Articles

Affinity Labeling of the Galactose/*N*-Acetylgalactosamine-Specific Receptor of Rat Hepatocytes: Preferential Labeling of One of the Subunits†

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ABSTRACT: The galactose/*N*-acetylgalactosamine-specific receptor (also known as asialoglycoprotein receptor) of rat hepatocytes consists of three subunits, one of which [43 kilodalton (kDa)] exists in a greater abundance (up to 70% of total protein) over the two minor species (52 and 60 kDa). When the receptor on the hepatocyte membranes was photoaffinity labeled with an ^{125}I-labeled high-affinity reagent [a triantennary glycopeptide containing an aryl azide group on galactosyl residues; Lee, R. T., & Lee, Y. C. (1986) *Biochemistry 25*, 6835–6841], the labeling occurred mainly (51–80%) on one of the minor bands (52 kDa). Similarly, affinity-bound, *N*-acetylgalactosamine-modified lactoperoxidase radioiodinated the same 52-kDa band preferentially. In contrast, both the photoaffinity labeling and lactoperoxidase-catalyzed iodination of the purified, detergent-solubilized receptor resulted in a distribution of the label that is comparable to the Coomassie blue staining pattern of the three bands; i.e., the 43-kDa band was the major band labeled. These and other experimental results suggest that the preferential labeling of the minor band and inefficient labeling of the major band on the hepatocyte membrane resulted from a specific topological arrangement of these subunits on the membranes. We postulate that in the native, membrane-bound state of the receptor, the 52-kDa minor band is topologically prominent, while the major (43 kDa) band is partially masked. This partial masking may result from a tight packing of the receptor subunits on the membranes to form a lattice work [Hardy, M. R., Townsend, R. R., Parkhurst, S. M., & Lee, Y. C. (1985) *Biochemistry 24*, 22–28].

There exists on the surface of mammalian hepatocytes a transmembrane receptor that recognizes galactose (Gal)[1] or GalNAc [known as asialoglycoprotein (ASGP) receptor]. Though exact physiological function of this receptor is not clearly understood, intracellular movement of the receptor and its ligands is one of the better studied of the receptor systems [for a review, see Ashwell and Harford (1982) and Breitfeld et al. (1985)]. According to the current concept, the major mode of operation of this receptor system is as follows: An asialoglycoprotein (ligand) is initially bound by the receptors on the hepatocyte surface, the receptor–ligand complex is endocytosed in vesicles via coated pits, the receptor and the ligand dissociate from each other at one of the stages before reaching lysosome, and the receptor then shuttles back to the cell surface while the ligand proceeds to lysosome to be degraded. The receptor is also present in the internal membranes of rat hepatocytes in a quantity that is 2–4 times more abundant than the surface receptor (Breitfeld et al., 1985). Internal receptors appear to be biochemically and immunochemically similar to the surface receptors (Pricer & Ashwell, 1976), but conflicting views exist for the functional relationship between the surface and internal receptors (Ashwell & Harford, 1982).

The receptor in a detergent-solubilized form has been purified by affinity chromatography from the liver of several mammalian species (Hudgin et al., 1974; Pricer & Ashwell, 1976; Baenziger & Maynard, 1980; Bezouska et al., 1985). All the mammalian Gal/GalNAc receptor molecules thus far studied share a structural similarity in that they are composed of subunits of 40–60 kDa that are not held together by disulfide bonds. The rat liver receptor which is the subject of this study consists of three polypeptide chains, of which the smallest one (43 kDa) is predominant (~70%) over the other two (52 and 60 kDa). All the chains share some structural homology, but the 52- and 60-kDa subunits are more closely related to each other (Warren & Doyle, 1981; Schwartz et al., 1981; Drickamer et al., 1984). The amino acid sequence of the major polypeptide (43 kDa) has been elucidated (Drickamer et al., 1984).

The receptor apparently binds specifically only the Gal/GalNAc portion of a ligand, since a variety of simple, synthetic galactosides and linear oligosaccharides, including β-Gal(1→4)GlcNAc and β-Gal(1→4)β-GlcNAc(1→2)Man, which represent portions of high-affinity oligosaccharide ligands (Lee et al., 1983), all had similar affinity toward the receptor (Lee et al., 1982). The receptor binds these galactosides rather weakly (K_d ~ 0.5 mM), but a properly aligned cluster of a mere three Gal residues in a ligand can increase its affinity to the receptors on the hepatocyte surface up to a millionfold, suggesting that there exists a specific arrangement of Gal-

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[1] Abbreviations: ASGP, asialoglycoprotein; ASOR, asialoorosomucoid; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; LPO, lactoperoxidase; PMSF, phenylmethanesulfonyl fluoride; TAGP, triantennary glycopeptide; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Gal, galactose; GalNAc, *N*-acetylgalactosamine; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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combining sites on the hepatocyte surface (Lee, Y. C., et al., 1984). This proper spatial arrangement of the Gal-combining sites is apparently perturbed in the "isolated" receptor, since the Triton-solubilized, affinity-purified receptor manifests a much weaker cluster effect (Lee, R. T., et al., 1984).

Recently we described the preparation of a high-affinity, photolabeling reagent for this receptor and showed that the reagent labeled the isolated rabbit and rat receptors in a photo- and affinity-dependent manner (Lee & Lee, 1986). In the process, all the subunits of the receptors were readily labeled, implying that each subunit contains at least one Gal-combining site. In this paper, by using two different methods of labeling, we show that the subunit labeling pattern of the receptor on the cell membranes is distinctly different from that of the Triton-solubilized receptor. One of the minor subunits (52 kDa) was predominantly labeled when the labeling was carried out on the hepatocyte membranes either by the photolabeling reagent or by iodination via LPO catalysis. From experimental results presented, we postulate that the 52-kDa band is much more exposed to the aqueous environment than the 43-kDa band (major band) on the cell membrane.

Materials and Methods

N-Hydroxysuccinimide ester of 4-azidobenzoic acid and 6-[(4-azido-2-nitrophenyl)amino]hexanoic acid were from Pierce Chemical Co. Pepstatin A and leupeptin were from Peptide Institute, Inc. (Osaka, Japan). Lactoperoxidase (LPO; EC 1.11.1.7), glucose oxidase (EC 1.1.3.4) from *Aspergillus niger*, Freund's complete and incomplete adjuvants, insoluble protein A (lyophilized cell powder of *Staphylococcus aureus*), phenylmethanesulfonyl fluoride (PMSF), iodoacetamide, and the molecular weight standards were from Sigma Chemical Co. A cluster lactoside, TyrAsp(LacAHT)$_2$, which has six lactosyl residues per molecule, was prepared as described (Lee, R. T., et al., 1984). Fetuin (Calbiochem) was desialylated at pH 5 for 48 h using *Arthrobacter ureafaciens* neuraminidase (Uchida et al., 1977) coupled to Sepharose 4B (Stults et al., 1983). Asialofetuin (90% of sialic acids removed) was coupled to partially hydrolyzed Sepharose 4B according to Stults et al. (1983). LPO was modified with GalNAc using the imidate method (Lee et al., 1976). The sugar-modified LPO contained on the average 25 mol of monosaccharide/mol. Enzymatic activity (L-tyrosine iodination) of LPO preparations was measured photometrically (Morrison & Bayse, 1970). The activity of the GalNAc-modified LPO was ~77% of the unmodified LPO. The affinity of GalNAc–LPO to the Gal/GalNAc-specific receptor on the rat hepatocyte surface was assessed by using the inhibition assay (Connolly et al., 1982) with ^{125}I-ASOR as the reference ligand. The $[I]_{50}$ value of GalNAc–LPO was 2 nM. The Gal/GalNAc-specific receptor from rat liver was prepared according to the method of Hudgin et al. (1974) using asialofetuin–Sepharose as affinant. For the preparation of antisera against the rat liver receptor, rabbits (white New Zealand, female, ca. 9 lbs; Bunnyville, PA) were injected subcutaneously with ethanol-precipitated receptor (~200 μg/rabbit) which had been suspended in phosphate-buffered saline and homogenized with an equal volume of the complete Freund's adjuvant. After a week, the second injection of the receptor suspended in the incomplete adjuvant was given. After 2 weeks, the sera gave a single precipitin band against the Triton-solubilized receptor in an immunodiffusion assay (Ouchterlony & Nilsson, 1973). Antisera for two hepatocyte cell surface antigens (ASGP receptor and CE-9; Bartles et al., 1985) and protein A coupled to Sepharose 6B were kindly provided by Drs. A. L. Hubbard and J. R. Bartles (The Johns Hopkins University).

