Identification of $^{125}$I-labeled Rat Reticulocyte Membrane Proteins With Affinity for Transferrin

By Albert L. Sullivan and Lewis R. Weintraub

The present studies were performed to identify reticulocyte membrane receptors for transferrin. Rat reticulocytes were labeled in vitro with $^{125}$I via lactoperoxidase; cell ghosts were then prepared and solubilized with Triton X-100. Binding of soluble membrane-$^{125}$I components to transferrin conjugated to Sepharose (Seph-tfn) was three times greater than to albumin conjugated to Sepharose (Seph-alb). Soluble membrane-$^{125}$I components released from Seph-tfn by 8 M urea re-bound to Seph-tfn but not to Seph-alb. Polyacrylamide gel electrophoresis (PAGE) of membrane components released from Seph-tfn showed three proteins with estimated molecular weights of 76,000, 95,000, and 145,000 daltons. Antibody to transferrin formed a precipitate upon incubation with soluble reticulocyte membrane components but not with soluble membrane components from erythrocytes or reticulocytes exposed to trypsin. PAGE of precipitates formed by incubation of antitransferrin and soluble $^{125}$I-reticulocyte membrane components showed three nonantibody proteins and corresponding $^{125}$I peaks with molecular weights of 76,000, 95,000, and 145,000 daltons. All three proteins increased in quantity when the amount of transferrin was increased in the immune precipitate by addition of "cold" exogenous transferrin, but only the 95,000- and 145,000-dalton components had increased $^{125}$I. These data indicate that two reticulocyte membrane components (95,000 and 145,000 daltons) can be iodinated and shown to have affinity for transferrin (76,000 daltons). The lack of components with such properties in membranes from erythrocytes or trypsinized reticulocytes, cells lacking the ability to bind transferrin, suggests that the two reticulocyte membrane proteins function as the transferrin receptor.

ERYTHROBLASTS AND RETICULOCYTES have surface membrane receptors for transferrin that disappear as reticulocytes transform into mature erythrocytes. The decrease in transferrin binding by erythroid precursors following exposure to trypsin indicates that these receptors are at least partly protein in nature. In the present study, reticulocyte membrane proteins were labeled with $^{125}$I by lactoperoxidase, then solubilized with 1%, Triton X-100 and subsequently incubated with antibody to transferrin or transferrin linked to Sepharose in the attempt to separate and identify the membrane proteins that bind transferrin.

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MATERIALS AND METHODS

Transferrin and antitransferrin. Rat transferrin and rabbit IgG antibody specific for rat transferrin were purified by salt precipitation, ion exchange chromatography, gel filtration, and affinity chromatography. All of the transferrin used in these experiments was fully saturated with iron by the addition of ferrous ammonium sulfate to rat plasma (63 μg/ml) and incubation for 12 hr at 4°C prior to the isolation of transferrin.

Preparation of red cell membranes and soluble membrane components. Red cells were obtained from normal rats with 0%-2% reticulocytes (hereafter called erythrocytes) or rats with phenylhydrazine-induced hemolytic anemia with 30%-60% reticulocytes (hereafter called reticulocytes). Whole blood containing 10 units heparin/ml was centrifuged and the plasma and buffy coat removed. Red cell membrane components were labeled with 125I using the lactoperoxidase method.

Cells were washed three times in 310 mOsm sodium phosphate pH 7.2 (310 PB); 10 ml of this incubation mixture contained 2 ml packed cells (1.6 x 10^11 cells), 200 μg lactoperoxidase (Sigma Chemical, St. Louis, Mo.), 20 μl glucose oxidase (Sigma), 1 mg glucose, and 310 μCi carrier-free Na125I (New England Nuclear, Boston, Mass.). The reaction was carried out at 37°C for 30 min with constant agitation. The reaction was stopped by adding 10-20 vol 10^{-3} M sodium thiosulfate in 310 PB at 4°C. Cells were centrifuged and rinsed four times in 310 PB.

Red cell ghosts were prepared by hypotonic lysis. Red cells were suspended in 40 vol 20 mOsm sodium phosphate pH 7.4 (20 PB) for 15 min at room temperature. The lysed cells were centrifuged at 27,000 g for 10 min at 4°C. The pellet obtained from erythrocytes was fluffy white, whereas the pellet from reticulocytes consisted of a fluffy white upper layer and a pasty greenish-brown bottom layer. In each case the fluffy white layer was rinsed four times in 20 PB.

