

Oligomeric Structure of the Human Asialoglycoprotein Receptor: Nature and Stoichiometry of Mutual Complexes Containing H1 and H2 Polypeptides Assessed by Fluorescence Photobleaching Recovery

Yoav I. Henis,*[‡] Ziva Katzir,* Michael A. Shia,[‡] and Harvey F. Lodish*[§]

*Department of Biochemistry, the George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel;

[‡]Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142; and [§]Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Abstract. The interactions between H1 and H2, the two polypeptides comprising the human asialoglycoprotein receptor (ASGP-R), were investigated by immunofluorescence and lateral mobility measurements combined with antibody-mediated crosslinking and immobilization. Immunofluorescence microscopy revealed two ASGP-R populations on the cell surface, one homogeneously distributed and the other in micropatches. This was observed both in stably transfected NIH 3T3 lines expressing H1 and/or H2, and in the human hepatoma cell line HepG2. In transfected cells expressing both polypeptides (the 1-7-1 line), H1 and H2 were colocalized in the same microaggregates. Moreover, enhancement of the patching of, e.g., H1 by IgG-mediated crosslinking was accompanied by copatching of H2.

To quantify H1-H2 complex formation, the lateral diffusion of H1 and H2 was measured at 12°C (to

avoid internalization) by fluorescence photobleaching recovery. H1 (or H2) was immobilized by crosslinking with specific IgG molecules; the other chain was labeled with fluorescent monovalent Fab' fragments, and its lateral mobility was measured. In HepG2 cells, immobilization of either H1 or H2 led to an equal immobilization of the other, indicating that all the mobile H1 and H2 are in stable heterooligomers. In 1-7-1 cells, immobilization of H2 immobilized H1 to the same degree, but immobilization of H1 reduced the mobile fraction of H2 only by 2/3. Thus, in 1-7-1 cells all surface H1 molecules are associated with H2, but 1/3 of the H2 population is independent of H1. From these data and from measurements of the relative surface densities of H1 and H2, conclusions are drawn regarding the oligomeric structure and stoichiometry of the ASGP-R.

THE asialoglycoprotein receptor (ASGP-R)¹ has served as a model for studying various aspects of receptor-mediated endocytosis (Ashwell and Harford, 1982; Breitfeld et al., 1985; Schwartz, 1990). The ASGP-Rs from mammalian parenchymal hepatocytes, which bind and internalize serum glycoproteins bearing terminal galactose or *N*-acetylgalactosamine residues (Geuze et al., 1983a, b, 1987), are all comprised of two different polypeptide chains (Drickamer et al., 1984; Breitfeld et al., 1985; Spiess and Lodish, 1985; McPhaul and Berg, 1986; Bischoff and Lodish, 1987; Halberg et al., 1987; Shia and Lodish, 1989). They therefore provide a particularly attractive system for studies on the role of interactions between multiple polypeptide chains in receptor structure and function.

The human ASGP-R activity involves two polypeptides, H1 (46 kD) and H2 (50 kD) (Spiess et al., 1985; Spiess and Lodish, 1985; Bischoff and Lodish, 1987). There are several

indications for functional interactions between H1 and H2 or their equivalent counterparts from rat, designated rat hepatic lectins 1 and 2/3 (RHL-1 and RHL-2/3). Thus, expression of the cDNAs of both H1 and H2 (or RHL-1 and RHL-2/3) is required to reconstitute ASGP-R activity (ligand binding and internalization) at the surface of transfected cells (McPhaul and Berg, 1986; Shia and Lodish, 1989). The existence of dimer and trimer species of human ASGP-R, some of which react with both anti-H1 and anti-H2 antibodies, was demonstrated in the human hepatoma cell line HepG2 (Bischoff et al., 1988) and in NIH 3T3 cells transfected with the cDNAs of H1 and H2 together (Shia and Lodish, 1989). Furthermore, Sawyer et al. (1988) employed specific antibodies directed against the extracellular COOH-terminal region of RHL-1 and RHL-2/3 to show that they form mutual complexes in the plasma membrane. On the other hand, Halberg et al. (1987) concluded from chemical crosslinking and galactose affinity chromatography studies that the rat ASGP-R polypeptides form only homooligomers of RHL-1 or RHL-2/3.

H1 expressed alone in transfected NIH 3T3 cells forms at

1. *Abbreviations used in this paper:* ASGP-R, asialoglycoprotein receptor; ASOR, asialoorosomucoid; FPR, fluorescence photobleaching recovery.

least homo-trimers and is transported normally to the cell surface; however, coexpression together with H2 is required to achieve binding and internalization of the ligand asialoorosomucoid (ASOR) (Shia and Lodish, 1989). In accord with these findings, rat RHL-1 expressed alone in rat hepatoma tissue culture (HTC) cells fails to bind and internalize ASOR, although it does bind and internalize to some degree a highly derivatized multivalent ligand (poly-L-lysine containing numerous galactose residues) (Braiterman et al., 1989).

H2 appears as two subspecies, H2A and H2B (Spiess and Lodish, 1985). H2B is most likely a spliced version of H2A, missing five amino acids (15 nucleotides) in the extracellular domain immediately following the membrane-spanning region (nucleotide positions 244–258) (Spiess and Lodish, 1985). H2A expressed singly in NIH 3T3 cells undergoes only core glycosylation and is mostly degraded (Shia and Lodish, 1989; Amara et al., 1989; Lederkremer and Lodish, 1990), while in the case of H2B a significant amount (10–20%) is correctly processed and gets to the cell surface. Its transport to the cell surface is enhanced to 40–60% upon coexpression with H1 (Lederkremer and Lodish, 1990; Shia, M. A., and H. F. Lodish, unpublished observations). At this point it is not known which subtype (H2A, H2B, or both) is synthesized in HepG2 cells; however, it is clear that H2B can couple with H1 to form a fully active receptor, as shown in transfected NIH 3T3 cells (Shia and Lodish, 1989; Lederkremer and Lodish, 1990).

The indications that the functional ASGP-R is a heterooligomer of unknown composition raise several questions. What is the nature of the interactions between H1 and H2? Do they form stable, long-lived complexes, or labile, short-lived ones? What is the stoichiometric relationship between H1 and H2 in the active ASGP-R complex? In the present study, we investigated these questions in cells expressing H1 and H2 (NIH 3T3 cells transfected with H1 and/or H2 cDNA, or HepG2 cells), by combining immunofluorescence microscopy and fluorescence photobleaching recovery (FPR) measurements of the lateral mobility of H1 and H2 in the plasma membrane with antibody cross-linking experiments.

Materials and Methods

Materials

TRITC and FITC were obtained from Molecular Probes (Junction City, OR). Affinity-purified goat IgG directed against rabbit IgG (GAR IgG) was a generous gift from Dr. Benny Geiger (Weizmann Institute of Science, Rehovot, Israel). Monovalent Fab' fragments were prepared from these antibodies as described earlier (Henis et al., 1985). Both the goat Fab' preparations, and the Fab' from the rabbit anti-peptide antibodies described below, were free of contamination by F(ab')₂ or IgG, as judged by SDS-PAGE under nonreducing conditions. GAR IgG or their Fab' fragments were tagged with TRITC and FITC by established procedures (Brandtzaeg, 1973). Succinyl concanavalin A (SConA; a dimeric derivative of the lectin Con A) was prepared according to Gunther et al. (1973) and conjugated with TRITC as described earlier (Eldridge et al., 1980).

Cell Culture

The cell lines 1-7-1 (expressing both H1 and H2) and 1-7 (expressing only H1) were derived from mouse NIH 3T3 fibroblasts using the retroviral vector pDO-L (Shia and Lodish, 1989). The 1-7-1 line expresses the H2B subtype (Lederkremer and Lodish, 1990), and is fully active (with the same parameters characterizing HepG2 cells) in the binding and internalization

of ASOR (Shia and Lodish, 1989). The 3-8 line (expressing H2B alone) was prepared similarly, using pDO-L containing the entire coding region of H2B to transfect the NIH 3T3 cells. The transfected NIH 3T3 cell lines were grown in DME supplemented with 10% calf serum and penicillin/streptomycin (100,000 U/L penicillin, 100 mg/L streptomycin) (Gibco Laboratories, Grand Island, NY) as described by Shia and Lodish (1989). The human hepatoma cell line HepG2 was grown in MEM containing 10% FCS, 2 mM glutamine, and antibiotics (penicillin/streptomycin) as described previously (Knowles et al., 1980).

