Shedding of Transferrin Receptor From Rat Reticulocytes During Maturation In Vitro: Soluble Transferrin Receptor Is Derived From Receptor Shed in Vesicles

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Measurements of circulating transferrin (Tf) receptor are useful in assessing erythropoiesis; however, steps involved in the generation of soluble Tf receptor from cellular receptor are incompletely understood. To obtain a better understanding of this process, we investigated the loss of Tf receptor during terminal maturation of reticulocytes in vitro. Previous studies have identified Tf receptor-containing vesicles in the culture medium of maturing reticulocytes. In the present study, vesicle-free reticulocyte culture medium was found to contain functional and immunoreactive soluble Tf receptor, which increased over time. During a 44-hour incubation, Tf receptor on reticulocytes decreased by approximately 69%, while, of the Tf receptor shed to the medium, 65% was present in vesicles and 35% was in a soluble form. Isolated vesicles reincubated in fresh medium released soluble Tf receptor to the medium. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the isolated soluble receptor protein was mainly 190 Kd and 95 Kd under nonreducing and reducing conditions, respectively, similar in size to the vesicular and cellular receptor. Our studies show that loss of Tf receptor from reticulocytes during maturation in vitro involves shedding of cellular Tf receptor in vesicles and release of soluble receptor from these vesicles.

UNDER PHYSIOLOGIC conditions in vivo, iron is transported bound to transferrin (Tf) and the cellular uptake of iron by cells is mediated through specific cell surface receptors for Tf-Fe. Since hemoglobin production in developing erythroid cells requires iron, Tf receptor expression on these cells is highest during stages of active hemoglobin synthesis. With the cessation of hemoglobin production, Tf receptors on erythroid cells are progressively diminished and can no longer be detected beyond the early reticulocyte stage.

Studies examining the loss of cellular Tf receptor during the maturation of sheep reticulocytes in vitro have shown that the Tf receptor is shed from these cells in vesicles. Such vesicles have been isolated from the plasma of sheep and rats and have been shown to contain Tf receptor identical in size to the cellular receptor. A soluble form of the Tf receptor has also been isolated directly from rat and human plasma; however, controversy exists regarding its size.

Current studies suggest that Tf receptor present in the circulation is derived predominantly from maturing red blood cells. However, the origin of plasma Tf receptor is probably not limited to erythroid cells, since none erythroid cells shed a soluble form of the Tf receptor that is smaller in size than the cellular receptor.

Although the physiologic significance of circulating plasma Tf receptor remains to be defined, measurement of plasma Tf receptor can provide clinically useful information. Several studies have shown that plasma Tf receptor levels are decreased in aplastic anemia and are increased in autoimmune hemolytic anemia, polycythemia vera, and iron-deficiency anemia.

While the measurement of plasma Tf receptor may have potential clinical application in the assessment of erythropoiesis, the intermediate steps involved in the generation of free Tf receptor at the cellular level have not been clearly defined. To obtain a better understanding of this process, we have examined Tf receptor released during rat reticulocyte maturation in vitro. We show that Tf receptor shed by these cells exists in both soluble and vesicular forms, and that the soluble Tf receptor is derived from Tf receptor in vesicles. Furthermore, soluble Tf receptor appears to be similar in size to its cellular counterpart. This study attempts to further characterize the sequence of events leading to the appearance of free soluble Tf receptor in the plasma.

MATERIALS AND METHODS

**Materials.** Human Tf, Triton X-100, Sephadex G150, Sepharocryl S300, cyanogen bromide-activated Sepharose 4B, molecular weight standards, bovine serum albumin (BSA), and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co (St Louis, MO). Tachisorb R IgG immunoadsorbent (goat antibody to rabbit IgG conjugated to Staphylococcus aureus cells) was obtained from Calbiochem (La Jolla, CA). 125I-Sodium iodide was obtained from New England Nuclear (Boston, MA). Iron-saturated Tf was prepared as described by Bates and Schlabach. Tf-Fe and protein A were iodinated by the chloramine T method. IgG fraction of rabbit antiserum raised against rat placental Tf receptor was purified using Affi-Gel protein A affinity chromatography (Bio-Rad, Richmond, CA). Alpha-minimum essential medium (MEM) and newborn calf serum were purchased from Whittaker MA Bioproducts (Walkersville, MD).

