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Characterization of a Membrane-associated Receptor from Rat Sinusoidal Liver Cells That Binds Formaldehyde-treated Serum Albumin*

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When treated with formaldehyde, serum albumin is known to be taken up and degraded by sinusoidal liver cells via adsorptive endocytosis. The present study aimed at characterization and identification of the membrane-associated receptor on rat sinusoidal liver cells. Kinetic studies of binding of ^{125}I-labeled formaldehyde-treated serum albumin (^{125}I-f-Alb) with the membranes of sinusoidal liver cells demonstrated the presence of specific, high-affinity, saturable membrane-bound receptors with an apparent K_d = 8 μg of f-Alb/ml and the optimal pH at around 8.0. The ^{125}I-f-Alb binding to the membranes was not inhibited by either native albumin, asialofetuin, methylamine-treated α_2-macroglobulin, mannan, or immune complexes. The binding process exhibited independence of calcium and susceptibility both to heat treatment and to destruction by proteases. The binding was inhibited by concanavalin A and the inhibition was effectively reversed by the presence of α-methyl-D-glucoside, a haptenic inhibitor for this lectin, indicating the glycoprotein nature of the receptor. The binding protein was extracted from the membrane preparations with octyl β-D-glucopyranoside and immunoprecipitated by anti-ligand antibody as a complex with the ligand. Sodium dodecyl sulfate-gel electrophoresis of the immunoprecipitate revealed two polypeptide chains with molecular weights of approximately 53,000 and 30,000, respectively.

As early as 1965, Mego and McQueen (1) found that formaldehyde-treated ^{131}I-serum albumin was rapidly cleared from the blood stream and taken up by the liver when injected intravenously into mice (1). Subsequent studies by Nilsson and Berg (2) showed that f-Alb[1] was exclusively taken up and degraded by sinusoidal liver cells both *in vivo* and *in vitro*. The kinetic studies on *in vitro* uptake using isolated rat sinusoidal liver cells have indicated the presence on the surface membranes of a specific receptor or binding protein which might serve in clearing of this ligand from the blood stream by receptor-mediated endocytosis (2). f-Alb has since been used either as a marker protein targeting sinusoidal liver cells (3-5) or as a useful probe to elucidate the general mechanism of endocytosis (6, 7).

Nothing has been known, however, about the physiological role(s) of the putative receptor which specifically recognizes this particular ligand molecule. It was argued without any experimental evidence that a structure alteration might not occur *in vivo* to circulating serum albumin, producing a molecular species which could be recognized by the receptor just like f-Alb. It is equally possible that there might be other natural ligand(s) which could be recognized by the receptor for the artificial ligand, f-Alb. For understanding the physiological role of this receptor on sinusoidal liver cell membranes, it is requisite to characterize the binding specificity of this receptor.

Like several ligands such as asialoglycoprotein (8), high-mannose type glycoprotein (9), and low-density lipoprotein (10) known to be endocytosed by a receptor-mediated process, endocytosis of f-Alb is believed to be composed of at least three steps: the initial binding of a ligand to its receptor on the surface membranes, internalization of receptor-ligand complexes, and subsequent delivery to lysosomes, where the degradation of the endocytosed ligand takes place (2, 11). Since it is generally accepted that the specificity of receptor-mediated endocytosis resides exclusively on the initial binding step between a ligand and its receptor (12), we directed our attention to the initial binding process by analyzing the interaction of f-Alb with the membrane fractions isolated from sinusoidal liver cells.

The present paper describes first some characteristics of binding of ^{125}I-labeled f-Alb to sinusoidal cell membranes, and, second, the identification of the binding protein in the nonionic detergent extract of sinusoidal cell membranes as a binding protein-ligand complex with monospecific anti-ligand antibody.

MATERIALS AND METHODS

Chemicals—Collagenase (type I and type IV), bovine serum albumin (Fraction V), Pronase, fetuin, and proteinase K were purchased from Sigma. Mannan was obtained from Nakarai Chemical Co. (Osaka, Japan). Na^{125}I (15.8 mCi/μg of iodine or 661 mCi/ml) was obtained from Amersham or New England Nuclear. *Staphylococcus aureus*, Cowan strain A, was cultured and fixed with formalin according to the method reported by Kessler (13). Octyl β-D-glucopyranoside (Dotite) was purchased from Dojindo Laboratory (Kumamoto, Japan). Human α_2-macroglobulin was purchased from Cappel Laboratory (Cochranville, PA). All reagents used were of the best grade available from commercial sources.

Preparation of Ligands and Iodination—f-Alb was prepared from bovine serum albumin by a modification of the methods reported (1, 2). Briefly, 0.4 g of bovine serum albumin was dissolved at room temperature in 5.2 ml of 0.45 M sodium carbonate buffer (pH 10.0), followed by centrifugation to remove insoluble debris. To the super-

