Micropinocytosis of Transferrin by Developing Red Cells: An Electron-microscopic Study Utilizing Ferritin-conjugated Transferrin and Ferritin-conjugated Antibodies to Transferrin

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Electron-microscopic examination of rat reticulocytes and normoblasts incubated with transferrin conjugated to ferritin or ferritin-labeled antitransferrin revealed binding of ferritin conjugates to the surface membrane, and uptake of ferritin conjugates in micropinocytotic vesicles. No binding or endocytosis of ferritin was visualized when rat reticulocytes or normoblasts were incubated with ferritin alone or ferritin conjugated to nonspecific rabbit IgG. These observations support the concept that transferrin binds to a surface membrane receptor and is subsequently internalized by the developing red cell. Time course and temperature dependence studies suggest the endocytosis of transferrin may be an important mechanism in delivery of iron to the developing red cell.

The mechanism by which iron is delivered from the plasma protein, transferrin, to the mitochondria of erythocyte precursors for incorporation into heme is not completely understood. Erythroblasts and reticulocytes have surface receptors for transferrin that disappear as reticulocytes transform into mature erythrocytes. Comparison of the half-lives of 59Fe- and 131I-labeled transferrin in vivo suggest that transferrin is recycled to the plasma following iron delivery. Studies in vitro utilizing 131I-transferrin have demonstrated that (1) the binding of iron-saturated transferrin to reticulocytes is greater than that of iron-free transferrin and (2) 131I-transferrin that has been allowed to saturate reticulocyte binding sites is fully exchangeable with unlabeled transferrin. These studies have led to the conclusion that a molecule of iron-laden transferrin attaches to a surface membrane receptor, releases its iron to the red cell precursor, and is displaced by another molecule of iron-laden transferrin.

On the other hand, several studies have raised the possibility that transferrin enters the developing red cell and may be involved in the intracellular transport of iron. Electron-microscopic radioautographs of rabbit reticulocytes incubated with 125I-transferrin have been interpreted to show transferrin within the cell. A subsequent study demonstrated micropinocytosis of ferritin conjugated to transferrin by rabbit reticulocytes. In addition, reticulocyte fractionation studies have localized transferrin within the cell as well as on the surface membrane. The following electron-microscopic studies were performed utilizing...
ferritin conjugated to transferrin as well as ferritin conjugated to antibodies to transferrin in an attempt to follow the fate of transferrin in developing rat red cells.

MATERIALS AND METHODS

Transferrin

Rat transferrin was isolated from serum which was saturated with iron by the addition of ferrous ammonium sulfate. Ammonium sulfate was added to obtain the serum proteins which precipitated between 30°c and 50° saturation. The precipitate was resuspended in water, equilibrated with 0.01 M Tris-HCl buffer, pH 8, and applied to a DEAE-cellulose column equilibrated with 0.05 M Tris-HCl buffer, pH 8. The column was eluted with 0.05 M Tris-HCl, pH 8 and 0.1 M Tris-HCl, pH 8, in a stepwise fashion. Elution with the latter buffer produced two transferrin-containing peaks. The first transferrin peak was concentrated and passed over a Sephadex G-100 column. At 20 mg/ml (protein concentration determined by the Lowry method with an albumin standard) the transferrin preparation obtained from the G-100 column gave a single arc on immunoelectrophoresis with a polyvalent rabbit antiserum to rat serum. Acrylamide-gel electrophoresis of 100 μg of this preparation revealed a single band.

Preparation of Ferritin Conjugates

Antibodies to rat transferrin were raised in rabbits by biweekly subcutaneous or intraperitoneal injections of 0.5 mg of rat transferrin emulsified in 0.5 ml of complete Freund's adjuvant. Rabbit antirat transferrin antibody was isolated by affinity chromatography. Forty milligrams of rat transferrin were conjugated to 40 ml of settled Sepharose 4B (Pharmacia) by the cyanogen bromide technique. The 0°c, 33°c amonium sulfate precipitate of rabbit antiseraus was suspended in and equilibrated with borate buffer (0.15 M NaCl, 0.02 M borate, pH 8) and passed over a column containing the Sepharose 4B conjugated to transferrin. The column was eluted first with borate buffer to remove nonspecifically bound protein. The protein subsequently removed with 8 M urea at 4°C was equilibrated with borate buffer and shown to be exclusively rabbit IgG by immunoelectrophoresis. This IgG was labeled with 125I, and 125I-lgG was separated from unbound 125I by Sephadex G-25 gel filtration. Ninety-six per cent of the 125I-lgG was precipitated with rat transferrin.

