Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8863–8867, December 1986
Biochemistry

Formation of functional asialoglycoprotein receptor after transfection with cDNAs encoding the receptor proteins

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Contributed by Paul Berg, August 7, 1986

ABSTRACT **The rat asialoglycoprotein receptor (ASGP-R) has been expressed in cultured rat hepatoma cells (HTC cells) after transfection with cloned cDNAs. Fluorescence-activated cell sorting of transfected cells was used to identify the functional cDNA clones and to isolate cells expressing the ASGP-R. Simultaneous or sequential transfections with two cloned cDNAs that encode related but distinctive polypeptide chains were needed to obtain ASGP-R activity; transfection with either cDNA alone failed to produce detectable ASGP-R. The affinity of transduced ASGP-R for asialo orosomucoid is less than that of the native rat ASGP-R, and the number of surface receptors in clones expressing ASGP-R is about one-fifth that found on rat hepatocytes.**

The asialoglycoprotein receptor (ASGP-R) is thought to mediate the turnover of numerous senescent serum glycoproteins (1, 2). This receptor is tissue specific and is found only on hepatocyte membranes, where it binds glycoproteins that bear terminal galactose residues on their oligosaccharide side chains. These receptor–ligand complexes are internalized via specialized regions of the plasma membrane termed coated pits and, once internalized, the receptors deliver the bound ligands via several intracellular compartments to the lysosomes (3). The ASGP-R then recycles to the cell surface for reuse in subsequent endocytic events (4).

Purification of the ASGP-R from mammalian liver revealed that it consists of several distinct proteins. Electrophoresis of the rabbit receptor in $NaDodSO_4$/polyacrylamide gels shows that it contains two polypeptide chains with estimated molecular masses of 40 and 48 kDa (5). Judging from cloned cDNAs, there appear to be at least two polypeptide species that comprise the ASGP-R in the human hepatoma line HepG2 (6). The rat ASGP-R contains three polypeptides with apparent molecular masses of 42 (RHL1), 49 (RHL2), and 54 kDa (RHL3) (7). How these various polypeptide chains contribute to the structure and function of the various receptors is not entirely clear.

To approach these questions, we first cloned two cDNAs, each one encoding a distinctive polypeptide that is part of the rat ASGP-R. The cDNAs were obtained in expression vectors by the cloning procedures described by Okayama and Berg (8). Details of these isolations and the characterization of the cDNA sequences are being published elsewhere (unpublished results). This paper shows that transfection of HTC rat hepatoma cells with the two cDNA clones, together, or sequentially in either order, is needed to produce a functional ASGP-R; no ASGP-R is formed when HTC cells are transfected with either cDNA alone. The ASGP-R formed in doubly transformed cells is capable of binding and internalizing the appropriate ligand. However, the avidity and number of receptors produced by the doubly transformed cells are lower than in rat hepatocytes. Moreover, the ASGP-R formed in the doubly transformed cells contains RHL1 and RHL2 polypeptide chains but appears to lack RHL3.

MATERIALS AND METHODS

Cell Culture. The HTC cell line was obtained from G. Ringold (Stanford University) and was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum containing penicillin and streptomycin. Selection with G418 or hygromycin B was performed as described (ref. 9; A. Smith, D. Strehlow, A. Miyajima, and P.B., unpublished results).

Screening the cDNA Library. A cDNA library was constructed from poly(A)$^+$ RNA prepared from rat liver using the pcD expression vector described elsewhere (7, 8). cDNA clones encoding the protein RHL1 were identified by hybridization using degenerate oligonucleotide probes based on a published protein sequence (10). Five separate RHL1 cDNA clones were isolated, and the nucleotide sequences of the cDNAs established that each contained an intact coding region. Twenty-three independent cDNA clones were isolated that hybridized with an oligonucleotide probe corresponding to the protein sequence common to RHL2 and RHL3. The cDNA clone chosen for these studies had an intact open reading frame consistent with the partial amino acid sequence already reported (ref. 10; unpublished results).

