

The H1 and H2 Polypeptides Associate to Form the Asialoglycoprotein Receptor in Human Hepatoma Cells

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Abstract. Antibody-induced degradation and chemical cross-linking experiments have been carried out to assess the nature of the interaction between the two asialoglycoprotein-receptor polypeptides, H1 and H2, synthesized in HepG2 cells. Incubation of HepG2 cell monolayers with anti-H1 antibody caused a specific and equal loss of both H1 and H2 polypeptides. The same result was obtained with anti-H2 antibody. Control serum did not affect the level of H1 or H2 nor did anti-H1 or anti-H2 antibodies affect the level of the transferrin receptor. The chemical cross-linking reagent, difluorodinitrobenzene, has been used to demonstrate that H1 can be cross-linked to H2 in HepG2

cell microsomal membranes. Dimer and trimer species with apparent molecular masses of 93 and 148 kD, respectively, were readily observed upon chemical cross-linking and some dimers and trimers were immunoreactive with both anti-H1 and anti-H2 antibodies. The putative trimer, possibly two H1 and one H2 molecules, is a minimum estimate of the true size of the asialoglycoprotein receptor in intact HepG2 cell, and it is possible that larger hetero-oligomeric forms of the receptor exist. The results of both types of experiments indicate that H1 and H2 form an oligomeric complex in HepG2 cells and thus, both polypeptides constitute the human asialoglycoprotein receptor.

THE asialoglycoprotein receptor (ASGP-R)¹ is a liver-specific membrane glycoprotein that binds to terminal galactose and *N*-acetylgalactosamine residues on serum glycoproteins (for review, 2, 6). This receptor has been studied extensively in rabbit liver, rat liver, and human liver, as well as the human hepatoma cell line HepG2. In each of these species, the receptor activity appears to consist of multiple polypeptide chains. In rabbit liver, 40- and 48-kD proteins have been observed (12, 13). In rat liver, three polypeptide species of 41.5, 49, and 54 kD have been characterized (7, 22, 28). In human liver and in HepG2 cells (3, 23), a single polypeptide of 46 kD has been observed; more recently, a 50-kD protein has also been characterized (5). In all cases, the higher molecular mass species are less abundant than the lower molecular mass polypeptides. Protein sequencing (7) and cDNA cloning (11, 18, 26, 27) of the rat and human receptors has revealed that the polypeptides are distinct from each other. The human cDNAs and their protein products are referred to as H1 and H2 (26) while the rat hepatic lectin (RHL) cDNAs are referred to as RHL-1 and RHL-2/3 (7). RHL-1 is more homologous to H1 than to RHL-2/3, and RHL-2/3 is more homologous to H2 than to RHL-1 (18, 26). RHL-2/3 is so named because it consists of two polypeptide species of 49 and 54 kD, respectively; the increment in ap-

parent molecular mass has been attributed to differential oligosaccharide modifications (10).

The fact that the proteins are distinct molecules raised an important question about the structure and function of the ASGP-R. Do the two proteins represent subpopulations of ASGP-R(s) or do they associate to form a multicomponent receptor? Transfection of the two cDNAs into heterologous cell lines has been used to ascertain the role of the individual polypeptide species in cellular ASGP-R activity. McPhaul and Berg (17) have shown that expression of both rat ASGP-receptor cDNAs, RHL-1 and RHL-2/3, is required to enable rat hepatoma cells to accumulate fluorescein-labeled asialo-orosomuroid in the lysosomes. In this laboratory, Shia and Lodish (submitted for publication) have found that both of the human ASGP-R cDNAs, H1 and H2, are required to reconstitute ASGP-R binding activity at the cell surface of murine 3T3 cells.

In the present study, we used two different experimental protocols to show that H1 and H2 polypeptides are present in an oligomeric complex in the human hepatoma cell line HepG2. First, we examined the fate of H1 and H2 after incubation of HepG2 cell monolayers with anti-H1, anti-H2, or control serum. Many cell surface receptors and macromolecules have been shown to undergo "capping" and disappearance from the plasma membrane upon incubation of cells with specific antireceptor antibodies (1, 8, 19, 23, 29). This is thought to occur because the polyclonal antibodies cause extensive cross-linking of the receptors at the cell sur-

1. *Abbreviations used in this paper:* ASGP-R, asialoglycoprotein receptor; DFDNB, 1,5-difluoro-2,4 dinitrobenzene; DTSP, dithiobis (succinimidyl) propionate; RHL, rat hepatic lectin.

face and subsequent delivery of the complex to the lysosomes, or in some cases, a "prelysosomal compartment" (31) for degradation. Incubation of HepG2 cells with an antibody specific for H1 caused an equal loss of both H1 and H2 polypeptides, and conversely, incubation of HepG2 cells with an antibody specific for H2 caused an equal loss of both polypeptides.

The second approach was to determine whether the polypeptides could be specifically cross-linked to each other by homobifunctional cross-linking reagents. Since we are most interested in the structure of the receptor in the membrane and we know that the interaction between the two polypeptides is difficult to detect once the proteins are detergent-solubilized (5), the cross-linking experiments were carried out on microsomal membranes. After chemical cross-linking of microsomal membranes, solubilization with detergent, and immunoadsorption with anti-H1 or anti-H2 antibody, molecular mass species consistent with dimer and trimer forms of the receptor were observed. Using two different immunodetection protocols we have demonstrated that some of the higher molecular mass species formed by chemical cross-linking react with both anti-H1 and anti-H2 antibodies. Thus, although we do not know the stoichiometry, each species must contain both H1 and H2 polypeptides. Our results, together with the results of H1 and H2 cDNA transfection experiments (Shia and Lodish, submitted for publication), indicate that the human ASGP-R is an oligomeric complex made up of two distinct polypeptide species—H1 and H2.

