Reticulocyte Maturation and Exosome Release: Transferrin Receptor Containing Exosomes Shows Multiple Plasma Membrane Functions

By R.M. Johnstone, A. Bianchini, and Kathy Teng

Vesicles (exosomes) released during sheep reticulocyte maturation contain a number of plasma membrane functions. Using an antibody coated, magnetic core bead, it has been shown unequivocally that vesicles that contain the transferrin receptor also contain other plasma membrane activities, such as the nucleoside transporter and acetylcholinesterase. Lysosomal activities, normally found in the same pellet, are excluded from the transferrin receptor-containing exosomes, suggesting that there is a common mechanism to segregate and concentrating specific plasma membrane proteins. In addition to the sheep, electron micrographic studies show that exosomes can be retrieved from the circulation of anemic pigs, rats, and rabbits.

MATERIALS AND METHODS

Preparation of reticulocytes. Blood containing enriched reticulocytes was obtained from phlebotomized sheep as described by Benderoff et al.1 For preparation of anemic rats and rabbits with concomitant reticulocyte formation, phenylhydrazine treatment2 and phlebotomy were used. Blood from pigs less than 1-month-old was used directly.

Isolation of exosomes from the circulation. To determine the presence of exosomes in the circulation, heparinized blood was centrifuged twice at 7,700 × g for 8 minutes. The cell-free supernatant, diluted 1:1 with isotonic saline, was then centrifuged at 100,000 × g for 90 minutes over a 1 mL, 26% sucrose cushion. After complete removal of the supernatant, the tube was wiped dry and the small pellet stored frozen at -70°C until required or suspended immediately in glutaraldehyde fixation buffer for electron microscopy of the pellet.

Formation and harvesting of vesicles in vitro. Washed, enriched sheep reticulocyte suspensions (70% to 90%) were cultured at 3% hematocrit in MEM containing 5 mmol/L adenine and 10 mmol/L inosine as well as 4 mmol/L glutamine, penicillin, and streptomycin as described by Benderoff et al.3 and Pan et al.4 After overnight incubation with gentle rotation at 37°C (or at the given temperature), the cells were removed by centrifugation and vesicles collected. The pellets were frozen and stored or suspended in the medium specified in the text and used immediately.

Preparation of immobilized αTFR. Dynal beads, containing covalently linked sheep, anti-mouse antibody were washed by centrifugation to remove the original medium and suspended in PBS. To the washed beads (70 μL) was added 20 μg of mouse IgG containing a monoclonal antibody against the human transferrin receptor (αTFR). In all cases a second sample was treated identically with non-immune-mouse IgG to examine the nonspecific binding. The IgG was allowed to bind to the beads in a rotating device at room temperature for 4 hours. Then excess unbound antibody was removed by centrifuging the beads (20 seconds at 12,000 × g in a microfuge). After removal of the supernatant, the beads were resuspended in PBS containing 20 μg/mL ovalbumin using a 200-fold excess of wash medium over bead volume. Following the ovalbumin wash, the procedure was repeated twice with PBS alone. To the IgG-containing beads was added a suspension of vesicles in
PBS. After a number of trials, we found that if vesicles derived from 
~3 × 10^6 cells (approximately 50 µg protein) were applied to 70 µL 
beads (2.8 × 10^7 beads) coated with αTFR, greater than 85% of the 
TFR in the suspension of vesicles could be recovered in the beads. If 
vesicles from more cells were used, part of the receptor remained in 
the supernatant. Vesicles were incubated overnight with rotation at 
4°C with IgG-coated beads. On the following morning the magnetic 
beads were separated from the suspension with the Dynal magnet 
and both supernatant (S) and pellet (P) retained. The beads were 
washed twice with PBS and the wash added to the supernatant. The 
beads were suspended to the same volume as the pooled supernata 
s. Enzyme assays or SDS gel electrophoresis were carried out on 
the washed bead pellets and the supernatants derived therefrom. 

