Detection and Isolation of a Hepatic Membrane Receptor for Ferritin*

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A ferritin receptor has been detected on isolated rat hepatocytes and has been partially purified from rat liver using affinity chromatography. Isolated hepatocytes exhibit approximately 30,000 ferritin binding sites/cell with a binding association constant (Kd) of 1 x 10^10 mol^-1 liter. A binding assay has been developed which utilizes a hepatic ferritin receptor coupled to a microparticulate support to facilitate separation of bound and free ligand. This method yielded a Kd of 3 x 10^10 mol^-1 liter for the purified hepatic ferritin receptor. Binding of ferritin to the insolubilized receptor was partially inhibited by human lactoferrin but unaffected by 200-fold molar excess of bovine albumin, rat transferrin, or human asialoorosomucoid.

Ferritin is an ubiquitous iron storage protein found in all tissues of the body and there is a close correlation between body iron stores and plasma ferritin concentration (1, 2). Injected ferritin has a plasma half life of 4-30 min and is taken up by the liver (3-7), specifically by hepatocytes (3). Use of the isolated perfused rat liver has indicated that the liver releases and takes up plasma ferritin, maintaining a perfusate ferritin concentration close to the plasma ferritin concentration, in vivo, of the donor animal (8).

The precise mechanism by which ferritin is taken up by the liver is unknown, but the existence of a ferritin receptor has been suggested. This is supported by the finding that uptake of fluorescent-labeled ferritin by isolated rat hepatocytes occurs in a manner consistent with receptor-mediated endocytosis (9). The observations that after homogenization of whole liver some ferritin is membrane-associated (10) and in a detergent-solubilizable membrane fraction (11) further strengthen the concept that “hepatocytes have receptors for...ferritin” (12).

We have examined the binding of [125]I-labeled rat liver ferritin to both isolated rat hepatocytes and a partially purified ferritin receptor preparation isolated from detergent-solubilized liver plasma membrane.

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Materials and Methods

Male Sprague-Dawley rats weighing 250–350 g were used throughout. Bovine serum albumin and fetal calf serum were obtained from the Commonwealth Serum Laboratories (Australia). Collagenase was purchased from Worthington, Teric 12A9 from ICI Chemicals (Sydney, Australia), human orosomucoid from Calbiochem-Behring, soybean trypsin inhibitor and agaroase-coupled neuraminidase Type X-A from Sigma, and CNBr-activated Sepharose 4B from Pharmacia Fine Chemicals (Uppsala, Sweden). Matrex pel 102 was purchased from Amicon (Lexington, MA), Bolton and Hunter Reagent (NEX 120) from New England Nuclear, and Sac-cel (donkey anti-rabbit whole serum coupled to cellulose) from Wellcome Reagents (Sydney, Australia). All other chemicals were of reagent grade.

Human lactoferrin (13), rat transferrin (14), ferritin, and purified antibody to rat liver ferritin (7) were prepared and rat serum ferritin was assayed as previously described (8). The equilibrium binding constant (Kd) of the rabbit antibodies to rat liver ferritin was 1.2 x 10^10 mol^-1 liter. Human asialoorosomucoid was prepared by incubating 10 mg of orosomucoid with 1 unit of insoluble neuraminidase in 0.1 M acetate, pH 5.0, (10 ml) at 37 °C for 24 h. Asialoorosomucoid was purified by centrifugation at 11,000 x g for 10 min to remove the insoluble neuraminidase followed by chromatography on a Sephadex G-25M column (1.5 x 8 cm, Pharmacia PD10 column) eluting with 0.14 M NaCl.

Rat liver ferritin (10 mg) or affinity purified rabbit antibody to rat liver ferritin (5 mg) in 0.1 M borate, pH 8.0, containing 0.5 M NaCl was coupled to 5 g of CNBr-activated Sepharose 4B at 4 °C for 18 h. Reactive groups in the affinity gel were blocked by addition of 0.1 M ethanolamine, pH 8.0, followed by stirring at 4 °C for 2 h and extensive alternate washing with 0.1 M acetic acid, pH 4.0, containing 0.1 M NaCl, and 0.1 M borate, pH 8.0, containing 0.5 M NaCl.