Seventy-five milligrams of each of the following proteins were conjugated to 75 mg of horse ferritin (2 x crystallized, cadmium free, Miles Laboratories, Kankakee, Ill.) with tolylene-2, 4 diisocyanate under the conditions previously described; (1) specific rabbit antirat transferrin, (2) nonspecific rabbit IgG (Cohn Fr II, Miles Laboratories), and (3) purified rat transferrin. Specific conjugates were separated from precursors by electrophoresis as previously described, but with potato starch as a supporting medium. Each crude conjugate was placed in a 4½-inch vertical slit 3½ inches from the cathode buffer well of a 5½ x 12½ x ½-inch starch block. Each block was electrophoresed with 0.05 M barbital buffer, pH 8.6, for 15 hr at 380-400 V across the buffer wells. Each block was cut into ½-inch vertical sections and each section eluted with 5ml borate buffer through a sintered glass funnel. The absorbance of each eluate at 280 nm was determined and in each case the crude conjugate separated into three distinct fractions: (1) an anodally migrating fraction which contained only ferritin, (2) a cathodally migrating fraction which contained only the protein to be conjugated, and (3) an intermediate fraction containing the desired ferritin-protein conjugate as determined by immunoelectrophoresis.

Cells

Blood with 30°c, 60°c reticulocytes was obtained from rats with phenylhydrazine-induced hemolytic anemia. Blood containing 10 units heparin per ml was centrifuged and the plasma anduffy coat removed. Red cells were suspended and centrifuged three times in phosphate-buffered saline (PBS), pH 7.4.

Twenty-five microliters of reticulocyte-rich washed cells were incubated in 75 μl of PBS containing 125 μg of rat transferrin for 5 min at 37°C. The cells were then washed three times in PBS at 4°C and incubated at 37°C with either (1) 800 μg of ferritin-antitransferrin conjugate
for 30 sec or 5 min, (2) 800 µg of nonspecific ferritin-rabbit IgG conjugate for 5 min, or (3) 800 µg of horse ferritin for 5 min. Condition (1) including preincubation with transferrin was also carried out completely at 4°C. Twenty-five microliters of reticulocyte-rich washed cells were also incubated in 75 µl of PBS containing 80 µg of ferritin-transferrin conjugate for 30 sec or 5 min at 37°C. Marrow was obtained from the femurs of phenylhydrazine-treated rats, and cells were dissociated from stroma by repeatedly aspirating and expelling particles with a pasteur pipette. Marrow particles were allowed to settle, and the supernatant containing marrow cells was washed three times in PBS. Twenty-microliter aliquots of marrow cells were incubated in 80 µl of PBS containing 125 µg of rat transferrin for 5 min at 37°C. The cells were then washed three times in PBS at 4°C and incubated at 37°C for 5 min with either (1) 800 µg of ferritin anti-transferrin conjugate or (2) 800 µg of nonspecific ferritin rabbit IgG conjugate. Cells were then prepared immediately for electron microscopy unless noted otherwise.

Preparation for Electron Microscopy

Cells were washed three times in PBS at 4°C and fixed in a mixture of 4% paraformaldehyde and 5% glutaraldehyde in 0.06 M cacodylate buffer (pH 6.8) for 2 hr at room temperature. Cells were washed in cacodylate buffer, postfixed in 1% osmium tetroxide for 1 hr at 4°C before dehydration in graded ethanol solutions, and embedded in Epon. Sections were collected on copper grids, stained with uranyl acetate and lead citrate, and examined in an AEI-6b electron microscope.

RESULTS

Rat reticulocytes and normoblasts were examined to determine the extent and location of endogenous ferritin. In these cells, which had been washed and processed for electron microscopy, ferritin occurred exclusively in the cell interior, being present as various sized aggregates usually contained in a vacuole (Fig. 1). The outer surface of the plasma membrane, invaginating surface pits, and micropinocytotic vesicles were devoid of ferritin. This observation is in agreement with previous findings in rat erythroid cells.14 Because of its occurrence as larger clusters and its absence from surface structures, endogenous ferritin is distinguished readily from the exogenous horse ferritin conjugates used in these studies and did not interfere with interpretation of the results.