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[1] The abbreviations used are: f-Alb, formaldehyde-treated bovine serum albumin; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; Staph A, formalin-fixed *Staphylococcus aureus*, Cowan strain A; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

natant solution was added, dropwise under stirring, 5.4 ml of formaldehyde solution (37% w/v) to give a final concentration of 20% (w/v). After incubation at 37 °C for 1 h, the solution was dialyzed overnight against 0.15 M NaCl, and, then, against distilled water at 4 °C. After removing the insoluble materials by centrifugation at 20,000 × *g* for 30 min at 4 °C, the resulting supernatant was stored at −80 °C before use. f-Alb thus prepared was radiolabeled to a specific activity of 4,000–5,000 cpm/ng with ^{125}I with immobilized Enzymobead lactoperoxidase-glucose oxidase (Bio-Rad) according to the manufacturer's instructions. Free iodine was removed by a rapid gel filtration on a mini-column of Sephadex G-25 (Pharmacia Fine Chemicals) as reported by Tuszynski *et al.* (14). Before being employed in the binding studies as a radioactive ligand, each preparation of ^{125}I-f-Alb was tested for the biological activity by measuring both *in vivo* plasma clearance in rats and *in vitro* cellular uptake by sinusoidal liver cells. Briefly, upon intravenous injection of a trace amount of ^{125}I-f-Alb (1–2 μg/100 g of body weight), an apparent plasma clearance rate was 2–3 min. A semilogarithmic plot of the clearance curve exhibited a straight line. When a loading amount (1 mg/100 g of body weight) was administered, it was cleared from the circulation with a half-life of about 20–25 min. This value was consistent with the reported value (15). By contrast, ^{125}I-labeled native albumin was cleared from the circulation with a half-life of 48 h. The *in vitro* uptake rate of ^{125}I-f-Alb by the isolated sinusoidal liver cells also agreed well with the reported value (2). No appreciable differences were observed from one preparation to another. In addition, proof that the active ligand which is actually recognized by sinusoidal liver cells was derived from albumin rather than contaminant(s) in commercial preparations of BSA was verified by the following evidence: (i) over 90% of ^{125}I-f-Alb infused at a trace or loading amount was cleared from the circulation in a single exponential process and (ii) further purification of commercial albumin preparations by Sephacryl S-200 chromatography or by a Sepharose 4B column coupled with Cibacron blue dye did not alter the plasma clearance nor the *in vitro* uptake by the cells or the binding to the cells. Therefore, in the present study, the commercial preparation of BSA was used without further purification.

Methylamine-treated α_2-macroglobulin was prepared as described by Marynen *et al.* (16). Asialofetuin was prepared from fetuin by the method of Spiro (17). Concanavalin A was prepared as described previously (18). Denaturation of serum albumin was performed either in 8 M urea (pH 5.5) as described by Moore *et al.* (19) or in 6 M guanidine hydrochloride, followed by extensive dialysis against 20 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. Carboxymethylation or carboxamidomethylation of serum albumin under denaturing conditions was performed by the method of Hirs (20). After dialysis, these preparations exhibited a significant loss of α-helical content as determined by their circular dichroic spectra (data not shown). The protein concentration was determined by the method of Lowry *et al.* (21) or by a dye-binding method reported by Bradford (22) with BSA as a standard.

Preparation of Rat Sinusoidal Liver Cells—Sinusoidal liver cells were prepared from male Wistar strain rats (200–240 g) by a modification of the collagenase perfusion method of Berry and Friend (23). Briefly, rat liver was perfused first with preperfusion buffer (Ca^{2+}-Mg^{2+}-free Hanks' solution) for 10 min and then with Hanks' solution containing 0.05% collagenase (type I:type IV = 1:3) for less than 15 min. The gas phase was maintained at 95% O_2, 5% CO_2 and perfusion was performed at 37 °C at a flow rate of 20–25 ml/min. After the perfusion was discontinued, the liver was excised and deprived of the capsule membrane, followed by dispersing the cells by gentle stirring in an ice-cold Hanks' solution containing 1% BSA. The dispersed cells were filtered through nylon mesh sieves (pore size, 0.5 × 0.3 mm) twice, followed by centrifugation at 50 × *g* for 30 s. The pelleted cells (mainly hepatocytes) were discarded. The supernatant was similarly centrifuged two more times. The resulting supernatant was, then, centrifuged at 200 × *g* for 2 min. The pelleted cells were resuspended in an ice-cold solution containing BSA and recentrifuged at 200 × *g* for 2 min. The cell suspension was further washed three more times with Hanks' solution to remove BSA. The final cell pellet was immediately stored at −80 °C until use. The viabilty of isolated sinusoidal cells was greater than 99% when determined either by trypan blue exclusion or by contents of lactic dehydrogenase (24) and was contaminated with hepatocytes less than 0.1%. The yield of the cells was 5–7 × 10^7 cells/rat.

Preparation of Sinusoidal Liver Cell Membranes—Membranes were prepared by a modification of the method described by Basu *et al.* (25). In a typical preparation, frozen or freshly isolated sinusoidal cells (2 × 10^8 cells) were suspended in 10 ml of 10 mM Tris-HCl (pH 7.4), 1 mM $CaCl_2$, and 0.15 M NaCl. The cells were then disrupted in a Polytron homogenizer (Brinkmann Instruments) using homogenization conditions (setting 8, 6 s, 4 °C). The whole homogenate was then centrifuged at 800 × *g* for 4 min at 4 °C to remove the nuclear fraction. The supernatant was saved and the pellet was resuspended in 5 ml of the same buffer and centrifuged at 800 × *g* for 4 min. The combined supernatant was then centrifuged at 105,000 × *g* for 1 h at 4 °C. The resulting 105,000 × *g* pellet was resuspended in an adequate amount of the same buffer (ice cold) to give a protein concentration of 1.0–1.5 mg/ml. The yield of the membrane fraction was 2 mg of protein from 2 × 10^8 cells.