Transfection of HTC Cells. Cultured HTC cells were transfected with DNA by the standard calcium phosphate precipitation technique (11, 12). The cloned cDNA being tested was present in a 10-fold excess over a plasmid containing a selectable drug-resistance marker [pSV2-neo (9) or pSV2-hph (A. Smith, D. Strehlow, A. Miyajima, & P.B., unpublished results)].

Radioactive Ligand. Human asialo orosomucoid (ASOM) was prepared from native orosomucoid obtained from Sigma (see below). Iodination of the protein was performed as described (13). The specific activity of the ^{125}I iodinated ASOM was 60 μCi per μg of protein (1 Ci = 37 GBq).

Fluorescent Ligand. Orosomucoid was desialylated with agarose-bound neuraminidase (Sigma) in 0.2 M sodium acetate (pH 5.0) for 12 hr. The desialylated orosomucoid was recovered by passage over a G-25 Sephadex column equilibrated with phosphate-buffered saline (PBS; 137 mM sodium chloride/15 mM sodium phosphate, pH 7.4), filtered and stored at 4°C. ASOM (15 mg) in 200 ml of PBS was conjugated to fluorescein by reaction with 1 mg of fluorescein isothiocyanate in 200 μl of 0.5 M sodium carbonate buffer (pH 9.0) at 4°C for 12 hr. The fluorescein-conjugated ASOM was isolated by passage over a G-10 Sephadex column equilibrated with PBS. Protein was determined by the method of Lowry *et al.* (14).

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Abbreviations: ASGP-R, asialoglycoprotein receptor; ASOM, asialo orosomucoid; FACS, fluorescence-activated cell sorter.

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Fluorescent Ligand Binding and Uptake. Cells were grown on 35-mm tissue culture plates to ≈90% confluency, washed once with PBS, and then covered with DMEM containing 10 mM Hepes (pH 7.35) and 100 μg of cytochrome *c* per ml. Fluorescent ASOM was then added, the cells were incubated at 37°C, and at various times thereafter the cells were washed with 5 ml of Tris-buffered saline (137 mM sodium chloride/25 mM Tris/5 mM potassium chloride/0.1 mM sodium phosphate, pH 7.4) and removed from the culture dishes with 0.5% trypsin. The cells were pelleted, resuspended in DMEM containing 10% fetal calf serum, and analyzed for bound ligand by the fluorescence-activated cell sorter (FACS).

Surface Binding Studies. Cells were grown as described above, washed with PBS, and then covered with 1 ml of DMEM plus 40 mM Hepes (pH 7.35). After incubation for 10 min at 37°C to clear the surface of any occupied receptors, the monolayers were placed at 4°C for 20 min. Labeled ligand was then added in 0.5 ml of DMEM to the desired final concentration. After 1.5 hr at 4°C, the medium was removed, the dishes were washed three times with 3 ml of PBS, and the cells were triturated in 1 ml of 0.1 M NaOH. The entire sample was counted in a Beckman γ 4000 counter. Cell number was determined directly on duplicate samples.

Internalization of Ligand. After the ligand was added to the surface of monolayers at 4°C for 20 min as described above, the cultures were incubated at 37°C. Duplicate cultures were removed at different times, washed at 4°C as described above, and the amount of cell-associated radioactivity was determined. The values for the uptake of ligand at 4°C and 37°C measure surface binding and internalized ligand, respectively.

Labeling of Cell Proteins. HTC cells that had been transfected with cDNA clones encoding RHL1 and RHL2 proteins and then selected for expression of ASGP-R by three sequential passes through the FACS (see Fig. 2*C*) were used to characterize the ASGP-R proteins. A nearly confluent culture of the transfected HTC cells was washed with 5 ml of PBS and labeled by the addition of 300 μCi of [^{35}S]methionine (New England Nuclear; 800 Ci/mmol) in methionine-free medium for 4 hr at 37°C. After placing these cultures in fresh medium containing unlabeled methionine, they were incubated for an additional 30 min. Primary rat hepatocytes were cultured as described (D. M. Bissell, D. M. Arenson, J. J. Maher, and F. J. Roll, personal communication) and labeled in an identical fashion.

