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Identification of heat shock protein 70 in canine reticulocytes and mature erythrocytes

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Abstract

In the present study, we demonstrated that heat shock protein 70 (Hsp 70) was present in both canine reticulocytes and mature erythrocytes, and that the canine Hsp70 in reticulocytes was decreased along with the maturation of the cells into erythrocytes. These results suggest that the Hsp70 in canine reticulocytes might act as a chaperone to remove unnecessary proteins during reticulocyte maturation. We also demonstrated that Hsp70 was present in exosomes from reticulocytes during their maturation in *in vitro* culture. Furthermore, the concentration of Hsp70 in reticulocyte membranes was increased in proportion to an increase of the protein in exosomes until 48 hours after the incubation of reticulocytes *in vitro*. At 96 hours of the incubation, however, only a trace amount of Hsp70 was detected in the membrane, while a large amount of the protein was present in the exosomes. These results suggest that Hsp70 in canine reticulocytes might play an important role for exosome formation in reticulocytes, resulting in the maturation of the cells.

Key words : canine Hsp 70, erythrocyte maturation, reticulocyte lysate, exosome

Introduction

Heat shock proteins (Hsps) are a chaperones, and are known as stress proteins be-

cause heat stress enhances their transcriptional activation and biosynthesis¹⁸⁾. A number of cellular proteins, known as chaperones, recognize and stabilize partially folded inter-

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mediates during polypeptide folding, assembly and disassembly⁸). Hsps are a conserved group of inducible and constitutive proteins that are thought to protect cells from stresses that typically result in protein misfolding such as hypoxia, ischemia, reactive oxygen species, or heavy metals^{23,26}). The constitutive expression of these proteins indicates that, in addition to their protective function, they also have an important role in normal cell functioning, acting as molecular chaperones¹³). Therefore, Hsps are considered to be house keeping proteins for cell life.

Members of the 70-kDa heat shock protein family are among the most abundant soluble proteins in mammalian cells^{3,9}). This family includes Hsp70, which may be induced by stress, and Hsc70 (heat shock cognate 70-kDa protein), which may be constitutively expressed¹⁰). They play many roles such as targeting damaged or abnormal proteins for degradation^{28,29}), directing protein folding¹), and maintaining an unfolded protein structure^{4,7}). This family is present in a variety of cells^{19,30}), including erythrocytes⁶).

Previous studies have demonstrated that the Hsp70 is an important protein for the maturation of reticulocytes through the release of target proteins¹⁶) such as transferrin receptor (TfR), in rats^{5,10}) and sheep²⁴). In carnivores, however, the characteristics and role of Hsp70 in erythrocytes have been poorly understood. In many ways, the erythrocytes of carnivores (dogs and cats) are different from those of other species. For example, most mammalian erythrocytes have high intracellular K and low Na concentrations via the function of a membrane-bound enzyme, Na,K-ATPase. In contrast, dog and cat erythrocytes are known to contain low K and high Na concentrations in general, and to lack the membrane Na,K-ATPase. It is considered that Na,K-ATPase is an unnecessary protein for the

mature erythrocytes in carnivores, and that Hsp70 might remove this protein. To clarify the role of Hsp70 in mammalian erythrocytes, therefore, it seems necessary to know the characteristics of Hsp70 in red blood cells of carnivores. In the present study, we examined canine erythrocyte Hsp70, and demonstrated the abundant presence of Hsp70 in canine reticulocytes and its change during the maturation of the cells.

Materials and methods

Animals

Adult beagles, two males and two females, weighting 10-12 kg were used in all experiments. During the experiments, all beagles were handled in accordance with the guidelines for animal handling of Hokkaido University, which basically conform to the American Association of Laboratory Animal Control Guidelines issued by the National Institutes of Health.

