Mammalian hepatic asialoglycoprotein receptors (ASGP-R) are composed of two unique, but closely related polypeptides, which in the rat are designated rat hepatic lectins 1 and 2/3 (RHL 1, RHL 2/3). Despite numerous efforts to define a functional ASGP-R has remained unclear. We examined this question in rat hepatoma tissue culture (HTC) cells (which lack endogenous ASGP-R) that were co-transfected with cDNAs for both RHL 1 and RHL 2/3. The original population was cloned, but derivatives were unstable. We therefore used fluorescence-activated cell sorting to separate a subpopulation of cells (positive) that specifically endocytosed fluoresceinated asialo-orosomucoid (ASOR) from one that did not (negative). We then used indirect immunofluorescence with polypeptide-specific ASGP-R antibodies, immunoanalysis, and binding and uptake studies with two Gal ligands (ASOR and NAc-galactosylated poly-L-lysine (Gal-Lys)) to further define the ASGP-R status in these two populations. As reported by others, we found that both RHL 1 and RHL 2/3 in the positive cells resulted in binding, uptake and degradation of ASOR, the most commonly used ASGP-R ligand. The negative cells expressed only RHL 1 and neither bound nor processed ASOR. However, the presence of RHL 1 was sufficient for specific affinity binding and processing of the synthetic ligand, Gal-Lys, by negative cells. These results show that RHL 1 can function as an ASGP-R, given a highly galactosylated ligand, and that RHL 2/3 must play an important role in the organization of native ASGP-R in the membrane.

Mammalian hepatic asialoglycoprotein receptors (ASGP-R)* mediate the specific endocytosis and lysosomal degradation of a wide variety of glycoconjugates, all of which bear either a terminal galactose (Gal) or N-acetylgalactosamine (GalNAc) residue (for reviews, see Ashwell and Harford, 1982; Breitfeld et al., 1985). The basis of the ligand-receptor interaction has been investigated through biochemical characterization of the receptor, examination of the structural organization of ligands that bind the receptor, and cDNA transfection experiments.

The ligand affinity-purified ASGP-R of rat liver is composed of three polypeptides, a major subunit called rat hepatic lectin 1 (RHL 1) at 42,000 daltons and two minor subunits, RHL 2 and RHL 3, at 49,000 and 54,000 daltons, respectively. Examination of primary sequence (Drickamer et al., 1984) has indicated that the three polypeptides are closely related, and molecular cloning studies have identified two unique, full length ASGP-R cDNAs (Holland et al., 1984; Halberg et al., 1987). RHL 2 and 3 are products of one gene and presumably differ only in the presence of polypeptide amino acid sequences on RHL 3 (Halberg et al., 1987), giving rise to the RHL 2/3 designation. The major difference between RHL 1 and RHL 2/3, each of which has only one transmembrane segment, is the presence of a unique three-amino acid sequence near the NH-terminal cytoplasmic domain of the latter. Drickamer and colleagues have shown that each of the three RHL subunits has at least one cytoplasmic carbohydrate recognition domain (Halberg et al., 1987).

Despite extensive work, the precise composition of a functional ASGP-R has remained elusive. Binding studies using defined synthetic oligosaccharides, glycopeptides, and glycoproteins have resulted in a model for the ASGP-R in which occupancy of three Gal/GalNAc combining sites (a triad) is required for high affinity binding (Lee et al., 1984b; Townsend, 1987, for review). Results of cross-linking experiments to assess the oligomeric state of the rat ASGP-R have indicated that RHL 1 and RHL 2/3 are not physically linked and thus may be independent galactose-binding proteins (Halberg et al., 1987). However, more recent studies using antibody-induced surface receptor loss as well as chemical cross-linking suggest that the human analogues of RHL 1 and 2/3 are physically associated with each other in HepG2 cells (Bischoff et al., 1988). Finally, results of cDNA transfection experiments have indicated that both rat subunits are essential for receptor function (McPhaul and Berg, 1986).

In this study, we have examined two sub-populations of rat hepatoma tissue culture cells (HTC) derived from a population originally transfected with cDNA’s for both RHL 1 and 2/3 (McPhaul and Berg, 1986). We show that one subset of cells, which represents only the RHL 1 polypeptide, has lost its ability to bind ASOR but expresses a high affinity for, internalizes, and degrades a highly galactosylated synthetic ligand. Thus, RHL 1 can function as a receptor in the absence of RHL 2/3, but its organization in the membrane must be different than that of the native ASGP-R, implying that RHL
RHL 1 Functions as a Receptor

2/3 and RHL 1 do associate with one another when both are present.