Antipeptide Antibodies

The extracellular COOH-terminal regions of H1 and H2 were labeled using antibodies raised in rabbits against synthetic peptides corresponding to the 12 and 15 carboxy-terminal residues of H2 and H1, respectively (Bischoff et al., 1988). No cross-reactivity was detected between these antibodies by immunoadsorption of radiolabeled translation products (Bischoff et al., 1988). Both the IgG fractions and the monovalent Fab' fragments prepared from these antibodies were highly specific for their respective protein antigens in immunofluorescence experiments on intact cells under the conditions employed in the present studies. The level of nonspecific staining of cells that do not express H1 or H2 was negligible, and no significant labeling of H1 by anti-H2 or vice versa was observed (see Fig. 1). Thus, these antibodies bind only to the specific epitopes located at the carboxy-terminus of H1 or H2.

Immunofluorescence Microscopy

Cells from confluent plates were split 1:40 (transfected NIH 3T3 lines) or 1:3 (HepG2 cells) and grown 2–3 d on glass coverslips. They were washed twice with DME (NIH 3T3 cells) or MEM (HepG2 cells) containing 20 mM Hepes (pH 7.2; no serum present), and incubated 30 min at 37°C in the same medium to allow receptor recycling. After washing twice with HBSS with Earle's salts (Gibco Laboratories) supplemented with 20 mM Hepes and 2% BSA (Sigma Chemical Co., St. Louis, MO) (HBSS/Hepes/BSA, pH 7.2), the cells were incubated with normal goat IgG (50 µg/ml, 30 min, 22°C) in the same buffer to block nonspecific antibody binding. This was followed by incubations (4°C, in HBSS/Hepes/BSA) with anti-H1 or anti-H2 Fab' or IgG (intact IgG were employed when antibody-mediated cross linking was desired). After rinsing three times with the same cold buffer, the coverslips were incubated with TRITC-GAR Fab', FITC-GAR IgG, or unlabeled GAR IgG (depending on the specific experiment; for details, see the specific figure legends). All incubations were in the cold, in order to eliminate internalization and allow only surface labeling. The samples were washed three times with cold HBSS/Hepes, and fixed successively in methanol (–20°C, 5 min) and acetone (–20°C, 2 min). Fluorescence micrographs were taken using rhodamine or fluorescein filters with a Zeiss Universal fluorescence microscope (63× oil immersion objective, Kodak Tri-X film).

Fluorescence Photobleaching Recovery

Lateral diffusion coefficients (D) and mobile fractions (R_f) of H1 and H2 on the surface of transfected NIH 3T3 cells and HepG2 cells were measured by FPR (Koppel et al., 1976; Axelrod et al., 1976) using an apparatus described previously (Henis and Gutman, 1983). The bleaching conditions employed in the FPR studies were shown not to alter the lateral mobilities measured (Wolf et al., 1980; Koppel and Sheetz, 1981). After labeling H1 or H2 with fluorescent monovalent antibody fragments (as described in the former section and detailed in the appropriate figure legends), the live cells were washed three times with cold HBSS/Hepes/BSA, and the coverslip was placed (cells facing downward) over a serological slide (M 6229-2; Scientific Products [Baxter], McGraw, IL) filled with the same buffer equilibrated at 12°C. A thermostated microscope stage was utilized to keep the sample at 12°C throughout the FPR experiments, all performed within 1 h of the labeling. This temperature was chosen in order to avoid temperature-dependent receptor clustering and internalization, which may lead to lateral immobilization of the tagged receptors; no significant internalization of the antibody-labeled receptors or of the radio-labeled ligand ASOR occurs for at least 1 h at 12°C (not shown).

The monitoring laser beam (529.5 nm, 1 µW, argon ion laser) was focused through the microscope (Zeiss Universal) to a Gaussian radius of 0.61 µm with a 100× oil immersion objective. A brief pulse (5 mW, 60 ms) bleached 50–70% of the fluorescence in the illuminated region. The time

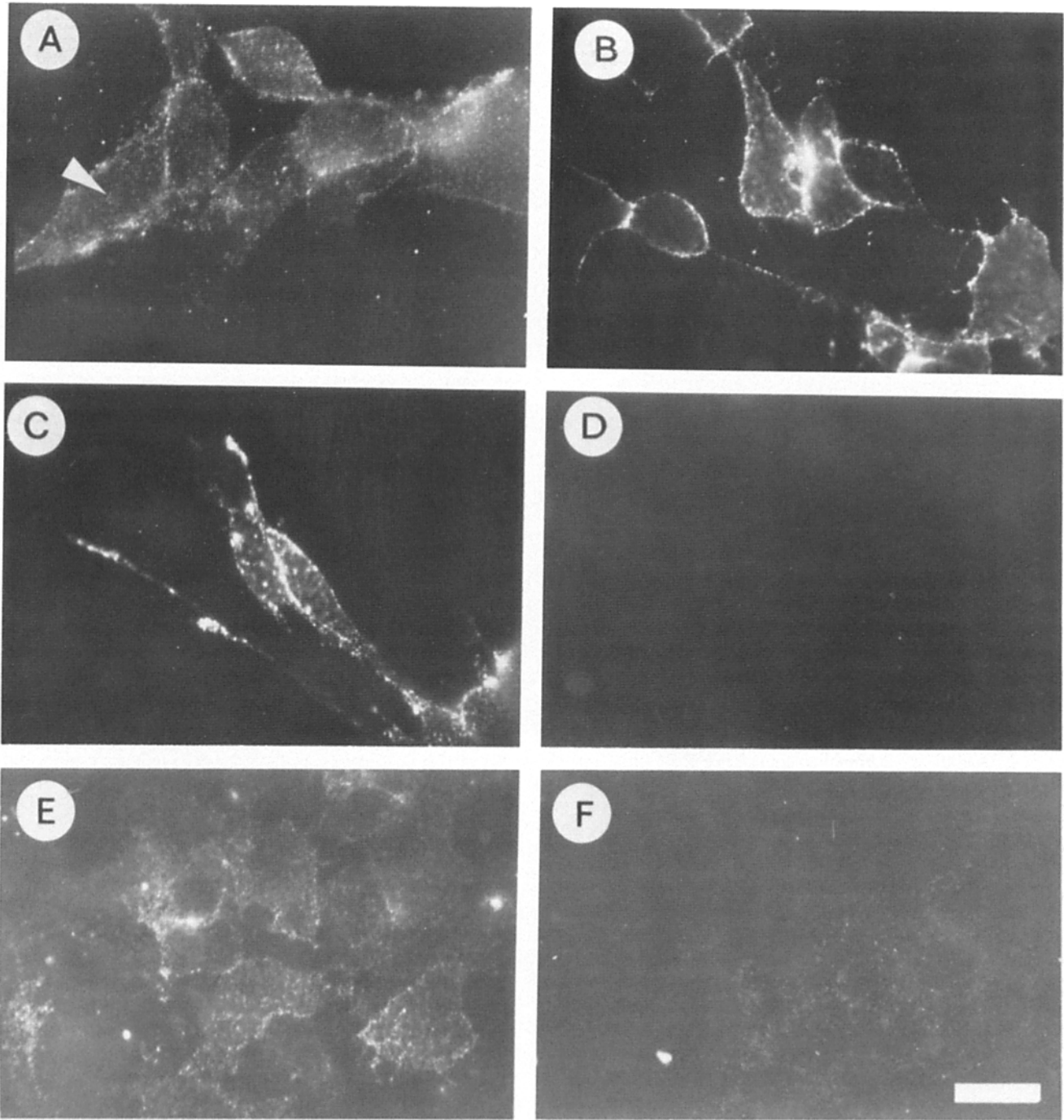


Figure 1. Immunofluorescence labeling of H1 and H2 on the surface of transfected NIH 3T3 cells and HepG2 cells using anti-peptide monovalent Fab' fragments. Cells grown on glass coverslips were washed and incubated with normal goat IgG as described under the immunofluorescence microscopy section (Materials and Methods). They were labeled in the cold with anti-H1 or anti-H2 Fab' (150 $\mu\text{g}/\text{ml}$, 45 min, in HBSS/Hepes/BSA). After washing three times with the same cold buffer, the cells were incubated (45 min, 1–4°C) with 100 $\mu\text{g}/\text{ml}$ TRITC-GAR Fab'. They were washed, fixed, and photographed using rhodamine filters as described under Materials and Methods. The arrowhead in *A* designates a group of four distinct micropatches. (*A*) 1-7-1 cells, expressing both H1 and H2 at the plasma membrane, labeled with anti-H1 Fab' followed by TRITC-GAR Fab'. (*B*) Same as *A*, except that anti-H2 Fab' replaced anti-H1 Fab'. (*C*) 1-7 cells, expressing H1 but not H2, stained as in *A*. (*D*) Same as *C*, except that anti-H2 Fab' was employed in place of anti-H1 Fab'. Similarly dark fields were obtained with either anti-H1 or anti-H2 on NIH 3T3 cells mock-infected with the retroviral vector devoid of ASGP-R cDNA inserts (0-1 cells; Shia and Lodish, 1989), or with anti-H1 Fab' on cells transfected with H2B alone (3-8 cells). (*E*) HepG2 cells labeled with anti-H1 Fab' followed by TRITC-GAR Fab'. (*F*) Same as *E*, except that anti-H2 Fab' replaced anti-H1 Fab'. Bar, 20 μm .