Binding of ^{125}I-f-Alb to Membranes—Binding assays were carried out in 1.6-ml polyethylene centrifuge tubes (Eppendorf). Unless otherwise stated, reaction mixtures for binding assay contained, in a final volume of 0.1 ml, buffer A (0.1 M NaCl, 0.5 mM $CaCl_2$, 50 mM Tris-HCl (pH 7.5), and 20 mg/ml of BSA), indicated amounts of membrane fraction, 0.37 μg of ^{125}I-f-Alb (4.65 × 10^6 cpm/μg), and indicated amounts of unlabeled f-Alb. The reaction was initiated by adding radiolabeled f-Alb and the mixture was allowed to incubate for 1 h at 0 °C in an ice-water bath with several intervals on a Vortex mixer. To determine the amount of ^{125}I-f-Alb bound to the membranes, the tubes were centrifuged at 35,000 × *g* for 1 h at 4 °C. The supernatant was removed by aspiration and the surface of the membrane pellet was washed gently by adding 1 ml of ice-cold buffer A without suspending pellet. The tube was recentrifuged at 35,000 × *g* for 30 min at 4 °C. After removing the supernatant, the bottom of the tube containing pellets of membranes was cut off with scissors and placed in a plastic tube for radioactivity determination in a Packard Auto-gamma scintillation spectrometer model 5130 (Packard Instrument Co.). Unless otherwise stated, each value in the figures and tables represents the data of duplicate assays. The nonspecific adsorption of ^{125}I-f-Alb to the tube wall was at a background level of radioactivity (less than 2% of nonspecific binding obtained in the presence of an excess amount of unlabeled f-Alb).

Preparation of Antibodies—Antibodies against native albumin and f-Alb were raised in rabbits by the method described previously (26). Antibodies were assayed by immunoprecipitation using either ^{125}I-labeled BSA or f-Alb by the method reported previously (27). Monospecific antibody against BSA were purified by passing antiserum through a column of Sepharose 4B coupled with BSA. Adsorbed fractions were eluted with 0.1 M glycine-HCl buffer (pH 3.0) as previously described (28). Antibodies against f-Alb were similarly purified by Sepharose 4B coupled with f-Alb. Each preparation exhibited two polypeptide chains corresponding to heavy and light chains of IgG molecule upon $NaDodSO_4$-gel electrophoresis.

To prepare the Staph A coated with each antibody, 200 μl of Staph A suspension (10% v/v) was incubated with a saturating amount of each antibody for 30 min at 4 °C, followed by extensive washing with 50 mM Tris-HCl, 0.1 M NaCl, then with buffer B (50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 0.5 mM $CaCl_2$, and ovalbumin, 40 mg/ml). The Staph A coated with each antibody was finally resuspended in 200 μl of buffer B and used for the experiment described below.

Identification of a Membrane-associated Receptor for f-Alb—The membrane fraction prepared from sinusoidal liver cells as described above was finally suspended in 20 mM sodium phosphate buffer (pH 7.2) and 0.15 M NaCl. To 200 μl of the membrane fraction (1.5 mg/ml) was added octyl β-D-glucopyranoside to a final concentration of 40 mM. The membranes were vigorously mixed on a Vortex mixer and kept on ice for 20 min, then centrifuged at 105,000 × *g* for 1 h at 4 °C. A portion of the resulting supernatant (solubilized membrane proteins) was radiolabeled with ^{125}I to a specific radioactivity of about 800 cpm/ng with Enzymobead as described above for ^{125}I-f-Alb. Binding of f-Alb to ^{125}I-labeled membrane proteins was performed as follows: The reaction mixture contained, in a final volume of 0.1 ml, ^{125}I-labeled membrane proteins (2 × 10^6 cpm) and 200 ng of f-Alb in buffer B. After incubation at 4 °C for 3 h, 100 μl of anti-f-Alb antibody-coated Staph A suspension, which was sufficient to trap all f-Alb present, was added to the reaction mixture. The mixture was then incubated on ice for 1 h with several intervals on a Vortex mixer, followed by centrifugation in an Eppendorf centrifuge for 1 min at 4 °C. The pellet was resuspended in 1 ml of ice-cold buffer B, followed by centrifugation. This washing procedure was repeated five more times. The final pellet was suspended in 80 μl of 0.05 M Tris-HCl (pH 6.8), 1% $NaDodSO_4$, and 1% 2-mercaptoethanol and boiled for 3 min, followed by centrifugation for 1 min. The supernatant was

electrophoresed on a $NaDodSO_4$-polyacrylamide slab gel (6–14%) as described previously (29) by using the buffer system of Maizel (30). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 and destained to locate the marker proteins for molecular-weight determination. The corresponding gel slot was cut into 2-mm thick slices, followed by determination of the radioactivity. Two control experiments were performed. One was conducted in exactly the same way as described above except for the omission of f-Alb as the ligand. In another control experiment, f-Alb was replaced by the same amount of native albumin (BSA), and then the mixture was reacted with anti-BSA antibody-coated Staph A.

RESULTS

Binding of ^{125}I-f-Alb to Sinusoidal Liver Cell Membranes—Fig. 1 shows the time course for binding of ^{125}I-f-Alb to membranes isolated from rat sinusoidal liver cells. The binding reaction appeared to reach equilibrium within 60–80 min at 0 °C. In the presence of a 1000-fold excess of unlabeled f-Alb, the binding of ^{125}I-f-Alb was reduced by more than 90%, indicating that the unlabeled f-Alb and radiolabeled f-Alb were competing for a limited number of the same binding sites.

Fig. 2 shows the amounts of ^{125}I-f-Alb bound at 0 °C as a function of the concentration of ^{125}I-f-Alb in the incubation medium. A double reciprocal plot of these data (Fig. 2*B*) indicated that the concentration of ^{125}I-f-Alb giving half-maximal binding was approximately 8 μg of protein/ml. A similar value for half-maximal ^{125}I-f-Alb binding was obtained when the binding assays were performed at 37 °C, although the maximal amount of ^{125}I-f-Alb bound to the membranes was 1.8–2.0-fold higher than that at 0 °C (data not shown).