Materials and Methods

Materials

Materials were obtained from the following sources: L-[³⁵S]cysteine (specific radioactivity, 983 Ci/mmol), New England Nuclear, Boston, MA; ¹²⁵I-labeled Protein-A (specific activity, >30 mCi/mg) and ¹⁴C-methylated protein standards, Amersham Corp., Arlington Heights, IL; keyhole limpet hemocyanin, Calbiochem Behring Corp., San Diego, CA; Protein-A-Sepharose Cl-4B, Sephadex G-25-150, oxidized glutathione, and aprotinin, Sigma Chemical Co., St. Louis, MO; sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate; dithiobis (succinimidyl) propionate (DTSP); 1,5-difluoro-2,4-dinitrobenzene (DFDNB); and BCA* protein assay reagent, Pierce Chemical Co., Rockford, IL; nitrocellulose filters, Schleicher & Schuell Inc., Keene, NH; goat anti-rabbit IgG, Boehringer Mannheim Diagnostics, Houston, TX; 2,5 diphenyloxazole, National Diagnostics Inc., Somerville, NJ; fetal calf serum, dialyzed fetal calf serum, Eagle's minimal essential medium, Dulbecco's modified Eagle's medium, glutamine (100X), penicillin/streptomycin (100X), and Freund's complete and incomplete adjuvant, Gibco Laboratories, Grand Island, NY. Anti-human transferrin receptor antibody was kindly provided by Dr. Caroline Enns, Syracuse University, Syracuse, NY. All other chemicals were reagent grade.

Preparation of Antipeptide Antibodies

15- and 12-amino acid peptides corresponding to the carboxy termini of H1 and H2, respectively, were synthesized by Dr. Peter Kim, The Whitehead Institute, Cambridge, MA by solid-phase synthesis (4) on an automated 430A peptide synthesizer from Applied Biosystems, Inc., Foster City, CA. In addition, a 10-amino acid peptide corresponding to the unique cytoplasmic domain of H2 was synthesized. The peptides were coupled to the carrier protein, keyhole limpet hemocyanin, using the chemical cross-linking reagent sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate following the procedure of Green et al. (9). Rabbits were immunized with the peptide conjugates in Freund's complete adjuvant and boosted every 2–3 wk for up to 3 mo. The specificity of the antisera was tested by their ability to immunoadsorb [³⁵S]methionine-labeled translation products of H1 and H2 as previously described (5). The anti-H1-COOH serum is specific for

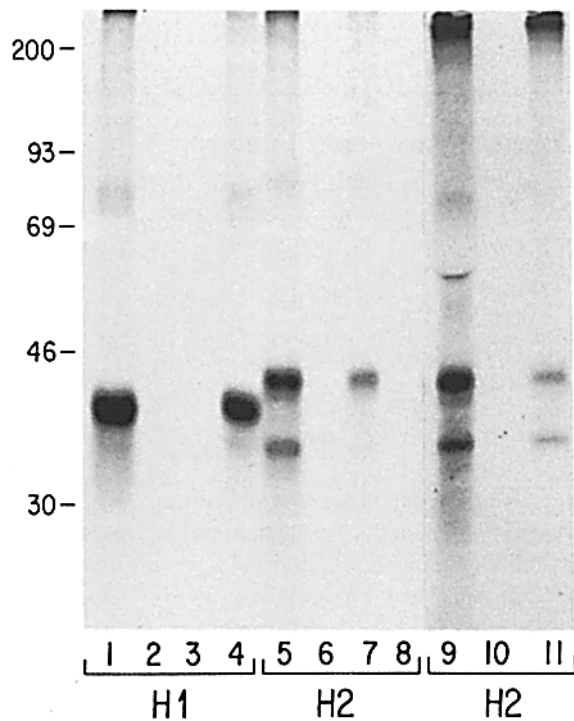


Figure 1. Specificity of anti-H1 and anti-H2 antibodies. Lanes 1–4 and 10 contained H1 translation products and lanes 5–8, 9, and 11 contained H2 translation products. Aliquots from the total translation reactions (lanes 1, 5, and 9) and immunoadsorbed translation products (lanes 2 and 6, control rabbit serum; lanes 3 and 7, anti-H2 cytoplasmic insert serum; lanes 4 and 8, anti-H1-COOH; and lanes 10 and 11, anti-H2-COOH) were subjected to SDS-PAGE. For immunoadsorption, the translation products were incubated in 50 μ l of immunoadsorption buffer with 2 μ l of the designated antiserum for 2 h on ice. The Protein A-Sepharose pellets were washed twice with immunoadsorption buffer and once with 10 mM Tris, pH 8.0, 0.2% Nonidet P-40. Lanes 5–8 and lanes 9 and 11 represent separate H2 translation reactions. The migration of molecular mass standards (kD) is indicated on the left.

H1 alone and the anti-H2-COOH and anti-H2 cytoplasmic insert sera are specific for H2 (Fig. 1). The anti-H1-COOH and the anti-H2 cytoplasmic insert antibodies were used for all immunoadsorption reactions presented in this paper.

Cell Culture

The growth of the human hepatoma cell line HepG2 in Eagle's minimal essential medium, 10% fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin was carried out as described (14). The cell monolayers were trypsinized and split 1/10 into 60-mm petri dishes 4–5 d before each experiment.

Metabolic Labeling of HepG2 Cells

Subconfluent (75–90%) monolayers were rinsed twice with DME devoid of cysteine but supplemented with 10% dialyzed fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (cys-free DME). The cells were preincubated in 2 ml of cys-free DME for 20 min at 37°C, rinsed again with cys-free DME, and then labeled overnight with [³⁵S]cysteine (200–300 μ Ci/ml) in 1.0 ml of cys-free DME supplemented with 5 μ M nonradioactive cysteine.