Experimental reticulocytosis in dogs

All dogs were bled about 20-24 ml/kg once daily from the jugular vein for 4 days²⁰). The hematocrit value in each dog was reduced from 46.8 to 23.7%, on average, in 7 days. Iron dextran (10 mg/kg) was given orally to each dog on the fifth day after the first bleed for the prompt recuperation of dogs from anemia. For separation of reticulocytes, about 100 ml of whole blood was taken into a heparinized syringe from each dog on the third day after the last bleed. At these times, the reticulocyte count in the peripheral blood from the dog was 7.0-9.8%. Heparinized whole blood was filtered through an α -cellulose/microcrystalline cellulose column to remove leukocytes and platelets²). The separation of canine reticulocytes was performed by using the method of Maede *et al.*²⁰) with some modification. The filtered cells were washed twice

with 10 mM phosphate-buffered saline (PBS, pH 7.4), then resuspended in the same buffer to yield a hematocrit value 25-30%. Two kinds of Percoll (GE Healthcare, Uppsala, Sweden) solution, 45 and 64.5% (v/v) Percoll in 150 mM NaCl, 0.1% (w/v) BSA, and 20 mM Hepes/Tris (pH7.5), were prepared for isolation of reticulocytes. The solutions had specific densities of 1.070 and 1.096 g/ml, respectively. The red cell suspension was carefully layered over the Percoll gradient, and centrifuged at $800 \times g$ for 15 minutes at room temperature. The cells distributed in the interface of the two Percoll solutions and the cells with a specific density of under 1.096 g/ml were collected. These cells were washed 3 times with PBS and the reticulocytes were counted after supravital staining with new methylene blue. The former cells consisted of reticulocytes (40% -97% ; average, 85%) and almost all of the latter were mature erythrocytes.

Culture of canine red blood cells

The separated canine RBCs, which included a large amount of reticulocytes, were washed twice with RPMI-1640 supplemented with sodium pyruvate (0.11 mg/ml), glutamine (0.3 mg/ml), sodium bicarbonate (2 mg/ml), penicillin (100 units/ml) and streptomycin (100 µg/ml). After washing, they were resuspended in medium consisting of 80% RPMI-1640 and 20% dog serum, to yield a hematocrit of 5%. Then 2 ml of suspension was placed in a well of a 6-well flat bottom plate (ϕ 1.7 cm, TPP, Transadingen, Switzerland) and incubated at 37°C under a humidified atmosphere containing 5% CO₂, 5% O₂, and 90% N₂ in an incubator. At days 0, 1, 2 and 4 of the culture, the culture suspension was collected and centrifuged at $12,000 \times g$ for 5 minutes at 4°C. The sedimented RBCs were collected and used for further analysis. Culture supernatants were also collected and

used for the isolation of vesicles.

Observation of recovery from experimental reticulocytosis in vivo

From dogs having experimental reticulocytosis, about 10 ml of whole blood was taken into a heparinized syringe from the day of the last bleed until the eighth day and washed as described above. After washing, RBCs were resuspended in PBS to yield a cell count of 1×10^8 cells/5µl, and the samples for immunoblot analysis were prepared as below.

Preparation of samples for immunoblot analysis

For the whole cell sample, the red blood cells (RBCs) were washed in PBS twice. After washing, RBCs were resuspended in PBS to yield a cell count of 1×10^6 cells/5µl. The RBC suspension was dissolved with 5µl of electrophoresis buffer consisting of 0.1 M Tris/Cl, 4% SDS, 12% 2-mercaptoethanol, 20% glycerol, and one drop of bromophenol blue, and filtered by QIAshredder™ (QIAGEN, Maryland, USA). The entire volume of the sample (containing 1×10^6 cells/10µl) was used for a single immunoblot analysis.