Immunoprecipitation of ASGP-R Proteins. Cells that had been labeled as described above were washed once with PBS and scraped into 2 ml of cold PBS with a rubber policeman. After centrifugation, the cell pellet was triturated in 2 ml of solubilization buffer (10 mM Hepes, pH 7.35/500 mM NaCl/10 mM EDTA, pH 8.0/1% Triton/0.2 mM phenylmethylsulfonyl fluoride/10 μg each of leupeptin and pepstatin per ml) and 100-μl aliquots of the lysates were frozen at −20°C. Monoclonal antibody (10 μg) to ASGP-R [HA107 (15) supplied by Ann Hubbard, or D3-5D3 (7) supplied by J. Harford and G. Ashwell] bound to IgSorb (The Enzyme Center, Malden, MA) coated with rabbit anti-mouse Ig was added to each 100-μl aliquot of labeled extract. After incubation at 4°C for 2 hr, the complexes were isolated by centrifugation and washed four times with PBS plus bovine serum albumin (250 μg/ml). The complexes were denatured and electrophoresed on 10% $NaDodSO_4$/polyacrylamide gels. The bands were visualized after enhancement with Enlightening (New England Nuclear) by exposure to Kodak XAR-5 film.

RESULTS

FACS provides a convenient way to detect and separate cells that bind and accumulate fluorescent labeled ligands. Fig. 1 shows the sorting profiles of HepG2 cells, which possess

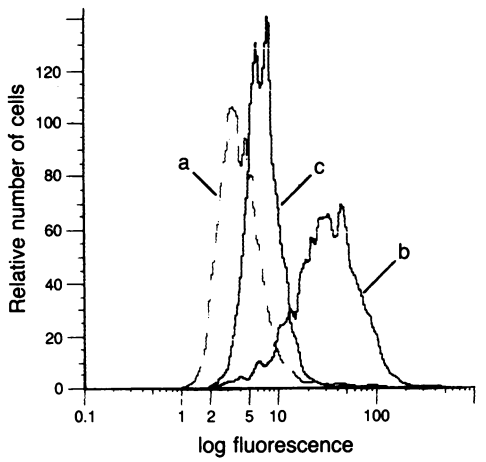

FIG. 1. Fluorescent ligand accumulation and competition. Subconfluent monolayers of Hep-G2 cells were washed with PBS and then incubated for 12 hr at 37°C in medium alone (curve a), with fluorescent ligand (curve b), or with fluorescent ligand plus a 100-fold excess of unlabeled asialo orosomucoid (curve c). Cells were analyzed by FACS as described.

functional ASGP-R, after incubation in the presence and absence of fluorescein-tagged ASOM. Accumulation of the labeled ligand is shown by the presence of cells exhibiting markedly increased fluorescence compared to the starting cells. The addition of a 100-fold excess of ASOM reduces the fluorescent population to nearly that of the starting cells; bovine serum albumin, cytochrome *c*, or native ASOM, each of which lacks the terminal galactosyl residues required for binding to the ASGP-R, fail to quench the uptake of the fluorescein-labeled ASOM.

The rat hepatoma line HTC lacks the ASGP-R and, consequently, does not accumulate fluorescent-tagged ASOM. Sequential sorts of HTC cells after exposure to the fluorescent ligand have failed to detect even a minor population capable of accumulating the ligand (see Fig. 2 *A* or *B*, for example). The HTC cells are, therefore, suitable recipients for transfections designed to detect cDNA clones capable of producing functional ASGP-R.

