Origin of a Soluble Truncated Transferrin Receptor

By Jinhi Ahn and Rose M. Johnstone

It has recently become evident that elevation of reticulocytes in the circulation of several species, including humans, leads to the formation of a noncellular transferrin receptor (TFR). In humans, the majority of the released receptor is in truncated form (Shih et al: J Biol Chem 265:19077, 1990). In other species (sheep, rat, chicken) the receptor is associated with a vesicle (exosome) and is full length (Johnstone et al: J Cell Physiol 147:27, 1991). In this report we show that in sheep reticulocytes incubated in vitro, the majority (~75%) of the released receptor is of native size and is exosome associated. A fraction (~25%) is a truncated form of ~80 Kd corresponding to the exofacial domain of the TFR. Herein we also address the question of whether the truncated receptor originates by proteolytic cleavage directly from the cell surface or by cleavage from exosomes. Using surface ~125I-labeled sheep reticulocytes as the experimental model, we show that during in vitro maturation, ~125I-TFR of native size appears in exosomes before the soluble, truncated, exofacial domain of the receptor is detected in the medium. Because cleavage and release of the exofacial domain would likely leave the truncated cytoplasmic and transmembrane domains in the originating membrane (plasma membranes or exosomes), both fractions were probed with antibodies specifically generated against the cytoplasmic domain of the receptor. Only exosomes, not plasma membranes, show the presence of a ~17-Kd peptide recognized by the antibody to the cytoplasmic domain of the transferrin receptor. Thus, it is concluded that the truncated, soluble receptor originates from exosomes in sheep. A 17-Kd cytoplasmic domain of the TFR was also detected in exosomes from the reticulocytes of an anemic man, suggesting that the truncated receptor in man may also originate from exosomes. Using in vitro cultures of surface ~125I-labeled sheep reticulocytes, it is concluded that exosome formation is the principal route for maturation-associated loss of the TFR. A similar conclusion was made earlier (Johnstone et al: J Cell Physiol 147:27, 1991) for the nucleoside transporter of maturing sheep reticulocytes.

Nearly a decade ago it was shown that sheep reticulocytes maturing in vitro shed a population of vesicles containing two readily distinguishable proteins, the transferrin receptor (TFR) and a 70-Kd protein later identified as the clathrin uncoating adenosine triphosphatase (ATPase). It soon became clear that the released vesicles (named exosomes) contained a variety of plasma membrane proteins that were known to diminish in the red blood cell (RBC) during the maturation process. Rather than budding directly from the cell surface, these exosomes were formed intracellularly in multivesicular sacs and were released after fusion of the sacs with the plasma membrane. The presence of the characteristic multivesicular sacs and/or circulating exosomes has now been demonstrated in nine species of immature RBCs from anemic animals, including humans. The anemias in which loss of the TFR during reticulocyte maturation in sheep occurs by exosome formation, and/or cleavage, we undertook to: (1) improve recovery of the sheep transferrin receptor lost during maturation; (2) assess in a controlled experimental situation, in the sheep, what fraction of the TFR lost during maturation occurs by surface cleavage from the cell; and (3) assess which mechanisms, other than exosome formation, contribute in a substantial way to the loss of the TFR during reticulocyte maturation in sheep RBCs.

The TFR was found to be an integral component of the exosomes in several species as detected by direct chemical analysis or by electron microscopic techniques. The TFR in the exosomes appeared to have a native size (rat, sheep, humans, and chicken) in the sheep, identical iodotyrosyl peptide maps were obtained for the cellular and exosomal receptor. However, failure to obtain a near (~70%) quantitative recovery of shed receptor in exosomes from sheep reticulocyte cultures left open the possibility that other mechanisms could also contribute significantly to the total loss of receptor. In contrast, ~80% of the maturation-associated loss of the nucleoside transporter could be recovered in exosomes that contained both TFR and the nucleoside transporter. Because the majority of the peptide chain of TFR is exposed at the surface, whereas the nucleoside transporter is largely inaccessible to proteases at the surface, the differences in recovery could arise from surface proteolysis of cells and/or vesicles, thus leading to an underestimate of TFR recovery.

Evidence for surface cleavage of the TFR from human reticulocytes has recently been obtained. These reports showed that the majority of the circulating TFR in humans was a soluble truncated form (~85 Kd). However, in vitro and in vivo studies with sheep and rat reticulocytes had suggested that the released receptor which was recovered from the medium was intact (~94-Kd monomer on reducing gels).