Incubation experiments. Reticulocytes were exposed to trypsin, which depleted their ability to bind transferrin. 125I-labeled reticulocytes were incubated with 250 μg/ml trypsin (twice crystallized; Nutritional Biochemicals) for 30 min at 37°C. Soybean trypsin inhibitor (Nutritional Biochemicals) was added at 500 μg/ml to terminate the reaction. The cells were pelleted and rinsed three times in 310 PB before lysis for preparation of ghosts.

Of the total erythocyte 125I label, 85%-90% was associated with the red cell ghosts, whereas 60%-70% of the total reticulocyte 125I label was associated with the cell ghosts. Ghosts prepared from trypsinized reticulocytes had 10%-30% less 125I than those from untrypsinized reticulocytes.

Membrane components of the cell ghosts were solubilized by adding Triton X-100 (Sigma) to a final concentration of 1% (v/v). Tritonized samples were centrifuged at 100,000 g for 1 hr and the supernate (soluble membrane components) removed with a Pasteur pipette and retained.

Studies of soluble red cell membrane components. Immune precipitation. Optimal antigen-antibody ratios for precipitation were determined by establishing precipitin curves in the presence of 1% Triton X-100 with (1) rat transferrin and affinity column-purified rabbit anti-rat transferrin, (2) dinitrophenylated bovine albumin (DNP-alb) and rabbit antiserum to dinitrophenylated bovine IgG (anti-DNP-IgG, kindly given by Dr. F. Mooltan, Dept. of Microbiology, Boston University School of Medicine). Precipitin curves were established with transferrin and antitransferrin in the presence and absence of Triton X-100 (1%). Triton X-100 had no effect on the amount of transferrin precipitated by antitransferrin. Triton X-100 1% has previously been noted to have no effect on antigen-antibody precipitation.

Incubation of antibody and 100,000-g supernates of triton-treated 125I-labeled ghosts (soluble components of 125I membrane) was carried out in 10 x 75 mm glass test tubes for 30 min at 37°C and overnight at 4°C. In certain experiments transferrin was added to soluble red cell membrane components for 10 min at 37°C prior to the addition of antibody to transferrin. To control for nonspecific precipitation, the soluble components of 125I reticulocyte membranes were incubated in the presence of anti-DNP-IgG and DNP-alb. Precipitates were sedimented at 800 g for 5 min. The supernate was removed, and precipitates were vortexed to resuspend them in borate-buffered saline (0.15 M NaCl-0.02 M borate, pH 8) containing 1%. Triton X-100 (v/v). The suspensions were centrifuged at 800 g for 5 min, and this procedure was carried out four times.

Polyacrylamide gel electrophoresis (PAGE). Equal volumes of the sample to be electrophoresed and 10 mM EDTA-10 mM β-mercaptoethanol pH 7.5 were mixed. After 24 hr at 4°C, sodium dodecyl sulfate (SDS) was added to the samples (0.1-3 mg protein) at a final concentration of...
and the suspension heated for 3 min at 100°C. Sucrose (final concentration 8%) and bromophenol blue (final concentration 0.005%) were added and the sample was applied to 90-95 mm x 5 mm 5% or 7.5% polyacrylamide gels (20:1 acrylamide to N,N'-methylenebisacrylamide, 0.1% SDS, 0.02 M EDTA, 0.1 M phosphate buffer pH 7.2). The gels were electrophoresed at 4-8 mA/gel in a buffer of 0.1 M sodium phosphate pH 7.2, 0.02 M EDTA, and 0.1% SDS until the bromophenol blue reached the end of the gel. The gels were stained with Coomassie blue for protein or the PAS procedure for carbohydrate, and patterns were obtained with a Gilford gel scanner at 550 nm. The gels were sliced into 0.5- or 1-mm sections for determination of \(^{125}\text{I}\) radioactivity in a gamma spectrometer.