EM of vesicles adsorbed to magnetic beads. To examine by EM 
the population of vesicles immobilized by the Dynal beads, the 
following procedure was adopted. Vesicles derived from ~15 mL of 
blood of phlebotomized rats (~30% circulating reticulocytes) were 
attached to the Dynal-bead specific antibody complex or nonim-
mune antibody complex. After removal of the unbound material by 
washing, the vesicle-Dynal bead complex was treated at room 
temperature with a solution of 4 mol/L urea, 0.2 mol/L glycine (pH 
2.3) for 3 minutes and quickly neutralized with Na2PO4 (pH 12) 
to bring the pH to 7.0. The beads were removed by centrifugation 
and the supernatant was fixed in buffered glutaraldehyde and processed 
for thin sectioning.4

Detection of TFR. To measure the presence of the transferrin 
receptor in the vesicles bound to Dynal beads, SDS gel buffer, 
containing mercaptoethanol, was added to the vesicle-containing 
bead pellet (P), heated for 5 minutes at 100°C, and the whole 
suspension including beads added to the SDS gels and electropho-
resed according to Laemmli.13 The beads stayed in the stacking gel 
during electrophoresis. The supernatant (S) from the beads was 
processed for SDS gel electrophoresis in an identical way. 

To measure 125I-transferrin (TF) binding to the beads (P) and 
supernatants (S) respectively, the ammonium sulphate precipitation 
procedure described by Klausner et al44 was used. TFR containing 
(P) and (S) were incubated with 125I-TF for 10 minutes at room 
temperature. Excess human TF (500-fold) was used to assess 
nonspecific binding. For filtration of the 125I-TF-TFR complex 
formed, the glass fiber filters were soaked in 30% (NH4)2SO4 with 
0.8% bovine albumin to assure adherence of the magnetic beads to 
the filters.

Nucleoside Binding

[1H]-Nitrobenzylthioinosine (NBMPR) was used to measure the 
presence of the nucleoside transporter.15 The procedure for binding 
to vesicles was described before3 and is based on the method by 
Hammond and Martin.16 Both P and S obtained as above 
were assayed for activity.

Lysosomal Enzymes

To measure N-acetyl β-glucosaminidase, β-glucuronidase, and 
acid phosphatase, the umbelliferyl derivatives of N-acetyl β-
glucosaminidase, β-glucuronide, and the phosphate were used. The 
fluorescent product obtained after hydrolysis was measured as 
described by Peters et al,17 using an excitation wavelength of 365 nm 
and emission at 460 nm.

Acetylcholine Esterase

The procedure used was described before and is an adaptation of 
the method by Ellman et al14 and both P and S were assayed. 
Because the beads are intensely colored, continuous measurements 
of acetylcholine hydrolysis with the suspension of beads was not 
possible. Instead, at intervals the suspending medium was with-
drawn using the Dynal magnet to immobilize the beads, the absorb-
bance of the suspending medium at 412 nm measured, and then 
returned to the beads for continued incubation at room temperature. 
The acetylcholinesterase in the supernatant was monitored by 
continuous assay of the change in absorbance at 412 nm.

Materials

Sheep transferrin was isolated following the procedure of Morgan et al19 
and iodinated as described by Hunter and Greenwood.20 Human transferrin was purchased from Sigma Biochemicals (St 
Louis). The monoclonal antibody against the sheep transferrin 
receptor was a gift from J. Larrick of Genelabs, Redwood, CA. The 
antibody against rat TFR (DOX-26) was a gift from Dr A. 
Williams, MRC Cellular Immunology, Sir Thomas Dunn School of 
Pathology, University of Oxford. Mouse IgG was isolated from 
mouse serum by protein A chromatography. Pig blood from young 
animals was obtained courtesy of Dr C. Chavez, MacDonald Col-
lege, McGill University.14-NBMPR was purchased from Moravec 
Biochemical Inc, Brea, CA and stored in methanol. Unlabeled 
NBMPR was purchased from Sigma Biochemicals. Acetyltihocho-
line and reagents for acetylcholinesterase, umbelliferyl N-acetyl 
β-glucosaminidase, umbelliferyl β-glucuronide, and umbelliferone 
phosphate were purchased from Sigma Biochemical. Magnetic 
beads conjugated to an anti-mouse sheep antibody were obtained 
from Dynal Inc (Great Neck, NY).