100 μg of rat liver ferritin was radio labeled with [125]I using 0.5 mCi of Bolton and Hunter Reagent (15). Labeled ferritin and free [125]I were separated by chromatography on a Sephadex G-25M column (1.5 x 8 cm, Pharmacia PD10), eluting with 20 mM KPO4, pH 7.4, containing 100 mM NaCl.

Protein concentration was determined by the deoxycholic acid/trichloroacetic acid precipitation method of Peterson (16).

Preparation of Isolated Rat Hepatocytes—Isolated rat hepatocytes were prepared by a modification of the collagenase perfusion method of Berry and Friend (17) as adapted by Seglen (18). Single-pass perfusion was commenced in situ via the portal vein with a calcium-free Krebs-Ringer bicarbonate buffer, containing 20 mM d-glucose and gassed with 95% O2, 5% CO2 (KRB). The liver was rapidly removed to a cabinet maintained at 37 °C (19) and perfusion was continued for a total of 10 min before commencing recirculating perfusion with KRB containing 5 mM CaCl2, 0.01% soybean trypsin inhibitor, and 0.05% collagenase for a further 20 min.

The liver capsule was removed and the cells were carefully teased out into oxygenated MEM at 37 °C. The resulting cell suspension was filtered sequentially through a single layer of gauze followed by 100-μm Nylon mesh and allowed to settle at room temperature for 10 min. The supernatant was removed, the cells were resuspended in warm MEM, and the settling process repeated twice more. This yielded 200 x 10^9 to 700 x 10^9 cells with a viability of 85-95% as assessed by trypan blue exclusion.

Assay for Binding of [125]I-Ferritin to Hepatocytes—Equilibrium binding studies were carried out at 4 °C. 1 x 10^6 hepatocytes were added to pre-chilled tubes containing MEM, varying amounts of ferritin (trace labeled with 1 μg of [125]I-labeled ferritin), and 10% FCS in a final volume of 1 ml. The tubes were gently swirled at 4 °C for 2 h. The cells were then separated from the incubation medium by vacuum filtration onto Whatman GF/A filter discs previously moistened with MEM, 10% FCS followed by washing with 5 x 2 ml of ice-cold MEM. The filter discs were then dried and bound [125]I-labeled ferritin was determined on a Kontron MR229 γ counter.

1 The abbreviations used are: MEM, Eagle’s minimum essential Medium, with Earles salts buffered with 25 mM 4-(2-hydroxyethyl)-1-piperazenemethanesulfonic acid, FCS, fetal calf serum; PMSF, phenylmethylsulfonyl fluoride; AsOR, asialoorosomucoid.

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Purification of Rat Liver Ferritin Receptor—Fresh rat livers weighing a total of 150 g were homogenized in 400 ml of 20 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM NaCl and 1 mM phenylmethylsulfonyl fluoride in a Waring Blender for 120 s. The homogenate was centrifuged at 30,000 g for 30 min and the supernatant discarded. The pellet was resuspended in 300 ml of PBS/PMSF and recentrifuged twice.

The pellet was then suspended in 200 ml of PBS/PMSF and solid Teric 12A9 was added to a final concentration of 1% (w/v). The sample was then sonicated (MSE ultrasonic homogenizer, 8 mm probe) for 5 min at 0 °C, filtered through a layer of glass wool, and recentrifuged at 10,000 g for 20 min. The supernatant was carefully removed from under the floating cap of precipitate and discarded.

The supernatant was then applied to a column (1.6 x 20 cm) containing 20 ml of anti-rat liver ferritin antibody coupled to Sepharose 4B equilibrated with B8BT at 4 °C. 200 ml of B8BT were applied and the ferritin-binding protein fraction was then eluted using 100 ml of 20 mM glycine, NaOH, pH 10, containing 0.5 mM NaCl and 0.5% Teric 12A9 (B8BT). After dialysis against the same buffer at 4 °C for 24 h, the preparation was centrifuged at 30,000 g for 20 min. The supernatant was then applied to a column (1.6 x 20 cm) containing 17 ml of anti-rat liver ferritin antibody coupled to Sepharose 4B equilibrated with B8BT at 4 °C. 200 ml of B8BT were applied and the ferritin-binding protein fraction was then eluted using 100 ml of 20 mM glycine, NaOH, pH 10, containing 0.5 mM NaCl and 0.5% Teric 12A9 (elution buffer). The column was then regenerated for further use by elution of bound ferritin with 100 ml of 10 mM glycine, HCl, pH 2, containing 150 mM NaCl, followed by 200 ml of B8BT.

