ASOR, then permeabilized on ice and washed with buffers of different pH (Fig. 8). ASGP-R complexes containing either H2b or H2c showed essentially identical profiles for the dissociation of bound 125I-ASOR as the pH was decreased from 7.4 to 5.0.

**Continuous Uptake and Degradation of 125I-ASOR by Cells Expressing ASGP-Rs Containing H1-H2b or H1-H2c Complexes**—Cells expressing H1 and H2b internalized and degraded 125I-ASOR at a rate similar to cells expressing H1 and H2c (Fig. 9). These results indicate that the inclusion or deletion of the 19-aa cytoplasmic insert in H2 does not alter the rate at which the ASGP-R is able to endocytose ligand, or the rate of ligand delivery to lysosomes, at least in SK-Hep-1 cells. Specific uptake of ASOR was undetectable in mock transfected SK-Hep-1 cells or in cells transfected with H1 cDNA alone (not shown).

**DISCUSSION**

Alternative splicing is an effective mechanism for generating multiple protein isoforms with diverse functions from a single gene. In most cases, protein isoforms that arise from alternative splicing of a single gene transcript share extensive regions of identity, and vary only in one or more specific domains, thus allowing the fine tuning of specific protein functions (29, 30). Because the minor ASGP-R subunit exists as three isoforms, H2a, H2b, and H2c, our goal in this study was to determine whether these splice variants modulate the function of the human ASGP-R.
The cDNAs of the three H2 isoforms were previously isolated from human liver and HepG2 hepatoma cDNA libraries (7, 16). Using RT-PCR, we found that H2b and H2c transcripts were also produced by another hepatoma cell line HuH-7, whereas H2a transcripts were not detected (Fig. 2). Although the RT-PCR conditions may not have been sensitive enough to detect the less abundant H2a transcript, which accounts for only 7.7% of total H2 transcripts in HepG2 (31), it is also possible that the level of H2a transcripts is even lower in HuH-7 cells.

Due to the small size difference between H1 and H2, the two species are not readily resolved by SDS-PAGE, and earlier studies with the human ASGP-R (32) assumed that only one type of subunit was present, namely H1. Because the difference in size between H2b and H2c is even less, it is more difficult to separate and identify the two fully glycosylated H2 isoforms.

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However, the two H2 isoforms could be separated into two distinct bands by SDS-PAGE after they were de-N-glycosylated. Using the anti-H2-COOH antibodies, we confirmed the presence of both H2b and H2c proteins in active ASGP-Rs isolated from HepG2, Trf1, and HuH-7 cells. Furthermore, we detected roughly equal amounts of H2b and H2c transcripts in HuH-7 cells as well as similar staining intensities of the H2b and H2c proteins in Western analyses of several hepatoma cell lines. These results are in agreement with findings in HepG2 cells that the relative abundance of transcripts with or without the 57-nt region is 41 and 59%, respectively (16).

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Because human ASGP-Rs are hetero-oligomers composed of H1 and H2 subunits, it is intriguing, and possibly very important, that the H2b and H2c isoforms are not present with H1 in the same receptor complexes. Several studies have examined the subunit stoichiometry of the human ASGP-R.

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the intriguing possibility that each receptor type might have separately as active and functional receptors. These findings raise populations can be purified from human hepatoma cells and by the ASGP-R. In H2b may play a role in other aspects of endocytosis mediated diate ligand binding. Therefore, the unique cytoplasmic region ASOR complexes to dissociation at lower pH. We con- tion, the presence or absence of the 19-aa insert in the H2, which is consistent with observations from an on- sequence does not contain a recognition signal for palmitoyla- (Fig. 3). The above results indicate that the 19-aa cytoplasmic portion, however, both the 50- and 44-kDa H2 bands were reduced of H2, whereas the 44-kDa band is probably the immature high mannose form of H2, which is still in transit through the endoplasmic reticulum and Golgi, as described by Bischoff and Lodish (37). The 44-kDa bands may be more prominent in the transiently transfected COS-7 cells, because H2 is overex- pressed and, thus, the cellular glycosylation machinery may not have the capacity to process all the newly synthesized H2. A similar banding pattern is also observed in COS-7 cells transfected with cDNA encoding H1 (38). After N-deglycosyla- tion, however, both the 50- and 44-kDa H2 bands were reduced to single bands corresponding to the H2b and H2c polypeptides (Fig. 3). The above results indicate that the 19-aa cytoplasmic sequence does not contain a recognition signal for palmityla- tion of H2, which is consistent with observations from an on- going study4 that the majority of the cytoplasmic domain is not required for palmitoylation of the major subunit H1. In addi- tion, the presence or absence of the 19-aa insert in the H2 subunits of ASGP-R complexes did not significantly alter their binding affinity for ASOR or the sensitivity of these ASOR-ASGP-R complexes to dissociation at lower pH. We con- clude that the 19-aa cytoplasmic insert in the H2 subunit is not intrinsically involved in the overall ability of ASGP-Rs to me- diate ligand binding. Therefore, the unique cytoplasmic region in H2b may play a role in other aspects of endocytosis mediated by the ASGP-R.