In vitro maturation of reticulocytes and separation of cells, vesicles, and culture medium. Reticulocytes were harvested and isolated from thiamphenicol-treated rats as previously described. An aliquot of cells (0-hour reticulocytes) was removed for preparation of lysates (see below). The remaining cells were washed once with phosphate-buffered saline (PBS) and once with α-MEM containing 25 mmol/L HEPES, 20 mg/dL glucose, 5% newborn calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, pH 7.5. Cells (2 × 10⁷/mL) were incubated in the same medium at 37°C in
were removed by ultracentrifugation, and the cell-free culture medium was concentrated twofold using a Centricon-30 concentrator for autoradiography. Densitometry of the autoradiogram was performed using a Biotech EL-308 microplate reader (BioTech Instruments, Winooski, VT).

In a parallel experiment, 100 μg of nonradioactive Tf-Fe was incubated with the mixture of culture medium and 125I-Tf before gel filtration.

**Immunoprecipitation studies.** The peak radioactive fractions obtained on gel filtration were concentrated approximately 10-fold and incubated with rabbit anti-rat Tf receptor antiserum (1:10 dilution) for 90 minutes at 37°C. Control samples were incubated with nonimmune rabbit serum. Tachisorb R (1.2 vol) was then added to each tube and the incubation was continued for an additional 30 minutes at room temperature. Following this, the tubes were centrifuged and the radioactivity in the pellet (representing 125I-Tf-receptor-antibody complexes) was counted. Control immunoprecipitation experiments were also performed by incubating anti-rat Tf receptor antiserum with 125I-Tf alone. Under these conditions, no radioactivity could be immunoprecipitated, thus confirming that the antiserum did not cross-react with Tf.

**Western blot analysis.** Sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) of the detergent-solubilized cell and vesicle lysates were performed according to the method of Laemmli. Proteins were transferred from the gel onto nitrocellulose membrane as described by Towbin et al., using a Transblot system (Bio-Rad). As in the dot blot method described above, immunoreactive Tf receptor was detected by incubating the nitrocellulose membrane with purified anti-rat Tf receptor IgG (2 μg/mL) followed by 125I-protein A.

**Isolation of soluble Tf receptor from incubation medium.** Medium from the culture of isolated vesicles or from a 44-hour culture of reticulocytes (170,000 cpm supernatant) was concentrated by Amicon cell ultrafiltration and applied to a Sephadex S300 2.5 × 30 cm gel filtration column. Aliquots from each eluted fraction were analyzed for Tf receptor by dot blot method. A single peak fraction was found to contain immunoreactive Tf receptor. This fraction was concentrated fourfold using a Centricon 30 concentrator (Amicon). From this fraction, 50 to 100 μg of protein was iodinated at 4°C with 1 to 2 mCi 125I-Na using 1:3,4,6-tetrachloro-3-oxydiphenylglycouril (IODO-GEN, Pierce Chemical, Rockford, IL). Free 125I-Na was removed by filtration through a Sephadex G25 column and the sample containing radiolabeled proteins was preadsorbed (to minimize nonspecific binding of proteins to Sepharose) by mixing with Sepharose-hemoglobin for 2 hours at 4°C. Sepharose-hemoglobin was removed by centrifugation and iodinated Tf receptor in the supernatant was isolated by mixing the sample overnight at 4°C with iron-saturated Sepharose-Tf. The resin was then extensively washed with PBS and finally with 62.5 mmol/L Tris-HCl, pH 6.8. The Sepharose pellet was resuspended in Laemmli sample buffer, and analyzed by SDS-PAGE (7.5% polyacylamide) under reducing and nonreducing conditions. Autoradiography of the dried gel was performed using XAR-5 film.

**RESULTS**

**Identification of soluble Tf receptor in incubation medium.** Our prior studies have shown that cell surface Tf receptor density is decreased during the maturation of rat reticulocytes in vitro. The Tf receptor is shed from these cells in vesicles that appear in the 100,000 g pellet fraction obtained on ultracentrifugation of the incubation medium. To determine whether Tf receptor is also present in the medium in a nonvesicular, soluble form, the supernatant obtained on
ultracentrifugation of the culture medium was analyzed. As shown in Fig 1, an 125I-Tf binding protein was detected in cell-free medium by dot blot analysis using 125I-Tf as the probe. No binding of 125I-Tf to control wells (medium incubated without cells) was seen.