Reticulocytes and normoblasts incubated with horse ferritin alone or horse ferritin conjugated to nonspecific rabbit IgG never had ferritin on the surface membrane, in surface pits, or lining micropinocytotic vesicles. Thus, there was

Fig. 1. Reticulocyte showing endogenous rat ferritin contained within a vacuole (fb). × 79,800.
Fig. 2. Reticulocyte incubated sequentially for 5 min with rat transferrin and rabbit antirat transferrin-ferritin conjugate at 37°C. Ferritin is associated with the surface membrane (arrows). Two vacuoles devoid of ferritin are indicated (v). × 79,800.

no evidence of nonspecific binding of ferritin or nonspecific rabbit IgG to the membrane of red cell precursors.

Reticulocytes which were incubated at 37°C for 5 min with ferritin-conjugated transferrin (Ft-Tfn) or with transferrin followed by ferritin-conjugated antitransferrin (Ft-anti Tf) revealed an identical distribution of ferritin. Patches of ferritin were observed adherent to the plasma membrane (Fig. 2) and contained in surface pits (Fig. 3) and micropinocytotic vesicles (Figs. 3-5). Serial sections revealed that the ferritin-containing vesicles were not connected to the surface membrane as elongated invaginations. Erythroblasts from rat

Fig. 3. Reticulocyte incubated as in Fig. 2. Ferritin is associated with the surface membrane (1), in forming micropinocytotic vesicles (2 and 3), and within an internalized vesicle (4). × 94,050.
Fig. 4. Reticulocyte incubated as in Fig. 2 showing ferritin at the surface membrane (arrows), and internalized in micropinocytotic vesicles (fv). m, mitochondrion. × 47,500.

Fig. 5. Reticulocyte incubated with rat transferrin-ferritin conjugate for 5 min at 37°C showing ferritin associated with a forming micropinocytotic vesicle (arrow) and within internalized vesicles (fv). × 47,500.
Erythroblast incubated sequentially for 5 min with rat transferrin and rabbit antirat transferrin-ferritin conjugate at 37°C. Ferritin is associated with the surface membrane (arrows) and within vesicles (fv). x 52,250.

Fig. 6

Marrow which were incubated with Ft-anti Tfn also revealed ferritin on the surface membrane and in micropinocytotic vesicles (Fig. 6). There was no binding of these ferritin conjugates to mature red cells in the same sections. When transferrin and Ft-anti Tfn were incubated with reticulocytes at 4°C, ferritin binding to the surface membrane was not affected (Fig. 7), but micropinocytotic uptake was greatly diminished as compared to the observations at 37°C.

Experiments were carried out to determine the time course of micropinocytosis of the transferrin-specific ferritin conjugates as well as the ultimate fate of the internalized conjugates. Within 30 sec of incubation at 37°C with Ft-Tfn or Ft-anti Tfn, ferritin conjugates were found within micropinocytotic vesicles. Following 5–10 min of incubation with Ft-anti Tfn, 90% of 50 reticulocyte sec-
Mean ± 1 SEM of 100 reticulocyte sections.

Table 1. Characteristics of Vesicles in Reticulocytes Sequentially Incubated With Ferritin Antitransferrin and Buffer

<table>
<thead>
<tr>
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<th>Per Cent of 50 Reticulocyte Sections With Vesicles</th>
<th>Per Cent of 50 Reticulocyte Sections With Ferritin in Vesicles</th>
<th>Number of Vesicles per Reticulocyte*</th>
<th>Number of Vesicles Containing Ferritin per Reticulocyte*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin anti-Tfn for 5 min</td>
<td>100</td>
<td>90</td>
<td>9.56 ± 1.02†</td>
<td>3.62 ± 0.47†</td>
</tr>
<tr>
<td>Buffer for 45 min</td>
<td>100</td>
<td>52</td>
<td>9.48 ± 0.80†</td>
<td>1.34 ± 0.25†</td>
</tr>
</tbody>
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*Mean ± 1 SEM of 50 reticulocyte sections.
†No significant difference by t test.
‡ p < 0.001 by t test.