Immunoadsorption of H1 and H2

All steps were carried out at 0–4°C unless otherwise noted. Total microsomal membrane proteins were solubilized and subjected to immunoadsorption with anti-H1 or anti-H2 serum as previously described

(5). The antigen-antibody complexes were eluted from the Protein-A-Sepharose, denatured by boiling for 5 min in 60 μ l of 125 mM Tris pH 6.8, 2% SDS, 20% glycerol, 2% 2-mercaptoethanol, and 0.002% bromophenol blue (SDS-gel sample buffer), and subjected to SDS-10%-PAGE (15). Fluorography using 20%, 2,5 diphenyloxazole in acetic acid (wt/wt) was carried out as described (25). The dried gels were exposed to pre-flashed Kodak XAR or SB-5 film at -70°C .

Incubation of HepG2 Cells with Anti-H1 and Anti-H2 Antibodies

The cell monolayers were washed once with 2 ml PBS, 5-mM EDTA for <1 min at 4°C and then two times with 3 ml of MEM medium (without serum), 2 mM glutamine, and 20 mM Hepes, pH 7.4 (MEM/Hepes). The cells were then incubated with 0.2 ml of control serum, anti-H1-COOH serum, or anti-H2-COOH serum in a total volume of 0.8 ml of MEM/Hepes for 90 min at 37°C . The monolayers were rinsed again three times with MEM/Hepes and then further incubated in the presence or absence of 35 μ g of affinity-purified goat anti-rabbit IgG in a total volume of 0.75 ml at 37°C for various lengths of time. At the end of the incubation, the cell monolayers were rinsed three times with ice-cold PBS and the solubilized microsomal membranes were prepared as described (5). To denature any IgG molecules that might have been present, the samples were adjusted to 1% SDS and 2 mM dithiothreitol and boiled for 5 min. The samples were diluted approximately sixfold into PBS, 1% Triton X-100, 0.5% sodium deoxycholate, 2.5 mg/ml bovine serum albumin (immunoabsorption buffer), and adjusted to 2 mM oxidized glutathione. The diluted samples were incubated with 75 μ l of a 1:1 suspension of Protein-A-Sepharose in PBS, 0.02% sodium azide for 1 h at 4°C with constant rotation. The Protein-A-Sepharose was sedimented by brief centrifugation. The supernatant was divided into three equal parts (0.45 ml) and subjected to immunoabsorption with 10 μ l of control serum, anti-H1-COOH serum, or anti-H2 serum at $0-4^{\circ}\text{C}$ for 14 h. The aliquot taken was adjusted so that equal protein (~ 85 μ g) was subjected to immunoabsorption for each experimental condition; this corrects for slight differences in recovery of cell protein from each petri dish. The antigen-antibody complexes, adsorbed to 25 μ l of packed Protein-A-Sepharose, were washed two times with 1 ml of immunoabsorption buffer and two times with 1 ml of 10 mM Tris, pH 8.0, 0.2% Nonidet-40 and then subjected to SDS-PAGE and fluorography.

Chemical Cross-linking

Solutions (100X) of DFDNB and DTSP in absolute ethanol were made fresh for each experiment. Total microsomal membranes were isolated as described (5) and resuspended in 0.1 M NaPO_4 pH 7.0. The cross-linking reactions were carried out for 1 h at room temperature and then quenched by adjusting the samples to 2 mM glycine. Membrane proteins were solubilized by addition of Triton X-100 and sodium deoxycholate to 1% and 0.5%, respectively, and incubation of ice for 1 h. The samples were centrifuged at 12,000 g for 20 min, and the supernatants subjected to immunoabsorption as described above.

Immunoblot with Anti-H1 Antibody

The samples were subjected to electrophoresis on a 5-10% polyacrylamide gradient SDS-gel and then transferred to a nitrocellulose membrane. The membrane was incubated with a 1:100 dilution of anti-H1 antibody, and then with ^{125}I -labeled Protein A following an established protocol (20). ^{125}I -Protein A-labeled bands were visualized by autoradiography at -70°C .

Two-Dimensional-SDS-PAGE (+/- 2-Mercaptoethanol)

Immunoabsorbed proteins were loaded on a 7.5% polyacrylamide tube gel (0.3 \times 15 cm) and subjected to electrophoresis as described (15). The tube gel was then equilibrated in 10 ml of SDS-gel sample buffer including 2% 2-mercaptoethanol. The tube was layered on the top of a standard 10%-polyacrylamide SDS gel, secured with agarose, and subjected to electrophoresis as described (15).

Protein Determination

The Pierce BCA* protein assay reagent was used to determine protein concentrations following the manufacturer's protocol. BSA was used as a standard.

Densitometry

Fluorograms were scanned (three times per lane) using an LKB 2202 Ultrascan laser densitometer with an LKB recording integrator (LKP Producter, Bromma, Sweden).

Results

Specificity of Anti-H1 and Anti-H2 Antibodies

The human ASGP-R polypeptides, H1 and H2, are 58% homologous at the amino acid sequence level (26) and have many structural features in common. Both polypeptides have an amino-terminal cytoplasmic domain, a single membrane-spanning domain, and a large exoplasmic carboxy-terminal domain. The unique features of H2 are an apparent insertion of 18 amino acids relative to H1 in its NH_2 -terminal cytoplasmic domain and a third asparagine-linked oligosaccharide near the COOH terminus. The proteins also differ in amino acid sequences at their extreme COOH termini. The last 13 amino acids of H1 and 10 amino acids of H2 are distinct (H1 is three amino acids "longer" than H2). We generated antipeptide antibodies directed against the COOH-terminal domains of each polypeptide, as well as the unique cytoplasmic domain of H2. The specificity of these antisera was examined by testing their ability to immunoabsorb [^{35}S]methionine-labeled translation products of H1 and H2 synthesized in the presence of dog pancreas microsomes (Fig. 1). The anti-H1 antiserum will recognize only H1 polypeptide (lane 4), and not H2 (lane 8), while the anti-H2 antisera will recognize only H2 polypeptide (lanes 7 and 11), and not H1 (lanes 3 and 10). The polypeptides were not adsorbed by control rabbit serum (lanes 2 and 6). Since the COOH-terminal domains of H1 and H2 are exoplasmic, anti-COOH antibodies are able to bind to receptors on the plasma membrane and thus, they provide useful tools for studying the interaction of H1 and H2 in intact HepG2 cells.