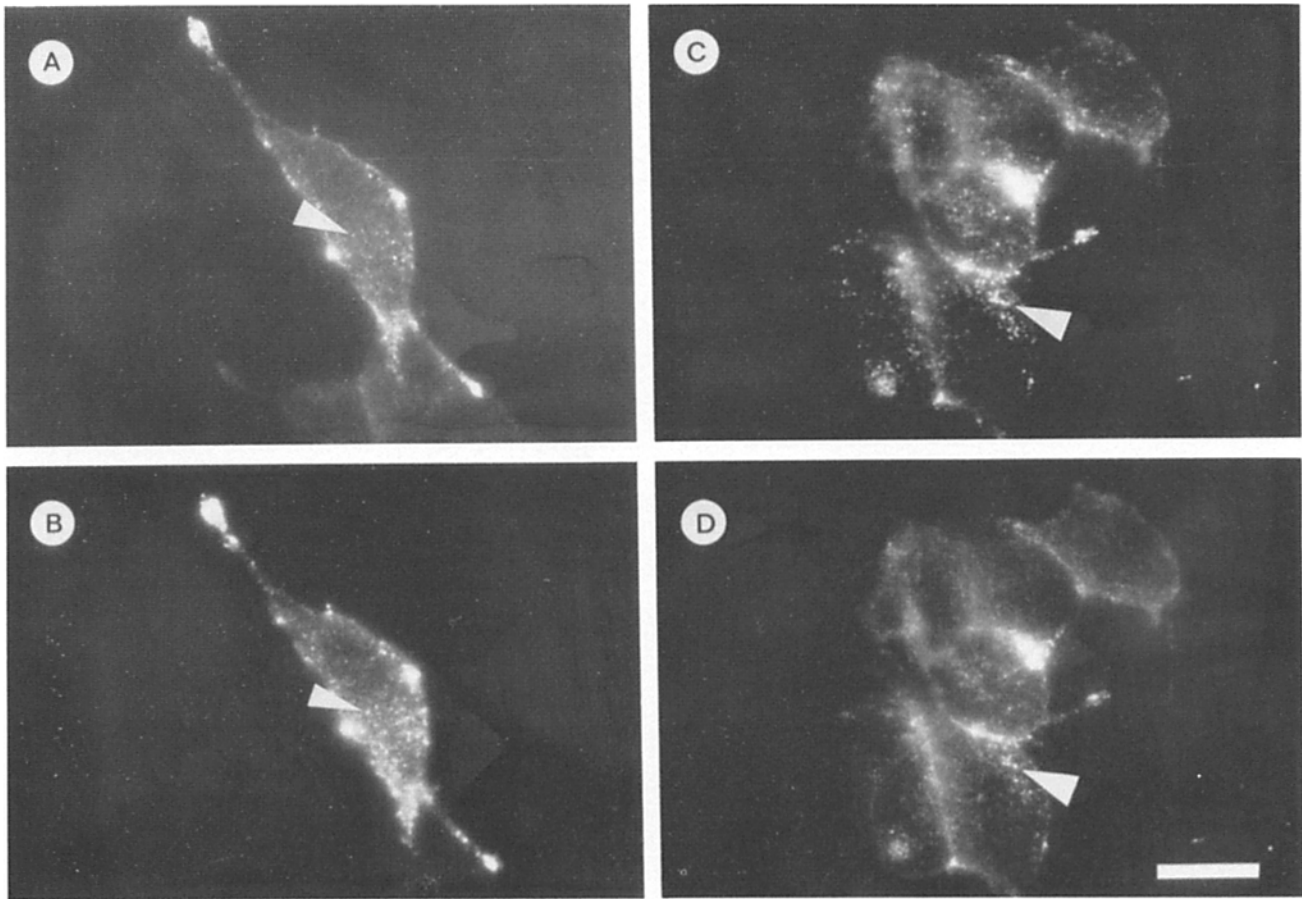


Figure 2. Double-labeling immunofluorescence of H1 and H2 on the surface of 1-7-1 cells using Fab' fragments. The cells were grown and treated up to and including the stage of incubation with normal goat IgG as in Fig. 1. The coverslips were then incubated successively for 45-min periods in cold HBSS/Hepes/BSA (washing three times between steps) with the following Fab' fragments (1) anti-H2 rabbit Fab' (150 $\mu\text{g/ml}$); (2) TRITC-GAR Fab' (100 $\mu\text{g/ml}$); (3) unlabeled GAR Fab' (100 $\mu\text{g/ml}$; this step was used to ensure complete blockade of the anti-H2 Fab' by GAR Fab'); (4) anti-H1 rabbit Fab' (150 $\mu\text{g/ml}$); and (5) FITC-GAR Fab' (150 $\mu\text{g/ml}$). This procedure yields H2 labeled with TRITC and H1 labeled with FITC, and was employed in A and B. In C and D, the labeling order with anti-H1 and anti-H2 was reversed, yielding TRITC-labeled H1 and FITC-labeled H2. The cells were fixed and photographed (first under fluorescein conditions, since FITC bleaches faster than TRITC) as described under Materials and Methods. Control experiments (not shown) verified that the first anti-ASGP-R Fab' is fully saturated by GAR Fab' before step 4 (no FITC labeling was detected when the anti-H1 Fab' in step 4 was omitted or replaced by preimmune rabbit Fab'). No significant contribution of TRITC to FITC fluorescence and vice versa was observed under the photography conditions employed. The arrowheads indicate groups of micropatches that can be detected under both FITC and TRITC photography conditions. (A) FITC-labeled H1. (B) Same field showing H2 labeled with TRITC. (C) TRITC-labeled H1. (D) FITC-labeled H2 on the same cells shown in C. Bar, 20 μm .

course of fluorescence recovery was followed by the attenuated monitoring beam. D and R_f were extracted from the fluorescence recovery curves by nonlinear regression analysis (Petersen et al., 1986). Bleaching by the monitoring beam for the duration required to obtain a complete FPR curve was below 5%. Incomplete fluorescence recovery was interpreted to represent fluorophores that are immobile on the FPR experimental time scale ($D \leq 5 \times 10^{-12} \text{ cm}^2/\text{s}$).

Results

Immunofluorescence Visualization of H1 and H2 on the Cell Surface

H1 and H2 present on the surface of live cells were visualized by immunofluorescence microscopy. The extracellular COOH-terminal regions of H1 and H2 were labeled in the cold (to avoid internalization) by anti-H1 or anti-H2 Fab' fragments, followed by TRITC-GAR Fab'. Monovalent Fab' fragments

were employed to avoid any possible antibody-mediated cross-linking. The labeling of H1 and H2 on the surface of several cell lines (1-7-1, a line of transfected NIH 3T3 cells expressing functional H1 and H2; 1-7, transfected with H1 only; and HepG2 cells, the human hepatoma cell line from which H1 and H2 were originally cloned) is shown in Fig. 1. In all cases, focusing on flat cell regions reveals a labeling pattern characterized by apparent diffuse staining along with distinct dot-like aggregates (micropatches), distributed rather evenly on the cell surface (Fig. 1, *arrowhead*). The majority of the micropatches are not induced by the binding of the Fab' fragments, since a similar pattern is observed when cells are prefixed with 3% paraformaldehyde before labeling with the Fab' fragments (not shown); this treatment reduces the fraction of laterally mobile H1 and H2 from >60% (see Fig. 5) to <20%.