EXPERIMENTAL PROCEDURES

Materials

Reagents were obtained from the following sources: Na[I][125I], [H]thymidine, and PD-10 columns containing Sephadex G-25M from Amersham Corp.; orosomucoid, poly-L-lysine with a degree of polymerization of 240, Fluorescein isothiocyanate (FITC), digitonin (composition 80%), p-phenylenediamine dihydrochloride, and N-acetylgalactosamine from Sigma; Durbecco's modified Eagle's medium (DMEM), Geneticin, and 0.05% trypsin, 0.02% EDTA from Gibco; fetal calf serum from Hyclone Laboratories; FITC-goat anti-rabbit from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD); Aqausol and Protosol from Du Pont-New England Nuclear; and Labtek chamber slides from Miles Scientific. Orosomucoid was dside-vlated to yield the asialo-derivative (ASOR) (Wall et al., 1980).

For immunofluorescence studies, we used two antisera: one designated anti-RHL 1 that has been described (Mueller and Hubbard, 1986) and reacts with RHL 1 by immunoblot; the second, designated anti-RHL 2/3 was the kind gift of Kurt Drickamer (Columbia University College of Physicians and Surgeons) and reacts with RHL 2/3 by immunoblot (Haiberg et al., 1987). When these antisera were analyzed by immunofluorescence using cells that were singly transfected with CDNAs to RHL 1 and RHL 2, we found that each antibody was reactive with both cell types. To deplete each antibody of cross-reactive determinants, purified RHL 1 (240 µg) and RHL 2/3 (85 µg) prepared as described (Haiberg et al., 1987), were each coupled to 200 µl of CNBr-activated Sepharose 4B beads as described (March, 1974).

Antibody Preparation

For immunofluorescence studies, we used two antisera: one designated anti-RHL 1 that has been described (Mueller and Hubbard, 1986) and reacts with RHL 1 by immunoblot; the second, designated anti-RHL 2/3 was the kind gift of Kurt Drickamer (Columbia University College of Physicians and Surgeons) and reacts with RHL 2/3 by immunoblot (Haiberg et al., 1987). When these antisera were analyzed by immunofluorescence using cells that were singly transfected with CDNAs to RHL 1 and RHL 2, we found that each antibody was reactive with both cell types. To deplete each antibody of cross-reactive determinants, purified RHL 1 (240 µg) and RHL 2/3 (85 µg) prepared as described (Haiberg et al., 1987), were each coupled to 200 µl of CNBr-activated Sepharose 4B beads as described (March, 1974). The beads were incubated with 7% gelatin in 0.1 M NaCl, 0.025 M NaNO3, pH 7.4 (gel-PBS), for 20 min at 22°C and recovered by centrifugation at 500 × g for 10 min (Beckman TJ-6R, Beckman Instruments, Inc., Palo Alto, CA). Whole 2/3 antiserum (1.0 ml at 1:40 dilution in 0.02% NaN₃ (w/v)-PBSa (PBSa)) and affinity-purified anti-RHL 1 (1.0 µl at 20 µg/ml in PBSa containing 0.1% BSA) were mixed with 200 µl of RHL 1-Sepharose or RHL 2/3-Sepharose, respectively, and incubated for 16 h at 4°C on a wheel. The beads were recovered by centrifugation as described above, the supernates retained and designated "anti-2/3-depleted of RHL 1 determinants (anti-2/3D)" and "anti-RHL 1 depleted of RHL 2/3 determinants (anti-1D )."

For the immunoblot analysis, we used anti-RHL 2/3 and an antibody raised in rabbits to detergent-solubilized and ASOR affinity-purified ASGP-R (Kawasaki and Ashwell, 1976). This antiserum, designated anti-ASGP-R 6, reacted with each of the ASGP-R subunits on immunoblots (see Fig. 3).