Accordingly, HTC cells were transfected with either pcD-RHL1 and pSV2-neo or with pcD-RHL2/3 and pSV2-neo. After selection for G418-resistant transformants, the pooled clones were analyzed for expression of ASGP-R activity by the uptake of fluorescein-labeled ASOM measured by FACS. Transfections with cDNA plasmids encoding either RHL1 or RHL2/3 did not yield cells that showed enhanced binding of the tagged ligand (Fig. 2 *A* and *B*). Even after cells from the most fluorescent end of the distribution (the top 0.5–1.0% of cells) were grown and resorted, they behaved like the starting HTC cells in their apparent lack of ASGP-R activity. By contrast, when cells were simultaneously transfected with a mixture of pSV2-neo, pcD-RHL1, and pcD-RHL2/3, ≈10% of the population showed enhanced accumulation of the tagged ligand (Fig. 2*C*). When the cells from the top 8% of the distribution were grown up and resorted, a substantial proportion of the cell population were able to accumulate the fluorescent ligand; binding of fluorescein ASOM by this population was virtually eliminated by the presence of excess unlabeled ASOM.

The dependence on the expression of both RHL1 and RHL2/3 for production of the ASGP-R was also shown by sequential transfections (Fig. 3). HTC cells were transfected first with pcD-RHL1 and pSV2-neo and G418-resistant clones were selected. This population, which produced no detectable ASGP-R activity, was then transfected with pcD-RHL2/3 plus pSV2-hph, a plasmid that confers resistance to

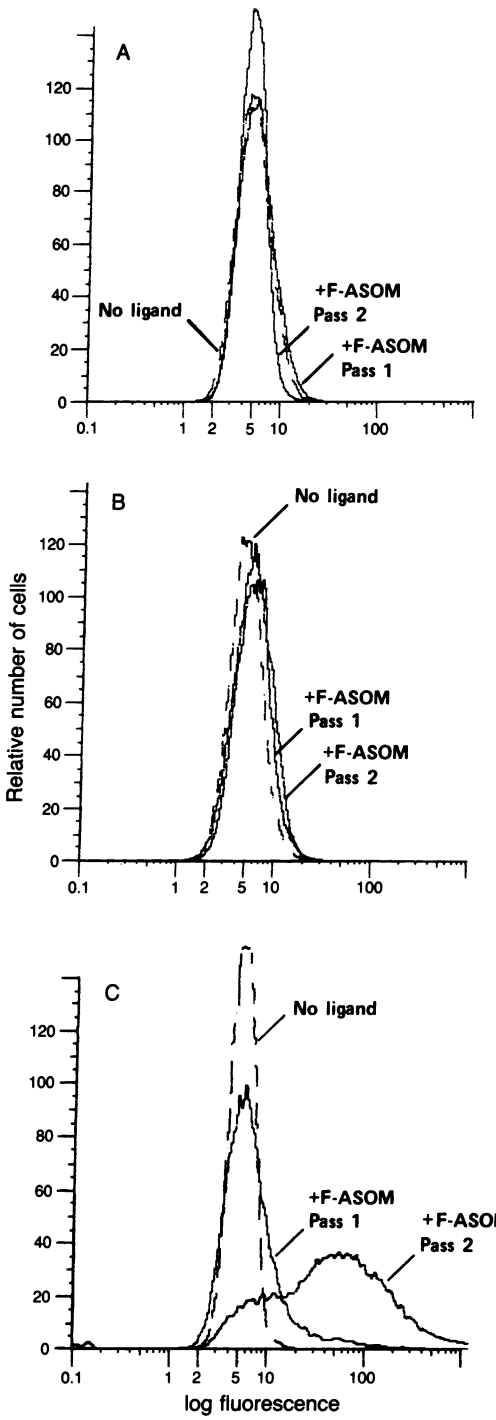

FIG. 2. Analysis of cells transformed with cDNAs encoding either RHL1 or RHL2 or both together. (*A*) HTC cells cotransfected with a mixture of pcD-RHL1 and pSV2-neo. G418-resistant colonies were pooled and analyzed by FACS. Cells from the top 1% of the fluorescence distribution were grown up and resorted 2 weeks later. (*B*) HTC cells after transfection with a mixture of pcD-RHL2/3 and pSV2-neo. The pooled G418-resistant clones were analyzed as in *A*. Cells from the top 0.5% of the fluorescent distribution were cells grown up and resorted 4 weeks later. (*C*) HTC cells cotransfected with pcD-RHL1, pcD-RHL2/3, and pSV2-neo. G418-resistant colonies were pooled and analyzed as described above. The top 8% of cells were collected, grown up, and resorted 2 weeks later.