Because loss of the cellular transferrin receptor may occur by exosome formation, and/or cleavage, we undertook to: (1) improve recovery of the sheep transferrin receptor lost during maturation; (2) assess in a controlled experimental situation, in the sheep, what fraction of the TFR lost during maturation occurs by surface cleavage from the cell; and (3) assess which mechanisms, other than exosome formation, contribute in a substantial way to the loss of the TFR during reticulocyte maturation in sheep RBCs.
Fig 1. Loss of TFR from sheep reticulocytes during in vitro maturation: Recovery of TFR in the culture medium. (A) Reticulocytes were labeled with ^125I and cultured for 4, 24, and 48 hours. After incubation TFR was immunoprecipitated from the isolated cell membranes, the 100,000g pellet (exosomes) or the PES as described under Materials and Methods. The immunoprecipitates were electrophoresed on SDS reducing gels (A), or nonreducing gels (B). A single experiment is shown that is representative of 12 separate experiments (see also Table 1). (A) Left block: Coomassie Blue stains of the immunoprecipitates. The band at 94 Kd is the TFR monomer. The higher molecular weight band is the unreduced dimer. Right block: Autorads corresponding to the Coomassie Blue stained gels. m, molecular weight standards; 0, 4, 24, and 48 represent the hours of incubation of the reticulocytes. Lanes marked membranes show the diminishing level of TFR in the immunoprecipitates of cell membranes with time. Lanes marked vesicles show the appearance of TFR in exosomes with time. PES shows the soluble TFR in the post exosome fraction with time. (B) Presence of cleaved receptor in PES: Effect of metabolic and proteolytic inhibitors. After 24 hours incubation at 37°C with surface ^125I-labeled sheep reticulocytes, the PES was immunoprecipitated and electrophoresed on nonreducing gels. The incubation medium contained: control (no additions); 2 mmol/L DOG and 1 μg/mL rotenone (lane marked rotenone + DOG); PMSF (10 μmol/L) and aprotinin 0.5U/mL (lane marked PMSF) or 0.85% albumin (lane marked albumin). The arrow points to a single labeled band of 160 Kd (the truncated dimer of the TFR).

MATERIALS AND METHODS

Cell Preparation

Sheep reticulocyte fractions. These were obtained after differential centrifugation of blood from phlebotomized, iron-supplemented sheep as described. Generally, reticulocyte levels of 75% to 90% were used in these experiments. The cells were either used directly after isolation or stored overnight in culture medium at 4°C before use. For culturing, a 3% to 10% suspension of cells in Eagle’s minimal essential medium containing 0.2 mmol/L nonessential amino acids, 4 mmol/

Table 1. Recovery of Labeled Species of TFR After Incubation of Sheep Reticulocytes

<table>
<thead>
<tr>
<th>Hours</th>
<th>0</th>
<th>4</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin of receptor</td>
<td>cpm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>10,640 7,460 4,250 1,280</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exosomes</td>
<td>—    740 1,750 2,200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant (PES)</td>
<td>—    100 600 180</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% RR as cleaved product:</td>
<td>Single value 12 25 5</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Average value</td>
<td>13 (n = 7) 25 (n = 12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>5-30 12-35</td>
<td></td>
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</tbody>
</table>

After incubation of surface iodinated sheep reticulocytes for the times given, the cells were centrifuged, washed, and plasma membranes prepared. After solubilization in Triton X-100, immunoprecipitation, and SDS-gel electrophoresis, the bands corresponding to the TFR were excised from the gels and counted. Both 190-Kd and 94-Kd species were processed. Similarly, after recovery of exosomes from the medium, they were immunoprecipitated and subjected to SDS-PAGE and the receptor bands were counted. The supernatant from the 100,000g pellet (PES) was also immunoprecipitated (~80-Kd peptide) and treated identically. A result from one complete experiment is shown. The percent of externalized receptor in the truncated form (~80 Kd) in PES is given for this experiment. In addition, the average value of the fraction of total released receptor present in the truncated form from 7 to 12 experiments is also given. The % RR as cleaved product is calculated as follows, using the values from the 24-hour incubation:

\[ \frac{600}{1,750 + 600} \times 100 = 25\%. \]

* RR, released receptor.
Table 2. Albumin Improves the Recovery of ¹²⁵I-TFR During Long-Term Incubation of Reticulocytes