RESULTS

Detection of Transferrin Receptor Containing Vesicles in 
the Circulation

Formation of vesicles (exosomes) during maturation of 
reticulocytes and their recovery in the medium has until now 
been detected only with sheep cells maturing in vitro.2,4 
The precursor structures located in multivesicular bodies have 
also been seen in rat reticulocytes by Harding et al21,22 as well as 
as in sheep reticulocytes by Pan et al.4 That vesicle formation 
is restricted to neither the in vitro situation nor to the sheep 
is shown in Fig 1A-D. Blood from phlebotomized animals or 
phenylhydrazine-treated animals was processed to collect a 
100,000 × g pellet from the cell-free plasma. It is evident 
that the plasma pellets from all four anemic species show the 
presence of exosomes. Significantly, however, exosomes can 
also be found in the plasma of normal, unbled sheep, 
although the number of vesicles detected is small relative to 
the number of normal (10%) (Fig 1E).

We have shown that the transferrin receptor can be found 
in exosomes from the circulation of a phlebotomized sheep,2 as 
well as from exosomes formed during in vitro incubation of 
reticulocytes, suggesting that this is a normal route for 
receptor shedding. To assess whether other species release 
the transferrin receptor into the circulation, the 100,000 × g 
pellet, derived from plasma of phlebotomized anemic and 
normal rats was probed with a monoclonal antibody against 
the rat receptor. The data in Fig 2A show the presence of 
TFR in the high speed pellet from plasma of phlebotomized 
but not unbled rat plasma. Control experiments (Fig 2B) 
show that the antibody against the rat transferrin receptor 
does not recognize other plasma membrane proteins and that 
onimmune serum even in great excess (60 µg IgG) does not 
bring down the transferrin receptor. To verify that the rat 

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Fig 1. (A-D) Presence of exosomes in the circulation of anemic animals. Heparinized blood (50 mL) from phlebotomized sheep and rabbits was centrifuged at 7,700 \( \times \) g for 8 minutes. The cell-free supernatant was recentrifuged in an identical way. Following these centrifugations, the supernatant was diluted with an equal volume of isotonic saline and centrifuged at 100,000 \( \times \) g for 90 minutes. The pellet was collected, fixed in glutaraldehyde, and processed for EM as described. Blood from 7- to 14-day-old piglets (~10 mL) was treated in an identical manner. With rats the animals were placed on a regimen of phenylhydrazine and blood collected by heart puncture, 5 to 7 days after the first injection. The cell-free plasma was treated as above. Ten to 15 milliliters of blood was used. More cellular breakdown products are evident in the plasma from phenylhydrazine-treated animals, but ~50 nm vesicles are evident. (A) Rabbit. (B) Sheep. (C) Rat. (D) Pig. Bar = 200 nm. (E) Presence of exosomes in the circulation of normal sheep. Fifty milliliters of blood from a normal, unbled sheep was processed as described in Fig 1A-D. The number of vesicles in the normal circulation were not quantified relative to phlebotomized animals, but sections with vesicles were much less frequent (<25%) of bled animals. No vesicles were detected with normal blood from rabbit or rat. Bar = 200 nm.

vesicles contain TFR, the vesicles obtained from the circulation of a phlebotomized rat were attached to Dynal beads containing immobilized rat \( \alpha \)TFR. Nonimmune IgG was used as control. After binding the vesicles to the beads and removal of unbound material by washing, the beads were treated with urea/glycine to release any bound vesicles. The data show the presence of vesicles released from (rat) \( \alpha \)TFR containing beads (Fig 3A). No vesicles were detectable in the nonimmune rat controls. Additional studies have also shown that after fixation for EM the vesicles bind rat \( \alpha \)TFR. Fixed sections of vesicles derived from the circulation of phlebotomized rats were treated either with rat \( \alpha \)TFR or nonimmune serum. The sections were rinsed and then treated with colloidal gold conjugated to protein A. The data in Fig 3B show gold grains dotting exosomes pretreated with immune serum. An equal number of sections (6) treated with immune and nonimmune sera was examined, and a total number of 500 vesicles from each treatment was counted. Despite the problems of postembedding treatment with protein A, with immune serum the number of gold grains associated with vesicles exceeded the nonimmune (338 ± 150 grains) and was higher in each of the six sections. Lack of antibodies that react with the TFR of other species precluded extension of this observation to additional species at this time. Nonetheless, these data suggest that vesicle formation may occur naturally in many mammalian species and that these vesicles carry obsolete transferrin receptors.