**Molecular weight determinations.** Molecular weights of membrane components were estimated from polyacrylamide gels using rabbit muscle phosphorylase A (92,000 daltons, Sigma), bovine liver catalase (57,700, Sigma), H chains from rabbit IgG (53,000, Miles Laboratory), and pepsin (34,500, Sigma) as markers with known molecular weights. A straight line was obtained in a semilogarithmic plot of molecular weight versus distance migrated into the gel, except for transferrin. A change in the electrophoretic mobility of transferrin has previously been described when transferrin was reduced with \(\beta\)-mercaptoethanol prior to SDS-PAGE.

**Affinity chromatography.** Rat transferrin or bovine serum albumin were conjugated to Sepharose 4B in a concentration of 0.5-1 mg/ml of packed Sepharose as previously described. Aliquots (200 \(\mu\)l) of conjugated Sepharose (50%, borate-buffered saline/50% Sepharose in a beaker with constant magnetic stirring) were added to the bottom of 10 x 75 mm glass tubes with disposable 200-\(\mu\)l pipettes. To be certain that equal aliquots could be delivered, 200 \(\mu\)l Sepharose conjugated to \(^{125}\text{I}\)-labeled albumin was pipetted into 10 x 75 mm glass tubes. The radioactivity in each tube did not vary by more than 5%.

From 25 to 900 \(\mu\)l of the soluble fraction of \(^{125}\text{I}\)-reticulocyte membrane was added to a series of tubes containing 200 \(\mu\)l of either transferrin linked to Sepharose (transferrin-Sepharose) or albumin linked to sepharose (albumin-Sepharose). Incubation was carried out for 10 min at 37°C with gentle agitation. The Sepharose beads were washed four times with 1\% Triton-borate-buffered saline (no radioactivity was detected in the supernate of the final wash). The beads were removed from the original tubes and placed in new tubes and the radioactivity was determined.

**RESULTS**

**Electrophoresis of soluble red cell membrane components.** SDS-solubilized membranes from \(^{125}\text{I}\)-labeled reticulocytes, \(^{125}\text{I}\) reticulocytes exposed to trypsin, and \(^{125}\text{I}\)-labeled erythrocytes were electrophoresed with SDS-PAGE. The only difference consistently noted on spectrophotometric scans or direct observation of gels from multiple preparations of the three types of membranes occurred with PAS staining. A band with an estimated molecular weight of 70,000-80,000 daltons (1.5 cm) was present in PAS-stained acrylamide gels of reticulocyte membranes (Fig. 1A, arrow) but not in similar gels of trypsinized reticulocytes or mature erythrocyte membranes. There was no major consistent difference in the distribution of \(^{125}\text{I}\) between the three different membrane preparations (Fig. 1).

**Affinity chromatography of soluble reticulocyte membrane components.** The binding of the soluble fraction of reticulocyte membrane \(^{125}\text{I}\) to transferrin-Sepharose was studied to determine if one or more of the iodinated reticulocyte membrane components had an affinity for transferrin. The soluble fraction of \(^{125}\text{I}\) reticulocyte membrane was incubated in varying amounts with aliquots of transferrin-Sepharose or albumin-Sepharose. The binding of reticulocyte membrane \(^{125}\text{I}\) to transferrin-Sepharose was three times greater than to Sepharose-albumin (Fig. 2), indicating that a reticulocyte membrane component with affinity for transferrin was iodinated under the present experimental conditions.
Experiments were performed to determine which reagent would be most suitable for detaching $^{125}$I-labeled reticulocyte membrane components from transferrin-Sepharose. Aliquots (200 $\mu$l) of transferrin-Sepharose saturated with the soluble components of $^{125}$I reticulocyte membrane were placed in the bottom of a series of 10 x 75 mm glass tubes. Then 2-ml aliquots of various reagents were added and allowed to incubate for 10 min at 4°C with gentle agitation. The tubes were then centrifuged at 800 g for 5 min; the supernate was removed and radioactivity was determined. The percent of radioactivity detached was determined by dividing the radioactivity in the supernate by the initial total radioactivity added.

Certain acids (0.05 M glycine HCl pH 2.3, 0.1 N acetic acid, 0.1 N HCl, 1.0 N HCl), 6 M guanidine HCl pH 2.9, 8 M urea, rat transferrin (2.8 mg/ml in borate-buffered saline), and albumin (10 mg/ml in borate-buffered saline) were utilized as potential dissociating agents. None of the acids detached more than 3% of $^{125}$I; 5% of the $^{125}$I was detached by albumin, 18% by urea, 24% by guanidine, and 30% by transferrin.