<table>
<thead>
<tr>
<th>Activity at 3 h (albumin %)</th>
<th>Activity at 24 h (albumin %)</th>
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<tbody>
<tr>
<td>cpm/Fraction</td>
<td></td>
</tr>
<tr>
<td>Membrane</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>36,150</td>
</tr>
<tr>
<td>0.5</td>
<td>45,000</td>
</tr>
<tr>
<td>1</td>
<td>49,180</td>
</tr>
<tr>
<td>2</td>
<td>48,670</td>
</tr>
<tr>
<td>Exosomes</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2,460</td>
</tr>
<tr>
<td>0.5</td>
<td>2,950</td>
</tr>
<tr>
<td>1</td>
<td>2,400</td>
</tr>
<tr>
<td>2</td>
<td>3,020</td>
</tr>
<tr>
<td>PES</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>130</td>
</tr>
<tr>
<td>0.5</td>
<td>460</td>
</tr>
<tr>
<td>1</td>
<td>660</td>
</tr>
<tr>
<td>2</td>
<td>730</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>77</td>
</tr>
<tr>
<td>0.5</td>
<td>95</td>
</tr>
<tr>
<td>1</td>
<td>103</td>
</tr>
<tr>
<td>2</td>
<td>103</td>
</tr>
<tr>
<td>Average recovery % (n = 5)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>51</td>
</tr>
<tr>
<td>0.5</td>
<td>85</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Range of values

(32-66) (57-100)

Sheep reticulocytes, surface labeled with ¹²⁵I, were incubated at 37°C. Samples were taken at 0, 3, and 24 hours of incubation. Cells were harvested, plasma membranes isolated, solubilized, and immunoprecipitated. The high-speed pellet (100,000g) from the cell-free incubation medium was also harvested and immunoprecipitated and the PES was collected and immunoprecipitated. All immunoprecipitates were subjected to SDS-PAGE. All samples had control nonimmune precipitates. After radioautography, the specific ¹²⁵I-labeled bands were cut from the gel and counted. Thus, for the membrane, the TFR monomer and dimer were pooled to give the total value in immunoprecipitates corrected for the values in nonimmune controls from the corresponding areas of the gels. The radioactivity in the immunoprecipitate of original membranes was 50,600 cpm. Complete details from a single experiment, representative of five experiments, are shown. In addition, pooled data, giving the average recovery from five experiments after 24 hours of incubation are also presented. All values have been corrected for their respective, nonimmune controls (≤5% of corrected values). All values are based on recovery from 10⁶ cells.

L glutamine, 200 U/mL penicillin, and 200 μg/mL streptomycin (complete MEM) was used. The medium was supplemented with 10 mmol/L inosine and 5 mmol/L adenosine, and the mixture was gassed with 95% O₂/5% CO₂.

**HL-60 cells.** A human promyelocytic leukemia cell line, HL-60 cells, were maintained at 2 x 10⁶ to 1.5 x 10⁶ cells/mL in RPMI 1640 containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine, 10% fetal bovine serum (complete RPMI).

**Human blood.** Blood was derived from hemochromatosis patients undergoing periodic phlebotomy and was discarded blood from the outpatient clinic. The heparinized blood was centrifuged and the cell-free plasma was diluted 1:1 with saline and centrifuged at 120,000g for 1 to 2 hours using a 20% sucrose cushion to obtain an exosome pellet. Alternatively, the plasma was incubated with iron-core Dynal beads (Dynal Inc, Great Neck, NY) cross-linked to sheep anti-mouse IgG and coated with mouse monoclonal antibody (MoAb)
ORIGIN OF TRUNCATED TRANSFERRIN RECEPTOR

Fig 3. Externalization of a trypsin-generated cytoplasmic domain of TFR from sheep reticulocytes. Reticulocytes were treated without or with 0.1% trypsin in PBS for 10 minutes at 37°C. The cells were washed with PBS containing 0.2% soybean trypsin inhibitor, then cultured for 16 hours and cells and exosomes harvested. The cell membranes and exosomes were subjected to SDS-PAGE, blotted, and probed with the antibody to the cytoplasmic domain of the TFR. Lane 1, prestained molecular weight markers (see Fig 3); lane 2, untreated reticulocyte membranes (20 μL equivalent of packed cells); lane 3, trypsin-treated reticulocyte membranes (20 μL equivalent of packed cells); lanes 4 and 5, exosomes from the culture medium derived from 20 μL and 50 μL equivalents, respectively, of untreated, packed reticulocytes; lanes 6 and 7, exosomes from the culture medium from 20 μL and 50 μL equivalents of trypsin-treated reticulocytes. Arrows indicate the position of the TFR. Similar results were obtained in three separate experiments.

to the TFR. The immobilized material was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Iodination of the Reticulocyte Cell Surface
To obtain a quantitative measure of the lost TFR during maturation, RBCs were iodinated using lactoperoxidase and glucose oxidase as described by Reichstein and Blostein. After washing to remove most of the unincorporated 125I, the labeled cells were cultured as described above. The radioactivity recovered in immunoprecipitates of the released TFR was compared with the amount of immunoprecipitable radioactivity lost from the cell membranes.