RBCs were resuspended in PBS to yield a cell count of 1×10^8 cells/5µl for the RBC membrane ghost and lysate samples. The RBC suspension was mixed to an equal volume of hypoosmotic solution (5 mM Tris/Cl, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.8) containing 0.8 mM phenylmethylsulfonyl fluoride (PMSF) for the preparation of hemoglobin-depleted RBC membrane ghosts. The lysed RBC suspension was centrifuged at $18,000 \times g$ for 10 minutes at 4°C. The resultant supernatant was collected and mixed with an equal volume of the electrophoresis buffer described above. The entire volume of the mixture (containing the cytoplasm from 1×10^8 cells) was used for a single analysis as an

RBC lysate sample. The resultant pellet was washed in 250 mM sucrose, 1 mM EDTA solution (pH 7.4) by centrifuging at $18,000\times g$ for 10 minutes at 4°C . The obtained RBC membrane ghosts were dissolved in 10 μl of the electrophoresis buffer described above. The entire volume of the RBC membrane ghosts (containing the membrane ghosts from 1×10^8 cells) was used for a single analysis as an RBC membrane ghost sample.

Isolation of exosomes from culture supernatant

Exosomes were isolated from the culture supernatant of the RBC culture according to Johnstone *et al.*¹⁷⁾. The obtained culture supernatants described above were centrifuged twice at $12,000\times g$ to ensure the removal of cells, and cell-free supernatants were obtained. For the isolation of exosomes, the cell-free supernatant was centrifuged at $100,000\times g$ for 90 minutes at 4°C . After removing the supernatant, the obtained pellets were washed with saline and centrifuged for a further 90 minutes at 4°C . The washed pellets were dialyzed overnight against water, lyophilized, and subjected to electrophoresis. All of the pellets obtained from supernatants were used for a single analysis as an exosome sample.

Antibodies

A mouse anti-Hsp70 monoclonal antibody (SPA-810, Stressgen, Victoria, Canada), which recognizes Hsp70 from various mammals, was used for immunoblot analysis. A horseradish peroxidase-conjugated anti-mouse IgG antibody (Bio-Rad Laboratories, Hercules, CA, USA) was used as the secondary antibody.

Immunoblot analysis

Immunoblot analysis was performed by using the method of Sambrook²⁷⁾. The samples

for immunoblot analysis described above were electrophoresed (8% polyacrylamide gels, 0.75 mm thick) and transferred onto polyvinylidene difluoride (PVDF) filters (Millipore, Bedford, MA, USA) by using the wet electrophoretic transfer method²⁷⁾. The anti-Hsp70 monoclonal antibody was diluted 1 : 1,000 in blocking buffer (25 mM Tris, 192 mM glycine, 20% methanol, 5% skim milk). The anti-mouse IgG antibody was diluted 1 : 2,000 in blocking buffer. For visualizing the bands of protein, the ECL system (GE Healthcare, Buckinghamshire, England) was used according to the supplied protocols.

Results

Detection of canine Hsp70 from reticulocytes and mature erythrocytes.

The immunoblot analysis using the anti-Hsp70 antibody for detection of canine Hsp70 from RBCs showed the presence of a protein with a molecular weight of about 70,000 in both canine reticulocytes and erythrocytes. The 70-kDa protein was more abundant in reticulocytes than in mature erythrocytes (Figure 1A). The protein was also detected in lysate samples from both reticulocytes and erythrocytes (Figure 1B). All four experiments performed using four different dogs had similar results (data not shown). On the other hand, the anti-Hsp70 monoclonal antibody cross-reacted with two additional proteins (about 49 kDa and 65 kDa) only in reticulocyte lysate (lanes 1 and 3 of Figure 1).

Change of the concentration of 70-kDa protein in RBCs in anemic dogs in vivo.