Identification of ASGP-receptor Subunits in BC6 Cells

Immunofluorescence Analysis—Chamber slides were coated with poly-L-lysine (200 µg/slide according to manufacturer's instructions) and then plated with 5 × 10⁵ cells. After 2 days in culture, the cell monolayers were fixed (20 min, 2% paraformaldehyde, 0.075 M lysine, 0.01 M NaOH, 0.037 M NaPO₄, pH 7.4). The slides were subsequently processed at 22°C with 0.22 µm filtered solutions and 5 min incubations except as indicated. The cell monolayers were rinsed twice in PBS, quenched in 0.25% NH₄Cl (w/v)-PBS, permeabilized with 0.06% digitonin-PBS, and then nonspecific sites blocked with 0.2% gel-PBS. The first antibody (either anti-1D (10 µg/ml), anti-2/3D (1:120) or diluent (0.2% gel-PBS)) was applied for 15 min followed by three rinses in PBS. The second antibody (FITC-goat anti-rabbit in 0.2% gel-PBS) was then applied for 15 min, followed again by three rinses in PBS. The slides were mounted with freshly prepared anti-fade solution (Johnson et al., 1982) made with 25% glycerol. Cells were observed by epifluorescence and phase contrast with an Axioscop Universal Microscope (Zeiss, West Germany). Photographs were taken with Kodak T MAX 400 film at an ASA setting of 800.

Ligands

ASOR and Nac-Tyr-Gal₃αpoly-L-lysine (Gal-Lys) were iodinated using the chloramine-T method as described (Greenwood et al., 1963). The specific activities for both ligands were 3–6 µCi/µg of protein. ASOR was fluoresceinated (F-ASOR) as follows. ASOR (1 mg) and FITC (5 mg) in 2.6 ml of 0.35 M NaHCO₃, pH 9, were incubated at 23°C for 4 h, and then at 4°C for 16 h in the dark. F-ASOR was recovered in the void volume of a PD-10 column equilibrated and run in 0.15 M NaCl, 0.02 M NaPO₄, pH 7.4.

Endocytosis Studies

F-ASOR as Ligand—Routine, 5 × 10⁵ cells were plated in 35-mm wells and grown for 2 days. The cell monolayers were rinsed with DMEM, and then 1 µg/ml F-ASOR in DMEM was added. After incubation at 37°C for 2.5–5 h, the cells were rinsed two times with GKN, removed from the wells with 0.05% trypsin, 0.02% EDTA, and reseeded in 10% fetal calf serum/dMEM. 100-fold excess unlabelled ASOR in the presence of F-ASOR was added to assess nonspecific uptake by the cells. The radioactivity in the cells then was analyzed in an EPICS 752 flow cytometer (Coulter Electronics Inc., Hialeah, FL). When positive and negative populations were separated, the labeled cells were analyzed in a FACS II flow cytometer (Becton-Dickinson FACS Systems, Mountain View, CA). To prevent ligand degradation, the cell monolayers were rinsed twice with 0.01 M NaHCO₃, pH 9, and then scraped into 0.1 M NaOH. A 100-fold excess of unlabelled ASOR was added to assess nonspecific uptake of [125I] ASOR. Nonspecific uptake of [125I]-Gal-Lys was determined by incubation of the cells for 10 min in 25 mM GalNIC in DMEM + 0.1% BSA, prior to addition of the [125I]-Gal-Lys.

Binding Studies

Surface and total ASGP-R were detected using a modification of the digitonin permeabilization assay as described previously (Weigel et al., 1983). Cells were plated as described for the endocytosis studies. The medium was removed and replaced with 2 ml of DMEM. The cell monolayers were incubated at 37°C for 4 h, then at 4°C for 30 min. After one rinse in DMEM-0.1% BSA, the radioactive ligand in DMEM-0.1% BSA without or with 0.075% (w/v) digitonin was added. After incubation at 4°C for 2.5–4 h, the radioactive medium
was removed, the cell monolayer rinsed twice with DMEM ± 0.1% BSA, twice with DMEM, then scraped into 0.1 M NaOH. The radioactivity in the cell lysates plus two rinses from each plate was determined as above. Nonspecific binding of 125I-ASOR was defined as binding in the presence of 100-fold excess of unlabeled ASOR. Nonspecific binding was determined by incubating the cells for 10 min in 25 mM GalNAc in DMEM ± 0.1% BSA with or without 0.075% (w/v) digitonin before addition of the 125I-Gal-Lys.