The ferritin-binding protein fraction was precipitated by the addition of solid ammonium sulfate (31.5 g/100 ml) stirred for 20 min, and centrifuged at 10,000 x g for 20 min. The precipitate was resuspended in B8BT and dialyzed.

The dialyzed ferritin-binding protein fraction was then applied to a column (20 cm) containing 15 ml of rat liver ferritin Sepharose 4B equilibrated with B8BT. After washing with 200 ml of B8BT, the ferritin receptor protein was eluted at pH 10 using 100 ml of the above elution buffer. The column was then re-equilibrated with B8BT.

The ferritin receptor fraction was concentrated by ammonium sulfate precipitation and dialyzed against B8BT. After washing with 200 ml of B8BT, the ferritin receptor protein was eluted at pH 10 using 100 ml of the above elution buffer. The column was then re-equilibrated with B8BT.

RESULTS AND DISCUSSION

The binding of 251-ferritin to isolated rat hepatocytes was essentially complete after 2 h of incubation. While a high level of nonspecific binding was observed (up to 60% of total binding) with hepatocytes, specific binding was nonetheless demonstrated (Fig. 1) and Scatchard plots (20) of the specific binding yielded a binding association constant (Kd) of 1.1 ± 0.4 x 10^10 mol⁻¹ liter (mean ± S.D.) with 3.2 ± 1.6 x 10^10 binding sites/hepatocyte (Fig. 2). Experiments were difficult to repeat in rats with normal or increased iron stores and reproducible results were obtained largely with hepatocytes from rats with relatively low iron stores (Serum ferritin concentration 30-150 ng/ml).

The purification of the ferritin receptor was undertaken in an attempt to overcome the high nonspecific binding encountered using isolated hepatocytes. The preparation was based on the methodology developed by Seligman et al. (21) for purification of a placental transferrin receptor. Advantage was taken of the fact that the relatively high intracellular ferritin levels in liver would result in the presence of little or no unoccupied ferritin receptor after homogenization of the liver. Binding of ferritin to the insolubilized receptor was maximal between pH 7.5 and 8.0 and was greatly reduced below pH 6 and above pH 9. Ferritin-anti-ferritin complexes on the other hand have been shown to be unstable below pH 6.0 but stable above pH 9.0. Therefore, advantage was taken of this relative stability of the anti-ferritin-ferritin complex and elution of the receptor from ferritin after binding to the anti-ferritin column was accomplished at pH 10. Contamination of the receptor preparation with rabbit antibodies to rat liver ferritin was excluded by incubation of the receptor preparation with donkey anti-rabbit whole serum coupled to cellulose.

Assay of Binding of 125I-Ferritin to Insolubilized Ferritin Receptor—Polypropylene 1.5-ml microcentrifuge tubes containing 0.06 ml of PBS/PMSF plus ferritin (0.2-50 μg/ml) or other protein, 0.004 ml of 125I-labeled ferritin (200 ng), 0.8 ml of B8BT, and 0.1 ml of inosolubilized receptor suspension in B8BT were allowed to stand at 4 °C for 18 h. The assay tubes were then centrifuged at 11,000 x g for 2 min (Hettich Microfiter centrifuge) and the supernatant carefully removed. The pellet was resuspended in 1 ml of ice-cold B8BT, recentrifuged, and the supernatant again removed. The radioactive content of the pellet was then determined on a Kontron MR252 γ counter.

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coupling of the partially purified ferritin receptor to a micro-
vellicular support resulted in an insolubilized receptor which
allowed effective separation of bound and free ferritin by
centrification.

The binding of 125I-labeled rat liver ferritin to the insolubi-
lized receptor in the presence of increasing amounts of unla-
beled ferritin after 18-h incubation is shown in Fig. 3. From
Scatchard plots of the binding of ferritin to the insolubilized
receptor (Fig. 4) a $K_a$ of $2.7 \pm 0.7 \times 10^4$ mol$^{-1}$ liter was derived,
which is in agreement with the $K_a$ obtained in the hepatocyte
binding studies. This provides additional evidence that the
receptor is present on hepatocytes (3, 8).