Antibody-induced Degradation

The fate of H1 and H2 polypeptides was examined after incubation of HepG2 cells with anti-H1-COOH or anti-H2-COOH serum and then further incubation with goat anti-rabbit IgG (Fig. 2). HepG2 monolayers, metabolically labeled with [^{35}S]cysteine for 21 h, were incubated for 90 min at 37°C in the presence of control serum (A and B, lanes 1, 4, and 7), anti-H1-COOH serum (A and B, lanes 2, 5, and 8), anti-H2-COOH serum (A and B, lanes 3, 6, and 9), buffer alone (A and B, lane 10), or anti-H1 and anti-H2 COOH together (A and B, lane 11). The unbound IgGs and serum proteins were rinsed off and the cell monolayers were further incubated with goat anti-rabbit IgG at 37°C for 0 h (A and B, lanes 1-3), 1 h (A and B, lanes 4-6), or 2 h (A and B, lanes 7-11). Solubilized membrane proteins were isolated and denatured by boiling in SDS and dithiothreitol and subjected to immunoabsorption with anti-H1-COOH (A), anti-H2 (B), or control serum (data not shown). The denaturation step was sufficient to inactivate any anti-H1 or anti-H2 molecules in the membrane extract because no H1 or H2 was observed upon subsequent immunoabsorption with control serum.

In this experiment, anti-H1 antibody caused a 75-80% decrease in immunoprecipitable H1 and H2 after a 1-h incuba-

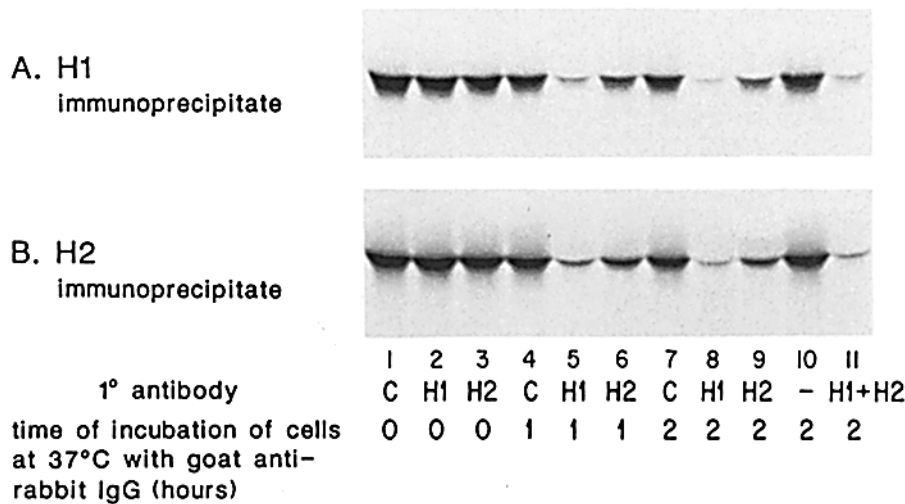


Figure 2. Antibody-induced degradation of H1 and H2 polypeptides. HepG2 cell monolayers were incubated with 200 μ l of control (lanes 1, 4, and 7), anti-H1 (lanes 2, 5, 8, and 11), or anti-H2 (lanes 3, 6, 9, and 11) serum or with buffer alone (lane 10) in a final volume of 0.8 ml for 90 min at 37°C. The cells were further incubated with 35 μ g of goat anti-rabbit IgG antibody for 0 (lanes 1-3), 1 (lanes 4-6), or 2 (lanes 7-11) h. Cells were lysed and prepared for immunoadsorption with anti-H1 (A) or anti-H2 (B) antibody as described in Materials and Methods. The fluorograph shown in A was exposed for 22 h whereas the fluorograph in B was exposed for 92 h.

tion with goat anti-rabbit IgG (lane 5). 90% of each polypeptide had disappeared by 2 h (lane 8). Anti-H2 antibody caused a 40% loss of H1 and H2 polypeptides by 1 h (lane 6) and a 70% loss by 2 h (lane 9). Loss of H1 and H2 polypeptides after incubation with both anti-H1 and anti-H2 serum (A and B, lane 11) was equivalent to that observed with anti-H1 serum alone (A and B, lane 8). Goat anti-rabbit IgG alone had no effect on H1 and H2 (A and B, lane 10) but it was required to obtain the antibody-induced degradation seen here (data not shown). This is probably due to the fact that the antipeptide antibodies are directed against single epitopes on the proteins and therefore may not cross-link sufficiently. Similarly, some monoclonal antibodies do not cause the extensive cross-linking necessary to bring about degradation (1) and second antibodies have been used to achieve this effect (19). Anti-H1-COOH, anti-H2-COOH, and control serum did not affect the level of biosynthetically labeled transferrin receptor in these HepG2 cells (data not shown) indicating that the antibody-induced degradation of H1 and H2 was specific.

Table I summarizes the results of four separate experiments carried out as described in Fig. 2. It is clear that the effect of anti-H1-COOH on the level of H1 and H2 polypep-

ptides is nearly identical and conversely, the effect of anti-H2-COOH on H1 and H2 is very similar. The extent of degradation due to anti-H2-COOH antibody was not as great as with anti-H1-COOH, although it was identical for both H1 and H2 polypeptides. Increasing the amount of anti-H2-COOH antibody did not result in greater degradation (data not shown).