Since soluble protein was lost by digitonin permeabilization (Weigel et al., 1983) and cells were lost during the rinsing procedure after digitonin treatment, we determined the number of cells remaining in digitonin-treated monolayers as follows. The cell number from untreated wells was determined by measuring total cell protein (Lowry et al., 1951) (357 μg of protein/1 × 10⁶ HTC cells). The resulting cell number was multiplied by the fraction of incorporated [3H]thymidine remaining in digitonin-treated wells (40–60%). This latter value was obtained from cells in companion wells (to those used for 125I-ligand binding) that were labeled with 0.5 μCi of [3H]thymidine over a 16-h period just prior to the binding studies. These wells were processed with or without digitonin as for the radioactive ligand, except that the 125I-ligand was omitted. Aliquots of cell lysates were precipitated in 10% (w/v) trichloroacetic acid, the precipitates solubilized in Protosol according to the manufacturer’s instructions and the radioactivity determined (LS 7000, Beckman). The amount of incorporated [3H]thymidine remaining in digitonin-treated cells was compared to that in untreated cells and expressed as a fraction, which was used to obtain accurate cell numbers for those treated with digitonin.

** Autoradiography **

Cells were plated on uncoated chamber slides and grown for 2 days. For the 4 °C binding studies, cells were incubated with 0.5 μg/ml 125I-ASOR or 2 μg/ml 125I-Gal-Lys in the presence and absence of digitonin as described above. After rinsing, the cells were fixed for 30 min in 2% glutaraldehyde, 0.1 M sodium cacodylate, 2 mM CaCl₂, pH 7.4. The slides were brought to room temperature during fixation, rinsed for 5 min in 0.1 M sodium cacodylate then dehydrated in a graded EtOH series and allowed to dry-air prior to emulsion coating.

Endocytosis experiments were carried out at 37 °C. Cells were rinsed with DMEM, incubated for 4 h with one of the 125I-ligands, rinsed four times with DMEM at 4 °C, then incubated for 15 min at 4 °C with 2 ml of GKN ± 2 mM EGTA, pH 7.4. Nonspecific binding was assessed using 25 mM GalNAc or excess ASOR as described above. The cells were fixed, dehydrated, and dried. Processed slides were coated with KS emulsion, exposed for 3–8 days and developed as described earlier (Zeitlin and Hubbard, 1982). They were mounted, examined on a Zeiss photomicroscope, and photographed as described above.

** RESULTS **

Desialylated plasma glycoproteins have been used routinely in studies of ASGP-R-mediated endocytosis. ASOR was reported by Ashwell and colleagues to exhibit one of the highest affinities for this receptor among the many naturally-occurring plasma glycoproteins tested (Pricer and Ashwell, 1971) and consequently has been the ligand used most frequently to study this receptor. McPhaul and Berg (1986) used the uptake of F-ASOR as a criterion for expression of a functional ASGP-R and found that transfection of both RHL 1 and RHL 2 cDNAs into HTC cells was required to obtain such expression. In their study, doubly transfected cells were incubated with F-ASOR at 37 °C for 12 h and then analyzed by FACS. Cells exhibiting the brightest fluorescence (top 8%) were collected, grown up for 2 weeks, resorted, and the top 8% again selected. Upon obtaining this sorted population from McPhaul, we examined them by light microscopic immunofluorescence using affinity-purified antibody to RHL 1 (not depleted of RHL 2/3 cross-reactive determinants) and found a variable level of staining (data not shown), indicating that the quantity of receptor from cell to cell was quite different. To obtain a homogeneous population, we cloned the doubly transfected cells. Clones were selected by their ability to bind 125I-ASOR at 4 °C, and one with high ligand-binding activity, BC6, was analyzed further. Upon passage in culture, the level of 125I-ASOR bound to BC6 cells decreased (data not shown). When the BC6 cells, passage 13, were analyzed in the FACS for their ability to accumulate F-ASOR at 37 °C, a bimodal distribution of fluorescence intensity was observed (Fig. 1A). One population accumulated significant amounts of F-ASOR in a ligand-specific manner (Fig. 1B) and was designated ASOR-positive. One population did not take up the ligand, since its fluorescence was coincident with cells that were incubated with unlabeled competitor and F-ASOR. We designated this the ASOR-negative population (negative, Fig. 1C). As reported previously (McPhaul and Berg, 1986), we found that the parent HTC cell line did not appear to take up the ligand (data not shown). Cells from the top 45% and the bottom 45% were separated (Fig. 1A), and the two populations were characterized further by immunological procedures, binding and uptake studies, and light microscopic autoradiography. Due to the instability of these transfected populations, all studies reported here were performed using cells passaged from two to six times after FACS separation.