Confirmation that the 125I-Tf binding protein in the medium represented the Tf receptor was provided by the gel filtration and immunoprecipitation studies shown in Fig 2. When applied to the column, 125I-Tf eluted with a peak radioactivity in fraction 29 (not shown). However, when 125I-Tf was first incubated with culture medium, the peak radioactivity eluted at fraction 21, consistent with the binding of 125I-Tf to another protein (Fig 2). Radioactivity in fractions comprising this peak could be immunoprecipitated by antiserum to rat Tf receptor, identifying this peak as containing an 125I-Tf-Tf receptor complex. Since we did not attempt to immunoprecipitate all the Tf receptor in this peak, the immunoprecipitation values should be interpreted as a qualitative rather than a quantitative index of the presence of an 125I-Tf-Tf receptor complex. It should be appreciated that the total radioactivity in each fraction comprising the first peak consisted of 125I-Tf-Tf receptor complex plus free 125I-Tf and that only cpm representing the receptor-ligand complex were subject to immunoprecipitation by the anti-Tf receptor antibody. Figure 2 also shows that in the presence of excess nonradioactive Tf-Fe, the binding of 125I-Tf to Tf receptor (in the first peak) was completely inhibited so that 125I-Tf eluted at fraction 29 (similar to the elution profile of free 125I-Tf).

Comparison of soluble Tf receptor in the medium at 20 and 44 hours of incubation of reticulocytes showed that the amount of soluble receptor released by cells increased over time. Culture medium at 44 hours contained approximately twice the amount of Tf receptor as that present at 20 hours (Fig 3).

Relative distribution of vesicular and soluble Tf receptor in culture medium. The loss of Tf receptor from reticulocytes and the relative amount of Tf receptor appearing in vesicles and soluble form in the medium was examined by dot blot analysis. During a 44-hour incubation of reticulocytes, cellular immunoreactive Tf receptor decreased by approximately 69% (Fig 4, left panel). Analysis of the 44-hour culture medium showed that approximately 30% of the total Tf receptor present in the incubation medium was in a soluble form, while the remainder was present in vesicles (Fig 4, right panel).

Release of soluble Tf receptor from vesicles. To determine whether soluble Tf receptor was released from vesicles, isolated vesicles were incubated in medium and the medium was analyzed for soluble Tf receptor. Figure 5 shows that after a 24-hour incubation of vesicles, immunoreactive soluble Tf receptor could be readily detected in the unconcentrated medium. These studies strongly indicate that soluble Tf in the medium originates from Tf receptor present in vesicles.

Isolation of soluble Tf receptor from culture medium. To determine the size of the soluble Tf receptor, proteins in
the culture medium of reticulocytes or the culture medium of vesicles were first separated by gel filtration. Using either ^1^2^I-Tf or antibody to Tf receptor as the probe, a single peak eluted fraction was found to contain Tf receptor (not shown). Proteins in this fraction were iodinated and radio-

Fig 3. Comparison of soluble Tf receptor in culture medium after 20 and 44 hours of incubation of reticulocytes. One-milliliter samples of unconcentrated culture medium were harvested by ultracentrifugation at the time points shown. Soluble Tf receptor was detected by dot blot method using ^1^2^I-Tf as the probe. Control represents medium (containing 10% fetal calf serum) alone.

Fig 4. Relative distribution of immunoreactive Tf receptor in cells, vesicles, and culture medium. Tf receptor was detected by dot blot method using antiserum against rat Tf receptor. (Left panel) Lysates from equivalent numbers of cells before and after a 44-hour incubation. (Right panel) Tf receptor in the corresponding culture medium at 44 hours. vTFR, Tf receptor present in vesicles (170,000g pellet); sTFR, soluble Tf receptor present in the cell-free, vesicle-free culture medium (170,000g supernatant). All samples were loaded onto nitrocellulose and probed for Tf receptor simultaneously.

Fig 5. Release of soluble Tf receptor from vesicles. Vesicles released from reticulocytes were isolated and reincubated in fresh medium. After 24 hours, vesicles and culture medium were separated by centrifugation and analyzed for immunoreactive Tf receptor. vTFR, Tf receptor present in vesicles; sTFR, Tf receptor present in culture medium.
labeled Tf receptor was isolated by Sepharose-Tf. Analysis of the soluble Tf receptor by SDS-PAGE and autoradiography showed major protein bands of approximately 190 Kd and 95 Kd under nonreducing and reducing conditions, respectively (Fig 6). Identical bands were seen for Tf receptor isolated from the culture medium of reticulocytes (Fig 6, left panel) or the culture medium of vesicles (Fig 6, right panel). These protein bands were similar to those of the cellular and vesicular Tf receptor identified by Western blots (Fig 7).

In addition to the major bands present on SDS-PAGE analysis of the soluble Tf receptor, minor polypeptide bands of 70 Kd and 55 Kd were detected under nonreducing and reducing conditions, respectively (Fig 6). These minor bands may represent degraded Tf receptor, since they persisted even after several cycles of preadsorption and extensive washing of Sepharose-Tf during the isolation procedure.