The formation and release of exosomes bearing the transferrin receptor is an event dependent on metabolic activity as illustrated in Fig 4. Sheep reticulocytes were incubated for 18 hours under three conditions: (1) at 4°C, (2) at 37°C with rotenone and 2 deoxyglucose to deplete ATP, and (3) in normal medium at 37°C. It is evident that considerably less
TFR is detected in the 100,000 \times g pellet from cells incubated under adverse metabolic conditions (compare lane 4 with lanes 1 and 2 in Fig 4). It has already been shown\textsuperscript{23,24} that reticulocytes incubated overnight at 4°C shed less protein and lose little transferrin binding activity in contrast with the loss seen in cells incubated at 37°C.

Selective separation of a vesicle population containing TFR. Although multiple functions can be recovered in the exosome fraction formed during in vitro incubation of sheep reticulocytes, our earlier studies failed to address the distribution of these activities within the population. Does a single vesicle contain all activities known to be released? To answer this question, we attempted to separate sheep vesicles bearing the TFR from the total vesicle population with an immobilized αTFR. We have found that magnetic core beads coated with αTFR can nearly completely remove TFR-containing exosomes from the total vesicle suspension (Fig 5, lane 5). In contrast, beads coated with nonimmune IgG (C\textsubscript{\textalpha}) (Fig 5, lane 4) bring down little TFR. Conversely, it is also clear that the supernatant from I\textsubscript{\textalpha} (P\textalpha) contains little TFR (Fig 5, lane 3), whereas, the C\textsubscript{\textbeta}, the supernatant from the nonimmune IgG, retains most of the TFR (Fig. 5, lane 2 compared with lane 1 [total vesicle protein]).

In Fig 5 it is also worth noting that a \textasciitilde 70 kDa peptide is removed from the exosome suspension with the beads coated with αTFR. We have already demonstrated that this protein, the clathrin uncoating ATP-ase,\textsuperscript{26} is externalized with the transferrin receptor.\textsuperscript{2} (The peptide at \textasciitilde 50 Kd found in both I\textsubscript{\textalpha} and C\textsubscript{\textalpha} appears to be a portion of the covalently bound IgG, released the magnetic beads themselves during processing and/or gel electrophoresis.)

As reported earlier by Johnstone et al,\textsuperscript{1} some hemoglobin (Hb) is found with the vesicle population. On the basis of insensitivity to trypsin digestion, we concluded that the Hb was occluded, perhaps trapped inside vesicles. Using the magnetic beads it was determined that the majority of Hb stays in the P\textalpha and that I\textsubscript{\textalpha} is relatively free from Hb.

To substantiate the conclusion that the population of vesicles associated with I\textsubscript{\textalpha} is enriched in transferrin receptors, both the bead and supernatant fractions were assayed for \textsuperscript{125}I-TF binding. The data in Table 1 show that the immune beads remove all the \textsuperscript{125}I-TF binding activity. No specific \textsuperscript{125}I-binding remains in the immune supernatant. This result should be contrasted with the data using nonimmune IgG-coated beads where all detectable TF binding remains in the supernatant.

Multiple Functions Are Found in TFR Containing Vesicles

To determine whether other functions found in the exosomes are associated with the TFR containing population, enzyme or binding assays were performed on immune and nonimmune IgG-coated beads (P) and the respective supernatants (S).