Double-labeling immunofluorescence experiments were

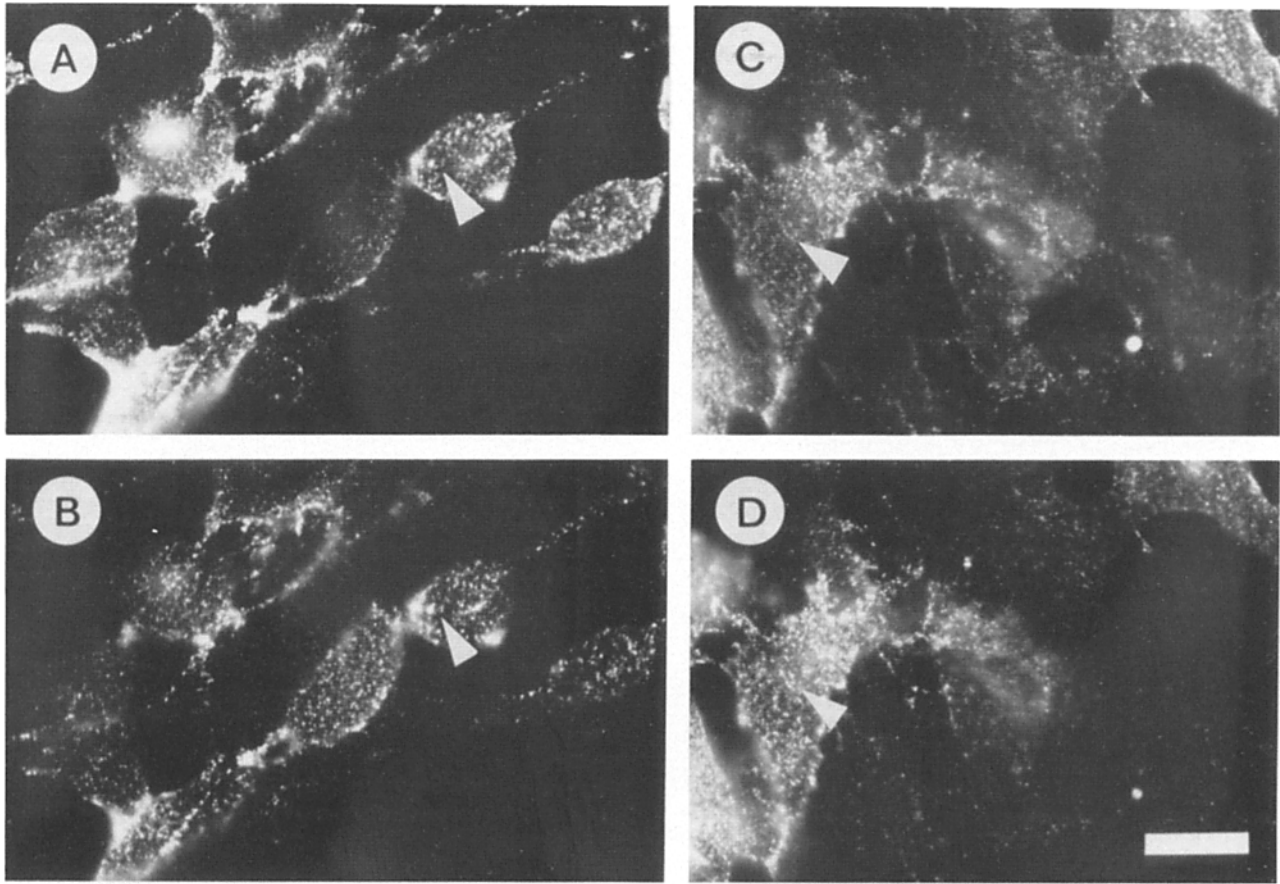


Figure 3. Copatching of H1 and H2 on the surface of 1-7-1 cells following antibody-mediated crosslinking of one polypeptide type. Cells were grown and treated up to the stage of incubation with normal goat IgG (inclusive) as in Fig. 1. This was followed by incubating the coverslips for successive 45-min periods in cold HBSS/Hepes/BSA (with three washes between steps) with the following antibodies (1) anti-H1 or anti-H2 rabbit Fab' (150 $\mu\text{g/ml}$); (2) TRITC-GAR Fab' (100 $\mu\text{g/ml}$); (3) unlabeled GAR Fab' (100 $\mu\text{g/ml}$, to ensure full blockade of the first rabbit Fab' by monovalent GAR Fab'); (4) 150 $\mu\text{g/ml}$ anti-H2 IgG (when anti-H1 Fab' was employed at step 1) or anti-H1 IgG (when anti-H2 Fab' was used at step 1); and (5) FITC-GAR IgG (20 $\mu\text{g/ml}$). Microphotography was performed as in Fig. 2. The arrowheads indicate selected groups of micropatches observed under both FITC and TRITC photography conditions. (A) TRITC fluorescence of H2 labeled exclusively by monovalent Fab' fragments (anti-H2 Fab' followed by TRITC-GAR Fab'). (B) FITC fluorescence of crosslinked H1 on the same cells (H1 labeled by anti-H1 IgG followed by FITC-GAR IgG). (C) TRITC fluorescence of H1 labeled by monovalent Fab' fragments (anti-H1 Fab' followed by TRITC-GAR Fab'). (D) FITC fluorescence of IgG-crosslinked H2 on the same cells (anti-H2 IgG followed by FITC-GAR IgG). Bar, 20 μm .

conducted on 1-7-1 cells to determine whether H1 and H2 are colocalized in the same micropatches, as expected if the two polypeptide chains reside in mutual hetero-complexes. These cells were chosen for the double-labeling studies since they express ASGP-Rs that are fully active in ligand endocytosis (Shia and Lodish, 1989), and since they display a high and comparable labeling intensity of H1 and H2; on HepG2 cells, the labeling intensity of H2 was significantly weaker (Fig. 1), ruling out accurate double-labeling immunofluorescence studies. Visualization of both H1 and H2 (labeled exclusively by monovalent Fab' fragments) on the cell surface demonstrated that most, if not all, microaggregates in the plasma membrane of these cells contain both H1 and H2 (Fig. 2). Some of the micropatches appear weaker in the micrographs of FITC fluorescence as compared with TRITC fluorescence; this is most likely due to the higher sensitivity of FITC to bleaching, as indicated by the fact that the same phenomenon is observed regardless of whether H1 or H2 are labeled with FITC (Fig. 2).

To explore the relationship between H1 and H2 molecules that are not originally in micropatches, we employed immunofluorescence experiments based on antibody-mediated crosslinking and patching of H1 or H2. In these experiments, the entire population of one polypeptide chain (e.g., H1) on the cell surface was crosslinked by a saturating concentration of intact bivalent anti-H1 IgG, followed by a second layer of FITC-GAR IgG. The other ASGP-R chain (in this case, H2) was labeled with a noncrosslinking monovalent anti-H2 Fab', followed by TRITC-GAR Fab'. Under these conditions, H1 (FITC-labeled) is cross-linked by a double layer of intact IgG molecules, while H2 (TRITC-labeled) is not cross-linked and is tagged exclusively by monovalent Fab' fragments. The antibody-mediated crosslinking of H1 leads to conspicuous patching, manifested by depletion of the diffuse labeling and augmentation of the micropatches (Fig. 3). The disappearance of the homogeneously distributed H1 population was verified by FPR experiments, which demonstrated that the above treatment results in lateral immobilization of the

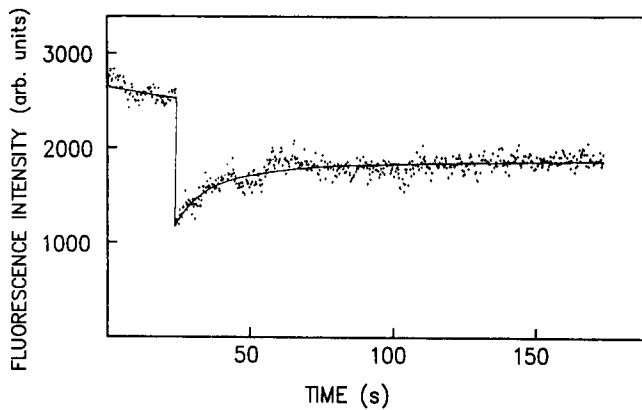


Figure 4. A representative FPR curve depicting the lateral mobility of H1 in the plasma membrane of 1-7-1 cells. Cells were grown and treated with normal goat IgG as in Fig. 1. H1 molecules on the cell surface were labeled by saturating concentrations of monovalent Fab' fragments in HBSS/Hepes/BSA. The labeling was achieved by the following successive incubations (45 min each, 4°C, with three washes between steps) (1) anti-H1 Fab' (150 µg/ml); and (2) TRITC-GAR Fab' (100 µg/ml). The cells were then wet-mounted (in HBSS/Hepes/BSA) and taken for the FPR experiments, performed at 12°C (see Materials and Methods). The points represent the experimentally determined fluorescence intensity (photons counted over a dwell time of 300 ms per point). The solid line represents the best fit to the lateral diffusion equation using nonlinear regression (Petersen et al., 1986). The specific curve shown yielded $D = 1.06 \times 10^{-10}$ cm²/s, and $R_f = 0.58$.

crosslinked ASGP-R polypeptides (described further on, see Figs. 7 and 8). Fig. 3 demonstrates that when H1 aggregation is induced by antibody-mediated crosslinking, H2 molecules that were not crosslinked are swept into the same aggregates. An analogous situation (copatching of H1 upon antibody-induced aggregation of H2) is observed when H2 is cross-linked by specific antibodies (Fig. 3). These experiments provide a qualitative indication that most of the H1 and H2 molecules present on the surface of 1-7-1 cells reside in mutual complexes.