hygromycin B, or with pSV2-hph alone. Cells transfected with only pSV2-hph failed to yield cells with enhanced uptake of fluorescent-tagged ASOM even after the top 1% of the distribution was grown up and resorted (Fig. 3*A*). However, ≈10% of the RHL1 transformed cells that were transfected

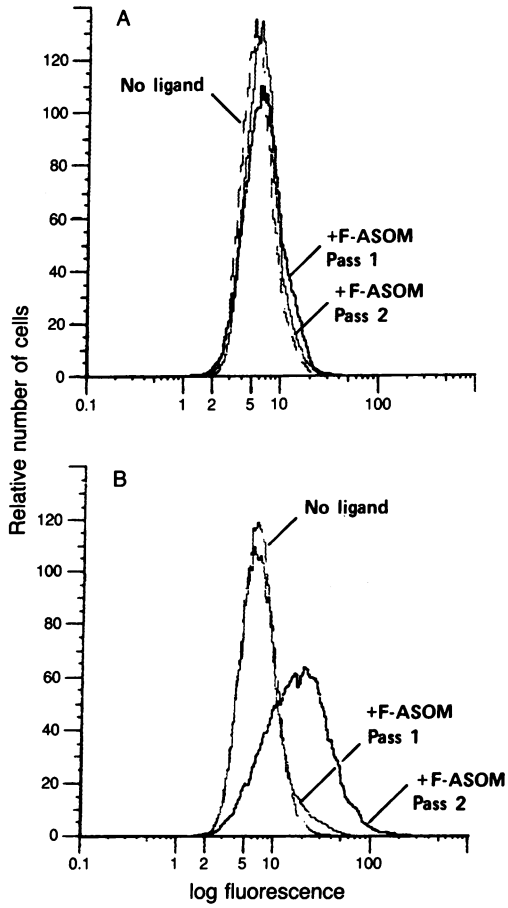

FIG. 3. HTC cells transfected first with pcD-RHL1 and subsequently with pcD-RHL2/3 produce functional ASGP-R. After obtaining G418-resistant colonies by transfection with pcD-RHL1 and pSV2-neo, the pooled clones were cotransfected with pSV2-hph alone (*A*) or with pcD-RHL2/3 plus pSV2-hph (*B*). The analysis of the pooled hygromycin-resistant colonies for ASGP-R by FACS (pass 1) and the subsequent resorting of the top 1% of fluorescent cells 3 weeks later is shown.

with both pcD-RHL2/3 and pSV2-hph and then selected for hygromycin resistance showed increased fluorescence after exposure to the tagged ASOM. Growth and resorting of the top 1% of the distribution in the first sort yielded cells that were clearly positive for expression of the ASGP-R (Fig. 3*B*).

Cells capable of even greater uptake of the tagged ligand could be obtained by a third sort of cells grown up from the top 8% of cells from the second pass shown in Fig. 2*C*. These cells were used to characterize the ASGP-R produced by RHL1 and RHL2/3 double transformants. To measure the surface binding activity of the double transformants, the cells were incubated with various amounts of ^{125}I-labeled ASOM at 4°C in the absence or presence of a 100-fold excess of unlabeled ASOM (Fig. 4). Under these conditions, HTC cells or cells transformed with either RHL1 or RHL2/3 alone do not bind the ^{125}I-labeled ASOM. However, the doubly transformed cells show a concentration-dependent binding of the tagged ligand. This binding is completely blocked by unlabeled ASOM but not by cytochrome *c*, bovine serum albumin, or native orosomucoid (data not shown). Plots of the binding data indicate that there are $\approx 2 \times 10^4$ surface receptors per cell and that half-maximal binding occurs with 0.7 μg of ligand per ml.