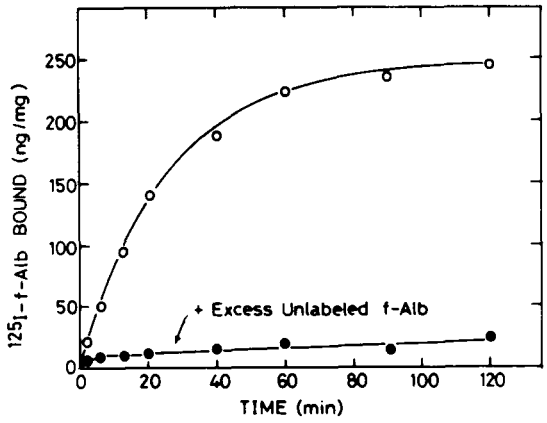

FIG. 1. **Time course of ^{125}I-f-Alb binding to sinusoidal liver cell membranes.** Each tube contained, in a total volume of 0.1 ml, 16.5 μg of membrane protein, 0.37 μg of ^{125}I-f-Alb (4670 cpm/ng), and buffer A in the absence (O——O) or presence (●——●) of 570 μg of unlabeled f-Alb. After incubation at 0 °C for the indicated interval, the total amount of ^{125}I-f-Alb bound to the membranes was determined as described under "Materials and Methods."

Fig. 3 shows the effect of unlabeled f-Alb and several preparations of denatured albumins on ^{125}I-f-Alb binding to the sinusoidal cell membranes. In the presence of 3.7 μg of protein/ml of ^{125}I-f-Alb, 50% competition with unlabeled f-Alb was achieved at a concentration of less than 18 μg of protein/ml. In contrast, albumin preparations denatured either by 8 M urea or 6 M guanidine hydrochloride did not compete with ^{125}I-f-Alb binding to the membranes. Albumin preparations which were *S*-carboxymethylated or *S*-carboxamidomethylated in the presence of 6 M guanidine hydrochloride were also ineffective at a concentration of 200 μg/ml (data not shown). These findings indicate that the drastic denaturation of albumin molecules is not required for the specific recognition by the membrane-associated receptor.

At a fixed concentration of ^{125}I-f-Alb, the amounts bound to the membranes were approximately proportional to the amounts of membranes present in the assay medium up to a concentration of 250 μg of membrane proteins/ml (Fig. 4). When the reaction was performed at different pH values ranging from 6.0 to 9.0, the maximal binding of ^{125}I-f-Alb occurred at pH 8.0 (Fig. 5). The binding was similar in phosphate or Tris buffer.

To inquire into the nature of f-Alb receptor(s) on membranes, the association of f-Alb with the membranes was examined for heat stability and susceptibility to the proteases. Prior to binding of ^{125}I-f-Alb, the membranes were subjected either to heat treatment or to protease treatment. The binding capacity was reduced by more than 84 and 98% upon incubation for 10 min at 55 and 80 °C, respectively (Table I). The binding was also affected by treatment with either Pronase or proteinase K. A possibility that proteolytic cleavage of ^{125}I-f-Alb might abolish its ligand activity seems unlikely: (i) an increase in trichloroacetic acid-soluble radioactivity was negligible when ^{125}I-f-Alb alone was incubated either with Pronase or proteinase K, and this protease treatment did not alter the electrophoretic mobility of ^{125}I-f-Alb on a $NaDodSO_4$-polyacrylamide gel; and (ii) when the binding reaction was performed after washing the membranes to remove proteases, a similar result was obtained (data not shown).

To test for the divalent cation dependency of ^{125}I-f-Alb binding to its putative receptor, the effect of Ca^{2+}, Mg^{2+},

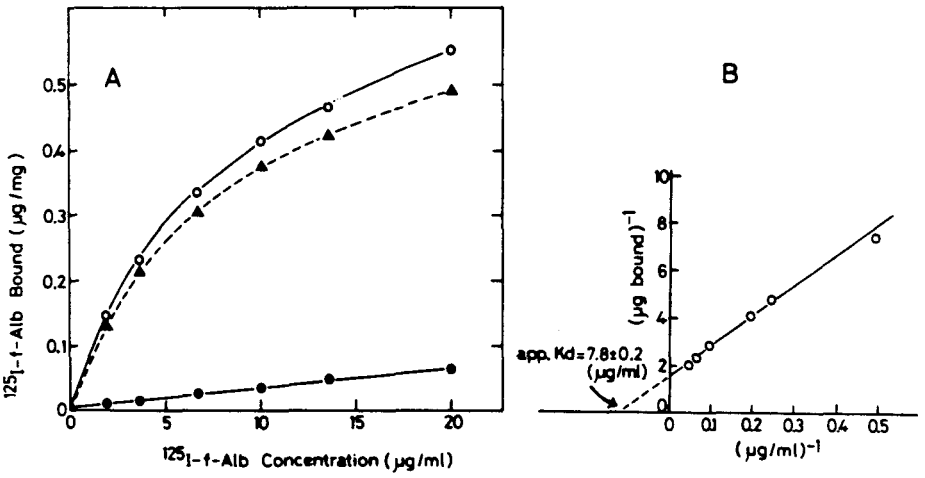

FIG. 2. **Binding of ^{125}I-f-Alb to sinusoidal liver cell membranes as a function of its concentration.** *A*, each tube, containing, in a final volume of 0.1 ml of buffer A, 16.5 μg (in protein) of membranes, and the indicated concentrations of ^{125}I-f-Alb (4670 cpm/ng), was incubated for 1 h at 0 °C. The total binding (O——O) was determined as described under "Materials and Methods." Nonspecific binding (●——●) was determined by parallel incubations in the presence of 570 μg of unlabeled f-Alb. Nonspecific binding was less than 10% of the total binding. Assays were run in duplicate. Specific binding (▲——▲) was plotted after correction for nonspecific binding as described under "Materials and Methods." *B*, the double-reciprocal plot of specific binding data in *A*.