Lateral Mobility of H1 and H2

The immunofluorescence experiments supply qualitative evidence for hetero-complexes containing both H1 and H2 on the surface of 1-7-1 cells. However, they do not enable a quantitative estimate of the degree of mutual complex formation, and cannot be adequately performed on HepG2 cells due to the lower level of H2 in these cells. To overcome these limitations, we employed FPR measurements to determine the lateral mobility of H1 and H2 in the plasma membrane; combination of these measurements with specific antibody-mediated crosslinking and immobilization of H1 or H2 enabled us to obtain a quantitative measure for the formation of H1-H2 complexes. The FPR measurements were performed on cells expressing only H1 (the 1-7 line) or H2B (the 3-8 line; Shia and Lodish, 1989; Lederkremer and Lodish, 1990), as well as on cells expressing both H1 and H2 (1-7-1 and HepG2 cells). After labeling H1 or H2 with monovalent Fab' fragments (anti-H1 or anti-H2 Fab' followed by TRITC-GAR Fab'), D and R_f of the labeled ASGP-R polypeptides

were determined by FPR experiments carried out at 12°C (a temperature chosen to avoid internalization during the time of the measurement). The indirect labeling protocol outlined above was preferred over labeling with TRITC-tagged anti-H1 or anti-H2 Fab' due to the superior signal-to-noise ratio (nearly threefold higher labeling); the use of a secondary TRITC-GAR Fab' did not lead to any measurable inhibition of H1 or H2 mobility, since similar D and R_f values were measured on 1-7-1 cells employing direct labeling with TRITC-tagged anti-H1 or anti-H2 Fab' (not shown). A typical FPR curve (depicting the lateral mobility of H1 on a 1-7-1 cell) is shown in Fig. 4. The average results of many such experiments are given in Fig. 5. These experiments demonstrate that on both 1-7-1 and HepG2 cells, the lateral mobility of H1 and H2 is characterized by similar dynamic parameters (D and R_f). Moreover, comparison between the D values of each polypeptide chain (H1 or H2) in the various cell types does not show significant cell type-specific differences.

The prebleach fluorescence level in the FPR experiments is directly proportional to the density of the labeled protein on the cell surface. This enabled us to quantify the relative surface densities of H1 and H2 on the different cell types (Fig. 6). On HepG2 cells, which naturally express H1 and H2, the ratio between the surface densities of H1 and H2 was around 3:1, while on 1-7-1 cells it was close to 1:1. This indicates an excess of H2 (relative to H1) in the plasma membrane of 1-7-1 cells as compared with the situation in HepG2 cells. This conclusion is in accord with the ratios of H1 to

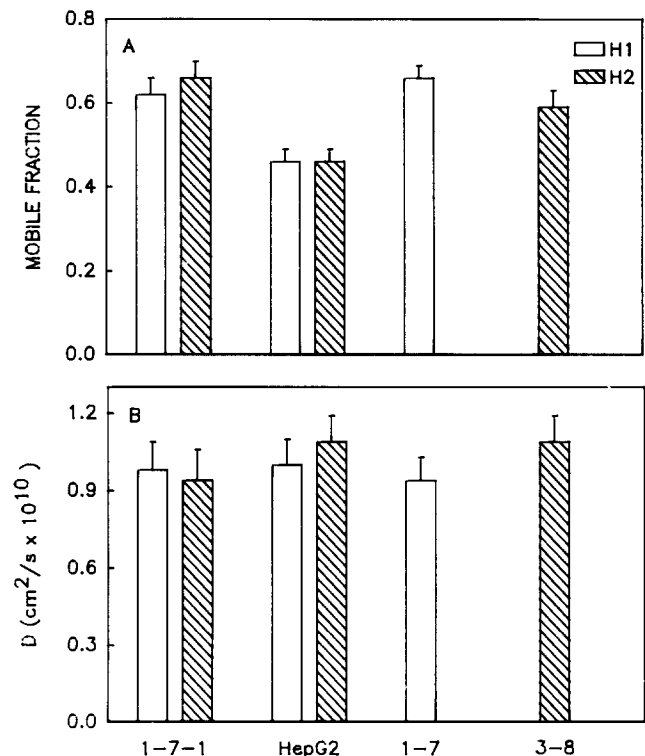


Figure 5. Lateral diffusion of H1 and H2 on the surface of several cell types. The experiments were performed as described in Fig. 4, labeling either H1 (with anti-H1 Fab' followed by TRITC-GAR Fab') or H2 (with anti-H2 Fab' followed by TRITC-GAR Fab'). (Blank bars) H1; (cross-hatched bars) H2. Each bar is the mean \pm SEM of 25-30 measurements.

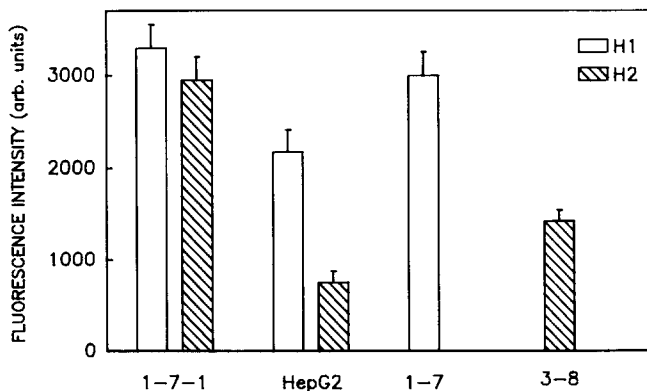


Figure 6. Quantification of the fluorescence intensity of Fab'-labeled H1 and H2 on the surface of 1-7-1, HepG2, 1-7, and 3-8 cells. The labeling of the cells by the appropriate Fab' fragments was performed as in Figs. 4 and 5. The fluorescence intensities were taken from the prebleach levels of the FPR experiments (i.e., under non-bleaching conditions). (Blank bars) H1; (cross-hatched bars) H2. The results are the means \pm SEM of 25-30 measurements in each case.

H2 found by quantitative immunoprecipitation (compare 1:1 in 1-7-1 cells and 5-6:1 in HepG2 cells; Bischoff and Lodish, 1987; Shia and Lodish, 1989). The somewhat higher H1:H2 ratio determined in HepG2 cells by immunoprecipitation as compared with immunofluorescence most likely reflects the fact that the first method measures both intracellular and surface receptors, while only the latter are measured by the quantitative immunofluorescence protocol employed (Fig. 6).

The immunofluorescence copatching experiments (Fig. 3) provide a qualitative indication for mutual H1-H2 complexes. The similar dynamic parameters of H1 and H2 in both 1-7-1 and HepG2 cells are in accord with the notion that they move together as a complex in the plasma membrane; however, the above similarity could also be coincidental and merely indicate that H1 and H2 experience analogous mobility-restricting interactions. To directly determine and quantify the interactions between H1 and H2 at the cell surface, we employed antibody-mediated crosslinking to immobilize one type of ASGP-R polypeptide chain (e.g., H1), and examined the effects on this immobilization on the lateral mobility of the other chain type (H2). In principle, immobilization of H1 can have one of several effects on the lateral mobility of H2 (and vice versa). (a) If the two proteins do not reside in the same complex, the lateral mobility of the protein that was not crosslinked (H2) will be unaltered. (b) If they form mutual complexes whose lifetime is long relative to the characteristic diffusion time, τ_D ($\tau_D = w^2/4D$, where w is the Gaussian radius of the laser beam on the cell surface), the mobile fraction of H2 is expected to drop along with that of H1, due to the association of H2 molecules with immobile crosslinked H1 molecules for the entire duration of the FPR measurement. (c) In case the complex formed has a lifetime comparable to or shorter than τ_D , an H2 molecule will undergo several association-dissociation cycles with immobile H1 molecules during the FPR measurement, leading to a reduction in the effective D value measured for H2 but not in R_f (Elson and Reidler, 1979; Petersen et al., 1986; Duband et al., 1988).