** Identification of ASGP-receptor Subunits in Positive and Negative Cells **

**Immunofluorescence **—Indirect immunofluorescence was performed on positive and negative cells using subunit-specific immunological reagents. As shown in Fig. 2, both positive (panel A) and negative (panel B) cells reacted with RHL 1-specific antibody. Upon examination of cells with RHL 2/3-specific antibody, the positive cells showed reactivity (panel C); however, the negative cells gave a signal that was close to background (panel D). A small percentage, usually 2–5%, but sometimes up to 10%, of the negative cells (passages 2–6) reacted with anti-2/3D, giving a signal similar to that found on the positive cells (data not shown). These cells were judged to be a small spillover of positive cells within the negative population. Therefore, the ASOR-positive cells expressed

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**Fig. 1. F-ASOR accumulation by BC6 cells (A) and by ASOR-positive (Pos) (B) and ASOR-negative (Neg) (C) populations.** The cells were labeled with F-ASOR and analyzed by FACS as described under "Experimental Procedures." A. cells in the two regions indicated by the solid bars (top 45%, ASOR-Pos, and bottom 45%, ASOR-Neg) were collected, grown up, and used for further analysis. B, and C, analysis of the positive (B) and negative (C) populations in the presence and absence of a 100-fold excess unlabeled ASOR.
both subunits, while the ASOR-negative cells expressed only RHL 1. When present, labeling was detected at both the cell surface and in a perinuclear location.

**Immunoblot Analysis**—To determine the levels of RHL 1 and RHL 2/3 in the two populations, we applied anti-ASGP-R 6 and anti-RHL 2/3 antibodies to nitrocellulose transfers of purified rat liver ASGP-R and HTC cell extracts (Fig. 3). The anti-ASGP-R 6 antibody bound to each subunit from a purified ASGP-R preparation, but in a slightly different ratio than did Coomassie Blue R (Fig. 3A). Less RHL 2/3 was detected on immunoblots than was present on protein-stained gels, giving RHL 1 to 2/3 ratios of 4:1 by immunoblot but 3:1 by Coomassie blue. Thus, the level of RHL 2/3 that we detected by immunoblot may be an underestimate of the quantity actually present. When the positive cell homogenate was examined (Fig. 3B), RHL 1 and RHL 2 had comparable mobilities to those from a rat liver homogenate used as a standard (lane not shown). Low levels of RHL 3 were detected in the positive cells (see Fig. 3C for clarification). By immunoblot, the stoichiometry of RHL 1 to RHL 2/3 in the positive population was 4:1, which is comparable to that in both the purified receptor and homogenate from rat liver.

When the negative cell homogenate was examined, a sig-
 significant amount of the RHL 1 subunit was detected (Fig. 3B). By densitometry, the level of RHL 1 in negative cells was 33% of that in the positive cells. There was no detectable RHL 2 or 3, even when four times more cell protein was analyzed. The high level of RHL 2 and RHL 3 detected by immunolocalization in 2–5% of the negative cells was undetectable at the level of cell homogenate used for immunoblotting.

The presence and absence of RHL 2/3 in the two HTC cell populations were confirmed by immunoblot analysis using anti-RHL 2/3 antibody (Fig. 3C). When RHL 2/3 subunit specific antibody was applied to nitrocellulose transfers of rat liver and HTC cell extracts, again there was no specific reactivity to proteins in extracts of the negative cells. However, RHL 2 and RHL 3 subunits were clearly present in both the liver and positive cell homogenates. The mobilities of RHL 2 and 3 in the positive cells corresponded to bands identified using anti-ASGP-R antibody 6 (Fig. 3B). With either antibody, RHL 3 from the positive cells appeared as a broad diffuse band with slower mobility than RHL 3 identified in liver.