Rat liver ferritin has been shown to contain approximately
2.5% carbohydrate (22). This carbohydrate consists of man-
nose, glucose, galactose, glucosamine, and galactosamine but
sialic acid and fucose were not detectable (22). Binding spec-
ificity studies, therefore, were carried out to determine
whether the characteristics of the ferritin-binding protein
resembled the previously described asialoglycoprotein recep-
tor (23, 24), the transferrin receptor (25), or the glycoprotein
receptor which has been described on macrophages (24, 26-
28). The asialoglycoprotein receptor shows specificity for ga-
lactose- or glucose-terminated glycoproteins such as human
asialoorosomucoid (23, 24), while the glycoprotein receptor
which has been previously described on macrophages is spe-
cific for fucose-, mannose-, and $N$-acetylglucosamine-termi-
nated glycoproteins (24, 26-28) including lactoferrin. Binding
studies were carried out using bovine serum albumin (a non-
specific protein), rat transferrin, AsOR, or human lactoferrin
in the assay system. The binding of ferritin was unaffected in
the presence of a greater than 200-fold molar excess of bovine
serum albumin, AsOR, or transferrin (Fig. 5), indicating that
the hepatocyte ferritin receptor differs from both the asialogly-
ycoprotein and transferrin receptors. Human lactoferrin
showed some degree of competition with ferritin binding to
the ferritin receptor (Fig. 6), however, the binding affinity of
ferritin to the receptor was 5-fold higher than the binding of
lactoferrin. This competition may reflect the presence of a com-
mon carbohydrate moiety on both the ferritin (22) and the lactoferrin molecules (29).

Accurate quantification of the total amount of hepatic re-
ceptor was not possible in these experiments because of the
difficulty in estimating recoveries at various stages of the
purification. However, calculation of the total binding capac-
ties in intact hepatocytes and in the final insolubilized recep-
tor preparation indicated an overall recovery of 5-15%. Prelim-
inary SDS electrophoresis of the receptor has shown three
faint bands with an $M_r$ of 30,000-50,000, but more complete
characterization must await the preparation of larger quanti-
ties of the binding protein.

The presence of a ferritin receptor has been reported pre-
viously on guinea pig reticulocytes (30). Pollack and Campana

Fig. 3 (left). Binding of 200 ng of
125I-labeled rat liver ferritin at 4 °C/18 h to insolubilized ferritin receptor in the presence of an increasing concentration of unlabeled ferritin.

Fig. 4 (right). Scatchard plot of the binding data derived from the experiment presented in Fig. 3. $K_a = 3 \times 10^4$ mol$^{-1}$ liter.

Fig. 5. Specificity of binding of 1 pg of 125I-labeled rat liver ferritin to insolubilized ferritin receptor in the presence of increasing concentrations of bovine serum albumin (a), human asialoorosomucoid (b), rat transferrin (c), and rat liver ferritin (d).

Fig. 6. Binding of 200 ng of 125I-labeled ferritin to insolubi-
lized ferritin receptor in the presence of increasing concentra-
tions of human lactoferrin (a) or rat liver ferritin (b).

in this study have reported a dissociation constant for the
binding of ferritin to reticulocytes ($K_a$) of $0.3 \times 10^{-10}$ M (or a
$K_a$ of $2 \times 10^4$ mol$^{-1}$ liter, if a correction is applied to allow for
the incubation volume of 1.45 ml and where $K_a = 1/K_d$). How-
ever, the large difference observed between binding at 37
and 0 °C suggests in these studies that internalization of
bound ferritin by reticulocytes occurs at 37 °C. In the presen-
t of internalization and in the short time period (20 min) used
for incubation, an equilibrium cannot be assumed, and anal-
ysis by Scatchard plot and derivation of binding equilibrium
constants may well be inappropriate.

We have demonstrated a hepatic ferritin receptor in rats by
the use of an insolubilized receptor preparation which permitted
analysis of the binding characteristics of the receptor. The
 technique should be generally applicable to the analysis of
ligand-receptor interactions. While the precise physiological
role of the ferritin receptor is not yet understood, we have
presented evidence for the existence of a specific mechanism responsible for the rapid uptake of ferritin from the circulation (8).

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