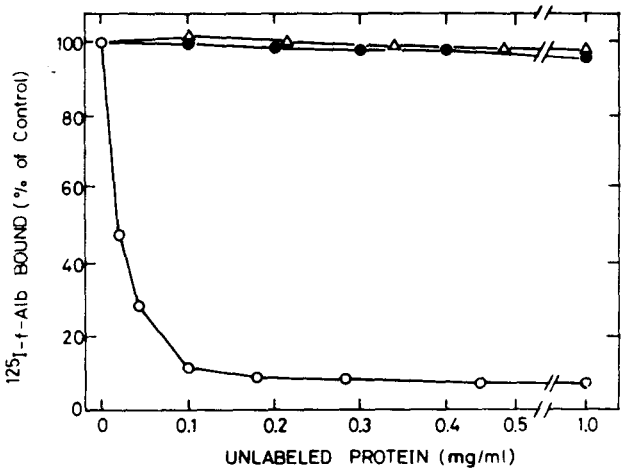

FIG. 3. **Effect of unlabeled f-Alb on ^{125}I-f-Alb binding to sinusoidal liver cell membranes.** Each tube contained, in a final volume of 0.1 ml of buffer A, 16.5 μg of membrane protein, 3.75 μg/ml of ^{125}I-f-Alb (4670 cpm/ng), and the indicated concentration of the unlabeled f-Alb (○——○). After incubation for 1 h at 0 °C, total amounts of ^{125}I-f-Alb bound to the membranes were determined as described under "Materials and Methods." The effect of BSA denatured either by 8 M urea (△——△) or 6 M guanidine chloride (●——●) was also examined by parallel incubations.

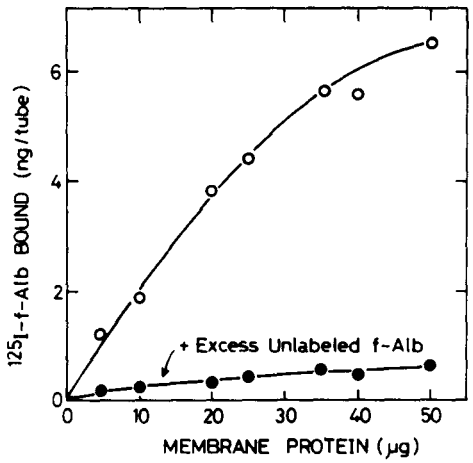

FIG. 4. **Dependency of ^{125}I-f-Alb binding to sinusoidal liver cell membranes upon amounts of membranes.** Each binding assay was carried out at pH 7.5. The reaction mixtures contained, in a volume of 0.1 ml, the indicated amounts of sinusoidal cell membranes, 0.37 μg of ^{125}I-f-Alb (4670 cpm/ng), and buffer A in the absence (○——○) or presence (●——●) of 5 mg/ml of unlabeled f-Alb. After incubation for 1 h at 0 °C, the total amount of ^{125}I-f-Alb bound to the membranes was determined as described under "Materials and Methods."

EGTA, and EDTA was examined. However, none of these reagents affected appreciably the binding of f-Alb with membranes (data not shown). Thus, the binding between f-Alb and its membrane receptor appeared to be Ca^{2+}-independent.

In an attempt to examine whether the receptor involved is of glycoprotein nature, the effect of concanavalin A, a lectin specific for mannose core of oligosaccharides, on the binding process was tested. As shown in Table II, the binding was substantially inhibited by this lectin (more than 70%) and, furthermore, this inhibition was protected by the presence of α-methyl-D-glucoside, a specific haptenic inhibitor for this lectin. Since f-Alb did not interact with this lectin (data not shown), this finding suggests that the f-Alb receptor could be a glycoprotein having mannose-type oligosaccharide structure.

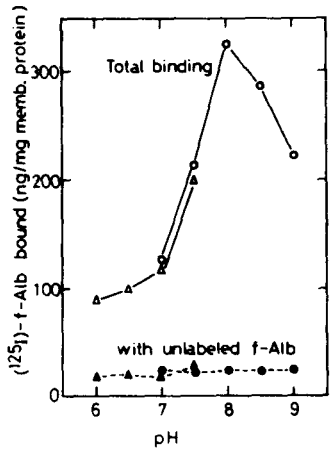

FIG. 5. **Effect of pH on ^{125}I-f-Alb binding to sinusoidal cell membranes.** Each tube contained 16.5 μg of membranes, 0.37 μg of ^{125}I-f-Alb (4670 cpm/ng), and buffer A in the absence (○——○, △——△) or presence (●——●, ▲——▲) of 5 mg/ml of unlabeled f-Alb. After incubation for 1 h at 0 °C at the indicated pH, the total amount of ^{125}I-f-Alb bound to the membranes was determined as described under "Materials and Methods." A 50 mM sodium phosphate buffer was used to adjust pH from 6.0 to 7.5 (△——△, ▲——▲). A 50 mM Tris-HCl buffer was used to adjust the pH from 7.0 to 9.0 (○——○, ●——●).