Acetylcholinesterase\textsuperscript{25} and nucleoside transport and binding\textsuperscript{26} are plasma membrane functions in red cells\textsuperscript{25} known to diminish on maturation.\textsuperscript{24,25} We have previously shown that acetylcholinesterase activity and nucleoside analogue binding (NBMPR) can be detected in exosomes.\textsuperscript{3} The results in Table 2 show that a substantial fraction of the acetylcholinesterase and \textsuperscript{3}H-NBMPR binding, are detected in the same population of vesicles as the TFR. For both acetylcholinesterase and \textsuperscript{3}H-NBMPR binding, approximately one half of the activity associates with the immune beads, with the remainder being in the immune supernatant. In contrast, almost 100% of both these activities remained in the nonimmune supernatant, with no significant activity being associated with the beads.

Because lysosomal enzymes have also been found in the 100,000 \times g pellet,\textsuperscript{9} we determined the distribution of three purported lysosomal enzymes, N-acetyl-β-glucosaminidase, acid phosphatase (pH 5.2), and β-glucuronidase.\textsuperscript{17} The results show that no β-glucosaminidase or β-glucuronidase is associated with the immune beads; almost all the recovered activity remained in the immune supernatant (Table 3).
Fig 3. Vesicles from the circulation of phlebotomized rats contain TFR. (A) Binding of vesicles from phlebotomized rat plasma to an immobilized anti-rat transferrin receptor. Vesicles from the circulation of phlebotomized rats were attached as described in Materials and Methods to Dynal beads carrying an antitransferrin receptor antibody against the rat receptor. After elution of the vesicles with 4 mol/L urea/0.2 mol/L glycine, the vesicles were processed for EM. No vesicles were detected in nonimmune controls. Bar = 200 nm. (B) Detection of aTFR binding to vesicles from the circulation of phlebotomized rats. Vesicles were collected and fixed for EM, and the cut sections were incubated with aTFR,. After washing to remove unbound antibody, the sections were treated with protein A conjugated to colloidal gold. Note the association of grains with vesicles. Bar = 200 nm.

With these enzymes there was no difference in distribution of activity with immune and nonimmune beads, suggesting that these activities are in a different population of vesicles, unassociated with the transferrin receptor.

Although acid phosphatase activity is normally considered a lysosomal marker, its distribution was unexpected and different from glucosaminidase activity. Phosphatase activity was partly removed by immune beads, characteristic of the plasma membrane functions noted in Table 2. The distribution of phosphatase activity between pellet and supernatant did not change if the assays were conducted at pH 6 rather than 5.2. Little activity remained at pH 9.0. Because rabbit red cell plasma membranes are known to possess fluoride sensitive acid phosphatase activity,” we verified that plasma membranes of mature red cells from sheep contain acid phosphatase, the activity being ~15% of that in the reticulocyte membranes (~800 fmol/min/µg protein). The specific activity in exosomes is approximately fourfold higher than mature membranes and is fluoride-sensitive. Thus, the acid phosphatase activity in TFR containing exosomes from sheep may originate, at least in part, from plasma membrane enzymes.

Attempts to measure cathepsin activity (both B and C), often associated with lysosomal membranes, was unsuccessful. Neither the reticulocyte plasma membranes nor the exosomes had significant cathepsin B or C activity under our conditions.

DISCUSSION

In the present work we have made use of a novel approach to selectively remove a population of subcellular organelles. Antibody-coated magnetic beads have been used to isolate cells and endosomes with specific antigenic markers by Ugelstad et al and Gruenberg and Howell. We have...
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Fig 5. Separation of transferrin receptor containing exosomes. Exosomes derived from an overnight culture of sheep reticulocytes were applied to αTRF-coated beads or beads coated with nonimmune IgG. After separation of beads from supernatant and a quick wash of the beads with PBS, SDS was added to both the bead fraction and the supernatant fraction and the material was subjected to SDS polyacrylamide gel electrophoresis. The gels were stained with Coomassie blue. An equal aliquot of the exosome suspension (total) was dissolved directly in SDS buffer without being treated with the magnetic beads. Lane 1, total exosomes. Lane 2, Cα = supernatant from nonimmune IgG-coated beads. Lane 3, γ' = supernatant from αTRF-coated beads. Lane 4, Cβ = bead pellet from nonimmune IgG-coated beads. Lane 5, Iγ = bead pellet from αTRF-coated beads.