Although we attribute the loss of H1 and H2 polypeptides to extensive cross-linking by the antibodies and subsequent delivery to the lysosomes, which is usually the case (1), we have not rigorously shown that the loss of antigens is due to degradation in the lysosomes. Gartung et al. (8) found that anti-mannose-6-phosphate receptor antibodies caused the 215-kD receptor to become detergent insoluble and thus, the degradation was only apparent. Furthermore, Schwartz et al. (21) reported that antibody-induced degradation of the ASGP-R occurred in a "prelysosomal compartment." They found, however, that the polyclonal antibody raised against the purified human liver ASGP-R caused a net loss of antigen from HepG2 cells and very little receptor had become detergent insoluble. The important point of the results presented in Fig. 2 and Table I is that both antibodies had the same effect on H1 and H2 polypeptides indicating that the two proteins are associated in a complex at the plasma membrane.

Table I. Effect of Anti-H1-COOH, Anti-H2-COOH, and Control Serum on HepG2 Cell H1 and H2

Exp. no.	Time	Anti-H1		Anti-H2		Control		Goat anti-rabbit IgG alone	
		H1	H2	H1	H2	H1	H2	H1	H2
	<i>min</i>	%							
1.	0	100	100	100	100	100	100	—	—
	90	26	30	52	51	120	122	90	107
2.	0	100	100	100	100	100	100	—	—
	60	26	18	60	49	105	79	—	—
	120	10	10	43	30	88	73	98	83
3.	0	100	100	100	100	100	100	—	—
	45	67	53	64	62	107	110	—	—
	90	30	49	—	55	102	89	—	—
	150	16	13	54	47	100	62	80	62

After incubation with anti-H1, anti-H2, or control serum, cells were incubated with 30-35 μ g of goat anti-rabbit IgG at 37°C. The amounts of [³⁵S]cysteine-labeled H1 and H2 polypeptides were determined by densitometric scanning of each fluorograph. Experiment 2 is the average of two separate experiments. —, not determined.

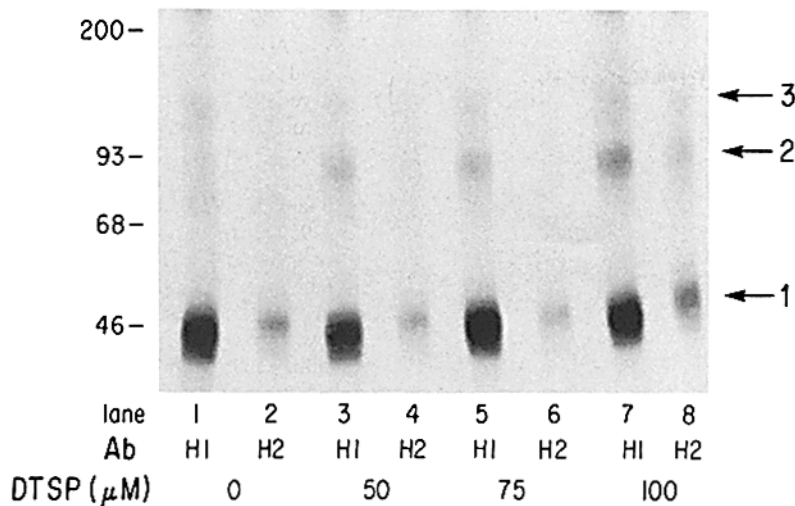


Figure 3. Cross-linking of microsomal membrane proteins with DTSP and immunoadsorption of H1 and H2. Microsomal membranes were isolated from HepG2 cells labeled with [35 S]cysteine for 20 h, and cross-linked with 0 (lanes 1 and 2), 0.05 mM (lanes 3 and 4), 0.075 mM (lanes 5 and 6), and 0.10 mM (lanes 7 and 8) DTSP as described in Materials and Methods. Solubilized membrane proteins were subjected to immunoadsorption with anti-H1 (lanes 1, 3, 5, and 7) or anti-H2 (lanes 2, 4, 6, and 8) serum and then SDS-PAGE and fluorography.

Chemical Cross-linking

The second experimental approach we employed to study the nature of the interaction(s) between H1 and H2 was chemical cross-linking. Two types of homobifunctional reagents were used, DTSP and DFDNB, to determine if H1 and H2 are non-covalently associated in microsomal membranes and thus can be chemically cross-linked to each other. In the study in Fig. 3, microsomes were treated for 1 h at room temperature with 0 (lanes 1 and 2), 0.05 mM (lanes 3 and 4), 0.075 mM (lanes 5 and 6), and 0.10 mM (lanes 7 and 8) DTSP; subsequently they were solubilized with detergent and immunoadsorbed with anti-H1 serum (lanes 1, 3, 5, and 7), anti-H2 serum (lanes 2, 4, 6, and 8), or control serum (data not shown). Higher molecular mass species of ~93 and 148 kD were detected with anti-H1 or -H2 serum but not with control serum. These 93- and 148-kD species were not detected in the non-cross-linked material immunoadsorbed with anti-H1 (lane 1) or anti-H2 (lane 2). Since the monomeric molecular masses of H1 and H2 are 46 and 50 kD, respectively, these higher molecular mass species probably represent dimeric and trimeric forms of the ASGP-R polypeptide chains.

The reagent DTSP contains a disulfide bond between the *n*-hydroxysuccinimide reactive groups so that the cross-linked proteins can be reduced with 2-mercaptoethanol to regenerate the monomeric forms. In Fig. 4, a sample equivalent to that shown in Fig. 3, lane 8 was subjected to two dimensional SDS-PAGE. The first dimension (see arrow at top) was a tube SDS-gel run in the absence of 2-mercaptoethanol. The tube gel was then equilibrated in SDS-sample buffer containing 2% 2-mercaptoethanol and layered on top of a slab gel and subjected to SDS-PAGE. The dimer and trimer species formed by DTSP comigrated with the monomer H2 after reduction with 2-mercaptoethanol. A similar result was obtained with dimers and trimers immunoadsorbed with anti-H1 antibody (data not shown).