Hepatocytes were prepared from rat (male Sprague-Dawley) by perfusing the liver with collagenase (Seglen, 1976). The hepatocyte preparations were routinely >85% viable (trypan blue exclusion) and >80% singles. All the hepatocyte handling was done in the cold. The washing procedure for hepatocytes involved (1) centrifuging the hepatocyte suspension at 300 rpm (Sorvall GLC-4) for 3 min, (2) aspirating the supernate, (3) suspending the cell pellet in the washing buffer, and (4) pelleting the hepatocytes once more. Preparation of the photoaffinity reagent from the desialylated triantennary glycopeptide of fetuin has been described in detail (Lee & Lee, 1986). Briefly, nonreducing terminal galactosyl residues of the tyrosylated glycopeptide were converted to 6-amino-6-deoxy-D-galactopyranosyl residues by a series of reactions. It was iodinated (^{125}I), and then a photolabeling group was introduced by using *N*-succinimidyl 6-[(4-azido-2-nitrophenyl)amino]hexanoate. Typically, the reagent contained 1.5 modified galactosyl residues per molecule.

Typical photolabeling of hepatocyte suspension was carried out as follows: 2.5×10^6 hepatocytes were washed once with 4 mL of buffer II (0.1 M HEPES, pH 7.6, 65 mM NaCl, 7 mM KCl, and 10 mM $CaCl_2$) and then suspended in 4 mL of the buffer containing the photoaffinity reagent [$(0.3–5) \times 10^{-7}$ M]. After incubation in the dark for 2 h with an end-over-end tumbling at 2 rpm in a 2 °C water bath, the cell suspension was transferred into a quartz tube (1.9 × 10 cm) into which a polished stainless-steel rod (0.8 cm diameter) was inserted for reflection of the irradiated light. The tube was flashed 6 times with three synchronously operated camera flash units without the protective cover (Ji, 1979) arranged in a triangle. The distance from the tube to any one of the flashes was ~3 cm. Care was taken to keep the tubes cold and the cells well suspended during the operation. After flashing, the cells were washed sequentially with 4 mL each of buffer II and the EDTA buffer (buffer II that contained 10 mM EDTA instead of $CaCl_2$). Noncovalently bound ligand was dissociated by suspending the cells in 4 mL of the EDTA buffer and incubating for 15 min with rotation at 2 rpm. The pelleted cells were shaken in 0.7 mL of the solubilization buffer [1% Triton X-100 buffered at pH 7.8 (10 mM HEPES) and containing 4.8 μM pepstatin A, 23 μM leupeptin, 10 mM iodoacetamide, and 0.1 mM PMSF] for ~1 h and usually left overnight in the cold. The solubilized cell suspension was centrifuged at 1000 rpm for 10 min to remove a small amount of debris. The supernate will be referred to as cell extract henceforth. To photoaffinity label the internal as well as surface receptors, digitonin (0.055%) was included in the incubation mixture (Weigel et al., 1983). To assess the labeling of cells under nonaffinity conditions, the EDTA buffer was substituted for buffer II during incubation. A portion of the cell extract was usually fractionated on a column (1 × 26 cm) of Sephadex G-150 with 5 mM HEPES buffer, pH 7.8, containing 0.2 M NaCl, 0.02% BSA, and 0.1% Triton X-100 as eluant to assess the distribution of radioactivity between the excluded and included peaks. SDS–PAGE was carried out using a 1.5-mm-thick 7.5% acrylamide slab gel according to Laemmli (1970). Each slab contained a lane of molecular weight standards and a lane of the soluble rat liver receptor preparation. After electrophoresis, the gel slabs were stained with Coomassie blue and then either sliced horizontally into $^1/_{16}$-in. slices to be counted or dried onto Whatman 3MM filter paper and used in radioautography. The stacking gel (~2.5 cm in length) was sliced into three sections horizontally and counted. Radioactive samples were counted with a Packard PDG γ counter.

The batchwise affinity adsorption of the cell extract to Sepharose was carried out as follows. The cell extracts (5000–20000 cpm) were shaken overnight in the cold with 0.3 g of Sepharose 4B in 0.8 mL of either buffer A (50 mM Tris-HCl buffer, pH 7.8, containing 1 M NaCl, 50 mM $CaCl_2$, 0.6% BSA, and 0.5% Triton X-100) or EDTA–buffer A (the same buffer containing 20 mM EDTA instead of $CaCl_2$). Gel was washed batchwise by centrifugation–aspiration with 0.8 mL each of the appropriate buffer (twice) and water (once), then eluted (gently shaking for 20 min) with 0.8 mL of EDTA–buffer A (twice), and washed with EDTA–buffer A (once).

Immunoprecipitation was carried out according to Hubbard et al. (1985), except that protein A–Sepharose or insoluble protein A was the solid phase used. Briefly, a radioactive sample [(1–5) × 10^4 cpm] was incubated with an antiserum (20 μL) in phosphate buffer (pH 7.4) for 2 h in the cold. Protein A–Sepharose (0.1 mL) or washed insoluble protein A (5 mg) was added, and the incubation was continued for an additional 2 h with gentle shaking. The solid material was washed several times with various buffers by centrifugation–aspiration, then heated at 100 °C in the dissociation buffer (60 mM Tris-HCl buffer, pH 6.8, containing 2.9% SDS, 5% mercaptoethanol, 10% glycerol, and 0.1 mg/mL bromophenol blue), and subjected to SDS–PAGE analysis.

Affinity iodination catalyzed by GalNAc–LPO was carried out as follows. Hepatocytes (5–6 million cells) in 2 mL of buffer II were incubated with 2–4 μg/mL GalNAc–LPO at 2 °C for 1.5 h to allow binding of the enzyme to the receptors. Hepatocytes were washed twice with the buffer (2 mL), suspended in 2 mL of buffer II containing glucose (1 mg/mL), glucose oxidase (2 units/mL), and $Na^{125}I$ (70–150 μCi/mL), and incubated at 2 °C for 40 or 90 min. Cells were washed once with EDTA buffer, incubated in EDTA buffer for 15 min, and then solubilized in 1 mL of the solubilization buffer. Affinity iodination of the soluble receptor was carried out similarly. The initial incubation of the receptor (34 μg) and GalNAc–LPO (5.5 μg) in 0.5 mL of buffer A without BSA was for 30 min at 25 °C. The components in the second incubation were added to the same level as in the hepatocyte experiments, and the iodination was carried out for 25 min at 25 °C. The nonaffinity control experiments were carried out by substituting 10 mM EDTA for 50 mM $CaCl_2$ in both the hepatocyte and soluble systems.