The change of the concentration of the 70-kDa protein in erythrocytes of dogs with severe anemia induced by bleeding was analyzed. The 70-kDa protein in the cells was decreased as the dogs recovered from anemia, and was proportional to a decrease of reticulo-

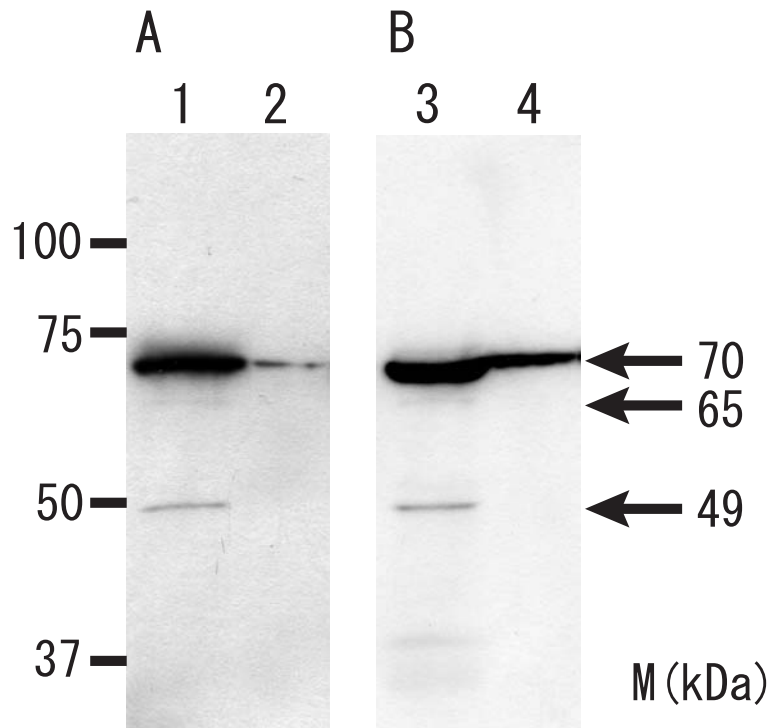


Figure 1 . Detection of 70-kDa protein in canine reticulocytes and erythrocytes by immunoblot analysis. (A) The 70-kDa protein in the whole cell samples (1×10^6 cells each) of reticulocytes (lane 1) and mature erythrocytes (lane 2) was detected by using an anti-Hsp70 monoclonal antibody (SPA-810). Molecular weight markers are indicated. The amount of the 70-kDa protein in reticulocytes (lane 1) was greater than in mature erythrocytes (lane 2). (B) The 70-kDa protein in the lysate samples (from 1×10^6 cells each) of reticulocytes (lane 3) and mature erythrocytes (lane 4) was detected using an anti-Hsp70 monoclonal antibody. The 70-kDa protein was more abundant in reticulocyte cytoplasm (lane 3) than in mature erythrocytes (lane 4). From the reticulocyte sample, the 49-kDa and 65-kDa proteins were also detected (lanes 1 and 3).

cyte counts in the peripheral blood of those animals (Figure 2). All three experiments performed using three different dogs had similar results (data not shown).

Change of the amount of 70-kDa protein in cultured reticulocytes during maturation in culture

When canine reticulocytes were cultured in vitro, the reticulocyte counts fell rapidly

within 48 hours after the onset of incubation, and then gradually decreased to less than 3.0% at 96 hours, while no decrease was observed in the total cell count. The 70-kDa protein in cultured reticulocytes was decreased along with the decreasing reticulocyte counts in the culture, and it almost disappeared from the cells at 96 hours (Figure 3A). The 49-kDa and 65-kDa proteins were detected in canine reticulocytes and the onset of incubation, but