TABLE I

Effect of heating and treatment with proteases on ^{125}I-f-Alb binding to sinusoidal liver cell membranes

Experiment A. The membrane fraction (18 μg of protein) suspended in 10 μl of 0.15 M NaCl, 1 mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.4) was subjected to heat treatment either at 55 or 80 °C. After 10 min incubation, each membrane fraction was rapidly chilled on ice and tested for the capacity to bind ^{125}I-f-Alb. The reaction mixture contained, in a total volume of 0.1 ml, heat-treated membranes, 0.37 μg of ^{125}I-f-Alb (4,670 cpm/ng), 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 0.5 mM $CaCl_2$, and 20 mg/ml BSA in the presence or absence of an excess amount of unlabeled f-Alb (570 μg). The mixture was placed on ice for 1 h with occasional intervals on a Vortex mixer, followed by washing the membranes as described under "Materials and Methods." Experiment B. To 10 μl of membranes (1.8 mg/ml) in 0.15 M NaCl, 1mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.4) was added 10–30 μl of a solution of protease (Pronase or proteinase K) dissolved in 30 mM sodium phosphate buffer (pH 7.3) to give a final amount of 15, 45, and 300 ng, followed by incubation at 37 °C for 10 min. The reaction mixture was rapidly chilled on ice and tested for ^{125}I-f-Alb binding capacity as described for Experiment A. After incubation for 1 h at 0 °C, the amount of ^{125}I-f-Alb bound to the membranes was determined as described under "Materials and Methods." For Experiment A and B, values for total and specific binding were obtained as described for Fig. 2.

Treatment	Total binding	Specific binding	Control
	cpm/system	*cpm/system*	%
Experiment A			
None	15,600	11,170	100
55 °C, 10 min	3,700	1,700	15.3
80 °C, 10 min	2,460	150	1.3
Experiment B			
None	16,110	14,630	100
Pronase (0.15 μg/ml)	5,810	4,390	30
Pronase (0.45 μg/ml)	3,270	2,190	15
Pronase (3 μg/ml)	2,120	440	3
Proteinase K (0.15 μg/ml)	2,270	1,020	6.9
Proteinase K (0.45 μg/ml)	1,890	330	2.2
Proteinase K (0.3 μg.ml)	1,460	90	0.6

Several ligands have been known to be endocytosed via a receptor-mediated pathway. As shown in Table III, no appreciable inhibition of ^{125}I-f-Alb binding to the membranes was

TABLE II

Effect of concanavalin A on ^{125}I-f-Alb binding to the sinusoidal liver cell membranes

Each assay contained, in a final volume of 0.1 ml, sinusoidal cell membranes (10 μg of protein), 0.35 μg of ^{125}I-f-Alb (4,670 cpm/ng), 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 20 mg/ml of BSA, and indicated amounts of either concanavalin A or α-methyl-D-glucoside or both, in the presence or absence of an excess amount of f-Alb (570 μg). After incubation for 1 h at 0 °C, radioactivity bound to the membranes was determined as described under "Materials and Methods." Values for the total and specific binding were obtained as described for Fig. 2.

Treatment	Total binding	Specific binding	Control
	cpm/system	*cpm/system*	%
None	10,070	9,070	100
ConA[a] (50 μg/ml)	5,490	4,900	54
ConA (100 μg/ml)	2,340	2,090	23
ConA (100 μg/ml) + α-MG[b] (0.1 M)	7,180	6,350	70
α-MG (0.1 M)	9,980	8,440	93

[a] ConA, concanavalin A.
[b] α-MG, α-methyl-D-glucoside.

TABLE III

Effect of several ligands on ^{125}I-f-Alb binding to sinusoidal cell membranes

Each assay contained, in a final volume of 0.1 ml, sinusoidal cell membranes (10 μg of protein), 0.37 μg of radioactive f-Alb (4,670 cpm/ng), 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 0.5 mM $CaCl_2$, 20 mg/ml BSA, and the indicated amount of ligand, in the presence or absence of excess unlabeled f-Alb (570 μg). After incubation for 1 h at 0 °C, the radioactivity bound to the membranes was determined as described under "Materials and Methods." Values for total and specific binding were obtained as described for Fig. 2.

Treatment	Total binding	Specific binding	Control
	cpm/system	*cpm/system*	%
None	9,980	9,020	100
Native albumin[a] (2 mg/ml)	9,810	8,840	98
Asialofetuin (0.5 mg/ml)	9,610	8,570	95
Methylamine-treated α_2-macroglobulin (0.5 mg/ml)	11,410	10,370	115
Mannan (0.5 mg/ml)	11,250	9,920	110
Rat IgG (1 mg/ml)	9,400	8,320	92
Rat IgG (1 mg/ml) + its antibody (0.2 mg/ml)[b]	9,240	8,250	91.5

[a] Assay mixture contained 20 mg/ml of ovalbumin instead of BSA.
[b] Soluble immune complexes were formed by adding rabbit anti-rat IgG antibody to rat IgG.

observed with native albumin (BSA), asialofetuin (31), methylamine-treated α_2-macroglobulin (32), mannan (33), and immune complexes (34). The latter three ligands were known to be cleared from the circulation by sinusoidal liver cells when injected intravenously. This strongly suggests that the receptor for f-Alb on sinusoidal liver cell membranes would be distinct from the receptors for these ligands tested.