Applied this approach to separate a specific population of vesicles of ~50 nm in diameter formed during reticulocyte maturation. Attempts to use density gradient fractionation or antibody coupled Sepharose beads failed to achieve the same results. The method is particularly useful to handle small amounts of material because the nonspecific entrapment volume in the magnetic beads, unlike porous material (eg, Sepharose), is minimal. The technique allows us unequivocally to conclude that exosomes released during reticulocyte maturation are multifunctional and that the activities in the vesicles originate from the plasma membrane either directly or indirectly (ie, the activities detected are

<table>
<thead>
<tr>
<th>Condition</th>
<th>Transferrin Bound (pmol/10⁶ cell equivalents) (typical experiment)</th>
<th>% of Total Binding (range of 5 experiments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total vesicles</td>
<td>10.1</td>
<td>—</td>
</tr>
<tr>
<td>Nonimmune supernatant</td>
<td>8.7</td>
<td>80–90</td>
</tr>
<tr>
<td>Immune supernatant</td>
<td>0.5</td>
<td>4–10</td>
</tr>
<tr>
<td>Nonimmune beads</td>
<td>0.3</td>
<td>1–3</td>
</tr>
<tr>
<td>Immune beads</td>
<td>8.2</td>
<td>80–90</td>
</tr>
</tbody>
</table>

Vesicles derived from overnight cultures of sheep reticulocytes were used. ¹²⁵I-transferrin binding was carried out as described for total binding by Klausner et al. After loading the vesicles on the magnetic beads overnight at 4°C, the beads were separated from the supernatant, washed, and transferrin binding measured using 10 μg/mL of ¹²⁵I sheep transferrin. Five hundred-fold excess of human transferrin was used to assess the nonspecific binding, which was less than 15% of the total binding. Values corrected for nonspecific binding are given. Transferrin binding ranged from 3 to 10 pmol in vesicles derived from incubating 10⁶ cells containing over 70% reticulocytes.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Condition</th>
<th>Activity (pmol/10⁶ cells/min) (typical experiment)</th>
<th>% of Total (mean ± SD) (no. of experiments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholinesterase</td>
<td>Total</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nonimmune supernatant</td>
<td>6.0</td>
<td>84 ± 5 (4)</td>
</tr>
<tr>
<td></td>
<td>Immune supernatant</td>
<td>2.9</td>
<td>47 ± 15 (4)</td>
</tr>
<tr>
<td></td>
<td>Nonimmune beads</td>
<td>0.3</td>
<td>6 ± 0.6 (4)</td>
</tr>
<tr>
<td></td>
<td>Immune beads</td>
<td>3.8</td>
<td>49 ± 7 (4)</td>
</tr>
<tr>
<td>³H-NBMPR binding</td>
<td>Total</td>
<td>902</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nonimmune supernatant</td>
<td>921</td>
<td>88 ± 5 (3)</td>
</tr>
<tr>
<td></td>
<td>Immune supernatant</td>
<td>656</td>
<td>59 ± 4 (3)</td>
</tr>
<tr>
<td></td>
<td>Nonimmune beads</td>
<td>197</td>
<td>9 ± 9 (3)</td>
</tr>
<tr>
<td></td>
<td>Immune beads</td>
<td>555</td>
<td>43 ± 5 (3)</td>
</tr>
</tbody>
</table>

Vesicles collected from overnight sheep reticulocyte cultures were used. The assays were conducted as described by Johnstone et al. To calculate the percent binding in the NBMPR assay, the calculations are based on the sums of the activity in the supernatant and corresponding pellet, which were always greater than the measured total binding.
Table 3. N-Acetyl-β-Glucosaminidase, β-Glucuronidase, and Acid Phosphatase in Transferrin Receptor Containing Exosomes