Nonspecific iodination of the hepatocyte by the Chloramine T method (Greenwood et al., 1963) was carried out in the cold as follows. All the solutions and hepatocyte suspensions were made in buffer II. To a hepatocyte suspension (6 million cells in 1 mL) were added 0.5 mCi of $Na^{125}I$ and 30 μL of Chloramine T (4 mg/mL). After 2 min, 50 μL each of sodium metabisulfite (1.23 mg/mL) and KI (10 mg/mL) was added, and the suspension was immediately centrifuged. Cells were washed 3 times with 5 mL of buffer II and dissolved in 0.7 mL of the solubilization buffer.

Results

Distribution of the Radiolabel in Photolabeling Experiments. Typically, the photoaffinity labeling of the hepatocytes incorporated 0.1–0.6% of the added radiolabel into the final suspension in 1% Triton. Centrifugation at 1000 rpm produced a small amount of insoluble debris which contained less than 20% of the total radioactivity in the suspension. Fractionation of the extract on Sephadex G-150 showed that the label was distributed almost equally between the excluded compounds with molecular weights >200K and the included compounds (Figure 1). When cells were incubated in the presence of digitonin (0.055%) to expose the internal receptors to the photolabeling reagent, incorporation of radioactivity increased 2–3-fold over the surface labeling, while the recovery of protein measured according to Lowry et al. (1951) decreased by ~20%. This increased labeling in the presence of digitonin occurred totally in the G-150 excluded peak which increased by 2–3-fold, while the included peak decreased slightly (Figure 1). Substitution of EDTA for calcium ion to abolish the receptor–ligand binding diminished radioactivities in the excluded and included peaks to 50% and 70% of the control, respectively.

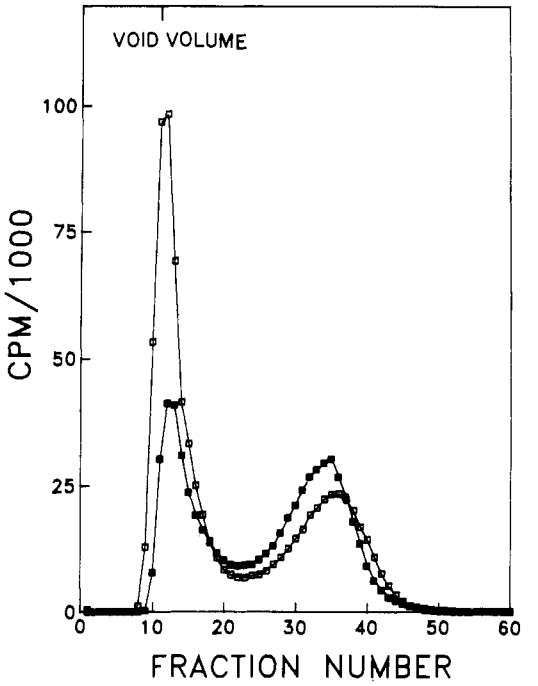

FIGURE 1: Elution profiles of photoaffinity-labeled cell extracts from a column (1 × 26 cm) of Sephadex G-150; 0.6 mL/fraction. (■) Labeled in the absence of digitonin; (□) labeled in the presence of digitonin.

Distribution of the radiolabel was further examined by SDS–PAGE in 15%, 10%, and 7.5% polyacrylamide gels. Comparison of the labeling pattern of the cell extract vs. the Sephadex G-150 excluded peak showed that the pattern was very similar in the region of molecular weights >30K (data not shown). Therefore, it appears that the Sephadex-included peak mainly contained molecular species smaller than 30 kDa. Typical SDS–PAGE profiles of the cells that were labeled on the surface (in the absence of digitonin) and cells that were labeled both internally and on the surface (in the presence of digitonin) are shown in Figure 2. Both the digitonin-treated and nontreated cells had major radiolabeled bands in the region corresponding to the receptor subunits (43–66 kDa), and the incorporation of the radiolabel in this region increased 2–3-fold with digitonin treatment. The digitonin-treated cells always had an extra radioactive band at ~80 kDa. Interestingly, such a band has been reported to occur in some preparations of the soluble rat receptor (Warrent & Doyle, 1981). Surface labeling of the hepatocytes that had been treated with EDTA or a 37 °C warming for 0.5 h to increase the surface receptors (Stults & Lee, 1986) had exactly the same SDS–PAGE pattern with a slightly higher incorporation of the radiolabel. Assuming that each rat hepatocyte contains approximately 200000 surface receptors (Weigel, 1980; Stults & Lee, 1986), we calculated from the incorporation of radiolabel in the 43–66-kDa region the efficiency of photolabeling to be ~15% at the reagent concentration of 5 × 10^{-7} M which is approxi-

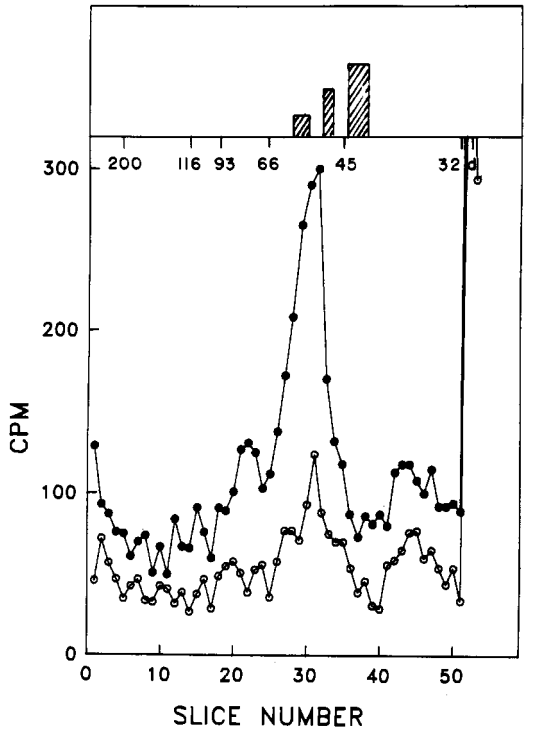

FIGURE 2: SDS–PAGE of photolabeled cell extracts. Gel slabs were sliced horizontally $^1/_{16}$-in. width per slice. Numbers in the upper portion of the figure are values of the molecular weight standards $\times 10^3$, and d stands for the dye front. Cell extracts equivalent to 3 $\times 10^5$ cells were applied. (○) Labeled in the absence of digitonin; (●) labeled in the presence of digitonin. Bargram in the upper panel shows the position and relative abundance of the soluble rat receptor subunits. In subsequent figures, only the positions of the subunits are indicated by arrows.

mately the $[I]_{50}$ of the reagent (Lee & Lee, 1986). Comparison of the SDS–PAGE pattern of the photolabeled hepatocyte extract with that of the Coomassie-stained, purified receptor (Figure 2, upper panel) indicated that the major band (43 kDa) was poorly photolabeled and that one of the minor bands (52 kDa) was most extensively photolabeled, whether or not the internal receptors were exposed by digitonin treatment (Figure 2). As in the case of photoaffinity labeling of the purified, Triton-solubilized receptor (Lee & Lee, 1986), each of the labeled bands appeared ~3 kDa larger than the parent band. For the convenience of discussion, however, the labeled bands will be identified by the size of the respective parent band. Table I shows the distribution of the radioactivity in the three subunit bands obtained in different experiments with both the purified, soluble receptor and the cell-associated receptor. Since the distribution of counts was similar for both the digitonin-treated and nontreated cells, in the subsequent experiments, unless otherwise noted, the hepatocytes were treated with digitonin to increase the radioactive signal.