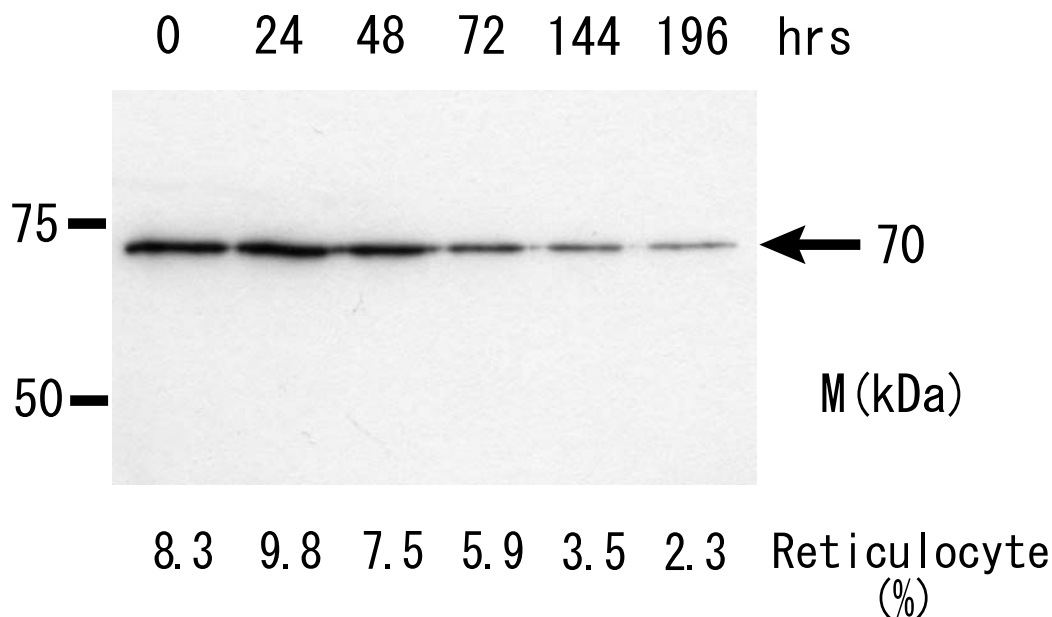


Figure 2. The change in the concentration of the 70-kDa protein in RBCs of dogs with experimental reticulocytosis. RBCs (1×10^8 cells each) were obtained from dogs with experimental reticulocytosis, and the 70-kDa protein was detected in the cells by using an anti-Hsp70 monoclonal antibody (SPA-810). The amount of 70-kDa protein in RBCs of the dogs decreased in proportion to the decrease in the percentage of reticulocytes in RBCs.

not detected at 24 hours after the incubation (Figure 3A). In contrast, the amount of 70-kDa protein in the vesicles isolated from the culture supernatant gradually increased during the maturation of reticulocytes to erythrocytes, and the maximum amount of the protein was detected at 96 hours (Figure 3B). On the other hand, the amount of 70-kDa protein in the reticulocyte membrane gradually increased as reticulocyte counts decreased in the culture, but the protein was scarcely detected in the membrane at 96 hours (Figure 3C). All four experiments performed using four different dogs had similar results (data not shown).

Discussion

In the present study, we demonstrated that Hsp70 was present in both canine reticulocytes and mature erythrocytes. Since the mouse anti-Hsp70 antibody used in this study

bound to the 70-kDa protein in the canine reticulocytes and erythrocyte lysate, and it is known that the amino acid sequence of Hsp70 is well conserved over species, the 70-kDa protein detected seemed to be the Hsp70 of canine RBCs. The presence of Hsp70 in erythroid cells has been detected in various species such as man¹¹⁾, sheep²⁴⁾, mice¹⁰⁾, rabbits¹²⁾ and chickens²²⁾. To our knowledge, however, the presence and characteristics of the protein in the red blood cells of carnivores have not been fully analyzed so far. Since there are many interspecies differences in the structure and metabolism of erythrocytes, it seems necessary to examine the protein in canine erythrocytes to understand the role of Hsp70 in erythrocytes from the comparative point of view. On the other hand, the mouse anti-Hsp70 antibody bound to two other proteins. It is possible that those proteins might be members of the family of heat shock pro-