The efficiency of chemical cross-linking with DTSP (Fig. 3) was too low to determine the composition of the dimer and trimer species. To overcome this problem, we tried a more efficient, but noncleavable cross-linking reagent—DFDNB. In Fig. 5, microsomal membranes were isolated from HepG2

cells labeled with [35 S]cysteine for 20 h and subjected to chemical cross-linking with DFDNB (lanes 3–11) or to mock cross-linking (lanes 1 and 2). The membrane proteins were solubilized and subjected to immunoadsorption with anti-H1 (lanes 1, 3–6), anti-H2 (lanes 2, 7–10) or control serum (lane 11). Antibody complexes were eluted from the Protein-A Sepharose by heating for 5 min at 100°C in 50 μ l of PBS, 1% SDS, and 2 mM dithiothreitol. For lanes 1–3, 7, and 11,

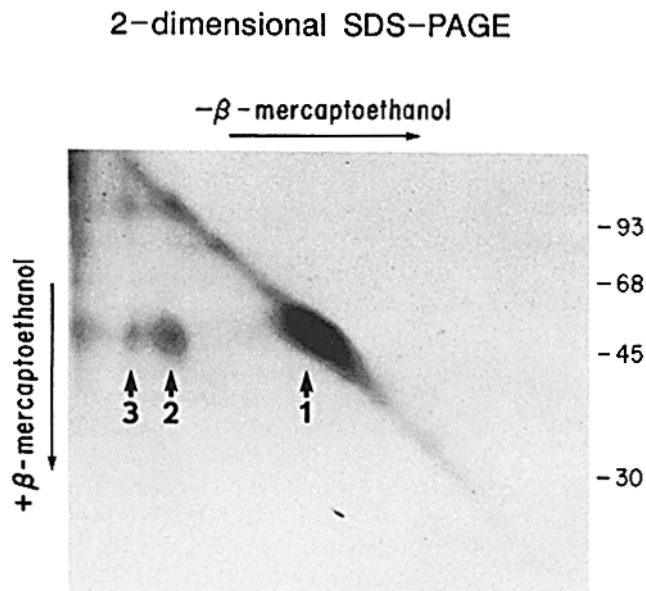


Figure 4. Two-dimensional SDS-PAGE of DTSP-cross-linked proteins that were immunoadsorbed with anti-H2 antibody. HepG2 cells were labeled for 20 h with [35 S]cysteine and the microsomal membranes were cross-linked with 0.1 mM DTSP and subjected to immunoadsorption with anti-H2 antibody. The antigen-antibody complexes were subjected to electrophoresis (arrow at top indicates direction of migration) on a tube gel under nonreducing conditions. The tube gel was then subjected to electrophoresis (arrow on the vertical axis indicates direction) on an SDS-slab gel under reducing conditions. The numbered arrows, 1, 2, and 3, indicate monomer, dimer, and trimer species, respectively.

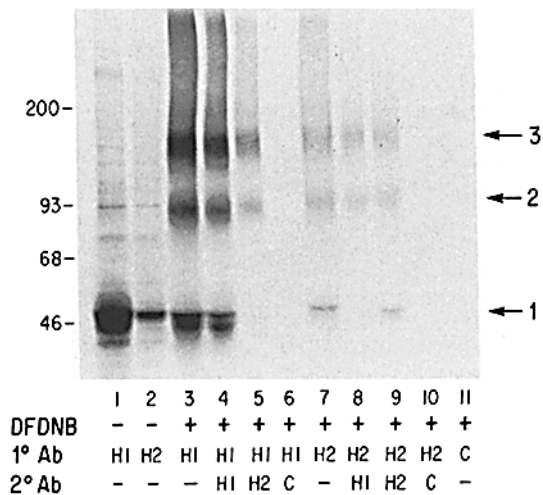


Figure 5. Individual dimer and trimer species formed by DFDNB cross-linking are recognized by both anti-H1 and anti-H2 antibodies. HepG2 cells were labeled for 20 h with [³⁵S]cysteine and microsomal membranes were treated without (lanes 1 and 2) or with (lanes 3-11) 1 mM DFDNB. The solubilized membrane proteins were immunoadsorbed with anti-H1 (lane 1, 3-6), anti-H2 (lane 2, 7-10) or control serum (lane 11). A portion of these immunoadsorbed proteins was analyzed directly on SDS-PAGE (lanes 1-3, 7, and 11). Denatured antigen-antibody eluates were re-adsorbed with anti-H1 (lane 4 and 8), anti-H2 (lane 5 and 9) or control (lane 6 and 10) serum. The immunoadsorbed proteins were subjected to SDS-PAGE on a 5-10% polyacrylamide gradient gel and then fluorography. The numbered arrows, 1, 2, and 3, indicate monomer, dimer, and trimer species, respectively.

the eluted samples were prepared for SDS-PAGE. Treatment of microsomal membranes with 1 mM DFDNB resulted in more efficient formation of dimer and trimer species that were recognized by anti-H1 (lane 3) and anti-H2 (lane 7) antibodies but not by control serum (lane 11).

In order to elucidate the composition of the 93- and 148-kD species observed after DFDNB cross-linking, individual dimer and trimer species were tested for immunoreactivity with both anti-H1 and anti-H2 antibodies (Fig. 5). The eluted and denatured samples that had first been adsorbed with anti-H1 (lanes 4-6) or anti-H2 (lanes 8-10) serum were diluted with 0.35 ml of immunoadsorption buffer, adjusted to 2 mM oxidized glutathione, and immunoadsorbed with anti-H1 (lanes 4 and 8), anti-H2 (lanes 5 and 9) or control serum (lanes 6 and 10). The 93- and 148-kD cross-linked species that were first immunoadsorbed with anti-H1 antibody were subsequently recognized by anti-H2 antibody (Fig. 5, lane 5) as well as by anti-H1 (Fig. 5, lane 4). Conversely, the 93- and 148-kD species that were first immunoadsorbed with anti-H2 antibody were subsequently recognized by anti-H1 antibody (Fig. 5, lane 8) as well as by anti-H2 (Fig. 5, lane 9). Importantly, the monomeric forms of H1 and H2 adsorbed initially by anti-H1 or anti-H2, respectively, were not recognized by the opposite antibody (lanes 5 and 8). Re-adsorption with control serum (lanes 6 and 10) shows that all of the antibody molecules used in the first immunoadsorption step were denatured by boiling in SDS and dithiothreitol. These results provide further evi-