Figure 3 shows the effect of incubation conditions on the resulting SDS–PAGE pattern. When the hepatocyte suspension was incubated with the photoaffinity reagent under nonaffinity conditions (e.g., with 10 mM EDTA and no $CaCl_2$), radioactivity in the 43–60-kDa area decreased uniformly to about 40% of the control value. Similarly, when $TyrAsp(LacAHT)_2$ (0.32 mM), a low molecular weight competitive inhibitor (Lee, R. T., et al., 1984), was included in the incubation mixture, the incorporation of radioactivity decreased to ~60% of the original level (not shown). However, when a large competitive inhibitor, Gal_{34}-BSA, was included in the mixture, there was a small but reproducible change in the labeling pattern (Figure 3); i.e., labeling of the

Table I: Distribution of Radiolabel among Three Receptor Bands in SDS–PAGE

experiment	distribution (%)[a] 43 kDa	52 kDa	60 kDa
(A) surface-photolabeled hepatocytes (–digitonin)	22	51	27
(B) surface + internally photolabeled hepatocytes (+digitonin)	16	60	24
(C) surface GalNAc–LPO-iodinated hepatocytes (40 min)	6	53	41
(D) surface GalNAc–LPO-iodinated hepatocytes (90 min)	19	49	32
(E) Chloramine T iodinated hepatocytes[b]	31	38	31
(F) photoaffinity-labeled soluble receptor[c]	63	27	10
(G) GalNAc–LPO-iodinated soluble receptor	63	26	11
Coomassie-stained soluble receptor	75[d]	16[d]	9[d]

[a] Percent of the total counts or absorbance in the three subunit bands. [b] Distribution of the counts in the immunoprecipitated material. [c] For details, see Lee and Lee (1986). [d] Percent of the staining measured by a densitometer.

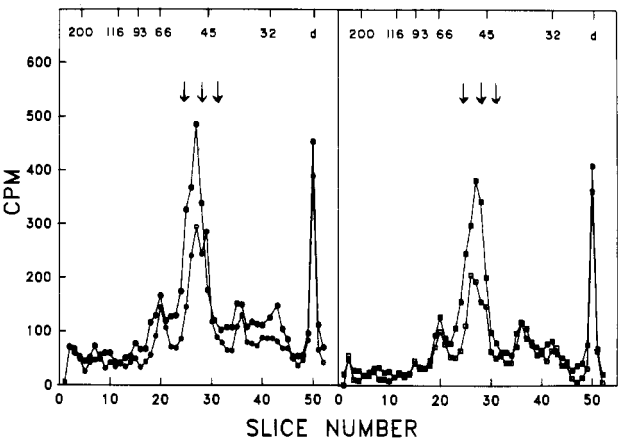

FIGURE 3: SDS–PAGE profiles of cell extracts photolabeled under various conditions. Cell extracts from 1.4 $\times 10^5$ cells were used. (●) Control, in the presence of 10 mM $CaCl_2$; (■) in the presence of 14 μM $GLcNAc_{35}$-BSA; (○) in the presence of 14 μM Gal_{34}-BSA; (□) in the presence of 10 mM EDTA instead of $CaCl_2$.

52-kDa band decreased to 53% of the control value, while that of the 43-kDa band remained approximately the same. The effect of Gal_{34}-BSA was sugar-specific, since $GlcNAc_{35}$-BSA did not lower or change the labeling pattern of the control significantly.

When the photolabeled cell extract (or the G-150 excluded material) was analyzed by SDS–PAGE, there was a significant but variable amount of counts trapped in the upper portion of the stacking gel (5% acrylamide). This radioactivity decreased to the background level, if the cell extract was first treated for 3 h at room temperature with a DNase preparation (data not shown), indicating that the material was mostly DNA in nature. Since the digitonin treatment did not affect the amount of radioactivity entrapped in the stacking gel, it is likely that these DNA molecules were present on the outside of hepatocytes, possibly originating from cells damaged during the hepatocyte preparation. It is known that DNA molecules are present on the surface of hepatocytes prepared by the collagenase perfusion method (N. L. Stults, personal communication).

Adsorption of the Photoaffinity-Labeled Cell Extract to Sepharose 4B. The following two approaches were used to demonstrate that the radioactive bands in the region of 43–60 kDa indeed belong to the Gal-specific receptor. In the first

Table II: Adsorption to and Desorption from Sepharose 4B of the Photoradiolabeled Hepatocyte Cell Extract

fraction[a]	radioactivity (cpm × 10^{-3}): adsorption in the presence of $CaCl_2$	adsorption in the absence of $CaCl_2$
supernatant after adsorption	47.9 (26.1)[b]	120.1 (45.2)
wash 1	48.6 (26.5)	97.4 (36.6)
wash 2	18.8 (10.2)	33.1 (12.4)
wash 3	7.9 (4.3)	10.3 (3.9)
eluate 1	44.6 (24.3)	3.6 (1.4)
eluate 2	11.2 (6.1)	1.0 (0.4)
eluate 3	4.5 (2.5)	0.5 (0.2)

[a] Each fraction contained ca. 0.8 mL as described in the text. [b] The values in parentheses represent the percentage distribution of radioactivity.

approach, antisera against the purified receptor were used to retrieve the labeled receptor molecules from the photolabeled hepatocyte extracts (see next section). In another approach, the Gal binding ability of the labeled receptor was utilized. It may be predicted that the photoaffinity labeling would severely compromise the Gal binding ability of the receptor. However, as much as 50% of the photoaffinity-labeled, purified receptor was bound to asialofetuin–Sepharose in a calcium-dependent manner (unpublished data). The partial retention of Gal binding ability is most likely due to the aggregative nature of the Triton-solubilized receptor, which has an apparent molecular size corresponding to 5 or 6 monomer units. Since the efficiency of photolabeling is only 10–15% (Lee & Lee, 1986), some aggregate molecules may have a sufficient number of unaffected Gal combining sites to allow its binding to affinity media.

Sepharose, which is a polymer consisting of D-Gal and L-3,6-anhydro-Gal, apparently has enough exposed Gal residues to function as an affinant for this receptor (unpublished observation). A batchwise adsorption of the cell extracts to Sepharose and desorption with the EDTA-containing buffer produced results as shown in Table II. About 33% of the radioactivity in the cell extract was bound and eluted in a calcium-dependent manner, suggesting that this portion of radioactive material belonged to the receptor. Comparison of the SDS–PAGE pattern of the EDTA-eluted material with that of the original cell extract showed that the radioactivity in the 52-kDa region was adsorbed preferentially (Figure 4), as the recoveries of radioactivity in the stacking gel (mainly DNA) and dye-front band (<30 kDa) were ~5% and 30%, respectively, of that of the 52-kDa band.

Immunoprecipitation of the Photolabeled Cell Extracts. SDS–PAGE of the immunoprecipitated cell extract showed that an antiserum against the soluble receptor immunoprecipitated the radiolabeled materials in the 43–60-kDa area, while the preimmune serum did not (Figure 5). Figure 5 also includes the SDS–PAGE pattern of the same amount of the cell extract, but without immunoprecipitation. About 30% of the radioactivity in the 43–60-kDa area was recovered by this immunoprecipitation, while a very small percentage of radioactivity in the stacking gel and the dye-front band was precipitated. When the photoaffinity-labeled, purified receptor (Lee & Lee, 1986) was immunoprecipitated similarly, the recovery of counts in the receptor bands was ~50%. In another experiment, antisera against ASGP receptor and another plasma membrane antigen of the rat hepatocyte, known as CE-9 (Hubbard et al., 1985), were compared. The antiserum against the receptor immunoprecipitated radioactivity in the 43–60-kDa region effectively, while that against CE-9 was only