The present results show that two different ASGP-R sub- populations can be purified from human hepatoma cells and that these two populations of ASGP-Rs can be expressed separately as active and functional receptors. These findings raise the intriguing possibility that each receptor type might have different functions or intracellular trafficking routes. Although it remains unknown whether these two human ASGP-R sub- populations, containing either the H2b or H2c isoform, differ in their subcellular localization in hepatocytes or hepatoma cells, it is interesting that ASGP-Rs mediate endocytosis in two distinct but parallel pathways, which were first discovered in 1983 (39) and later designated as the State 1 and the State 2 pathways (reviewed in Refs. 5, 40, and 41). The two pathways appear to be present in numerous cells types from a wide range of vertebrates and to be utilized by many endocytic recycling receptors, including the ASGP-R, transferrin, and mannose receptors and an aromatic amino acid transporter. Although roughly equal numbers of the total ASGP-R function in each pathway, the dissociation of internalized ligand is 20-fold faster in the State 2 pathway than the State 1 pathway (39); as a result the State 2 pathway accounts for the majority of ligand uptake. Receptors functioning in the State 2 pathway can also be distinguished from those in the State 1 pathway based on the sensitivity of cell surface receptor activity to modulation by a variety of agents, such as monensin, chloroquine, vanadate, microtubule drugs, and by lower temperature and ATP deple- tion (42–46). During the process of endocytosis, only the State 2 receptors undergo an inactivation/reactivation cycle (47, 48) that may be regulated by changes in the phosphorylation (49) and palmitoylation status (21, 48, 50) of these receptors. We have suggested that the less efficient State 1 uptake pathway may represent a residual, evolutionarily early process that developed before the appearance of large multicellular organisms (41).

Stockert et al. (22) independently validated the existence of this two-pathway endocytosis system for a variety of receptor types targeted to coated pits when they fortuitously isolated a trafficking mutant (designated Trf1) from HuH-7 cells. The Trf1 mutant is defective in endocytosis mediated by the State 2 pathway and its pleiotropic phenotype is exactly what was predicted for cells lacking a functional State 2 pathway (5, 40). The defect in the State 2 pathway in Trf1 cells did not affect H2 splicing or the assembly of ASGP-R complexes containing the H2b and H2c splice variants, because both isoforms were de- tected in purified receptor.

Casein kinase 2 (CK2) is a ubiquitously expressed eukaryotic Ser/Thr protein kinase that is involved in the phosphorylation of several growth factor receptors, transcription factors, and cytoskeletal proteins (51). An exciting discovery was recently made by Shi et al. (52), who found that both Trf1 and HuH-7 cells expressed similar amounts of two CK2 catalytic subunit isoforms, CK2α and CK2α′, but that the Trf1 mutant did not express a third and novel isoform, CK2α′. The lack of this latter isoform resulted in a 60% reduction of total CK2 expression and hypophosphorylation of ASGP-Rs in the Trf1 mutant. Transfection of a cDNA encoding the CK2α′ isoform into the Trf1 mutant restored these cells to the parental phenotype, in which ASGP-Rs were phosphorylated normally and both the State 1 and State 2 endocytic pathways were active (52). Hence the expression of the CK2α′ isoform is required to maintain a functional State 2 endocytic pathway in HuH-7 cells.

Because phosphorylation of H2 occurs only at Ser residues and requires the presence of the 19-aa cytoplasmic insert (18), this 19-aa region provides a possible structural explanation both for the existence of two ASGP-R sub-populations containing either H2b or H2c and the two functionally distinct sub- populations of ASGP-R in the two endocytic pathways. The two subunit isoforms enable the cell to create a subpopulation of ASGP-Rs that can be modulated by phosphorylation. Roughly half of the total ASGP-Rs operate in each of the State 1 and State 2 pathways, and this coincides with the similar levels of

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H2b and H2c protein expression. Therefore, we propose that the State 1 ASGP-Rs correspond to H1-H2c hetero-oligomeric complexes and the State 2 ASGP-Rs correspond to H1-H2b hetero-oligomeric complexes. In the case of the Trf1 mutant, the H1-H2b ASGP-R complexes that cannot be phosphorylated in the absence of CK2α behave like State 1 ASGP-Rs. Although we found here that the rates of ligand internalization and degradation were similar in SK-Hep-1 cells expressing ASGP-R complexes containing either H1-H2b or H1-H2c, this could be due to a low expression of the CK2α isoform. Using an antibody specific for CK2α (52), we did not detect CK2α in SK-Hep-1 cells, but it was present as expected in HepG2 cells (not shown). Apparently due to the lack, or low expression, of CK2α in SK-Hep-1 cells, the H1-H2b complexes may not be phosphorylated and targeted to the State 2 pathway in a normal or optimal manner, and thus the H1-H2b (State 2) complexes might function and traffic more like the H1-H2c complexes in the State 1 pathway.

Because the expression of CK2α results in the phosphorylation of the State 2 ASGP-Rs containing H2b, but not the ASGP-Rs containing H2c, the pS residue in H2b of State 2 ASGP-Rs may confer a new specific, as yet unknown, function or optimal manner, and thus the H1-H2b (State 2) complexes might function and traffic more like the H1-H2c complexes in the State 1 pathway.

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Jasper H. N. Yik, Amit Saxena and Paul H. Weigel

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