Next, we address the question of the composition of the ASGP-R made by cells transfected with both RHL1 and RHL2/3 (Fig. 5). ASGP-R positive cells, obtained by transformation with pcD-RHL1 and pcD-RHL2/3 and three suc-

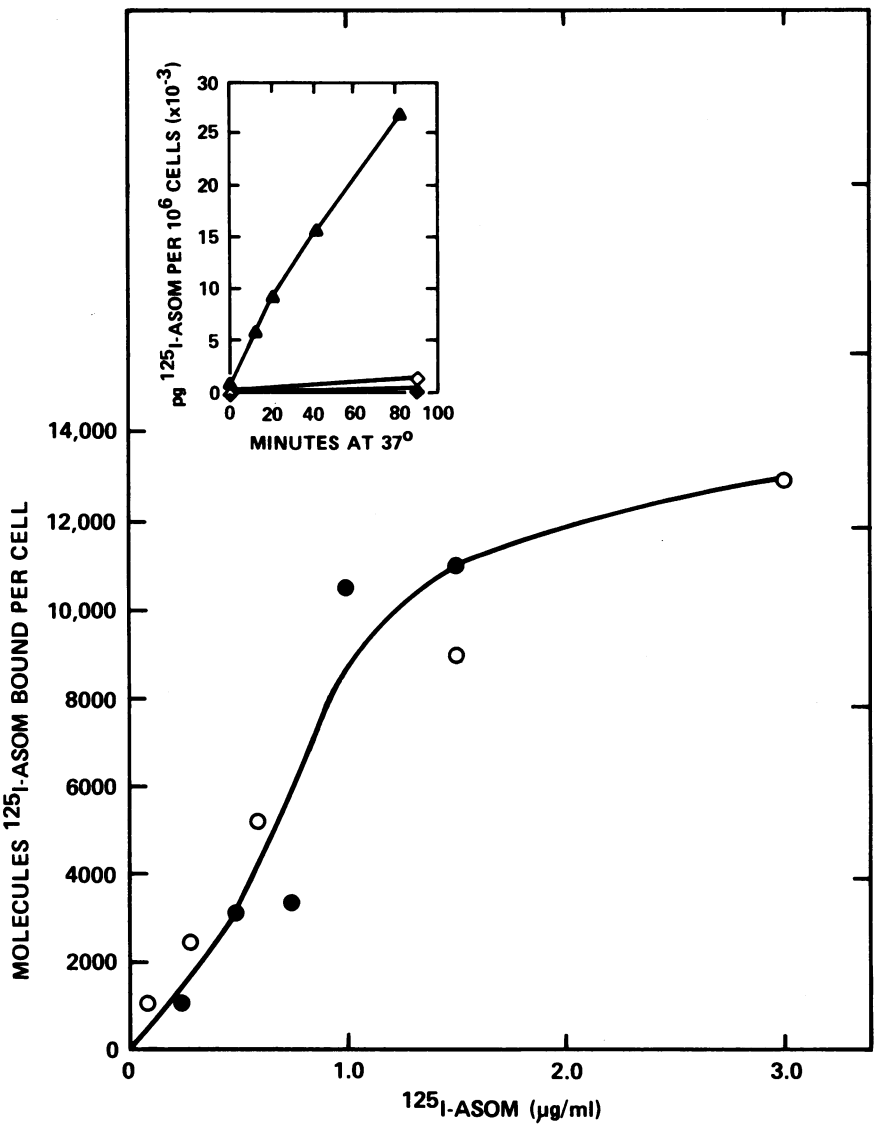

FIG. 4. Surface binding of ^{125}I-labeled asialo orosomucoid. Confluent monolayers of HTC cells that had been transformed for RHL1 and RHL2/3 and enriched for functional ASGP-R (see pass 2 in Fig. 2*C*) were incubated with ^{125}I-labeled ASOM at 4°C in the presence or absence of unlabeled ASOM. Results of two separate experiments are shown. The specific activities of the ligand used in experiments 1 (○) and 2 (●) were 1.8 and 1.4 μCi per μg of protein, respectively. The specific binding shown was the difference between the amount of ligand bound in the presence and absence of a 100-fold excess of unlabeled ASOM. (*Inset*) Internalization of ^{125}I-labeled asialo orosomucoid. Cells were incubated with ^{125}I-labeled ASOM at 4°C for 20 min and then allowed to internalize the ligand at 37°C for various lengths of time. Radioactive ligand accumulation by parental HTC cells is shown (◆). This is compared to cells transfected with both RHL1 and RHL2/3 and enriched for cells expressing the ASGP-R (pass 2 in Fig. 2*C*) in the presence (◇) or absence (▲) of a 100-fold excess of unlabeled ASOM. One picogram of ASOM is equivalent to $\approx 1.6 \times 10^7$ molecules.

cessive enrichments using the FACS (see Fig. 2*C*), were labeled *in vivo* with [^{35}S]methionine for 4 hr. After a chase with unlabeled methionine (see *Materials and Methods*), the cells were solubilized and aliquots of the extracts were either electrophoresed directly or after immunoprecipitation with a monoclonal antibody [HA 107 (15)] directed against the ASGP-R. For comparison, freshly obtained rat hepatocytes containing functional ASGP-R were labeled and analyzed in the same way. It is evident that the ASGP-R produced by the doubly transfected cells yields bands that correspond in apparent molecular mass to RHL1 (44 kDa) and RHL2 (50 kDa). On the other hand, the native receptor associated with rat hepatocytes contains the previously reported RHL3 band as well. The same bands were found when another monoclonal antibody against ASGP-R (D353) was used for the immunoprecipitations. Thus, HTC cells are either unable to produce RHL3 from the cloned RHL2/3 cDNA or RHL3 is encoded by a related but different cDNA sequence.

DISCUSSION

The ASGP-R of rat hepatocytes is composed of three glycosylated polypeptides, the major one of which (RHL1) has a unique sequence and an apparent molecular mass of 42 kDa (10). Two minor species, RHL2 and RHL3, have apparent molecular masses of 49 and 54 kDa, respectively, and share a common carboxyl-terminal fragment of 101 amino acids. In the present experiments, we have investigated whether the expression of cDNAs encoding RHL1 and RHL2/3 in HTC cells can produce a functional ASGP-R.

The cDNAs for the individual polypeptides of rat ASGP-R were constructed and isolated from mRNA extracted from rat liver using the pcD expression vector and methods described by Okayama and Berg (8). Their isolation and nucleotide sequences will be described elsewhere (unpublished results), but one clone containing the complete coding sequence of RHL1 and another containing a complete coding sequence of

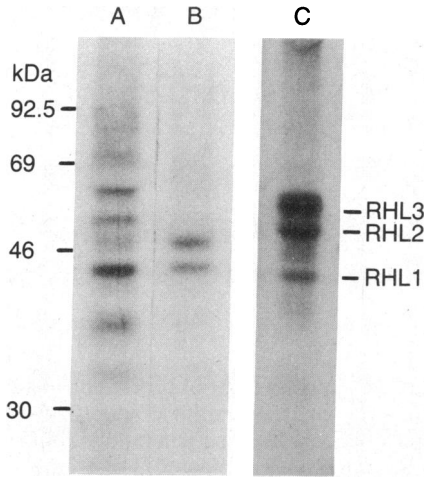

FIG. 5. Immunoprecipitation of ASGP-R in transfected HTC cells and in cultured rat hepatocytes. Transfected HTC cells (pass 2 in Fig. 2C) were labeled *in vivo* with [^{35}S]methionine. Lane A shows the pattern of total proteins labeled in this manner. Lane B shows the pattern following immunoprecipitation of a 10-fold larger sample with monoclonal antibody HA107. Lane C shows the pattern found when rat hepatocytes are labeled *in vivo* with [^{35}S]methionine and immunoprecipitated with antibody HA107.

either RHL2 or RHL3 (designated RHL2/3 because it contains the coding sequence corresponding to the shared amino acid sequence of RHL2 and RHL3) were used to determine whether their expression could reconstitute a functional ASGP-R.