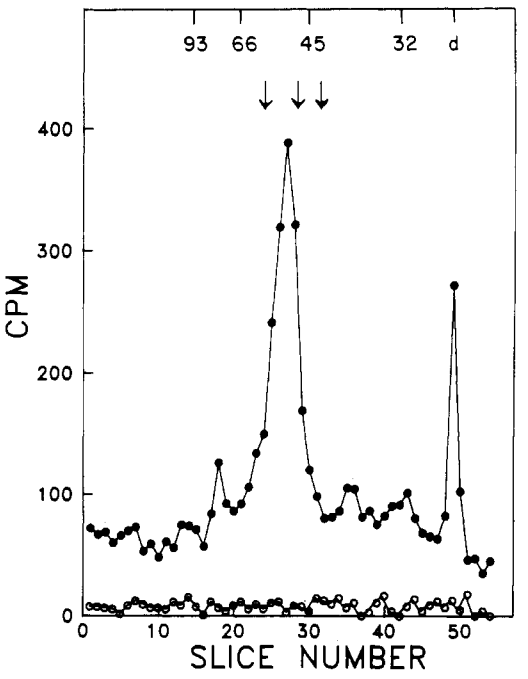

FIGURE 4: SDS–PAGE profiles of materials eluted from Sepharose. Initial adsorption to Sepharose: (●) under affinity conditions (+$CaCl_2$); (○) under nonaffinity conditions (+EDTA and no $CaCl_2$).

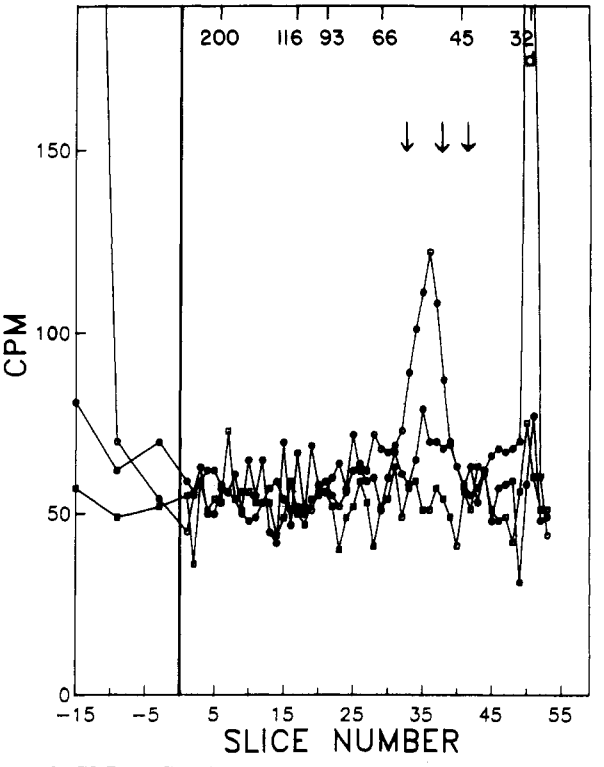

FIGURE 5: SDS–PAGE of immunoprecipitated cell extracts. Negative values (−15 to 0) on the x axis indicate the stacking gel area. (○) Cell extract without immunoprecipitation; (●) precipitated with an immune serum; (□) precipitated with the preimmune serum.

approximately one-eight as effective (data not shown).

Effect of Triton X-100 on Photolabeling. To study how Triton will affect the photoaffinity labeling of the cell-associated receptor, the following experiment was carried out. In one tube, 2.5 × 10^6 hepatocytes were gently shaken overnight in 2 mL of buffer II containing 1% Triton and proteolytic inhibitors (4.8 μM pepstatin A, 23 μM leupeptin, 10 mM iodoacetamide, and 0.5 mM PMSF). The photolabeling reagent was then added, and photolysis was carried out after

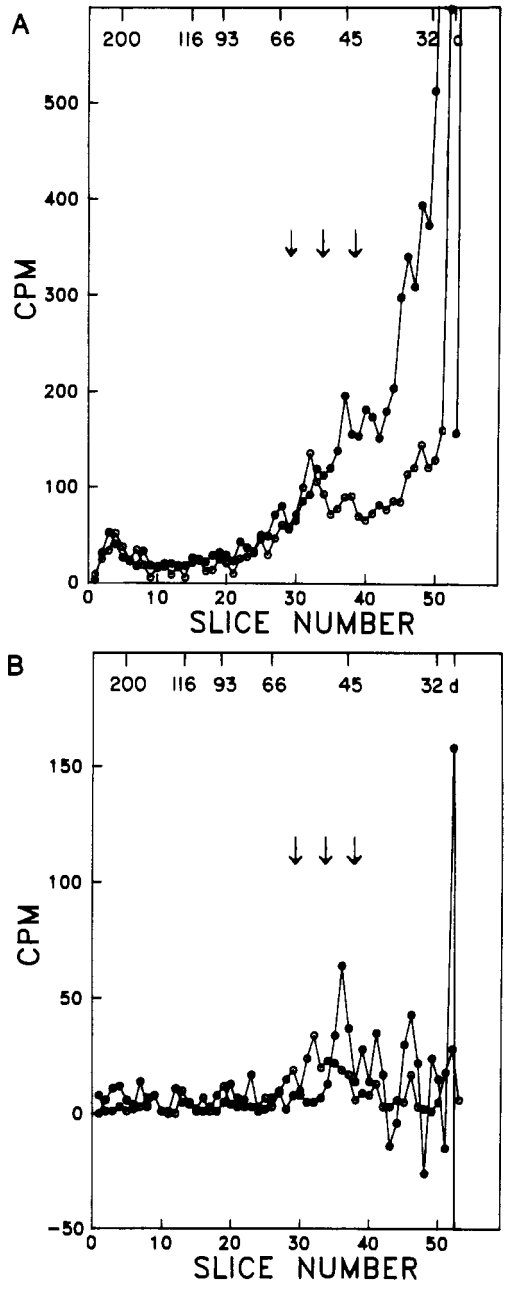

FIGURE 6: (A) SDS–PAGE of extracts from Triton-treated cells. (○) Photoaffinity reagent and Triton were coincubated with the hepatocytes; (●) hepatocytes were preincubated with 1% Triton X-100, and then the reagent was added. (B) SDS–PAGE of the immunoprecipitates of the extracts used in Figure 6A.

2-h incubation. In another tube, the reagent was added initially in the Triton buffer, and the mixture was photolyzed after 2-h incubation. After photolysis, the mixture was shaken overnight in the cold, so that both samples were exposed to 1% Triton for ~20 h. After centrifugation at 1000 rpm for 10 min to remove a small amount of debris, the supernatant solutions were fractionated on a column (1.5 × 28 cm) of Sephadex G-150. SDS–PAGE analysis of the void volume fractions is shown in Figure 6A. The cells that were exposed to the reagent and Triton together produced the SDS–PAGE pattern similar to the Triton-free experiments; i.e., the highest radiolabeled band in the 43–60-kDa region was at 52 kDa. However, incorporation of the radioactivity in the receptor polypeptide region (43–60 kDa) was much less efficient in the presence of Triton than in the Triton-free experiment. When the cells were solubilized with 1% Triton before the addition of the reagent, the incorporation of counts in the Sephadex-excluded peak increased dramatically (3-fold), and the SDS–PAGE pattern became considerably more complex, with much more counts incorporated in the molecules <40 kDa. Immunoprecipitation of the samples used in Figure 6A gave the SDS–PAGE patterns shown in Figure 6B. The ordinate in Figure 6B represents the difference counts of immunoprecipitation with the antiserum and its preimmune serum. The 52-kDa band was the major band labeled, if the cells were incubated simultaneously with Triton and reagent. In contrast, the 43-kDa band became the major labeled receptor band, when the cells were preincubated in Triton.