Immunoprecipitation of the TFR
RBC membranes were prepared by osmotic lysis using 30-fold excess of 5 mmol/L P5 containing 1 mmol/L EDTA, pH 8.0. The membranes were washed twice with the same buffer, once with 20 mmol/L P5 (pH 7.0), and then solubilized with stirring in 1% Triton X-100 (final concentration) containing aprotinin as described. The solubilized membranes were diluted with an equal volume of 20 mmol/L phosphate at pH 7.4 (PBS) and centrifuged at 27,000g for 30 minutes. The supernatant was immunoprecipitated overnight at 4°C with anti-TFR MoAb, followed by precipitation with protein A Sepharose (Pharmacia).

To retrieve the exosome-associated TFR released to the medium (or to the plasma), cells were removed by centrifugation twice at 12,000g and the cell-free medium was centrifuged at 100,000g (culture medium) or at 120,000 to 130,000g (human plasma) for 90 to 120 minutes to pellet any insoluble (membrane-associated) TFR. The pellet was solubilized with Triton X-100 (Fisher Scientific, Montreal, Quebec, Canada) and immunoprecipitated as outlined for the plasma membranes. Soluble receptor in the 100,000g supernatant (postexosome supernatant; PES) was also immunoprecipitated as described above. HL-60 cells (~10^7 cells/mL) were solubilized with 1% Triton X-100 containing 0.1 mmol/L phenylmethylsulfonylfluoride (PMSF) and 0.2 U/mL aprotinin and the lysate was immunoprecipitated as for RBCs (see above). The cell-free culture medium from HL-60 cells (with 0.1 mmol/L PMSF added) was fractionated into an insoluble fraction (100,000g pellet) and soluble fraction (PES) by centrifugation. The PES was concentrated 8 to 10× in an Amicon ultrafiltration cell (YM100 membrane; Amicon Div, Grace and Co, Beverly, MA) before immunoprecipitation.

SDS-PAGE and Quantitation of the TFR
SDS-PAGE was used to separate the TFR from the protein A-Ig complex. To solubilize the bound receptor, the protein A pellets were either incubated at 37°C for 2 hours in SDS sample buffer (for immunoblotting) or the samples were heated at 95°C for 10 minutes (for detection by staining with Coomassie Blue). The solubilized, protein A-free material was electrophoresed on reducing or nonreducing gels (5% to 15% acrylamide). With 35S-labeled preparations, the immunoprecipitated radioactive bands were excised from the gel and counted.

Processing of 35S-labeled TFR (HL-60 cells)
HL-60 cells were washed with PBS and the cells (10^7 cells/mL) incubated in complete MEM containing 10 μmol/L methionine, 10% fetal bovine serum (FBS), 20 mmol/L NaHEPES, pH 7.4, and 20 μCi/mL [35S]methionine for 1 hour at 37°C. The labeled cells were cultured 12 to 38 hours in complete RPMI under 95% O2/5% CO2. After culture, the cells were washed and solubilized with Triton X-100 and immunoprecipitated as given above for reticulocytes. The immunoprecipitates were subjected to SDS-gel electrophoresis. Fluorography was used to detect the 35S-labeled receptors.

Blotting Procedures
After SDS-PAGE, the proteins were transferred to nitrocellulose and subjected to blotting as described using alkaline phosphatase coupled second antibody. For blotting a 1/200 dilution of rabbit serum containing anti-CDTFR was used. Nonimmune rabbit serum served as control.

Materials
HL-60 cells were obtained from Dr C. A. Enns (Oregon Health Sciences University, Portland). Methionine-free MEM and [35S]-methionine and Na[35S]I were obtained from ICN Flow (Mississauga, Canada). MEM, RPMI 1640, FBS, nonessential amino acids, glutamine, streptomycin, and penicillin were from Gibco (Canada). Dynabeads M-450 were purchased from P & S Biochemicals (Gaithersburg, MD). Inosine, adenosine, bovine albumin, trypsin, soybean trypsin inhibitor, lactoperoxidase, glucose oxidase, rotenone, aprotinin, and PMSF were purchased from Sigma Chemical Co (St Louis, MO). 2-Deoxy-D-glucose was from Aldrich Chemical Co (Milwaukee, WI). Protein A-sepharose was obtained from Pharmacia (Quebec, Canada). Triton X-100 was obtained from Fisher Scientific (Montreal, Quebec, Canada). Three antibodies were used: A mouse anti-human TFR MoAb that recognizes the native and exofacial domains of the sheep TFR and two antibodies made against amino acids (Ala4-Arg25) or Met1-Ser124 of the cytoplasmic domain of the human TFR coupled to albumin (gifts of Dr T Yoshimori, Osaka University, Osaka, Japan and Dr R. K. Draper, Molecular and Cell Biology Program, University of Texas at Dallas, respectively). The latter antibodies recognize the intact receptor and its cytoplasmic domain and cross-react with sheep TFR.
RESULTS

Although both TFR and nucleoside transporters diminish during reticulocyte maturation, most (≤70%) of the nucleoside transporter, but not the TFR, lost from the cells is recovered in the released exosomes. The fractional recovery of the lost TFR in exosomes has generally been well below 50% (≈30%).