tions contained micropinocytosed ferritin conjugates. However, when reticulo-
cytes were incubated for 5 min at 37°C in the presence of Ft-anti Tfn, washed
with PBS at 4°C, and subsequently incubated for 45 min at 37°C in the absence
of Ft-anti Tfn, only 52% of 50 reticulocyte sections contained ferritin conjugates
in one or more micropinocytotic vesicles. There was no decrease in the total
number of micropinocytotic vesicles per cell following incubation in buffer
despite the fact that the number of vesicles containing ferritin decreased (Table
1). At no time and under no condition was the presence of ferritin observed in
mitochondria or as dispersed particles in the cytoplasm.

The failure to observe a reduction in the number of vesicles per reticulocyte
after incubation in transferrin-free buffer suggests that reticulocyte endocytosis
is not primarily induced by transferrin. In order to test this possibility, rat
reticulocytes were suspended in PBS alone or PBS containing rat transferrin
(1.4 mg/ml). Aliquots were fixed for electron microscopy at 10 min and 60 min
following incubation at 37°C, and the mean number of vesicles per reticulocyte
was determined. There was no significant difference between those reticulocytes
incubated for 10 min or 60 min in buffer versus transferrin (Table 2).

A prominent feature of reticulocytes irrespective of incubation conditions
was the presence of membrane-bound ribosomes associated with scattered
profiles of granular endoplasmic reticulum (Figs. 8 and 9). Elements of the
endoplasmic reticulum often were located in the cell periphery, occasionally in
proximity to the plasma membrane (Fig. 8). Ferritin conjugates were not seen
in the intracisternal space of the endoplasmic reticulum.

Table 2. Characteristics of Vesicles in Reticulocytes Incubated With or Without Transferrin

<table>
<thead>
<tr>
<th></th>
<th>Per Cent of 100 Reticulocyte Sections With Vesicles</th>
<th>Number of Vesicles per Reticulocyte*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tfn for 10 min</td>
<td>100</td>
<td>8.66 ± .417†</td>
</tr>
<tr>
<td>Buffer for 10 min</td>
<td>100</td>
<td>8.69 ± .56†</td>
</tr>
<tr>
<td>Tfn for 60 min</td>
<td>99</td>
<td>9.06 ± .632†</td>
</tr>
<tr>
<td>Buffer for 60 min</td>
<td>100</td>
<td>9.01 ± .603†</td>
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*Mean ± 1 SEM of 100 reticulocyte sections.
†No significant difference by t test.
DISCUSSION

These studies demonstrate reticulocyte and normoblast surface membrane binding and micropinocytosis of transferrin covalently attached to ferritin or ferritin-labeled antitransferrin antibodies. Evidence that binding of these ferritin conjugates is specific for membrane transferrin binding sites is twofold. First, there was neither cell surface membrane binding nor endocytosis of ferritin alone or ferritin covalently attached to rabbit IgG having no antitransferrin activity. Second, the ferritin-transferrin and ferritin-antitransferrin conjugates were bound and endocytosed by reticulocytes and normoblasts but not by mature erythrocytes, a result in agreement with previous experiments demonstrating that the transferrin receptor is lost as the erythroid cell matures from reticulocyte to erythrocyte.\textsuperscript{2}

Micropinocytosis of the transferrin-specific ferritin conjugates could result
from (1) cross linking of membrane transferrin receptors by divalent transferrin antibody or several transferrin molecules linked to a single ferritin molecule, (2) specific stimulation induced by the binding of transferrin itself to a membrane receptor site (or sites), or (3) a general membrane process of internalization of surface receptor molecules independent of attachment of specific ligands.

Cross linking of the cell membrane receptors or antigens by divalent antibody has been shown to produce spatial rearrangement and endocytosis of surface membrane markers in many cell types, including erythroid cells. Our present data do not rule out the possibility that cross linking of membrane transferrin receptors might induce endocytosis via the same mechanism. The induction of endocytosis stimulated artificially by a change in the manner in which transferrin is bound to its receptor might alter the usual in vitro pattern of transferrin uptake by reticulocytes. However, conjugation of 125I-labeled transferrin to ferritin does not interfere with its normal uptake by reticulocytes. In addition, the radioautographic demonstration of intracellular transferrin indicates that transferrin may enter the reticulocyte without being cross linked. These observations tend to rule out the possibility that endocytosis of transferrin-specific ferritin conjugates is an artifact.