Lactoperoxidase-Catalyzed Iodination of the Receptor. Triton extracts of hepatocytes radioiodinated by the prebound GalNAc–LPO were analyzed by Sephadex G-150 gel filtration and SDS–PAGE. The elution profile from the Sephadex G-150 column was similar to that shown in Figure 1, with the radioactivity distributed 3 to 2 in favor of the excluded peak. When the affinity binding of GalNAc–LPO was prevented by substituting EDTA for $CaCl_2$, the incorporation of radioactivity decreased by 80%, and both the excluded and included peaks were equally affected. SDS–PAGE of the affinity-iodinated cell extract showed that the three peptide bands belonging to the receptor were the most heavily labeled (Figure 7A,C). However, the affinity iodination was not as specific as the photoaffinity labeling, as the iodination produced many extra peptide bands which were not immunoprecipitated by the antiserum for the receptor (Figure 7A). The recovery of the radioactivity in the receptor region (43–66 kDa) was 40–60%. The position of the iodinated receptor bands corresponded exactly to the Coomassie-stained bands, since iodination would cause little change in the molecular weight. Note also that the labeled bands are sharper in the GalNAc–LPO-iodinated material than in the photolabeled material. Inclusion of digitonin in the initial incubation with GalNAc–LPO increased the incorporation of the radiolabel without affecting the distribution of label among the three receptor bands; i.e., the 52-kDa subunit was still the major band labeled (not shown). As shown in Figure 7A and Table I, distribution of the label among the three bands varied with the length of iodination time. At the shorter iodination time when the concentration of free iodine was probably still low, very little label had been incorporated into the 43-kDa band.

The soluble receptor from rat liver iodinated by GalNAc–LPO was fractionated on a column of Sephadex G-150 (1 × 26 cm). The SDS–PAGE pattern of the void volume fractions is shown in Figure 7B,C. The distribution of the radiolabel among the three subunit bands was similar to that of the Coomassie staining; i.e., the major portion of the total counts in the receptor was in the 43-kDa band (Table I).

Iodination of Hepatocytes by the Chloramine T Method. The Sephadex G-150 elution profile of the cell extract nonspecifically iodinated by the Chloramine T method showed that the excluded peak was only half the size of the included peak (data not shown). The SDS–PAGE profile of the cell extract showed that the receptor bands were no longer the predominantly labeled species in this nonaffinity mode of labeling (Figure 8). Immunoprecipitation showed that about an equal amount of radioactivity was incorporated into each of the receptor bands.

Isolation of the Gal/GalNAc-Specific Receptor from Isolated Rat Hepatocytes. Isolated hepatocytes from three rats (~1 × 10^9 cells) were combined and washed with the EDTA buffer containing proteolytic inhibitors (pepstatin A, 4.8 μM; leupeptin, 23 μM; iodoacetamide, 10 mM; and PMSF, 0.5 mM), resuspended in the same buffer, and frozen overnight.

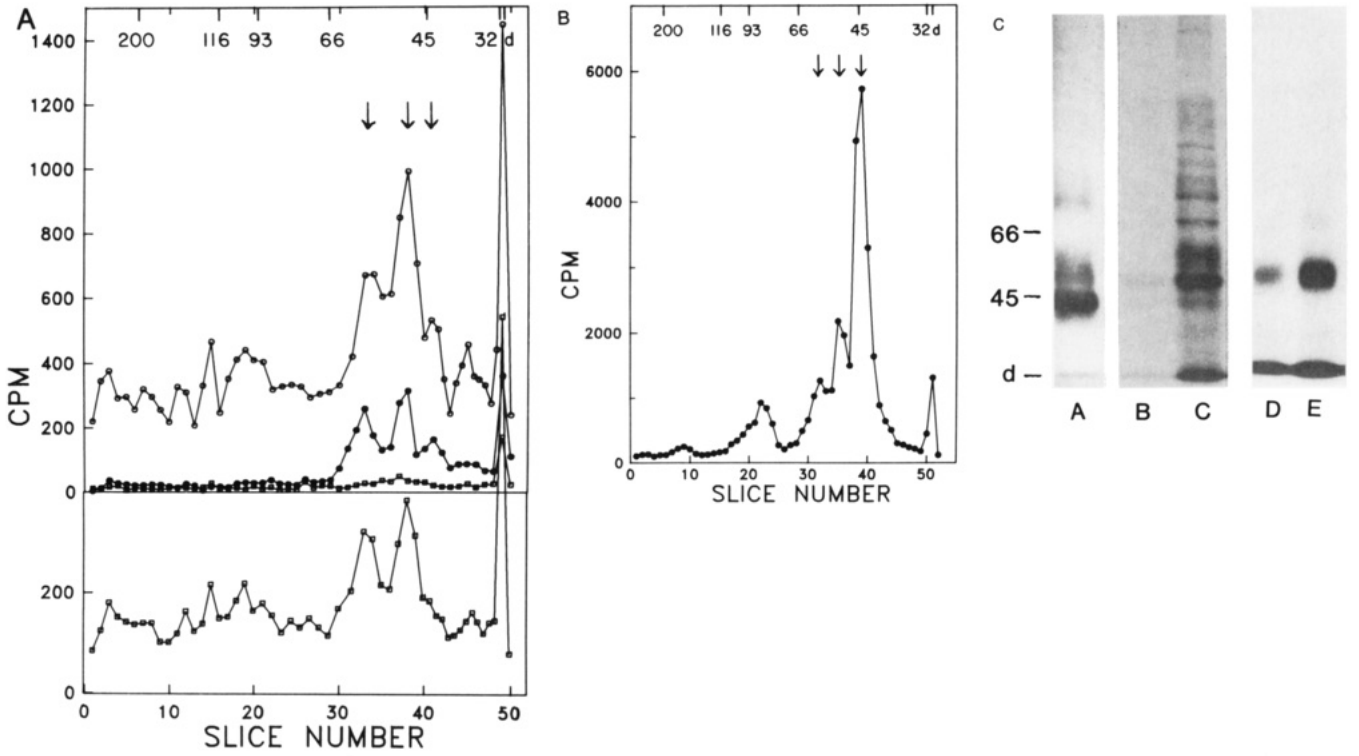

FIGURE 7: (A) SDS–PAGE of GalNAc–LPO-iodinated hepatocyte extracts. Upper panel: (○) iodinated for 90 min; the amount of the extract applied was equivalent to 1.2×10^5 cells; (●) the same material after precipitation with the immune serum; (□) the same material after precipitation with the preimmune serum. Lower panel: (□) iodinated for 40 min. (B) SDS–PAGE of the soluble receptor iodinated by GalNAc–LPO. (C) Autoradiograph. Lane A, purified receptor iodinated with GalNAc–LPO; lane B, Triton extract of digitonin-treated hepatocytes iodinated by GalNAc–LPO; GalNAc–LPO was incubated with hepatocytes in the absence of $CaCl_2$ and in the presence of EDTA; lane C, same as lane B, except incubation with GalNAc–LPO was carried out in the presence of $CaCl_2$; lane D, Triton extract of digitonin-treated hepatocytes photolabeled in the presence of EDTA; lane E, same as lane D, except photolabeling was carried out in the presence of $CaCl_2$.