Several reasons may account for this shortfall: (1) mechanisms other than exosome formation may also be involved in the loss of the TFR from the cell during maturation of the reticulocytes; (2) the TFR is inherently unstable and loses capacity to bind transferrin and antibody during long-term incubation in vitro; and (3) the receptor is cleaved from the cell and/or vesicles so that our retrieval procedures fail to harvest all the released receptor.

A cleaved, extracellular domain of the human TFR has been detected in the plasma of anemic individuals.

To address these questions we examined: (1) whether recovery could be improved if the duration of the incubation was reduced; (2) the effect of added albumin as a putative protective agent against nonspecific losses was tested because long-term incubations were performed in serum-free medium; (3) the loss of 125I-labeled TFR from 125I surface-labeled cells was followed instead of 125I-transferrin binding.

In these studies, the labeled receptor was immunoprecipitated from: (1) the membranes of the cultured cells; (2) the exosomes released to the medium; and as well as (3) the PES to assure recovery of all fractions containing TFR. The PES should contain the cleaved, soluble exofacial domain of the receptor. To assure that all the forms of the TFR were available for quantitative analysis by γ-counting after SDS-PAGE, both reducing and nonreducing gels were used to recover the four potential forms of the receptor (native dimer, native monomer, cleaved dimer, and cleaved monomer).

A Portion of the Released Receptor Is Cleaved

The pattern of labeled peptides obtained from these immunoprecipitates of the TFR is shown in Fig 1A and is characteristic of the pattern obtained with reducing gels. The stained immunoprecipitates and autoradiograms of reducing gels from the cell membranes showed residual dimer (≈190-Kd peptide) and monomer (≈94 Kd). The majority of the label was recovered in the intact monomer (Table 1), although some dimer was frequently found despite the presence of mercaptoethanol. In the exosome fraction, the labeled peptides were similar to those from the cell membranes, with the majority of the label in the ≈94-Kd peptide. The labeled peptide pattern in the residual PES was different, with the radioactivity appearing in a band ≈80 Kd, characteristic of a cleaved, monomeric form of the receptor. The cleaved peptide was similar to the peptide obtained by trypsin digestion of the reticulocyte surface. Reticulocytes, treated with trypsin,
In contrast to reports with cleaved, human receptor,²⁸,²⁹ the released, soluble TFR migrated as a peptide of ~160 Kd on non-reducing gels (Fig 1B). On reduction, monomers were found (Fig 1A). At present, we cannot distinguish whether the dimer is due to the presence of an additional S-S bridge or whether the cysteine residues of the sheep TFR are situated downstream of the protease cleavage site. It is known that not all species have S-S bridges with an identical disposition to those of the human receptor (ie, the chicken receptor).³⁰

**Time of Formation of Cleaved TFR**

If the formation and release of a truncated receptor from the cell occurred simultaneously with exosome formation, this process might be an additional mechanism for the maturation associated loss of the TFR. However, if cleaved receptor formation followed the formation of exosome-associated TFR, such a result would imply a secondary role for cleaved receptor formation. The results in Tables 1 and 2 (see also Fig 1) show that at 3 to 4 hours of incubation, native size receptor was detected in exosomes, but little truncated form was found in the PES. At 24 hours of incubation, when over 50% of the receptor was lost from the cells, both exosome-bound and soluble (~80 Kd) receptor were present. It is evident that exosome formation preceded the formation of the cleavage product as well as being the major product at all times. The radioactivity in the soluble receptor was always under 40% of the radioactivity in the exosome-associated receptor (>8 experiments).

Because the appearance in the medium of the truncated receptor follows the appearance of exosomes (Fig 1), attempts were made to generate truncated receptor directly from exosomes. However, exosomes reincubated in fresh or conditioned medium, disintegrated and no antibody-detectable, soluble TFR was found. These data should be contrasted with those of Chitambar et al.,³¹ in which reincubation of rat exosomes gave rise to intact, soluble receptor.