The initial requirement for these experiments is to establish conditions under which crosslinking of H1 or H2 with specific antibodies will immobilize most of the crosslinked proteins. In these experiments, H1 (or H2) on the cell surface were incubated with a saturating concentration (150 $\mu\text{g/ml}$) of TRITC-IgG (anti-H1 or anti-H2). Further crosslinking was achieved by a second layer of unlabeled GAR IgG (20 $\mu\text{g/ml}$). FPR measurements on cells labeled in this manner measured the lateral mobility of the crosslinked ASGP-R polypeptides (H1 or H2). The results (Figs. 7 and 8) demonstrate that antibody-mediated crosslinking under the conditions described above results in a nearly complete immobilization of the polypeptide chain against which the primary rabbit IgG is directed: R_f drops to $\sim 10\%$ (which is roughly the detection limit of R_f in the FPR experiments) on both 1-7-1 and HepG2 cells, while the D values characterizing the

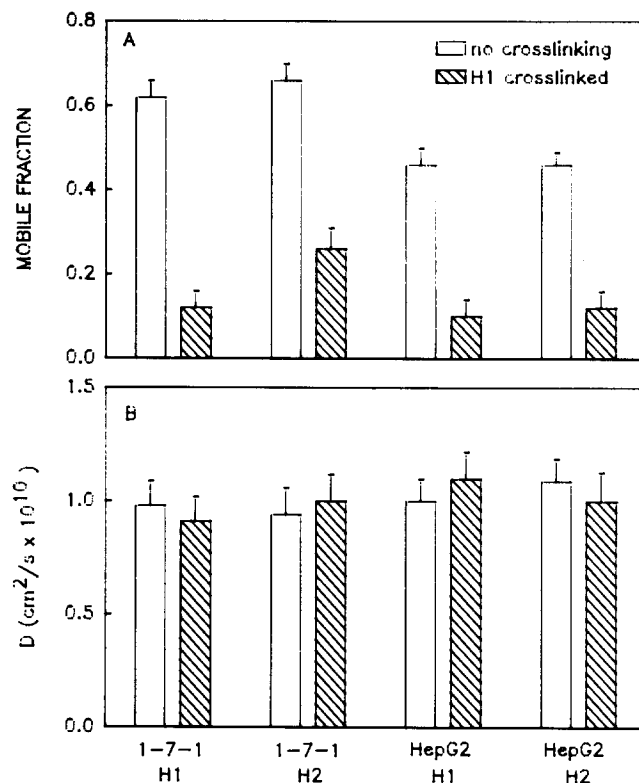


Figure 7. Crosslinking H1 on 1-7-1 and HepG2 cells: effect on the mobility of H1 and H2. Cells were grown and treated with normal goat IgG as in Fig. 1. Empty bars represent measurements of H1 or H2 mobility without crosslinking (performed as described in Fig. 5). Cross-hatched bars stand for measurements performed on cells whose surface H1 polypeptides were crosslinked by a double layer of IgG. For determination of the effect of crosslinking H1 molecules on their own lateral mobility, the cells were incubated successively in cold HBSS/Hepes/BSA (45 min each incubation, with three washes between steps) with (1) anti-H1 TRITC-IgG (150 $\mu\text{g/ml}$); and (2) unlabeled GAR IgG (20 $\mu\text{g/ml}$). For determination of the effect of crosslinking H1 on the lateral mobility of H2, the following successive incubations were employed (1) anti-H2 Fab' (150 $\mu\text{g/ml}$); (2) TRITC-GAR Fab' (100 $\mu\text{g/ml}$); (3) unlabeled GAR Fab' (100 $\mu\text{g/ml}$); (4) anti-H1 IgG, unlabeled (150 $\mu\text{g/ml}$); and (5) unlabeled GAR IgG (20 $\mu\text{g/ml}$). The FPR experiments were performed at 12°C as described in Fig. 4. Each bar is the mean \pm SEM of 25-30 measurements.

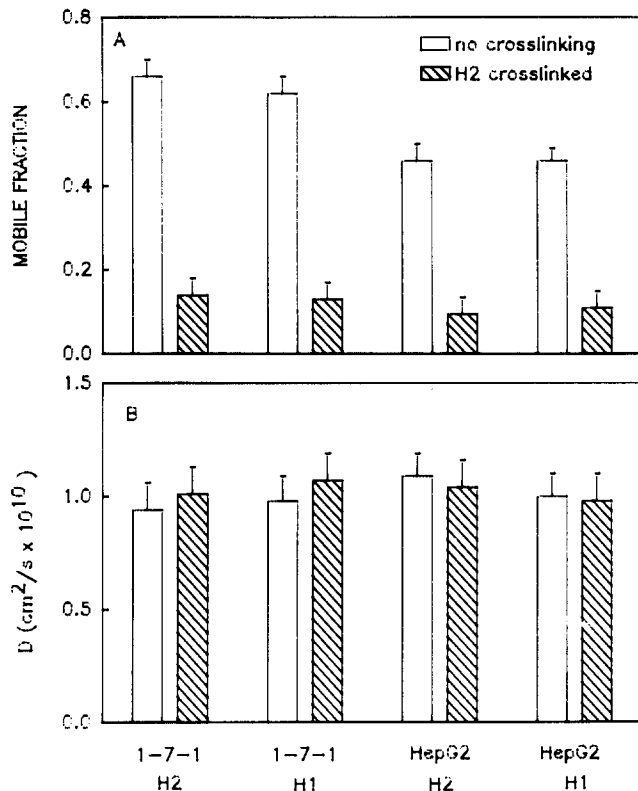


Figure 8. Crosslinking H2 on 1-7-1 and HepG2 cells: effect on the mobility of H1 and H2. Empty bars represent mobility measurements under normal conditions (in the absence of antibody-mediated crosslinking), performed as in Fig. 5. Cross-hatched bars stand for measurements on cells whose surface H2 proteins were crosslinked by a double layer of IgG. The labeling and antibody-mediated crosslinking protocols employed were as in Fig. 7, except that anti-H2 TRITC-IgG was used in place of anti-H1 TRITC-IgG (in measurements of the effect of crosslinking H2 molecules on their own lateral mobility), and anti-H1 Fab' replaced anti-H2 Fab' (for determination of the effect of crosslinking H2 on the lateral mobility of H1). The FPR measurements were performed at 12°C as in Fig. 4. Each bar represents the mean \pm SEM of 25–30 measurements.

motion of the 10% of the labeling that are still mobile are not altered.

After establishing conditions for lateral immobilization of H1 or H2, we proceeded to determine the effect that immobilizing one ASGP-R chain type has on the lateral mobility of the other. H1 (or H2) were crosslinked and immobilized as above, except that unlabeled anti-H1 or anti-H2 IgG were employed in place of TRITC-IgG. Instead of labeling the crosslinked polypeptide (e.g., H1), the other chain (H2) was fluorescently labeled with monovalent Fab' fragments, and its lateral mobility was measured by FPR (for detailed protocol, see Fig. 7). Figs. 7 and 8 depict the results of these experiments. In HepG2 cells, immobilization of H1 led to an identical reduction in R_f of H2, and vice versa. This effect is not due to general entrapment of membrane proteins by the surface-bound IgG network, since the same treatment with anti-H1 IgG and GAR IgG had no significant effect on the lateral mobility of Con A receptors labeled with TRITC-SConA (the mean \pm SEM values of R_f before and after crosslinking H1 were 0.46 ± 0.03 and 0.43 ± 0.04 , and the

respective D values were $(1.8 \pm 0.4) \times 10^{-10}$ and $(2.0 \pm 0.4) \times 10^{-10}$ cm²/s; 15 cells were measured in each case). In the case of 1-7-1 cells, the effect of IgG-mediated crosslinking of H1 on the mobility of H2 differed from the one observed on HepG2 cells; the reduction in R_f of H2 due to crosslinking H1 was only $\sim 2/3$ of the reduction in R_f of H1 itself (Fig. 7). This did not occur when the surface H2 molecules on 1-7-1 cells were crosslinked, in which case a concomitant and equal reduction in R_f of H1 and H2 was observed (Fig. 8). In all these cases, immobilization of H1 had no significant effect on the apparent D values measured for H2, and vice versa (Figs. 7 and 8). The parallel reduction in R_f of H1 and H2 upon crosslinking one polypeptide chain type suggests the existence of stable complexes containing both H1 and H2, while the lack of effect on the D values demonstrates the absence of detectable amounts of H1-H2 complexes that undergo association-dissociation on a time scale comparable to that of the FPR measurements (several minutes in the current experiments) (Elson and Reidler, 1979; Petersen et al., 1986; Duband et al., 1988).

Discussion

There are several indications for the existence of functional interactions between the two polypeptide chains comprising human or rat ASGP-R (McPhaul and Berg, 1986; Bischoff et al., 1988; Sawyer et al., 1988; Shia and Lodish, 1989). In the present communication, we employed antibody-mediated crosslinking combined with FPR studies on the lateral mobility of the ASGP-R polypeptides and with immunofluorescence microscopy, to explore the nature of the interactions between H1 and H2 in the functional ASGP-R complex. These studies demonstrate that H1 and H2 at the cell surface appear in mutual complexes, and that these complexes are stable for at least several minutes. Moreover, the FPR experiments on cells expressing different ratios of H1 and H2 in the plasma membrane enable us to draw conclusions on the oligomeric structure of the human ASGP-R.