Immunoblot with anti-H1 antibody of immunoadsorbed proteins

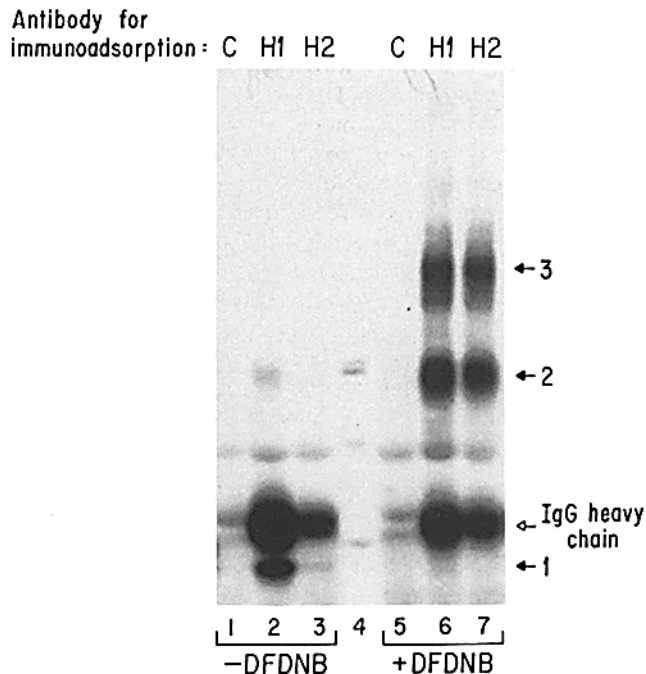


Figure 6. Immunoblot with anti-H1 antibody. HepG2 cell microsomal membrane proteins were treated without (lanes 1-3) or with (lanes 5-7) 1 mM DFDNB. Solubilized membrane proteins were immunoadsorbed with control (lanes 1 and 5), anti-H1 (lanes 2 and 6), or anti-H2 (lanes 3 and 7) and subjected to SDS-PAGE. The SDS-gel was blotted onto nitrocellulose and treated with anti-H1 antibody as described in Materials and Methods. ¹⁴C-labeled protein standards were run in lane 4. The solid arrows marked 1, 2, and 3, indicate monomer, dimer, and trimer species, respectively. The open arrow indicates the position of the 53-kD rabbit IgG heavy chain.

dence that H1 and H2 are associated in HepG2 membranes in a hetero-oligomeric complex.

To corroborate the results presented in Fig. 5, we used a different immunodetection method to demonstrate that at least some dimer and trimer species formed after chemical cross-linking react with both anti-H1 and anti-H2 antibodies. Microsomal membranes were prepared from confluent monolayers of HepG2 cells and subjected to chemical cross-linking with DFDNB (Fig. 6, lanes 5-7) or mock cross-linking (Fig. 6, lanes 1-3). The solubilized proteins were subjected to immunoadsorption with control serum (lanes 1 and 5), anti-H1 (lanes 2 and 6), or anti-H2 (lanes 3 and 7) and then SDS-PAGE. The proteins fractionated by SDS-PAGE were transferred electrophoretically to a nitrocellulose membrane and then treated with anti-H1 antibody as described in Materials and Methods. In lane 2, the 46-kD H1 monomer reacted with the anti-H1 antibody by the immunoblot procedure as expected. A trace amount of dimer (93 kD) was also observed in lane 2. The 53-kD heavy chain of rabbit immunoglobulin reacted with the ¹²⁵I-Protein-A and thus, was seen in varying amounts in every lane of the gel, as was one other nonspecific band. Lanes 5-7 are the DFDNB cross-

linked samples. The anti-H1 immunoadsorbed material (lane 6) contains 93- and 148-kD species that react with anti-H1 antibody by immunoblotting. Therefore, each species must contain at least one H1 polypeptide chain. The anti-H2 immunoadsorbed material (lane 7) also contains 93- and 148-kD species that react with the anti-H1 antibody and therefore, must contain both H1 and H2 polypeptides. Thus, the H1 and H2 ASGP-R proteins can be chemically cross-linked to each other in microsomal membranes. The converse experiment, immunoblotting with anti-H2 antibody, could not be done because we do not have an anti-H2 antibody that reacts with SDS-denatured H2 on an immunoblot.

Minor bands were detected by the immunoadsorption/immunoblot procedure that migrated as very high molecular species of 178, 213, and 280 kD (Fig. 6, lanes 6 and 7). These could represent tetra-, penta-, and hexameric forms of the ASGP-R polypeptides, but we cannot rule out the possibility that they contain unrelated proteins cross-linked nonspecifically to H1 and H2 polypeptides.

Discussion

Antibody-induced degradation and chemical cross-linking experiments demonstrate that the H1 and H2 ASGP-R polypeptides must be associated in a hetero-oligomeric complex and therefore both polypeptides constitute the ASGP-R in HepG2 cells. This result is in agreement with the cDNA transfection studies of McPhaul and Berg (17) on the rat ASGP-R polypeptides and with similar results from this laboratory on the human ASGP-R polypeptides (Shia, M. A., and H. F. Lodish, manuscript submitted for publication).