Our experiments show that HTC cells, which do not produce the ASGP-R, acquire that ability after simultaneous or sequential transformations with both the pcD-RHL1 and pcD-RHL2/3 cDNA clones. However, cells transformed with either the RHL1 or RHL2/3 coding sequence alone fail to produce functional ASGP-R—that is, singly transformed cells do not exhibit surface binding of ligand at 4°C, nor do they accumulate the ligand internally at 37°C. These results indicate that RHL1 or the product made from the RHL2/3 clone are insufficient to constitute a functional receptor.

The processes of maturation and assembly of the different chains constituting the ASGP-R are not known. Preliminary studies with singly transformed cells indicate that mRNAs corresponding to RHL1 and RHL2/3 are expressed in cells that are transformed with either pcD-RHL1 or pcD-RHL2/3, respectively. Additional experiments are needed to determine whether these mRNAs are translated and whether the proteins are stable, modified, and transported to their proper intracellular targets.

The analysis of the ASGP-R in the doubly transformed cells indicates that it is composed of just two polypeptide chains, RHL1 and RHL2. Since the receptor of rat hepatocytes, analyzed under the same conditions, contains RHL3 as well, the origin of RHL3 remains unaccounted for. Since hybridization of the RHL2/3 cDNA to different restriction digests of rat genomic DNA reveals patterns compatible with only a single gene, it is unlikely that there are separate genes encoding RHL2 and RHL3 (unpublished results). One possibility is that a single locus yields two differentially spliced mRNAs, one encoding RHL2 and the other encoding RHL3. This may be the case, since there are clones in the cDNA library that share the coding sequence common to RHL2 and RHL3 but differ in other respects. However, the molecular basis for these differences remains to be elucidated. Lastly, it is possible that RHL2 and RHL3 proteins in rat ASGP-R have the same amino acid sequence but differ in their posttranslational modifications. Thus, the HTC cells may be unable to make the type of modification that transforms either RHL2 or its precursor into RHL3. It is also possible that the absence of RHL3 is responsible for the lower ligand binding affinity of the ASGP-R produced by the double transformants. At this point, however, other deficiencies in the receptor structure cannot be excluded.

Our initial attempts to identify cDNA clones that encode the rat ASGP-R centered on the transfection of HTC cells with cDNA expression libraries and the examination of such transformants for cells capable of accumulating fluorescent ligand. When it became clear that the ASGP-R contained multiple polypeptide chains, our lack of success using this approach was readily explained. However, the availability of amino acid sequence data for RHL1 and partial sequence information for RHL2 and RHL3 made it feasible to use oligonucleotides as hybridization probes to screen the pcD library for candidate cDNA clones that encode the three ASGP-R polypeptides. The test of whether these clones contained the complete protein coding sequences and whether the two protein products could reconstitute a functional ASGP-R was greatly facilitated by the FACS analysis. The FACS assay permitted the isolation of cells expressing the ASGP-R and a progressive enrichment of these cells expressing sufficient receptors for biochemical analysis. The FACS methodology for identifying genes that encode other surface markers—whether detectable by labeled antibodies or ligands—and for studying the expression and subsequent metabolism of that receptor is invaluable.

We thank Joe Harford and Gilbert Ashwell for samples of the monoclonal antibody D353. We are indebted to Ann Hubbard for samples of the monoclonal antibody HA107 and for many helpful discussions. We express our appreciation to Dr. L. Hertzenberg for his help and cooperation in the use of the FACS facility. Eleanor Olson provided expert secretarial assistance in the preparation of this manuscript. This work was supported by Public Health Service Grant GM-13235 from the National Institute of General Medical Sciences and by a fellowship grant from the Helen Hay Whitney Foundation.

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