If the formation of soluble truncated receptor occurs naturally in sheep, the presence of an ~80-Kd peptide in the plasma of anemic sheep with 10% circulating reticulocytes should be detectable. With our procedures of immunoprecipitation and/or blotting we were unable to detect soluble TFR in the 100,000g supernatant of plasma from phlebotomized sheep. The exosome pellet from an equivalent volume of plasma had readily detectable, full-length TFR.³²

**Albumin Improves TFR Recovery**

Although the addition of a single or a cocktail of proteolytic inhibitors (including PMSF, aprotinin, soybean trypsin inhibitor, leupeptin, and pepstatin) failed to alter the recovery or the level of released truncated receptor, the presence of albumin in the incubation medium improved recovery of ¹²⁵I-labeled receptor. With as little as 0.5% albumin in the medium nearly all the initial label in the TFR could be accounted for after an overnight incubation of sheep reticulocytes (Table 2). With albumin, the majority (up to 85%) of the TFR lost from reticulocytes could be recovered in exosomes. Albumin did not influence the amount of truncated receptor formed (see Fig 1B), which in most experiments accounted for less than 25% of the total released receptor.
Fig 6. (A) Proteolysis of immunoprecipitated sheep TFR. Sepharose-Protein-A bound anti-TFR MoAb was used to precipitate TFR from sheep reticulocyte membranes as described in Materials and Methods. Except for the nonincubated control, the Sepharose-Protein-A bound TFR was incubated for 2 hours at 37°C with 100 μL of packed, washed blood cells derived from the following sources. Lane 1, original TFR immunoprecipitate (TFRIM), no incubation; lanes 2 through 6, 2 hours incubation with: lane 2, TFR-IM with chicken blood cells; lane 3, TFR-IM with human blood cells; lane 4, TFR-IM with mouse blood cells; lane 5, TFR-IM with rat blood cells; lane 6, TFR-IM with sheep blood cells. After incubation, the protein A pellets were centrifuged and subjected to SDS gel electrophoresis using reducing conditions.

(B) The experiment was performed identically to (A) except that samples of washed RBCs were also passaged through a column of cellulose/methyl cellulose to remove WBCs. Lanes 3 through 7 were all incubated for 2 hours at 37°C with TFR-IM and 100 μL of packed blood cells. Lane 1, molecular weight standards; lane 2, control, no incubation, initial TFR-IM; lane 3, washed, complete human blood cells; lane 4, washed, WBC-free human RBCs; lane 5, washed, complete sheep blood cells; lane 6, washed, WBC-free sheep RBCs; lane 7, control, incubated TFR-IM without blood cells.

(Table 2). It is noteworthy that with albumin, the sharp decrease in cellular 125I-TFR during incubation was reduced and the amount of label recovered from exosomes was increased. This level of TFR recovered is nearly twice that reported earlier7 and approaches the recovery of the nucleosome transporter.7 In the detailed experiment reported in Table 2, virtually all of the original 50,600 cpm in the TFR of the original plasma membranes could be accounted for after incubation with albumin. In contrast, the recovery at 24 hours without albumin was 67%. A composite of five different experiments (Table 2) summarizes these observations.

Origin of the Cleaved TFR: Detection of the Cytoplasmic Domain in Sheep Exosomes

The data above suggested that exosome release can account for the majority of the TFR lost during maturation of sheep reticulocytes in vitro. Whether the truncated peptide originated from the cells or exosomes was not established. If TFR was cleaved from the cells and/or the exosomes, the transmembrane and cytosolic domains of the receptor may be retained in the originating membranes. Recent studies have suggested that in cultured cell lines, the membrane-bound, cytoplasmic domain of the TFR is stable and recycled like an intact receptor.25 Using antibodies against the cytoplasmic domain of the TFR (anti-CDTFR), we probed sheep erythrocyte membranes, sheep reticulocyte membranes, and sheep exosomes for the presence of the N terminal (cytoplasmic) domain of the TFR. The results showed that anti-CDTFR recognizes native (94 Kd and 190 Kd) TFR in sheep reticulocyte membranes (Fig 2). No other reactive peptides were seen in the membrane preparations. However, in exosomes the presence of two low molecular weight antibody-reacting peptides was evident, in addition to the native form of the receptor. The faster migrating peptide (circa 17 Kd) corresponded approximately to the expected size of the human N terminal domain (∼15,000 Kd) if cleavage of native TFR had occurred at the exofacial domain at residue 130 (a tryptic cleavage site). The fact that a second peptide of approximately twice the size was also present suggested the possibility of a second cleavage site. Both anti-CDTFR antibodies gave identical results.
Recycling of the Cleaved Cytoplasmic Domain in Sheep Reticulocytes