**Binding and Uptake Studies**

Biochemical Analysis—We compared the 4 °C binding properties of the positive (RHL 1 + RHL 2/3) and negative (RHL 1 only) cells using ASOR and Gal-Lys, a high affinity synthetic ligand composed of a 40,000-dalton poly-l-lysine polymer to which an average of 100 galactose moieties were attached (Lee et al., 1987). This ligand binds to the ASGP-R with a 10-fold lower $K_d$ than does ASOR on isolated hepatocytes and presumably has more degrees of freedom for binding due to the high density of galactosyl residues and multiple confor mations attributable to flexible aglycon and lysine side chains.

When the total ligand binding activities in positive and negative populations were examined with 125I-ligands at 4 °C in the presence of digitonin, we found that the positive cells specifically bound 125I-Gal-Lys as well as 125I-ASOR. A representative Scatchard plot is shown in Fig. 4, and Table I summarizes information from multiple experiments. In the positive population, we measured ~390,000 specific binding sites/cell with 125I-ASOR ($K_d = 24 \pm 8.4 \times 10^{-9}$ M) and 340,000 specific binding sites/cell with 125I-Gal-Lys ($K_d = 3.6 \pm 1.8 \times 10^{-9}$ M). The binding sites for the two ligands appeared to be largely overlapping, since ASOR added before 125I-Gal-Lys effectively blocked (70–80%) the latter’s association with positive cells (data not shown). The negative population also bound 125I-Gal-Lys with a $K_d$ of 2.3 ± 0.9 × 10^{-9} M, which is in good agreement with that of the positive cells. However, we detected only 51,000 specific 125I-Gal-Lys-binding sites/cell or ~15% of the binding sites found in the positive cells. When the negative cells were examined using 125I-ASOR, <2% of the binding sites found in the positive cells were detected, even at 125I-ASOR concentrations as high as 400 nM.

The distribution of binding sites between the cell surface and intracellular sites was next determined for 125I-ASOR in the positive cells and for 125I-Gal-Lys in both cell populations. In all cases, ~20% of the total ligand-binding sites were found at the surface of both positive and negative cells (i.e. sites detected in the absence of digitonin), with the remaining 80% found inside (data not shown). These distributions are comparable to those found for isolated in situ hepatocytes (Weigel et al., 1983; Geuze et al., 1983).

We next examined the uptake and degradation at 37 °C of the 125I-ligands in both cell populations. 41,000 125I-ASOR molecules/cell were processed by the negative cells after 21 h of exposure to the ligand, which represented only 0.8% of the amount of 125I-ASOR processed by the positive population (data not shown). In contrast, we were able to detect significant levels of 125I-Gal-Lys uptake by the negative cells (Fig. 5). After only 90 min of exposure to the ligand, the negative cells had processed 35,000 molecules/cell, or ~10 times more than the number of 125I-ASOR molecules endocytosed by the same cells per unit of time. When the uptake of 125I-Gal-Lys by positive and negative cells was compared, the latter processed 16% that of the former, which is in agreement with the difference in 4 °C binding of 125I-Gal-Lys between the two cell populations. Both the time course of endocytosis and number of molecules endocytosed by the positive cells were comparable for both ligands (Fig. 5). Although we detected little degradation of 125I-Gal-Lys in either population after only 90 min of continuous endocytosis, degradation had occurred by later time points (data not shown).

Morphological Analysis—To determine the proportion of cells in the negative population that expressed an RHL capable of binding and processing ligand, we quantitated 125I-Gal-Lys and 125I-ASOR 4 °C binding and 37 °C uptake by light microscopic autoradiography. The results are presented

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1 R. T. Townsend and Y. C. Lee, unpublished results.
negative mental Procedures." The specific activities of '*'I-ASOR and 125I-Gal-uptake by the cells was calculated from the difference between the amounts of  '*'I-ligand taken up in the absence and presence of Lys were 5.5 \times 10^6 and 7.5 \times 10^6 cpm/mg, respectively. The specific uptake by the cells was calculated from the difference between the amounts of  '*'I-ligand taken up in the absence and presence of competitor.

FIG. 5. Endocytosis of 125I-ligands by positive (Pos) and negative (Neg) cells. Cell monolayers were incubated with 1 \mu g/ml 125I-ASOR and 0.1 \mu g/ml 125I-Gal-Lys as described under "Experimental Procedures." The specific activities of 125I-ASOR and 125I-Gal-Lys were 5.5 \times 10^6 and 7.5 \times 10^6 cpm/mg, respectively. The specific uptake by the cells was calculated from the difference between the amounts of 125I-ligand taken up in the absence and presence of competitor.