To determine whether or not soluble $^{125}$I components from reticulocyte membranes detached from the transferrin-Sepharose would rebind specifically to
Fig. 2. Binding of Triton X-100-solubilized reticulocyte membrane $^{125}$I components to Sepharose linked to transferrin (tfn-Sepharose) or Sepharose linked to albumin (alb-Sepharose). Incubation media contained 200 µl tfn-Sepharose or alb-Sepharose to which was added increasing amounts of soluble $^{125}$I reticulocyte membrane components. After incubation for 10 min at 37°C binding was determined as described in Materials and Methods.

transferrin-Sepharose, the detached radioactivity was dialyzed into 1% Triton-borate-buffered saline, divided into two aliquots, and passed over either transferrin-Sepharose or albumin-Sepharose packed into 2-ml columns (1 mm diameter). The columns were washed with 1% Triton-borate-buffered saline until radioactivity in the effluent reached background levels. The radioactivity of the beads in each column was then determined.

$^{125}$I detached from transferrin-Sepharose by 6 M guanidine HCl pH 2.9 would not rebind to transferrin-Sepharose, perhaps because of irreversible denaturation of the detached $^{125}$I membrane components. Fifty-three percent of an aliquot of the $^{125}$I detached by 8 M urea was able to rebind to transferrin-Sepharose, but only 9% of an identical aliquot was retained by albumin-Sepharose. The limited supply of transferrin did not permit its use in preparative experiments. Urea (8 M) was therefore the dissociating agent used in an attempt to recover reticulocyte membrane components from transferrin-Sepharose affinity columns. The soluble fraction of $^{125}$I membrane obtained from 8 ml of reticulocytes (total cpm 8 x 10⁹) was applied to a 5-ml transferrin-Sepharose column. The unbound eluate from this column was applied to a second transferrin-Sepharose affinity column, and the unbound eluate from the second column
was applied to a third column. The radioactivity bound to the initial column was 121,000 cpm, to the second column 64,000 cpm, and to the third column 6,000 cpm. The three columns were then eluted with 8 M urea at 4°C, and the eluates were pooled, concentrated, and prepared for SDS-PAGE. A total of 50,000 cpm was detached from the three affinity columns, but the majority of the radioactivity irreversibly attached to the collodian bag used for concentrating the sample. Three major Coomassie blue bands at 1.2, 3, and 4.1 cm (estimated mol wts 145,000, 95,000, and 76,000 daltons) were observed following electrophoresis (5% polyacrylamide) of the concentrated material obtained from affinity columns (Fig. 3A). The 1.2- and 3-cm proteins were associated with small ¹²⁵I peaks greater than 20 cpm at their maximum that did not occur at any other region of the gel.

**Immune precipitation of reticulocyte membrane components associated with transferrin.** Purified antibody to rat transferrin was incubated with the soluble membrane components of ¹²⁵I-labeled reticulocytes, erythrocytes, and ¹²⁵I reticulocytes exposed to trypsin. A precipitate formed when antitransferrin was incubated with the soluble components of reticulocyte membranes (Table 1). No precipitate occurred on incubation of antitransferrin and the soluble components of erythrocyte membranes or the soluble components of membranes from reticulocytes exposed to trypsin.

Table 1 lists the radioactivity from soluble membrane components that was associated with the washed immune precipitates in a typical experiment. The

![Fig. 3. Radioactivity and protein patterns from SDS-PAGE (polyacrylamide 5%) of (A) soluble reticulocyte membrane proteins released from Sepharose-transferrin affinity columns by 8 M urea, (B) precipitate (150 µg) formed by antitransferrin antibody (330 µg) and soluble ¹²⁵I reticulocyte membrane (300 µl), and (C) precipitate (180 µg) formed by antitransferrin antibody (330 µg), transferrin (48 µg), and soluble ¹²⁵I reticulocyte membrane (300 µl). In B and C the 8-cm peak (A550) comigrated with rabbit IgG light chain (23,000 daltons); 5.6-cm peak (A550) comigrated with rabbit IgG heavy chain (53,000 daltons). The 4.1-cm peak in A, B, and C (A550 and radioactivity) comigrated with rat transferrin. Peaks (A550 and radioactivity) at 3 and 1.2 cm in A, B, and C correspond to molecular weights of 95,000 and 145,000 daltons, respectively. Conditions for immune precipitation, affinity chromatography, sample preparation, electrophoresis, and gel scanning are described in Materials and Methods. Gels were sliced into 0.5-mm sections for determination of radioactivity.
Table 1. Membrane $^{125}$I Precipitated by Antitransferrin From Solubilized $^{125}$I-Labeled Reticulocyte, Trypsinized Reticulocyte, or Erythrocyte Membranes