The suspension was thawed, and the freezing–thawing was repeated again. The cell suspension (~45 mL) was centrifuged at 1000 rpm for 5 min, and the sedimented cells (~10 mL) were stirred for 2 h in the cold with 70 mL of the Triton buffer containing the same proteolytic inhibitors as above. After centrifugation at 15000 rpm for 25 min, the supernatant solution (~80 mL) was made 50 mM in $CaCl_2$ and shaken with 5 mL of asialofetuin–Sepharose overnight. The Sepharose was centrifuged, and the supernatant solution was discarded. The gel was packed into a column and washed overnight with the column loading buffer of Hudgin et al. (1974). The column was eluted with the elution buffer (10 mM HEPES buffer, pH 7.8, containing 1.25 M NaCl, 10 mM EDTA, and 0.1% Triton X-100), collecting 0.4 mL per fraction. Fractions were analyzed for the receptor activity by using ^{125}I-ASOR as ligand (Hudgin et al., 1974). The fractions containing the ASOR binding activity were combined and dialyzed against a buffer (10 mM HEPES buffer, pH 7.8, containing 1.25 M NaCl and 0.1% Triton X-100). About 30% of the ASOR binding activity in the Triton extract was recovered in the specifically eluted fractions from the asialofetuin–Sepharose column. The SDS–PAGE patterns of the receptor obtained in this fashion and that obtained from the whole liver acetone powder are shown in Figure 9. The patterns were quite similar; i.e., the 43-kDa band was the major band for both preparations. In a 7.5% acrylamide gel, preparations from the acetone powder generally showed a faint, diffuse band moving slightly ahead of the 43-kDa band. This band probably resulted from a limited proteolysis, as its intensity increased with storage. This band was absent in the receptor preparations obtained from the isolated hepatocytes. We noted that the purified receptor obtained from acetone powder usually contained a diffuse band of slower mobility corresponding to dimers. This band was also absent in the receptor prepared from the hepatocytes.

Discussion

The Gal/GalNAc-specific receptors isolated from mammalian livers are all composed of polypeptide chains of 40–60 kDa in size. These polypeptide chains are apparently not held together by disulfide bonds but exist in aqueous solution in the presence of nondenaturing, neutral detergents as aggregates ranging from 160 kDa (Bezouska et al., 1985) to 600 kDa (Anderson et al., 1982) in size. While human, pig, and rabbit receptors consist of only one subunit or two subunits in a simple ratio (Baenziger & Maynard, 1980; Bezouska et al., 1985; Kawasaki & Ashwell, 1976), the rat receptor has an unusual composition, having two minor polypeptide species (52 and 60 kDa) and one predominant species (43 kDa) which accounts for 60–75% of the total protein (Harford et al., 1982; Schwartz et al., 1981; Warren & Doyle, 1981). Our preparation of the rat receptor protein also had a similar polypeptide composition.

The high-affinity photolabeling reagent used in this study was derived from a triantennary glycopeptide of fetuin (Lee & Lee, 1986). Earlier studies showed that the reagent labeled all the subunit bands of the Triton-solubilized, purified rat and rabbit receptors in a photo- and affinity-dependent manner with 10–15% overall efficiency. Because of this low efficiency, it is expected that a majority of the labeled subunits are modified with only one reagent molecule, even if the subunit contains more than one Gal/GalNAc combining site. In the case of the soluble rat receptor, the major Coomassie stain band (43 kDa) was also the most heavily photolabeled band (Lee & Lee, 1986). Therefore, it was rather unexpected that the photolabeling of the receptor on the hepatocyte membranes produced the predominantly labeled band at 55 kDa, sug-

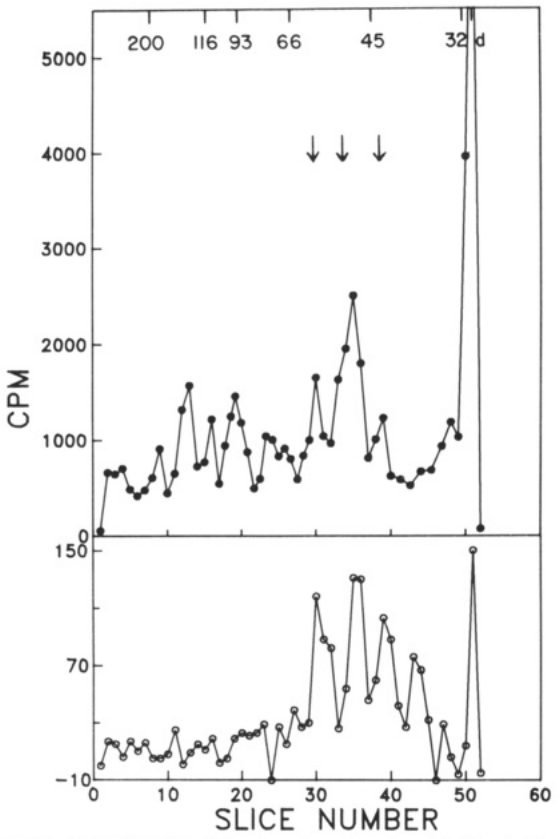

FIGURE 8: SDS–PAGE of the hepatocyte extract iodinated by the Chloramine T method. Upper panel: Cell extract equivalent to 1.1 × 10^6 cells. Lower panel: Difference cpm between the immune and preimmune sera.

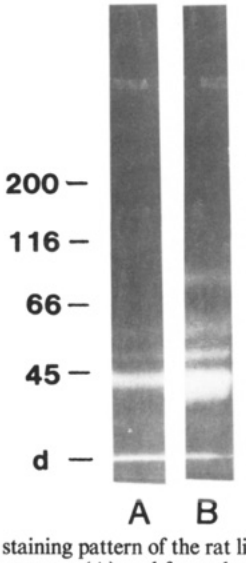

FIGURE 9: Coomassie staining pattern of the rat liver receptor prepared from the isolated hepatocytes (A) and from the acetone powder (B).

gesting that the labeling occurred mainly on the 52-kDa band (a minor band by Coomassie staining). This was true whether the labeling was aimed at the hepatocyte surface only or with the internal membranes exposed (Figure 2).

Three lines of evidence indicated that the labeled bands in the region of 43–60 kDa indeed belong to the receptor. First of all, the inclusion of competitive inhibitors [Gal_{34}-BSA and $TyrAsp(LacAHT)_2$] or exclusion of calcium ion during preincubation diminished labeling of these bands significantly without causing much change to the labeling of other areas (Figure 3). Secondly, a significant portion of the labeled material in the 43–60-kDa area was preferentially adsorbed to Sepharose and specifically eluted from Sepharose in a calcium ion dependent manner (Figure 4). As mentioned under Results, this phenomenon probably resulted from the oligomeric nature of the receptor. Thirdly, these bands could be immunoprecipitated with antisera against the receptor but not with the preimmune serum or with an antiserum against another rat hepatocyte cell surface antigen.

It has been reported by at least three groups that lactoperoxidase-catalyzed radioiodination of the hepatocyte surfaces and plasma membranes perferentially labeled the 52-kDa band (sometimes the 60-kDa band as well) of this receptor, while the 43-kDa band was poorly labeled (Warren & Doyle, 1981; Schwartz et al., 1981; Hubbard et al., 1985). In all three cases, the labeled receptor was obtained either by immunoprecipitation or by affinity adsorption. From such results, it was speculated that either the materials in the 43-kDa band were degradation products representing artifacts of isolation from the liver acetone powder or the 43-kDa material existed mainly on the internal membranes of the hepatocytes. We carried out iodination of rat hepatocytes with GalNAc-modified LPO which was first allowed to bind by affinity to the receptors on the hepatocyte to mask ~50% of the receptor sites. Analysis of the cell extract directly by SDS–PAGE without prior immunoprecipitation or affinity adsorption showed that the 52-kDa band was the major band iodinated both in the presence and in the absence of the digitonin (Figure 7). The strikingly similar labeling patterns observed for the photoaffinity labeling and LPO-catalyzed iodination suggest that there must be a common explanation for these phenomena.