<table>
<thead>
<tr>
<th>Assay</th>
<th>Condition</th>
<th>Activity (pmol/10^6 cells) (typical experiment)</th>
<th>% of Total (mean ± SD) (no. of experiments)</th>
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<tr>
<td>N-acetyl-β-glucosaminidase</td>
<td>Total</td>
<td>336.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Nonimmune supernatant</td>
<td>246.0</td>
<td>96 ± 10 (4)</td>
</tr>
<tr>
<td></td>
<td>Immune supernatant</td>
<td>280.0</td>
<td>90 ± 15 (4)</td>
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<td></td>
<td>Nonimmune beads</td>
<td>25.0</td>
<td>4 ± 2 (4)</td>
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<tr>
<td></td>
<td>Immune beads</td>
<td>23.0</td>
<td>4 ± 3 (4)</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>Total</td>
<td>24.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Nonimmune supernatant</td>
<td>21.0</td>
<td>90 (3)</td>
</tr>
<tr>
<td></td>
<td>Immune supernatant</td>
<td>19.5</td>
<td>82 (3)</td>
</tr>
<tr>
<td></td>
<td>Nonimmune beads</td>
<td>0.0</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td>Immune beads</td>
<td>0.2</td>
<td>(3)</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>Total</td>
<td>2,860.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Nonimmune supernatant</td>
<td>2,650.0</td>
<td>94 ± 5 (5)</td>
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<tr>
<td></td>
<td>Immune supernatant</td>
<td>1,350.0</td>
<td>50 ± 12 (5)</td>
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<td></td>
<td>Nonimmune beads</td>
<td>150.0</td>
<td>8 ± 2 (5)</td>
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<tr>
<td></td>
<td>Immune beads</td>
<td>1,150.0</td>
<td>43 ± 8 (5)</td>
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</table>

Exosomes collected after 40 hours of culture of sheep reticulocytes were used for the glucosaminidase and phosphatase assays. Vesicles from an overnight culture were used for β-glucuronidase. In all cases the umbelliferyl derivative of the substance was used as substrate at 0.2 mmol/L and the sample was incubated for 30 minutes at 37°C in an acetate buffer at pH 5.2. The extent hydrolysis was determined fluorimetrically.

Although the mechanism for selecting specific proteins for externalization is not known, the data presented suggest that these proteins may contain a common feature in their cytoplasmic termini, which targets them for selective removal as the cells age. Moreover, because different species are known to lose different functions during maturation (eg, pig, not humans, loses all glucose transporters32) there presumably exist species-specific differences in the cytoplasmic portions of these transmembrane proteins that may selectively target them for externalization during the maturation process.

Changes in the cytoplasmic portions of obsolescent proteins might target them for recognition by the 70-Kd clathrin uncoating ATPase, which is reported to bind to unfolded parts of proteins.33,34 This 70-Kd protein is present in high concentration in red cells.19 It is interesting to note that vesicles isolated by the coated, iron-core beads also contain the ~70-Kd protein (Fig 5). The close association of ~70-Kd with the transferrin receptor in externalized vesicles earlier led us to suggest a role for the 70-Kd protein in targeting specific proteins for externalization.19 It will be of interest to determine whether an immobilized antibody against another released membrane protein will also bring down some ~70-Kd peptide.

Although our data show that exosomes isolated by immune beads contain all the available transferrin receptor, it is not surprising that only a portion (~50%) of the other plasma membrane activities are co-isolated. It would be unlikely for every transferrin receptor-containing exosome to contain 100% of all other activities. It is more reasonable to expect a statistical distribution of functions, some vesicles being enriched in one function relative to another. Indeed, an immunocytochemical study showed that not all released vesicles contained TFR.4 One might reasonably expect that using an antibody against acetylcholinesterase, for example, only a fraction of transferrin binding activity would be pulled out. Significantly, however, the presence of multiple functions in one specific exosome population argues for a common recognition and processing system.

Using this nondestructive approach, we have been able to provide compelling evidence that each vesicle formed during reticulocyte maturation contains several plasma membrane functions. We have also demonstrated that little of the Hb or the externalized lysosomal functions are included in this population of the TFR containing exosomes. This simple technique has the potential to answer similar questions about intracellular segregation of functions in different populations of vesicles.

In conclusion, we have also presented evidence that exosomes are found in the circulation of anemic animals and that (at least in two species) the transferrin receptor is shown to be associated with the exosomes. These observations suggest that exosome formation, with concomitant release of the transferrin receptor, is a natural phenomenon.

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