<table>
<thead>
<tr>
<th>Membrane Type</th>
<th>Initial cpm</th>
<th>Protein Precipitated (μg)</th>
<th>cpm Precipitated</th>
<th>Percentage of Initial cpm Precipitated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reticulocytes (300 μl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plus antitransferrin (330 μg)</td>
<td>953,760</td>
<td>130</td>
<td>2150</td>
<td>0.225</td>
</tr>
<tr>
<td>plus antitransferrin (330 μg)</td>
<td>953,760</td>
<td>280</td>
<td>3849</td>
<td>0.404</td>
</tr>
<tr>
<td>and transferrin (48 μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plus anti-DNP-γ globulin and DNP albumin</td>
<td>953,760</td>
<td>456</td>
<td>311</td>
<td>0.032</td>
</tr>
<tr>
<td>Trypsinized Reticulocytes (300 μl)</td>
<td>846,020</td>
<td>0</td>
<td>475</td>
<td>0.056</td>
</tr>
<tr>
<td>plus antitransferrin (330 μg)</td>
<td>846,020</td>
<td>300</td>
<td>709</td>
<td>0.084</td>
</tr>
<tr>
<td>plus antitransferrin (330 μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>and transferrin (48 μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocytes (300 μl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plus antitransferrin (330 μg)</td>
<td>1,712,340</td>
<td>0</td>
<td>441</td>
<td>0.026</td>
</tr>
<tr>
<td>plus antitransferrin (330 μg)</td>
<td>1,712,340</td>
<td>280</td>
<td>764</td>
<td>0.043</td>
</tr>
</tbody>
</table>

Percentage of initial $^{125}$I precipitated by antitransferrin from the soluble components of $^{125}$I reticulocyte membranes was eight times greater than the percentage precipitated from erythrocyte membranes (0.225% versus 0.026%) and four times greater than the percentage precipitated from reticulocytes exposed to trypsin (0.056%). Addition of exogenous transferrin to the soluble components of $^{125}$I reticulocyte membranes and antitransferrin increased the amount of $^{125}$I in the precipitate by 80%. Precipitates of exogenous transferrin and antitransferrin formed in the presence of soluble erythrocyte or trypsinized reticulocyte membrane $^{125}$I components contained one-ninth and one-fifth the percentage of $^{125}$I, respectively, compared to those formed in the presence of the soluble fraction of $^{125}$I reticulocyte membranes. Nonspecific precipitation does not appear to explain the amount of $^{125}$I contained in precipitates occurring following the incubation of the soluble fraction of $^{125}$I reticulocyte membranes and antitransferrin. An unrelated control precipitate formed by anti-DNP-γ globulin and DNP-albumin in the presence of the soluble components of $^{125}$I reticulocyte membrane contained 1.6 times more protein but only 8% of the $^{125}$I found in the precipitate of transferrin, antitransferrin, and the soluble components of $^{125}$I reticulocyte membranes. These results indicate that a reticulocyte membrane component that can be iodinated is precipitable by an antibody that binds transferrin. Furthermore, incubation of the soluble components of $^{125}$I reticulocyte membranes with transferrin prior to the addition of antibody yielded a precipitate containing greater radioactivity than a precipitate formed without extra transferrin.

Immune precipitates were solubilized in SDS and electrophoresed on polyacrylamide gels so that the iodinated reticulocyte membrane components in the precipitates could be identified. Electrophoresis (5% polyacrylamide) of the precipitate formed by antitransferrin and the soluble components of $^{125}$I reticulocyte membranes (Fig. 3B) showed protein bands and coinciding $^{125}$I peaks with estimated molecular weights of 145,000 (1.2 cm), 95,000 (3 cm), and
RETICULOCYTE TRANSFERRIN RECEPTORS

76,000 (4.1 cm) daltons. In addition, there were protein bands of 23,000 (8 cm) and 53,000 (5.6 cm) daltons corresponding to light and heavy chains of the antibody. The 76,000-dalton band (4.1 cm) comigrated with both rat transferrin and the major $^{125}$I peak.