First, a highly specific antiserum that recognizes the carboxyl terminus of H1 caused a rapid and equal loss of cellular H1 and H2 polypeptides. Similarly, an antiserum specific for the carboxyl terminus of H2 caused an equal loss of H1 and H2. Thus, over the time course of the experiment, most of the cell-associated H1 must be in a complex with H2, and vice-versa. Antibody-induced degradation, or in some cases, detergent insolubility has been observed for a number of cell surface receptors and macromolecules including the low-density lipoprotein receptor (1), the 215-kD mannose-6-phosphate receptor (8), and the ASGP-R (21). Schwartz et al. (21) have shown that anti-ASGP-R antibody added to intact HepG2 cells caused a specific and rapid degradation of immunoreactive 46-kD ASGP-R. The polyclonal anti-ASGP-R antibody they used was raised against affinity-purified human liver ASGP-R but appears to recognize only the 46-kD H1 polypeptide. Thus, these investigators were analyzing only one component of the ASGP-R. For each of these receptors, and in this study, the effect of the antireceptor antibodies was shown to be specific because the antibodies did not cause degradation of other, nonrelated cell surface molecules nor did antibodies to unrelated cell surface molecules affect the receptor in question.

In an experiment similar to that presented in Fig. 2 and Table I, O'Neill et al. (19) have reported recently that two distinct cell surface molecules, the T-cell receptor and the L3T4 molecule, form the binding site on C6VL/1 cells for its cognate retrovirus by demonstrating that incubation of C6VL/1 cells with either anti-T-cell receptor antibody or anti-L3T4 antibody causes a specific loss of retrovirus binding sites. Hence, the composition of multicomponent cell surface re-

ceptors can be elucidated, in part, by studying the effect of antibodies specific for one component on the cellular fate of the other polypeptide components.

The results of our chemical cross-linking experiments support the notion that the human ASGP-R is a hetero-oligomer because membrane-associated H1 and H2 can be chemically cross-linked to each other to form a trimer. Dimer and trimer, but not monomer species that were initially immunoadsorbed with anti-H1 serum, subsequently reacted with an anti-H2 antibody; similarly, dimers and trimers that were initially immunoreactive with anti-H2 serum, subsequently reacted with anti-H1 antibody (Figs. 5 and 6). Although we have not determined the exact composition, we postulate that some of the trimers contain two H1 molecules and one H2 molecule because H1 appears to be more abundant than H2 in HepG2 cells. It is possible, however, that some cross-linked H1 homodimers and H1 homotrimers exist. The trimer is a minimum estimate of the true size of the ASGP-R in an intact HepG2 cell. Minor bands of 178, 213, and 280 kD were observed (Fig. 6, lanes 6 and 7) in cross-linked samples that might represent higher oligomeric forms of the ASGP-R. Schwartz and co-workers (24) reported that the HepG2 ASGP-R has a minimum functional size of 140,000 D and postulated that the functional receptor is a tetramer of receptor polypeptides.

Chemical cross-linking reagents have been used to study the association or interaction of various membrane bound proteins. Recently, Loeb and Drickamer (16) used chemical cross-linking with DFDNB to show that the detergent-solubilized chicken asialoagalactoglycoprotein-receptor, which is related to the ASGP-R, is a hexameric protein of six apparently identical subunits. When the DFDNB reaction was carried out on membrane-bound receptor, only dimer and trimer species were observed. They attributed this difference to the fact that the high concentration of lipids and proteins in the membrane compete for the cross-linking reagent and therefore inhibit hexamer formation. They proposed that multiple sugar-binding sites clustered together in an oligomeric receptor would endow the receptor with the high affinity binding activity that it does exhibit.

Very recently, the rat ASGP-R polypeptides, RHL-1 and RHL-2/3, were subjected to the same types of chemical crosslinking experiments by Halberg et al. (10). They found that when homogeneous preparations of RHL containing RHL-1, RHL-2, and RHL-3 polypeptides were treated with DFDNB, hexamers of RHL-1 and hexamers of RHL-2/3 were formed. When the chemical crosslinking was carried out on rat liver microsomal membranes, RHL-1 dimers and trimers were formed as well as RHL-2/3 dimers and trimers. They never observed RHL-1-RHL2/3 cross-linked species, and therefore concluded that the RHL-1 and RHL-2/3 polypeptides represent separate populations of ASGP-Rs. These results are not consistent with our data since we have demonstrated that H1 can be chemically crosslinked to H2 in microsomal membranes (Figs. 5 and 6). This discrepancy might be due to the fact that RHL-1 and RHL-2/3 polypeptides are slightly different than their human counterparts, H1 and H2. The amino acid sequences are very similar (18, 27) but not identical. In addition, RHL preparations contain a third polypeptide, RHL-3, which is not observed in human ASGP-R preparations. RHL-3 might alter the structure of the rat ASGP-R. Another possibility is that RHL heteroolig-

mers were not detected because the crosslinking reaction abolished asymmetrically an epitope required for antibody recognition. In summary, several lines of evidence suggest that the human ASGP-R is made up of two distinct polypeptide chains—H1 and H2. First, both H1 and H2 polypeptides were degraded when HepG2 cells were incubated with either anti-H1 or anti-H2 antibody (Fig. 2). Second, dimer and trimer species containing H1 and H2 polypeptides were formed upon chemical crosslinking (Figs. 5 and 6). And finally, cDNA transfection experiments demonstrate that expression of both H1 and H2 is required to reconstitute ASGP-R activity in murine fibroblasts (Shia and Lodish, submitted for publication). We conclude that the H1 and H2 polypeptides, associated in an oligomeric complex, form the human ASGP-R.

We thank Drs. J. Amara, E. J. Neufeld, and B. Thorens for critical reading of the manuscript and M. Boucher for excellent secretarial assistance.

Dr. Bischoff is a postdoctoral fellow of the Helen Hay Whitney Foundation and Dr. Shia was supported by a postdoctoral fellowship from the National Institutes of Health (GM-10203). This research was supported in part by National Institute of Health grant GM-35012.

Received for publication 24 September 1987, and in revised form 23 November 1987.

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