Although the cleaved cytoplasmic domain of the TFR was not detectable in fresh or incubated sheep reticulocytes, the possibility exists that any cytoplasmic domain in the cells would have been rapidly removed. In contrast, exosomes would be unlikely to remove the membrane-bound domain because the exosomes are unlikely to contain the necessary enzymes. To assess whether a cytoplasmic domain, if present in the cells, would survive long-term incubation, a cleaved, cytoplasmic domain was generated in the cell membranes by treatment of sheep reticulocytes with trypsin before incubation in vitro. The results showed that the cleaved, cell-associated cytoplasmic fragment generated by trypsin was recognized by the anti-CDTFR. This domain was retained in the cell membranes after overnight incubation (Fig 3). The exosomes produced by these cells also contained the cleaved, cytoplasmic domain of the receptor (Fig 3). It is evident that in exosomes from trypsin-treated cells, there was a substantial increase in the amount of cleaved, cytoplasmic domain with concomitant decrease of the native receptor. Thus, if the cleaved, soluble, receptor were generated directly from reticulocytes during normal maturation, the residual cytoplasmic domain would likely be detected in the cell membranes after overnight incubation. The absence of the truncated, cytoplasmic domain in fresh or incubated sheep reticulocytes argues that the released, exofacial domain seen in in vitro incubations came from the released exosomes.

Cleaved TFR in Human Cells

Although we were unable to detect a cleaved TFR in the circulation of anemic sheep or rats, the presence of a truncated form of TFR in the circulation of a phlebotomized, hemochromatosis patient was detected (Fig 4A) in agreement with other studies in humans. An intact, non-cell-associated, receptor was also found in the high-speed pellet of plasma in humans (Fig 4B) as well as an intact receptor in reticulocyte enriched blood from a hemochromatosis patient. Therefore, the possibility was considered that human blood may contain a protease that cleaved the TFR.

Studies on TFR synthesis with HL-60 cells (Fig 5) have shown the presence of an ~80-Kd 35S-labeled immunoreactive peptide in the 100,000g supernatant of the culture medium along with a native size, 35S-labeled TFR in the cell membranes. No intact receptor was ever found in the cell-free medium from HL-60. These data are in accord with studies done by Chitambar and Zivkovic, who also found a truncated, ~80-Kd fragment in the culture medium from HL-60 cells. The formation of a truncated, soluble, fragment with cultured cells suggested that some cells may contain surface proteases that cleave the receptor. Cell surface neutral peptidases have been described. Therefore, we examined whether there are differences in cell surface protease activities between sheep and human blood cells.

Using immobilized sheep TFR as proteolytic substrate, the results showed that human blood cells had higher proteolytic activity than blood cells from nine other species (dog, guinea pig, rat, sheep, chicken, mouse, rabbit, pig, and hamster). As seen in Fig 6A, the blood cells of chicken, sheep, rat, and mouse had little proteolytic activity. Only dog cells showed significant proteolytic activity against sheep TFR (not shown). Moreover, if the human RBCs were passed through cellulose to remove the white blood cell (WBC) fraction, little proteolytic activity remained in the residual RBCs from humans (Fig 6B). That the WBCs were the source of the protease activity was confirmed by studies using the WBC fraction remaining after ammonium lysis of the RBCs (not shown). The remaining WBCs had the majority of the original protease activity. Thus, formation of a truncated, circulating receptor in humans, but not in sheep or rats, may be caused by differences in WBC-associated protease activity.

Because of limited availability of reticulocyte enriched human blood, the source of the human, truncated receptor (reticulocytes or exosomes) remains inconclusive. However, the data to date argue that the exosomes are likely to be the precursors of the truncated receptors. Thus, (1) exosomes containing intact TFR have been detected in the circulation of hemochromatosis patients (Fig 4B). (2) Native TFR has been detected in plasma membranes from enriched reticulocytes of hemochromatosis patients (Fig 4C). The same preparations failed to show the presence of the cleaved, cytoplasmic domain of the TFR by immunoblotting. (3) Exosomes obtained from in vitro culture of human reticulocytes (Fig 2) or from the circulation of a hemochromatosis patient (not shown) both showed evidence for a truncated cytoplasmic domain of the TFR, in addition to native receptor.

The data are consistent with the conclusion that maturation and membrane remodeling of reticulocytes proceeds by exosome formation with the release of TFR.