In comparison to these results, incubation of the soluble components of $^{125}$I reticulocyte membranes with exogenous transferrin before addition of antitransferrin yielded a precipitate that on PAGE showed increased protein but decreased radioactivity in the 76,000-dalton region (4.1 cm) and increased protein and radioactivity in the 95,000- (3 cm) and 145,000-dalton (1.2 cm) regions (Fig. 3C).

$^{125}$I reticulocyte membrane proteins detached from Sepharose-transferrin affinity columns by 8 M urea were similar to those precipitated by antitransferrin antibody when compared by electrophoresis (Fig. 3A).

**DISCUSSION**

These studies show that two reticulocyte membrane proteins, which can be labeled with $^{125}$I and solubilized with Triton X-100, are able to bind specifically to transferrin. The affinity for transferrin is shown by the greater binding of solubilized reticulocyte membrane $^{125}$I to Sepharose-transferrin than to Sepharose-albumin. The specificity of this binding is shown by the fact that soluble reticulocyte membrane $^{125}$I released from transferrin-Sepharose affinity columns is able to rebind to transferrin-Sepharose but not to albumin-Sepharose. The ability of antitransferrin to precipitate soluble membrane protein or to coprecipitate soluble membrane $^{125}$I from reticulocytes, trypsinized reticulocytes, or erythrocytes parallels the ability of these cells to bind transferrin in vitro. Neither erythrocytes nor reticulocytes exposed to trypsin bind transferrin in vitro. Incubation of solubilized membrane from erythrocytes or trypsinized reticulocytes with antitransferrin fails to yield a precipitate, whereas incubation of a similar preparation of reticulocyte membrane yields a definite precipitate. The amount of soluble membrane $^{125}$I coprecipitable with transferrin and antitransferrin from erythrocytes or trypsinized reticulocytes is less than 20% of that from reticulocytes, despite the fact that the initial amount of radioactivity and its distribution are similar in the three preparations (Table 1, Fig. 1).

Three $^{125}$I-labeled reticulocyte membrane proteins are precipitated by antibody to rat transferrin and are separable by SDS-PAGE. The protein with an apparent molecular weight of 76,000 daltons comigrates with rat transferrin. The other two proteins, with apparent molecular weights of 95,000 and 145,000 daltons, appear to be closely linked to the 76,000-dalton protein, since they are coprecipitated with it by antitransferrin. Furthermore, both the radioactivity and protein concentration of the two higher molecular weight components are coprecipitated from the soluble components of $^{125}$I reticulocyte membranes in greater quantity when the amount of transferrin is increased in the immune precipitate with added transferrin. The increase of the two higher molecular weight $^{125}$I proteins roughly parallels the increase in the 76,000-dalton protein. Radioactivity at 76,000 daltons decreases in precipitates formed with additional transferrin.
These results suggest that “cold” exogenous transferrin (76,000 daltons) binds to “free” $^{125}$I-labeled transferrin receptor (95,000, 145,000 daltons) and substitutes for $^{125}$I-labeled endogenous membrane transferrin (76,000 daltons) in the immune precipitate. In addition, the $^{125}$I-labeled reticulocyte membrane proteins released from Sepharose-transferrin affinity columns by 8 M urea were virtually identical in mobility by SDS-PAGE to those contained in the transferrin immune precipitates from reticulocytes.

The two higher molecular weight components in the transferrin and anti-transferrin immune precipitates do not represent nonspecific trapping, since electrophoresis of a larger control precipitate formed by DNP-albumin and anti-DNP-$\gamma$ globulin in the presence of an identical aliquot of the soluble fraction of $^{125}$I reticulocyte membranes did not contain any $^{125}$I components.