Identification of f-Alb Binding Protein from Detergent Extract of Sinusoidal Liver Cell Membranes—To identify the putative membrane-bound receptor for f-Alb, the membrane fraction used for the binding studies as described above was solubilized with 40 mM octyl β-D-glucopyranoside. Solubilized membrane proteins were labeled with ^{125}I by lactoperoxidase-glucose oxidase-catalyzed iodination as described under "Materials and Methods." The iodinated preparation was reacted with f-Alb, followed by the addition of anti-f-Alb antibody-coated Staph A to trap the putative receptor-ligand complexes. After unbound materials were extensively washed out, the final pellet was boiled for 3 min in 0.05 M Tris-HCl (pH 6.8) containing 1% $NaDodSO_4$ and 1% 2-mercaptoethanol, followed by brief centrifugation. The resulting supernatant was analyzed by $NaDodSO_4$-polyacrylamide slab gel electrophoresis, which revealed two radioactive bands whose molecular weights corresponded to 53,000 and 30,000, as estimated from the mobility of the standard marker proteins (Fig. 6). Two control experiments were performed in parallel. One control experiment was conducted in exactly the same way as described above except for the omission of f-Alb. In another control experiment, ^{125}I-labeled membrane proteins were incubated with native albumin (BSA) instead of f-Alb, and then reacted with anti-BSA antibody-coated Staph A particles. Electrophoretograms for two control runs did not exhibit any significant bands. The two radioactive protein bands observed only when f-Alb was used as a ligand might represent two nonidentical subunits which constitute a membrane-binding protein for f-Alb, or, alternatively, two distinct f-Alb-binding proteins in sinusoidal membranes. As shown by one of the control runs, any binding protein for native albumin did not appear to be present in sinusoidal cell membranes.

DISCUSSION

It has long been known that, when treated with formaldehyde *in vitro*, serum albumin is recognized by sinusoidal liver cells and is rapidly taken up by the mechanism of adsorptive endocytosis. Thus, a suggestion was made on the involvement of a membrane-associated receptor of these cells (2). However,

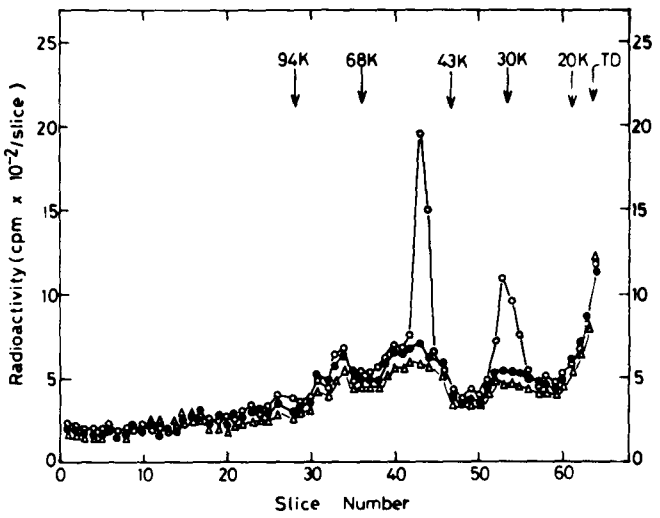

FIG. 6. **Identification of a membrane-associated receptor specific for f-Alb in ^{125}I-labeled membrane fractions of sinusoidal liver cells.** Proteins were solubilized from membranes by octyl β-D-glucopyranoside and labeled with ^{125}I to a specific radioactivity of about 800 cpm/ng. ^{125}I-labeled membrane proteins (2×10^6 cpm) were incubated with f-Alb for 3 h at 4 °C, followed by addition of a sufficient amount of anti-f-Alb antibody-coated Staph A suspension. The resulting Staph A particles were extensively washed by repeating centrifugation and suspension. The final pellet was immersed in 1% $NaDodSO_4$ and 2-mercaptoethanol and boiled for 3 min, followed by centrifugation. The resulting supernatant was electrophoresed on a $NaDodSO_4$-polyacrylamide slab gel (6–14%). See text for further details. After electrophoresis, the gel was cut into 2-mm thick slices, followed by determination of radioactivity (O——O). Two control experiments were as follows: one control experiment was conducted in the same way as described above except for the omission of f-Alb (Δ——Δ). In another control experiment, f-Alb was replaced by the same amount of native albumin (●——●). M_r standards used were: phosphorylase *b*, 94,000 (*94K*); bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; and soybean trypsin inhibitor, 20,000. Bromphenol blue was used as a tracking dye (*TD*). Their locations are shown by *arrows*.

no direct evidence has been presented for the nature of the receptor or binding protein which could specifically recognize this ligand. The present study has clearly shown the presence of a membrane-associated receptor for f-Alb in the membrane fractions of rat sinusoidal liver cells. Furthermore, two f-Alb-binding proteins having molecular weights of approximately 53,000 and 30,000, respectively, were found in nonionic detergent extracts of sinusoidal cell membranes. It is not known whether these two polypeptide chains may represent two nonidentical subunits which constitute an f-Alb receptor, two distinct proteins capable of binding f-Alb, or another proteolytic product of a single chain. These questions remain for further investigation.

Kinetics of ^{125}I-labeled f-Alb binding to sinusoidal cell membranes was similar to that obtained by using the isolated rat sinusoidal liver cells (2). This suggests that the f-Alb-binding protein isolated from membranes in the present investigation may reflect the receptor involved in the uptake of this ligand by the isolated cells, and lends further support to the idea that the specific recognition of the ligand by sinusoidal cells could be ascribed to the initial f-Alb binding to its receptor, a prerequisite event for the receptor-mediated endocytosis of this ligand.

The protein nature of the receptor was shown by both its heat lability and susceptibility to proteases (Pronase and proteinase K) (Table I). Similar data were also available on the receptor for low-density lipoprotein in human fibroblast (25). Although ^{125}I-f-Alb binding to the membranes was significantly inhibited by treating membranes either with heating or proteases, it could not be ruled out that these treatments result in global perturbation of the membrane structure which may abolish the receptor activity. It is not known whether or not the receptor requires lipid for its function, or whether interaction with other membrane protein(s) is essential for its activity. These points remain for further investigation.