The correlation of several of our electron-microscopic observations with previous in vitro studies on the interaction of radioactively labeled transferrin with reticulocytes supports the concept that endocytosis of transferrin is a normal physiologic mechanism in the iron transport pathway. When 125I-transferrin is added to reticulocytes in vitro, maximum binding is observed after 5-10 min, but the amount of 125I-transferrin bound subsequently decreased either spontaneously or upon incubation with unlabeled (“cold”) transferrin. Endocytosis of Ft-Tfn or Ft-anti Tfn in the present studies was observed within 30 sec of incubation and was maximal after 5-10 min. Significantly fewer micropinocytotic vesicles containing ferritin conjugates were noted after 45 min of incubation in buffer (Table I). The temperature dependence of iron and 125I-transferrin uptake by reticulocytes in vitro also parallel the endocytosis of Ft-anti Tfn conjugates observed in these studies. Both occur maximally at 37°C but are suppressed at 4°C. Also, colchicine and other metabolic inhibitors that have been shown to block transferrin uptake and iron delivery to reticulocytes are known to block endocytosis of cell surface receptors in other cell types.

The attachment of transferrin to its receptor may specifically trigger its endocytosis, or the transferrin receptor complex may simply be internalized as part of a continuous process of receptor endocytosis. The former possibility was suggested by previous studies demonstrating that rabbit reticulocytes, washed and incubated in transferrin-free medium, showed a marked decrease in micropinocytic activity. In contrast, our studies revealed no difference in the number of micropinocytotic vesicles per rat reticulocyte whether incubated in the presence or absence of transferrin. In addition, in cells incubated with ferritin-transferrin antibody conjugates for 10 min followed by buffer alone for 45 min there was no decrease in the number of micropinocytic vesicles despite a significant reduction in the number of vesicles containing ferritin conjugates. These observations suggest that micropinocytosis in rat reticulocytes is in-
dependent of transferrin binding and that vesicle formation may be induced by some other stimulus. Whether a distinct stimulus for reticulocyte endocytosis exists is unknown, but recent observations raise the possibility that micropinocytosis is a process necessary for several events in red cell development including membrane remodeling during maturation and the delivery of other essential metabolic components such as vitamin B₁₂.

The ultimate fate of the internalized ferritin conjugates is unknown. There was no indication of intramitochondrial accumulation of the transferrin-specific ferritin conjugates or dispersion of ferritin within the cytoplasm of the reticulocytes. Therefore, the decrease in the appearance of the horse ferritin label in the endocytotic vesicles with time, without its appearance in the cell cytoplasm or mitochondria, suggests either the subsequent exocytosis or intravesicular metabolism of the conjugate. Further studies are in progress to determine the fate of the internalized conjugates.

The membrane-bound ribosomes observed in association with the granular endoplasmic reticulum are of interest with regard to biochemical studies of protein synthesis in reticulocytes. The specific role of the endoplasmic reticulum in reticulocytes and erythroid cells in general has not been resolved. In rabbit reticulocytes, most of the newly synthesized protein associated with a membrane-bound ribosomal fraction was reported to be nonglobin. This result was not confirmed in other studies reporting that membrane-bound ribosomes were involved largely in globin synthesis. Recently, it was suggested that membrane-bound ribosomes may be responsible for the synthesis of membrane proteins associated with the external surface. Our present studies indicate that (1) membrane-bound ribosomes in intact reticulocytes are associated with the endoplasmic reticulum (Figs. 8 and 9), (2) there is no morphologic evidence of ribosomal binding to the plasma membrane, and (3) the endoplasmic reticulum, although occurring in the deeper regions of the cytoplasm, is often located in the periphery of the cell near the cell surface (Fig. 8). The topographical distribution of the granular endoplasmic reticulum raises the possibility that it may be engaged in the synthesis and regeneration of surface receptor molecules such as the transferrin receptor. Further studies are necessary to support this suggestion.

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MICROPINOCYTOSIS OF TRANSFERRIN


Micropinocytosis of transferrin by developing red cells: an electron-microscopic study utilizing ferritin-conjugated transferrin and ferritin-conjugated antibodies to transferrin

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