The 43-kDa band of Triton-solubilized, purified rat receptor typically appears as an unusually broad band in the Coomassie blue staining. We have also observed that the salt–EDTA extract and Triton extract of the rat liver acetone powder, which represent the first two steps in the preparation of the Triton-solubilized, purified receptor, efficiently degraded the affinity-labeled bands (data not shown). Although the amino acid sequence studies suggested the 43-kDa material to be a gene product distinct from the 52- and 60-kDa polypeptides (Drickamer et al., 1984; Holland et al., 1984), we investigated the possibility that materials in the 43-kDa band may also contain degradation products of the 52-kDa material. Huisman et al. (1974) have shown that proteolytic degradation in the rat hepatocytes can be effectively arrested by a combination of protease inhibitors, pepstatin A (an inhibitor of the carboxylic acid type proteases) and iodoacetate (an inhibitor of cysteine-type proteases). We used leupeptin (a powerful inhibitor of trypsin-type proteases, such as cathepsin B) and PMSF (an inhibitor of serine-type proteases) in addition to the aforementioned inhibitors to carry out the initial phases of the receptor isolation from the isolated rat hepatocytes. The results showed that the Coomassie-stained pattern was essentially the same as that of the receptor obtained from the acetone powder (Hudgin et al., 1984), though the 43-kDa band obtained from the hepatocytes was considerably less diffuse (Figure 9). Therefore, it appears that the 43-kDa band exists as the major component of the native receptor in the rat hepatocytes.

The receptor resides both on the external and on the internal membranes of hepatocytes, with the internal pool generally considered more abundant than the surface receptor (Steer & Ashwell, 1980; Baenziger & Fiete, 1980; Weigel & Oka, 1983). When the hepatocytes were photolabeled in the presence of digitonin so as to expose the internal receptors to the affinity reagent (Weigel et al., 1983), the labeling of the receptor bands increased 2–3-fold compared to the surface labeling while the labeling profile remained quite similar

(Figure 2 and Table I). These results suggest that the 43-kDa band is probably present as the major band both on the internal and on the plasma membranes but is always poorly labeled by the photoaffinity reagent in the membrane-bound state.

The fact that the 43-kDa band was most prominently labeled when the Triton-solubilized, purified receptor was photoaffinity labeled or iodinated by GalNAc–LPO catalysis [Figure 7C, Table I; also see Lee and Lee (1986)] indicated that the 43-kDa band was not deficient in the Gal combining sites or in the iodinatable tyrosine residues. Exposure of hepatocytes to Triton altered the photolabeling pattern of the receptor subunits in a time-dependent manner. The 52-kDa band was still the dominantly labeled band, if the hepatocytes were exposed to Triton for only 2 h together with the photoaffinity reagent. When the hepatocytes were exposed to Triton alone for a longer period, the 43-kDa band became dominantly labeled (Figure 6B). These results suggest that the total destruction of the membrane organization by Triton X-100 appeared to proceed gradually and that a partial retention of the receptor organization on the membranes was probably needed for the preferential labeling of the 52-kDa band.

The labeling pattern of the receptors on the hepatocyte membranes appeared to be influenced by the size of the labeling reagent. As shown in Table I, incorporation of the radiolabel into the 43-kDa band was only 6% when a macromolecular reagent was used (the short-term GalNAc–LPO-catalyzed iodination). The incorporation rose to 20% when the photolabeing reagent TAGP was used. Though the molecular weight of TAGP is only ~3K, its effective size may be quite large. The oligosaccharide portion of TAGP exists in aqueous solutions in a preferred conformation in which the nonreducing terminal, *N*-acetyllactosamine units are widely separated in space (Bock et al., 1982; Lee, Y. C., et al., 1984). As the size of reagent decreased further to iodine (Chloramine T iodination), the incorporation of the label into the 43-kDa band rose to 31% (Table I). However, uniform labeling of all the subunits was not achieved even with this small reagent. Putting all the results together, we conclude that the preferential labeling of the 52-kDa band occurs because the 52-kDa band is topologically prominent on the hepatocyte membranes and is easily accessible by aqueous phase reagents, while the 43-kDa subunit is severely hindered sterically. When the receptor molecules are incorporated into Triton micelles, this subunit organization is apparently perturbed, making all the subunits equally accessible to reagents.

While the photolabeling reagent produced only 5–20% nonspecific labeling of the Triton-solubilized, purified receptor (Lee & Lee, 1986), nonspecific labeling in the more complex cell environment was generally in the range of 30–50%. Though the incorporation of the label in all three bands seemed to decrease uniformly in the blanks containing either EDTA or a small multivalent inhibitor, TyrAsp(LacAHT)$_2$ (Figure 3), a large competitive inhibitor, Gal$_{34}$-BSA, reproducibly resulted in the reduction of the label in the 52–60-kDa area without affecting the 43-kDa area. This result can also be explained by the postulate that the 43-kDa subunits are sterically hindered on the hepatocyte membranes. A large ligand such as Gal-BSA, having a limited access to the Gal combining sites of the 43-kDa subunits, cannot block the binding of smaller ligands, such as the photolabeling triantennary glycopeptide and TyrAsp(LacAHT)$_2$. Indeed, it has been documented (Hardy et al., 1985) that the number of ligand binding sites on the hepatocyte surface varied depending on the size of the ligands. The hepatocyte surface that has been saturated with respect to a large ligand, ASOR, still possessed unoccupied sites available to a small ligand, such as Tyr-Asp(LacAHT)$_2$.

In addition to the 52-kDa band, the rat hepatic Gal/GalNAc receptor contains another minor band of approximately 60 kDa. Unlike the 52-kDa subunit, this band appears diffuse, and the reported molecular size (58–64 kDa) and relative abundance (0–15%) vary more widely (Warren & Doyle, 1981; Schwartz et al., 1981; Drickamer et al., 1984). Our soluble, pure receptor preparations usually contain the 60-kDa subunit in about half the quantity of the 52-kDa band. The 52- and 60-kDa subunits are more closely related to each other in their primary structure (Warren & Doyle, 1981; Schwartz et al., 1981; Drickamer et al., 1984) than the 43-kDa band. Since the 60-kDa band was usually labeled more heavily than the 43-kDa band (Table I, Figures 2 and 7A), it is likely that the 60-kDa subunit behaves similarly to the 52-kDa subunit.

The phenomenon of preferential labeling of the minor (52-kDa) band may also be related to the well-documented, cluster effect (Lee et al., 1983; Lee, R. T., et al., 1984). Monovalent glycosides of Gal are bound with a K_d of 0.3–1 mM to both the Triton-solubilized receptor and receptors on the hepatocyte surface (Connolly et al., 1983). However, clustering of three Gal residues within a ligand could increase the affinity to the hepatocyte surface receptor tremendously (up to a millionfold), while binding to the soluble receptor increased only 100–1000-fold (Lee, R. T., et al., 1984). It has been postulated from comparative studies of many cluster ligands that this cluster effect is produced by a specific arrangement of the Gal combining sites on the hepatocyte surface (Lee, Y. C., et al., 1984). It is likely that the subunits of the receptor are also organized and maintained in a definite spatial arrangement in order to accomplish a proper clustering of the Gal combining sites. We postulate here that the subunit organization that gives the maximal cluster effect also makes the 43-kDa band less accessible to the affinity reagents and especially to large molecules, such as LPO and BSA derivatives. Solubilization in Triton loosens the subunit organization so that both inaccessibility of the 43-kDa band and much of the cluster effect are lost. If this is true, a large ligand, such as ASOR, may be bound to the hepatocyte membrane receptors primarily by the 52-kDa (and the 60-kDa) bands. Recently, Ray et al. (1986) showed that certain detergents, such as Triton X-100, increased the number of ^{125}I-ASOR bound to permeabilized–fixed hepatocytes more than 2-fold. This increased binding capacity can be explained as the result of the Triton-induced, increased availability of the Gal combining site(s) of the 43-kDa subunit.

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