Measurements of the lateral mobility of H1 or H2 in the plasma membrane of several cell types in the absence of crosslinking demonstrate relatively high mobile fractions (over 60% in transfected NIH 3T3 cell lines, and near 50% in HepG2 cells) and essentially identical D values (Fig. 5). Interestingly, H1 and H2 display similar D and R_f values whether expressed singly or together in transfected NIH 3T3 cells (Fig. 5), indicating that H1-H2 interactions do not significantly alter the lateral diffusion of the ASGP-R polypeptides. Although hetero-complex formation may increase the size of the ASGP-R complex, a limited size increase is not expected to affect the lateral diffusion rate, since theoretical considerations predict a very weak dependence (logarithmic) of the lateral diffusion of integral membrane proteins on their molecular size (Saffman and Delbruck, 1975; Saffman, 1976). The lateral motion of many cell surface proteins appears to be constrained by factors beyond the viscosity of the membrane lipid bilayer (e.g., interactions with peripheral structures on either side of the membrane, or with other molecules within the membrane bilayer; reviewed in Edidin, 1987; Jacobson et al., 1987). Therefore, the above results indicate that H1 and H2 experience rather similar mobility-restricting interactions whether expressed alone or together.

At least part of the immobile fraction observed in the FPR studies summarized in Fig. 5 is likely to be due to the ASGP-R population that appears in micropatches (Figs. 1 and 2). Since the patches do not change their position on the cells for at least 10–15 min and cannot be internalized at the low temperatures employed in the current experiments, they should be immobile on the micrometer scale of the FPR studies, as was observed for visible fluorescent patches in other systems (Schlessinger et al., 1978; Henis and Gutman, 1983; Duband et al., 1988). The nature of these patches is not clear; they could represent preferential localization of some of the ASGP-R polypeptides in coated pits, or attachment to other membrane or cellular structures. In any event, the relatively high R_f values of H1 and H2 (Fig. 5) suggest that a significant fraction of the ASGP-R is not associated with such immobile structures for periods significantly longer than the duration of the FPR experiment (Elson and Reidler, 1979; Duband et al., 1988). This suggestion is in accord with the demonstration that in both HepG2 cells and transfected NIH 3T3 cells (the 1-7-1 and 1-7 lines), the ASGP-R polypeptides are found both along smooth plasma membrane regions and in coated pits (Geuze et al., 1984; Breitfeld et al., 1985; Geffen et al., 1989).

To obtain a quantitative evaluation of the interactions between H1 and H2 in the plasma membrane, we employed a combination of lateral mobility measurements with antibody-mediated crosslinking of the ASGP-R polypeptide chains. The experiments were based on lateral immobilization of one chain type (e.g., H1) by crosslinking with specific IgG molecules, followed by measuring the effects on the lateral mobility of the other chain (e.g., H2). These experiments yielded different results on HepG2 and on 1-7-1 cells (Figs. 7 and 8). In HepG2 cells, immobilization of H1 was accompanied by a concomitant and equal reduction in the mobile fraction of H2, and vice versa. As discussed under Results (see Elson and Reidler, 1979; Petersen et al., 1986; Duband et al., 1988), this phenomenon demonstrates that within the detection limit of the experiment, all of the laterally mobile H1 and H2 molecules at the HepG2 cell surface are present in mutual complexes. The lack of effect of immobilizing one chain type on the D value of the other suggests that these complexes are stable and do not undergo dissociation and reassociation on the experimental time scale (such processes with half times up to 5–10 min would have been detected, as they would lead to a measurable recovery of the fluorescence). In 1-7-1 cells, the IgG-mediated reduction in R_f of H2 led to an identical reduction in R_f of H1 (Fig. 8), suggesting the absence of free H1 molecules not associated with H2. However, when H1 was immobilized by antibody-mediated crosslinking, the reduction in the mobile fraction of H2 was only 2/3 of the reduction in R_f of H1 (Fig. 7). This demonstrates that part of the H2 molecules on the surface of 1-7-1 cells are free of H1, and only $\sim 2/3$ of the mobile H2 molecules are complexed together with H1. Furthermore, no significant exchange of complexed with free H2 molecules occurs on the FPR experimental time scale, since the D values of H2 are not reduced upon immobilization of H1. The existence of stable H1-H2 complexes is further supported by the immunofluorescence studies, which demonstrated that antibody-mediated crosslinking of one ASGP-R chain type (e.g., H1) was accompanied by copatching of the other chain type (H2), which was not crosslinked (Fig. 3).

The lack of detectable exchange in the ASGP-R hetero-complexes is in accord with the suggestion (based on the enhanced transport of H2 to the cell surface in the presence of H1) that H1 and H2 interact already early in biosynthesis (Shia and Lodish, 1989). Some or all of the H2 molecules that are free of H1 in 1-7-1 cells could arrive at the cell surface uncomplexed with H1, since 10–20% of H2B (the H2 subtype expressed in these cells) is transported to the cell surface in the absence of H1 (Lederkremer and Lodish, 1990; Shia, M. A., and H. F. Lodish, unpublished observations). It should be noted that the conclusions on H1-H2 complex formation derived from the FPR experiments apply to the population of laterally mobile ASGP-R polypeptides; however, the colocalization of H1 and H2 in the same micropatches (which most likely represent at least part of the immobile ASGP-R population) in the absence of antibody-mediated crosslinking (Fig. 2) supports the notion that they also are comprised mostly of H1-H2 complexes.

Earlier studies based on chemical crosslinking (Bischoff et al., 1988) and on radiation target size analysis (Schwartz et al., 1984) indicate that the functional ASGP-R in HepG2 cells is at least a trimer, and H1 homo-trimers were detected in 1-7 cells expressing H1 alone (Shia and Lodish, 1989). Thus, a plausible model is that the active receptor complex consists of a core of an H1 trimer which can associate with one, two, or three H2 molecules to form the high-affinity ligand binding site(s). Multiplications of this minimal structure are also possible. The information on the stoichiometric ratio of H1 and H2 obtained in the present study strongly supports such a model. In HepG2 cells, the H1:H2 ratio at the cell surface is around 3:1 (Fig. 6), and all of the H1 and H2 molecules appear to be complexed together. This supports a 3:1 stoichiometry between H1 and H2 in the complex. In 1-7-1 cells, the relative levels of H1 and H2 at the surface are close to unity (Fig. 6), but about a third of the H2 population is free of H1, suggesting a stoichiometry of three H1 and two H2 molecules in the complex. This ratio may be an average reflecting a mixture of complexes containing H1 and H2 at 3:1, 3:2, and 3:3 ratios. This proposal is in accord with the requirement for coexpression of H1 together with H2 or of RHL-1 with RHL-2/3 in order to achieve high-affinity binding of normal asialoglycoprotein ligands at the cell surface (McPhaul and Berg, 1986; Shia and Lodish, 1989; Braiterman et al., 1989). It is tempting to assume that the high-affinity binding involves interaction of the triantennary ligands with sites on one H2 and two H1 molecules in this complex, since high-affinity binding of typical *N*-linked oligosaccharides to the ASGP-R was shown to require simultaneous interactions with at least three galactose residues (Lee et al., 1983, 1984; Lee and Lee, 1987).

This work was supported in part by grant no. 88-00014 from the U.S.-Israel Binational Science Foundation (BSF, Jerusalem, Israel) to Y. I. Henis and H. F. Lodish; by a grant from the Fund for Basic Research administered by the Israel Academy of Sciences and Humanities (to Y. I. Henis); and by National Institutes of Health grant (GM35012) to H. F. Lodish. Part of this work was undertaken during the tenure of an Eleanor Roosevelt International Cancer Fellowship supported by the American Cancer Society (to Y. I. Henis). M. A. Shia is a fellow of the Charles A. King Trust.

Received for publication 5 April 1990 and in revised form 25 June 1990.