FIG. 6. Autoradiography of BC6 cells exposed to 125I-ligands at 4 and 37°C. Positive (POS, column 1) and negative BC6 (NEG, columns 2 and 3) cells were exposed for 4 h to 125I-ASOR (A, A', A") or 125I-Gal-Lys (G-L; B, B', B") at 4°C in the presence of digitonin or for 2 h to 125I-Gal-Lys at 37°C. In the 37°C experiment, cells were cooled to 4°C and the surface-bound ligand stripped with EGTA (see "Experimental Procedures"). The cells were fixed and processed for light level autoradiography. The developed grains represent 125I-ligands. It is apparent that the positive cells (column 1) recognize and internalize both 125I-ASOR (A) and the synthetic 125I-Gal-Lys (B, C), whereas the negative cells bind and internalize only the latter (A', B', C'). The controls in column 3 (A", B", C") were exposed to 125I-ASOR and GalNac (B", C", G-L + Gnac) was added, demonstrate that binding and uptake are specific. The high levels of background labeling in B, B" and C, C" reflect a 10-fold greater amount of radioactivity used for 125I-Gal-Lys than for 125I-ASOR and 8-day (in B and C rows) versus 3-day (in A) exposures. Each panel depicts >6 cells except C", where only 2 are seen. \times 300.

in Fig. 6. A majority (96%) of the negative BC6 cells not only exhibited substantial levels of specific Gal-Lys-binding activity at 4°C (Fig. 6B') but the majority (80%) was also capable of internalizing this ligand at 37°C (Fig. 6C'). The negative cells neither bound nor internalized significant amounts of 125I-ASOR under the conditions used.

**Discussion**

In the present study, we have demonstrated that RHL 1, the major subunit of the rat ASGP-R, can function as a carbohydrate receptor in the absence of RHL 2/3. The key to this finding was use of Gal-Lys, a ligand that contains 5–10 times more and differently organized galactosyl residues than does ASOR. We showed that cells expressing only the RHL 1 polypeptide were capable of binding, internalizing, and degrading this ligand. In agreement with the results of McPhaul and Berg (1986), we found that these cells did not recognize ASOR. Our results imply that: 1) the structure of the Gal-ligand dictates whether both subunits are required for binding; 2) the RHL 2/3 subunit plays an important role in the organization of native ASGP-R in the membrane; 3) the native rat ASGP-R is a functional hetero-oligomer; and 4) the biosynthesis, assembly, and participation in endocytosis of RHL 1 polypeptides do not depend on expression of RHL 2/3.

**Ligand Requirements for High Affinity Binding**—Drickamer and colleagues have demonstrated that both rat ASGP-R subunits have Gal-binding sites, as defined by the ability of each isolated polypeptide to bind specifically to Gal-Sepharose (Halberg et al., 1987). However, the binding of glycoproteins to the intact ASGP-R in a biological membrane is most likely different and clearly more complicated than the interaction of a solubilized receptor or individual subunits with immobilized Gal residues for the following reasons. First, glycoproteins bind the intact ASGP-R with nanomolar affinity while monosaccharides bind it with affinities in the millimolar range (Lee et al., 1983). Second, glycoproteins bear multiple oligosaccharide chains of the complex type, each located at a specific glycosylation site and each having two to four Gals (Schmid et al., 1979). ASOR, which is the most commonly used ASGP-R ligand, has five N-linked oligosaccharides, exhibits considerable microheterogeneity at each glycosylation site and therefore represents a mixture of glycoforms bearing an average of 10–25 galactosyl residues per polypeptide (Graham, 1972). Finally, the two non-identical ASGP-R polypeptides, RHL 1 and RHL 2/3, whose expressions and functions have been the focus of this study, most likely have different affinities for various Gal-structures, but these are currently unknown, as are their associations with one another in the membrane (see the Introduction).