DISCUSSION

Our present study shows that the loss of Tf receptor from reticulocytes during terminal maturation in vitro involves shedding of the receptor in vesicles and the release of a soluble form of Tf receptor from these vesicles. Prior studies show that 50-nm lipid vesicles enriched in Tf receptor are shed from reticulocytes during maturation in vitro and can be recovered in the 100,000g pellet fraction following ultracentrifugation of the culture medium. As an extension of these earlier observations, we examined the 170,000g supernatant (soluble protein fraction) of culture medium from maturing reticulocytes and found it to contain functional and immunoreactive soluble Tf receptor.

Two different approaches (anti-rat Tf receptor antibody and Tf binding) were used to detect soluble Tf receptor in the medium. The dot blot method using human 125I-Tf as a probe proved to be highly sensitive, despite the presence of bovine Tf in the medium (which could potentially interfere with 125I-Tf binding). The high affinity of human Tf for rat Tf receptor undoubtedly allowed for preferential binding of this probe to the receptor. The ability of nonradioactive Tf to inhibit 125I-Tf binding to soluble protein in the culture medium is compatible with specific receptor ligand interaction and clearly identifies this protein as the Tf receptor.

The appearance of increasing amounts of soluble Tf receptor in the culture medium during the 44-hour incubation of reticulocytes was accompanied by a marked decrease in immunoreactive cellular Tf receptor. These latter findings are consistent with the previously described decrease in 125I-Tf binding to rat reticulocytes during maturation. Tf receptor lost from cells over 44 hours was found in the culture medium in both vesicular and soluble forms. An interesting finding was that the total Tf receptor (vesicular and soluble) in the medium was greater that the amount apparently lost from cells during the 44-hour incubation. Synthesis of the Tf receptor has been shown to occur in the rabbit reticulocyte, and a likely explanation for our finding is that rat reticulocytes continue to synthesize new Tf receptor during the 44-hour incubation period and thus lose more Tf receptor than is appreciated by direct comparison of 0-hour and 44-hour cells.

The presence of two forms of Tf receptor in the medium raised questions regarding the relationship of these receptors and the origin of the soluble receptor. Following incubation of isolated vesicles, the culture medium was found to contain soluble Tf receptor that was similar in size to the Tf receptor present in the 44-hour culture medium. Comparison of the cellular, vesicular, and soluble Tf receptors showed the three forms of the receptor to be dimeric proteins of identical size on SDS-PAGE. Although we have not excluded the possibility that soluble Tf receptor may also be shed directly from reticulocytes, our results suggest that one pathway involved in the generation of soluble Tf receptor is the release of this form of receptor from vesicles shed by reticulocytes.
cells and vesicles were separated by 7.5% SDS-PAGE and transferred onto nitrocellulose filter. Tf receptor was detected using purified antibody against rat Tf receptor and 125I-protein A. (Left panel) Tf receptor from detergent-solubilized cells. Lane 1, reduced; lane 2, nonreduced. (Right panel) Tf receptor from detergent-solubilized vesicles. Lane 1, reduced; lane 2, nonreduced.

Our finding that the vesicular Tf receptor is identical to the cellular Tf receptor is in agreement with that reported by others.\textsuperscript{9,10} However, in contrast to our studies, analyses of soluble Tf receptor isolated directly from human and rat plasma have yielded conflicting results. Tf receptor in human plasma has been shown to exist as an intact protein,\textsuperscript{11} nicked dimers of 55 Kd,\textsuperscript{12} or monomers of 85 Kd lacking the transmembrane and cytoplasmic domains of the receptor.\textsuperscript{13} Beguin et al found the Tf receptor in rat plasma to be similar in size to placental Tf receptor,\textsuperscript{14} while the soluble Tf-binding protein recently isolated from rat plasma by Nair et al was found to be mainly an 85-Kd fragment.\textsuperscript{15} Tf receptor isolated directly from plasma could be substantially altered by proteolytic activity or by other events and may circulate intact or as proteolytic fragments. Furthermore, it is possible that circulating Tf receptor may represent a mixture of receptors originating from erythroid and nonerythroid cells. Human myeloid HL60 cells release soluble Tf receptor, smaller in size than its cellular counterpart.\textsuperscript{16,17} All these factors may explain, in part, the differences in the size of the circulating Tf receptor reported by different investigators.

Our experimental system has enabled us to examine the fate of the cellular receptor in a synchronized population of reticulocytes during maturation. However, this system does have obvious limitations in that the use of rat reticulocytes generated by thiamphenicol treatment and in vitro incubation conditions could potentially influence vesicle formation and the relative amounts of soluble and vesicular Tf receptor. Clearly, further in vivo studies will be needed to define the process of reticulocyte shedding of the Tf receptor and the generation of soluble receptor under physiologic and pathologic conditions.

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