References

- Amara, J. F., G. Lederkremer, and H. F. Lodish. 1989. Intracellular degradation of unassembled asialoglycoprotein receptor subunits: a pre-Golgi, non-lysosomal endoproteolytic cleavage. *J. Cell Biol.* 109:3315-3324.
- Ashwell, G., and J. Harford. 1982. Carbohydrate-specific receptors of the liver. *Annu. Rev. Biochem.* 51:531-554.
- Axelrod, D., D. E. Koppel, J. Schlessinger, E. L. Elson, and W. W. Webb. 1976. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* 16:1055-1069.
- Bischoff, J., and H. F. Lodish. 1987. Two asialoglycoprotein receptor polypeptides in human hepatoma cells. *J. Biol. Chem.* 262:11825-11832.
- Bischoff, J., S. Libresco, M. A. Shia, and H. F. Lodish. 1988. The H1 and H2 polypeptides associate to form the asialoglycoprotein receptor in human hepatoma cells. *J. Cell Biol.* 106:1067-1074.
- Braiterman, L. T., S. C. Chance, W. R. Porter, Y. C. Lee, R. R. Townsend, and A. L. Hubbard. 1989. The major subunit of the rat asialoglycoprotein receptor can function alone as a receptor. *J. Biol. Chem.* 264:1682-1688.
- Brandtzaeg, P. 1973. Conjugates of immunoglobulin G with different fluorochromes. I. Characterization by anionic-exchange chromatography. *Scand. J. Immunol.* 2:273-290.
- Breitfeld, P. P., C. F. Simmons, Jr., G. J. Strous, H. J. Geuze, and A. L. Schwartz. 1985. Cell biology of the asialoglycoprotein system: a model of receptor-mediated endocytosis. *Int. Rev. Cytol.* 97:47-95.
- Drickamer, K., J. F. Mamon, G. Binns, and J. O. Leung. 1984. Primary structure of the rat liver asialoglycoprotein receptor. *J. Biol. Chem.* 259:770-778.
- Duband, J.-L., G. H. Nuckolls, A. Ishihara, T. Hasegawa, K. M. Yamada, J. P. Thiery, and K. Jacobson. 1988. Fibronectin receptor exhibits high lateral mobility in embryonic locomoting cells but is immobile in focal contacts and fibrillar streaks in stationary cells. *J. Cell Biol.* 107:1385-1396.
- Edidin, M. 1987. Rotational and lateral diffusion of membrane proteins and lipids: phenomena and function. *Curr. Top. Membr. Transp.* 29:91-127.
- Eldridge, C. A., E. L. Elson, and W. W. Webb. 1980. Fluorescence photobleaching recovery measurements of surface lateral mobilities on normal and SV 40-transformed mouse fibroblasts. *Biochemistry.* 19:2075-2079.
- Elson, E. L., and J. A. Reidler. 1979. The analysis of cell surface interactions by measurements of lateral mobility. *J. Supramol. Struct.* 12:481-489.
- Geffen, I., H. P. Wessels, J. Roth, M. A. Shia, and M. Spiess. 1989. Endocytosis and recycling of subunit H1 of the asialoglycoprotein receptor is independent of oligomerization with H2. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2855-2861.
- Geuze, H. J., J. W. Slot, G. J. Strous, H. F. Lodish, and A. L. Schwartz. 1983a. Intracellular site of asialoglycoprotein receptor-ligand uncoupling: double-label immunoelectron microscopy during receptor-mediated endocytosis. *Cell.* 32:277-287.
- Geuze, H. J., J. W. Slot, G. J. Strous, and A. L. Schwartz. 1983b. The pathway of the asialoglycoprotein-ligand during receptor-mediated endocytosis: a morphological study with colloidal gold/ligand in the human hepatoma cell line, HepG2. *Eur. J. Cell Biol.* 32:38-44.
- Geuze, H. J., J. W. Slot, G. J. A. M. Strous, J. Peppard, K. von Figura, A. Hasilik, and A. L. Schwartz. 1984. Intracellular receptor sorting during endocytosis: comparative immunoelectron microscopy of multiple receptors in rat liver. *Cell.* 37:195-204.
- Geuze, H. J., J. W. Slot, and A. L. Schwartz. 1987. Membranes of sorting organelles display lateral heterogeneity in receptor distribution. *J. Cell Biol.* 104:1715-1723.
- Gunther, C., J. L. Wang, I. Yahara, B. A. Cunningham, and G. M. Edelman. 1973. Concanavalin A derivatives with altered biological activities. *Proc. Natl. Acad. Sci. USA.* 70:1012-1016.
- Halberg, D. F., R. E. Wager, D. C. Farrell, J. Hildreth, M. S. Quesenberry, J. A. Loeb, E. C. Holland, and K. Drickamer. 1987. Major and minor forms of the rat liver asialoglycoprotein receptor are independent galactose-binding proteins: primary structure and glycosylation heterogeneity of minor receptor forms. *J. Biol. Chem.* 262:9828-9838.
- Henis, Y. I., and O. Gutman. 1983. Lateral diffusion and patch formation of H-2K^b antigens on mouse spleen lymphocytes. *Biochim. Biophys. Acta.* 762:281-288.
- Henis, Y. I., O. Gutman, and A. Loyter. 1985. Sendai virus envelope glycoproteins become laterally mobile on the surface of human erythrocytes following fusion. *Exp. Cell Res.* 160:514-526.
- Jacobson, K., A. Ishihara, and R. Inman. 1987. Lateral diffusion of proteins in membranes. *Annu. Rev. Physiol.* 49:163-175.
- Knowles, B. B., C. C. Howe, and D. P. Aden. 1980. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science (Wash. DC).* 209:497-499.
- Koppel, D. E., and M. P. Sheetz. 1981. Fluorescence photobleaching does not alter the lateral mobility of erythrocyte membrane glycoproteins. *Nature (Lond.)* 293:159-161.
- Koppel, D. E., D. Axelrod, J. Schlessinger, E. L. Elson, and W. W. Webb. 1976. Dynamics of fluorescence marker concentration as a probe of mobility. *Biophys. J.* 16:1315-1329.
- Lederkremer, G. Z., and H. F. Lodish. 1990. An alternatively spliced minixon alters the subcellular fate of the human asialoglycoprotein receptor H2 subunit: endoplasmic reticulum retention and degradation or cell surface expression. *J. Biol. Chem.* In press.
- Lee, R. T., and Y. C. Lee. 1987. Affinity labeling of the galactose/N-acetyl galactosamine-specific receptor of rat hepatocytes: preferential labeling of one of the subunits. *Biochemistry.* 26:6320-6329.
- Lee, R. T., P. Lin, and Y. C. Lee. 1984. New synthetic cluster ligands for galactose/N-acetylgalactosamine-specific lectin of mammalian liver. *Biochemistry.* 23:4255-4261.
- Lee, Y. C., R. R. Townsend, M. R. Hardy, J. Lonngren, J. Arnap, M. Haraldson, and H. Lonn. 1983. Binding of synthetic oligosaccharides to the hepatic Gal/GalNac lectin. *J. Biol. Chem.* 258:199-202.
- McPhaul, M., and P. Berg. 1986. Formation of functional asialoglycoprotein receptor after transfection with cDNAs encoding the receptor proteins. *Proc. Natl. Acad. Sci. USA.* 83:8863-8867.
- Petersen, N. O., S. Felder, and E. L. Elson. 1986. Measurement of lateral diffusion by fluorescence photobleaching recovery. In *Handbook of Experimental Immunology*. D. M. Weir, L. A. Herzenberg, C. C. Blackwell, and L. A. Herzenberg, editors. Blackwell Scientific Publications Ltd., Edinburgh. 24.1-24.23.
- Saffman, P. G. 1976. Brownian motion in thin sheets of viscous fluid. *J. Fluid Mech.* 73:593-602.
- Saffman, P. G., and M. Delbruck. 1975. Brownian motion in biological membranes. *Proc. Natl. Acad. Sci. USA.* 72:3111-3113.
- Sawyer, J. T., J. P. Sanford, and D. Doyle. 1988. Identification of a complex of the three forms of the rat liver asialoglycoprotein receptor. *J. Biol. Chem.* 263:10534-10538.
- Schlessinger, J., Y. Shechter, M. C. Willingham, and I. Pastan. 1978. Direct visualization of binding, aggregation, and internalization of insulin and epidermal growth factor on living fibroblast cells. *Proc. Natl. Acad. Sci. USA.* 75:2659-2663.
- Schwartz, A. L. 1990. Asialoglycoprotein receptor: intracellular fate of receptor and ligand. In *Intracellular Transport*. J. Hanover and C. J. Steer, editors. Cambridge University Press, Cambridge, UK. In press.
- Schwartz, A. L., L. J. Steer, and E. S. Kempner. 1984. Functional size of the human asialoglycoprotein receptor as determined by radiation inactivation. *J. Cell Biol.* 259:12025-12028.
- Shia, M. A., and H. F. Lodish. 1989. The two subunits of the human asialoglycoprotein receptor have different fates when expressed alone in fibroblasts. *Proc. Natl. Acad. Sci. USA.* 86:1158-1162.
- Spiess, M., and H. F. Lodish. 1985. Sequence of a second human asialoglycoprotein receptor: conservation of two receptor genes during evolution. *Proc. Natl. Acad. Sci. USA.* 82:6465-6469.
- Spiess, M., A. L. Schwartz, and H. F. Lodish. 1985. Sequence of human asialoglycoprotein receptor cDNA: an internal signal sequence for membrane insertion. *J. Biol. Chem.* 260:1979-1982.
- Wolf, D. E., M. Edidin, and P. R. Dragsten. 1980. Effect of bleaching light on measurements of lateral diffusion in cell membrane. *Proc. Natl. Acad. Sci. USA.* 77:2043-2045.