DISCUSSION

Recent work with blood from anemic patients has shown the presence of a truncated form of the TFR in the circulation whose level is proportional to the degree of iron deficiency and the reticulocyte level. The existence of such a form of receptor in the circulation raises the question of its origin. Is shedding of receptor by cleavage from the cell surface a major mechanism for TFR loss from the RBC surface during maturation? Or is the cleaved receptor a consequence of exosome formation followed by cleavage from the surface of exosomes? Alternatively, is a soluble truncated receptor synthesized and released?

Exosomes were first detected with maturing sheep reticulocytes, and were absent in erythrocytes. The presence of exosomes has now been described in seven different species, including humans, both in vivo in anemic animals and during in vitro maturation of reticulocytes.

Using in vitro cultures of sheep, rat, and chicken reticulocytes, it was previously shown that the released exosomes contained a native size (~94 Kd) TFR. Moreover, in the circulation of various species of anemic animals (including humans), a full-length TFR was found in the exosomes.

A population of multivesicular bodies that appears to be the precursor of the circulating exosomes has been shown inside immature RBCs of several species. Based on this common behavior of reticulocytes, we have put forth the
proposal that exosome formation is a major route for the elimination of specific membrane proteins during RBC maturation.

Until now the incomplete recovery in exosomes of the TFR lost from sheep RBCs left some doubt whether exosome formation was sufficient to account for all TFR lost from these RBCs during maturation. In the present communication this uncertainty has been overcome. It was shown that in sheep RBCs, ~75% or more of the receptor lost from the cells during incubation can be recovered in exosomes from the medium. Thus, it is probable that exosome formation is the major, if not the sole, route for shedding TFR in sheep reticulocytes.

A small component (~25%) of the TFR released during in vitro maturation is a truncated, exofacial domain and not exosome-associated. This truncated form runs as an ~80-Kd monomer and as an ~160-Kd dimer on reducing and nonreducing gels, respectively. Three possible origins for this truncated receptor exist: (1) synthesis and secretion of a soluble form of receptor; (2) cleavage of the receptor from the cell surface; and (3) cleavage of the receptor from released exosomes.

1. The first possibility can be eliminated in the sheep system. In sheep reticulocytes, little de novo synthesis of TFR occurs. Moreover, the small amount of newly synthesized receptor is not released to the medium. Thus, it is unlikely that the origin of the soluble receptor is caused by de novo synthesis followed by secretion.

2. Cleavage of TFR from the cell surface would be expected to leave the cytoplasmic and transmembrane domains embedded in the plasma membrane. This domain (expected size ~15 Kd) is detectable by an antibody (anti-CDTFR) made against synthetic peptides corresponding to the cytoplasmic domain. In maturing reticulocytes, no such peptides were detected in the plasma membranes. Artificially generated cytoplasmic domains, made by trypsinization of the cell surface, were retained in the plasma membrane even after 20 hours of incubation. Moreover, the time course of appearance of the truncated, soluble receptor suggested that it arose after the appearance of the exosomes. No direct evidence was obtained that the exofacial domain was cleaved directly from the sheep reticulocyte surface.

3. The majority of the evidence to date suggests that in sheep reticulocytes, the receptor was cleaved from exosomes and that exosomes were the major source of the soluble TFR. Thus, (a) exosomes from in vitro maturation of sheep reticulocytes showed the presence of both native size receptor and the cytoplasmic domain of the receptor; (b) one of the TFR peptides retained in sheep exosomes had a size corresponding roughly to the expected size of the cytoplasmic domain of the TFR (~17 Kd); and (c) tryptic cleavage of the surface of sheep reticulocytes before in vitro maturation also yielded a peptide of ~17 Kd in the released exosomes.

The latter peptide was recognized by the anti-CDTFR.

The presence of the cytoplasmic domain in exosomes, but not in the plasma membranes of sheep reticulocytes argues that the cleavage of the TFR occurred from the surface of exosomes. Although extensive studies with human reticulocytes have not yet been performed, the preliminary data suggest that in humans a similar situation obtains. Nonetheless, given the modest number of studies with human reticulocytes, it is prudent to reserve judgement on the quantitative importance of the exosome route in maturation-associated loss of the TFR in humans.

In contrast, the quantification of the release of the TFR from sheep reticulocytes maturing in vitro coupled with earlier studies on the quantification of the release of the nucleoside transporter during in vitro maturation suggest that exosome formation plays a major role in membrane remodeling during maturation of sheep reticulocytes.

While many of our in vitro and in vivo observations are in accord, direct studies addressing in vivo maturation of reticulocytes have not yet been executed. Until the difficulties of obtaining quantitative yields of pure exosome fractions from large volumes of plasma are overcome, the quantitative correlation between in vitro and in vivo events remains unknown.

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Origin of a soluble truncated transferrin receptor

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