Several other investigators have undertaken experiments to identify a transferrin receptor on immature erythroid cells. Garret et al. incubated $^{125}$I transferrin with rabbit reticulocytes and solubilized the reticulocyte membranes with deoxycholate. Column chromatography showed a component with a molecular weight of 350,000–700,000 daltons containing $^{125}$I. Speyer and Fielding, in similar experiments, using human reticulocytes, reported a 150,000-dalton component that was associated with $^{125}$I transferrin in the Triton X-100 solubilized membrane. Van Bockxmeer and Morgan found a 275,000-dalton transferrin-binding component in rabbit reticulocyte membranes solubilized with Teric 12A9. Sly et al. separated a 200,000-dalton complex from Triton X-100-solubilized rabbit reticulocyte membranes that contained $^{125}$I transferrin. Separation of this component with PAGE in the presence of SDS, urea, and mercaptoethanol showed two fractions with molecular weights of 120,000 and 60,000 daltons in addition to transferrin. Nuñez et al. exposed rabbit reticulocytes incubated with $^{125}$I transferrin to reagents in order to cross-link transferrin to nearby membrane proteins (presumably receptor). PAGE showed $^{125}$I transferrin cross-linked to membrane proteins with apparent molecular weights of 60,000 and 145,000 daltons. Leibman and Aisen have separated $^{125}$I transferrin-binding proteins from Triton X-100-solubilized rabbit reticulocyte membranes by gel filtration; SDS-PAGE showed two glycoproteins in this fraction with molecular weights of 95,000 and 175,000 daltons.

The present and previous studies suggest that the transferrin receptor is composed of two components of unequal molecular weights. Differing values for molecular weights of the two components are probably related to differences in techniques or species used by the various investigators. For example, reticulocytes induced with phenylhydrazine appear to have certain membrane properties different from reticulocytes induced by phlebotomy. Phenylhydrazine-induced reticulocytes have altered transferrin binding and $^{125}$I labeling catalyzed by lactoperoxidase and galactose oxidase. Phenylhydrazine-induced reticulocytes were used in the present study, and these could have altered transferrin receptors in comparison to phlebotomy-induced reticulocytes.

Also in the present study the larger component of the receptor could not be assigned a definite molecular weight, since it migrated above the largest standard available to us for molecular weight determinations (phosphorylase, 92,000 daltons). We assumed that the molecular weight calibration line re-
mained linear above 92,000 daltons and derived a molecular weight of 145,000 daltons for the larger component of the receptor on that basis. It does not appear that the larger component is a polymer of the smaller one, since both appear in systems that should dissociate any disulfide and noncovalent bonds. It is not clear from the available data whether these two components are subunits of a single receptor molecule or are separate components of the membrane that both have affinity for transferrin. The results of the present study are consistent with either possibility. Both components are available at the cell surface, since both are accessible to lactoperoxidase for iodination; both components are precipitable by antitransferrin and bind to transferrin linked to Sepharose. Leibman and Aisen suggested that the larger of the two bears transferrin receptor function. This conclusion was drawn from experiments that showed that the smaller component is present in both reticulocyte and erythrocyte membranes as a glycoprotein, whereas the larger component is a glycoprotein in reticulocyte membranes but lacks carbohydrate in erythrocyte membranes. They suggested that loss of the carbohydrate moiety from the larger component is responsible for loss of transferrin binding as reticulocytes mature to erythrocytes. We were unable to detect definite PAS-stained bands at the 95,000- or 145,000-dalton region in SDS-PAGE of precipitates of antitransferrin and soluble reticulocyte membrane, perhaps owing to the limited amount of material available for electrophoresis and the faint staining produced by the PAS technique in comparison to Coomassie blue. The origin of the PAS-stained band present in reticulocyte but not trypsinized reticulocyte or erythrocyte membranes (Fig. 1A, arrow) is not clear. Although it migrates in the region of transferrin, it forms a much narrower and sharper band than serum transferrin on SDS-PAGE.

The present study shows that the rat reticulocyte membrane contains two proteins, estimated as 95,000 and 145,000 daltons, that have affinity for transferrin. Isolation of these two proteins in quantity will be helpful for characterization of their transferrin receptor function and membrane organization. It will be of particular interest to determine the relation of the transferrin receptor to other membrane components (such as spectrin and the actinlike protein), since micropinocytosis of transferrin appears essential for iron delivery; this system may provide molecular insight into the mechanism of micropinocytosis.

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Identification of 125I-labeled rat reticulocyte membrane proteins with affinity for transferrin

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