Lee and colleagues have clarified understanding of this complicated receptor-ligand interaction by using ligands of known structure to define the requirements for high affinity binding (K_d = 0.5 > 5 nM) to the ASGP-R on the surface of intact hepatocytes (reviewed by Townsend, 1987). They have proposed that the minimal "functional" unit of the ASGP-R is a triad of Gal-binding sites (Hardy et al., 1985; Lee et al., 1984). Two of the three binding sites in the triad are spaced ~15 Å apart in the membrane while the third site may be mobile to a limited (30 Å) extent. They also have proposed that the triads are themselves clustered in the membrane, since they found additional binding sites for a small triantennary glycopeptide on hepatocytes saturated with ASOR (Hardy et al., 1985). Both this higher level of triad organization in the membrane, as well as the triad itself, are most likely disorganized when the ASGP-R is solubilized, because there is more of a reduction (~100-fold) in affinity for triantennary oligosaccharides than for glycoproteins such as ASOR (~16-fold), which have more Gals in more orientations and...
thus can still make the essential three point interactions (Lee et al., 1984a).

A Proposed Role for RHL 2/3 in ASGP-R Organization.—In the present study we confirmed the results of McPhaul and Berg (1986) that HTC cells containing only RHL 1 do not bind ASOR. This suggests that RHL 2/3 is needed, but for what function? Since immunofluorescence showed that RHL 1 was at the surface of these cells, RHL 2/3 must not be serving a delivery function. However, since RHL 1 alone is able to bind Gal-Sepharose (Halberg et al., 1987), we reasoned that an active RHL 1 in these negative cells might recognize molecules such as Gal-lys that have a higher density of Gal residues (100 Gal residues per 250 lysine residues) than does ASOR. Indeed, Gal-lys exhibited nanomolar affinity when bound to cells bearing only the RHL 1 polypeptide. Such binding is understandable if RHL 1 subunits were clustered in such a way as to allow the essential three interactions by the multiple Gal of Gal-lys. Thus, the key findings of loss of ASOR-binding activity yet persistence of Gal-lys activity in cells bearing only RHL 1 strongly suggest that the absence of RHL 2/3 profoundly affects the organization of the proposed triads required for the binding of asialoglycoproteins but not the cell surface expression of clustered RHL 1 Gal-binding sites.

Several additional characteristics of RHL 2/3 suggest that it may be playing an important organizing role in the structure and therefore function of the native ASGP-R. RHL 2/3 is present in lesser amounts than RHL 1 (the mass ratio of rat liver RHL 1 to RHL 2/3 is 3 or 4/1), yet it is preferentially cross-linked to a photoaffinity-labeling reagent based on a triantennary glycopeptide found in asialo-fetuin (Lee and Lee, 1987). RHL 2/3 loses Gal-binding activity more readily than does RHL 1 (Halberg et al., 1987). Since, from the present study, RHL 2/3 is apparently not necessary for transport of RHL 1 through the biosynthetic or endocytic pathways, it must be serving another function. We postulate that the minor subunit is either itself forming a homodimer in the membrane with its Gal-binding sites ~15 Å apart, or it organizes or participates with RHL 1 subunits to form such a dimer. In either case, there must be additional RHL 1 subunits associated to accommodate the 3 or 4/1 mass ratio of RHL 1 to RHL 2/3 that we have observed. Therefore, we suggest that the native ASGP-R is a hetero-oligomer.

RHL 1 Can Act as a Receptor.—The two HTC populations we have studied were originally derived from co-transfection of cells with cDNAs for both ASGP-R subunits. Although the negative cells do not express detectable RHL 2/3 protein, the status of the RHL 2/3 cDNA is not presently known. With this caveat, our results show that RHL 1 is targeted through the biosynthetic pathway and reaches the cell surface in a form that can bind a specific type of ligand. Since RHL 1 was also capable of internalizing significant quantities of Gal-Lys, it alone seems to contain all the information necessary for participation in the endocytic pathway as well. Preliminary experiments suggest that recycling may occur, because more ligand can be processed than total functional receptors present. We have not performed the kinetic experiments that would indicate whether the efficiency of RHL 1 targeting through the various intracellular pathways is comparable in the presence and absence of expression of the minor subunit.

Acknowledgements—We would like to thank Arlene Daniel for word processing, Tom Uquhart for photographic assistance, Carol Renfew for critically reading the manuscript, Dr. Kurt Drickamer for antibody to RHL 2/3, and Dr. Michael McPhaul for the original transfected HTC cells.

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