Several receptors for natural ligands including asialoglycoproteins (35), high-mannose type glycoproteins (36), α_2-macroglobulin-protease complexes (37), and low-density lipoprotein (10) have been well characterized. However, f-Alb would not be a naturally occurring ligand since such modification of serum albumin seems unlikely to occur *in vivo*. Although the present results strongly suggest the presence of receptor protein(s) for f-Alb in the sinusoidal cell membranes, an important question to be answered is what the natural ligand for the receptor should be. In our preliminary experiment, the binding of ^{125}I-f-Alb to the sinusoidal cell membranes was found to be inhibited to a significant degree by the presence of rat serum, indicating the occurrence in the serum of a natural ligand structurally similar to f-Alb. A further study is in progress to identify a natural ligand(s) in the rat serum which is specifically recognized by the receptor.[2]

Although several possibilities have been raised, it remains unknown which type of modification endows the serum albumin molecule with structural features responsible for a specific recognition by the membrane-associated receptor on sinusoidal cells. Neither treatment with denaturing reagents such as 8 M urea and 6 M guanidine hydrochloride nor further *S*-carboxymethylation or *S*-carboxamidomethylation produced preparations which compete with ^{125}I-f-Alb for binding to its receptor (Fig. 3). Furthermore, treatment of f-Alb with the similar denaturing reagents did not result in any loss of its binding activity to the cells (data not shown). Therefore, drastic denaturation of the molecule may not be involved in the specific recognition by the receptor. Buys *et al.* (15) showed that modification of albumin by formaldehyde produced both polymeric and monomeric molecules. The former may probably be generated by intermolecular cross-linking via methylene bridge formation (38). However, both polymeric and monomeric forms did not differ from each other in their binding capacity to or uptake capacity by sinusoidal cells as well as plasma clearance rate. This indicates that polymerization of serum albumin is not the main factor responsible for the specific recognition by the receptor. Supporting evidence was presented by Moore *et al.* (19). Also unlikely is a contribution of the surface net charge of the molecule since formaldehyde treatment would not produce any difference in charge as judged from electrophoretic mobility.

It is not known whether the same phenomenon could take place with proteins other than BSA when treated with formaldehyde. Our preliminary experiment indicates that formaldehyde treatment also endows human and rat albumin as well as ovalbumin with the ligand specificity of being recognized by the receptor. However, no activity was observed with γ-globulin, RNase A or RNase B treated similarly with formaldehyde.[3]

The binding of f-Alb to its membrane receptor was demonstrated to be independent of extracellular concentration of Ca^{2+} in agreement with the results described by Hildenbrandt and Aronson (39). However, Pratten and Lloyd (40) observed a partial inhibition (46%) by 5 mM EGTA when assayed for intracellular digestion of this ligand in rat peritoneal macrophages (40). This apparent discrepancy could be reconciled if one assumes that Ca^{2+} is not required for at least the initial binding of f-Alb to its membrane-associated receptor. A similar Ca^{2+} independency was reported for the binding of high-density lipoprotein (41) and acetyl low-density lipoprotein (42).

Edelson and Cohn (43) reported that, in mouse peritoneal macrophages, concanavalin A prevented the infusion of pinocytotic vesicles with lysosomes and, hence, blocked proteolytic degradation of endocytosed proteins. Goldstein *et al.* (44) subsequently showed that the lectin inhibited the binding of low-density lipoprotein to its receptor on the surface membrane of cultured fibroblast and, hence, suppressed the intracellular degradation of the endocytosed ligand. Since the present experimental system allows us to observe solely the initial event of f-Alb endocytosis independently from subsequent events such as internalization and lysosomal degradation, the inhibitory effect of concanavalin A on f-Alb binding could be due to either its binding to the receptor itself or to its binding to other membrane component(s) which might affect secondarily the functioning of the receptor. This remains for further studies using a purified receptor.

Sinusoidal liver cells represent heterogeneous cell populations which mainly comprise Kupffer cells and endothelial cells (45). It is not known which type of cells is responsible for removal of f-Alb from the blood stream. In a preliminary experiment, it was found that the adhering cell population (mainly Kupffer cells) obtained by culturing isolated sinusoidal liver cells showed predominantly a high affinity for f-Alb but not the floating cell population.[4] Furthermore, in view of the fact that f-Alb was also endocytosed by rat peritoneal macrophages (7, 41, 46), it is tempting to speculate that the membrane-associated f-Alb receptor would reside on macrophages or macrophage-derived cells. Similarly, the receptor for acetyl low-density lipoprotein was postulated to be present on Kupffer cells (47).

[2] S. Horiuchi, K. Takata, and Y. Morino, manuscript in preparation.

[3] S. Horiuchi, T. Yu-ki, K. Takata, and Y. Morino, mansucript in preparation.

[4] S. Horiuchi, K. Takata, and Y. Morino, unpublished observations.

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FORMALDEHYDE /ALBUMIN /WOW
MACROPHAGE
A intravenous injection of a trace amount of f-ALB gave an apparent plasma clearance rate of 2-3 min When a loaded amount loading ammount 1mg/100g it was cleared from the circulation with a half-life of about 20-25 min. This value was fare less than the 48 hour half life of native albummin "However, f-Alb would not be a naturally occurring ligand since such modifications of serum albumin seems unlikely to occure in vivo.Although the present data strongly suggest the presence of receptor protein(s) for f-Alb in the sinusoidal cell membranes" Their preliminary study show the same phenomenon takes place with formaldehyde treatment also endows human albumin with the ligand specificity of being recognized by the receptor.
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