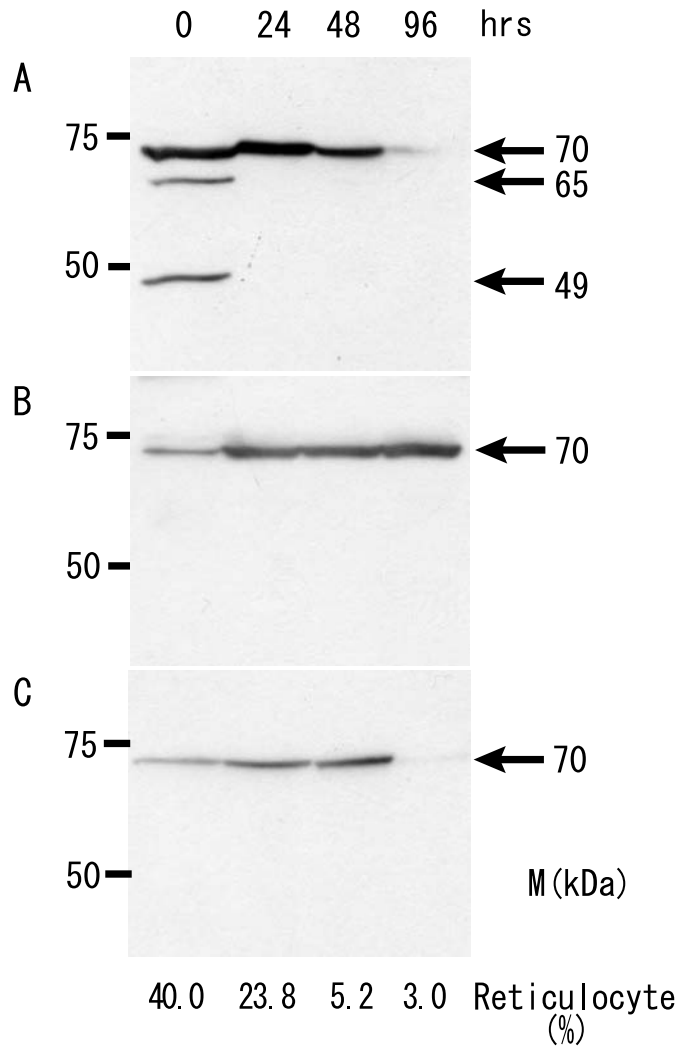


Figure 3. The change in the amount of 70-kDa protein in cultured reticulocytes during maturation *in vitro*. Samples were obtained at 0, 24, 48 and 96 hours after the onset of the culture. The 70-kDa protein was detected using an anti-Hsp70 monoclonal antibody. (A) In reticulocyte samples (1×10^6 cells each), the 70-kDa protein in cultured reticulocytes gradually decreased with the decrease of reticulocyte counts in the culture, and almost disappeared at 96 hours. The 49-kDa and 65-kDa proteins were also detected in the sample at 0 hours. (B) Vesicles were isolated from the supernatant of reticulocyte culture. The amount of 70-kDa protein in the vesicles isolated from the supernatant of reticulocyte culture gradually increased along with decreasing reticulocyte counts in the culture. (C) RBC membrane ghosts were prepared from RBCs (1×10^8 cells each) obtained from cultured reticulocytes. The 70-kDa protein in RBC membrane ghosts was detected using the anti-Hsp70 monoclonal antibody. The amount of 70-kDa protein in the RBC membrane ghosts increased until 48 hours, and it disappeared almost completely 96 hours after the incubation.

teins, which should have amino acid sequences similar to Hsp70, or might be split products of Hsp70³¹.

It is clear that the canine Hsp70 in reticulocytes gradually decreases during their maturation. It was reported that the Hsp70 could have a role for removing denatured and unnecessary proteins in the cell^{5, 10, 16, 24}. Therefore, the Hsp70 in canine reticulocytes might act as a chaperone to remove unnecessary proteins during reticulocyte maturation.

In the present study, we also analyzed vesicles released during *in vitro* reticulocyte maturation, and confirmed the presence of Hsp70 in the vesicles. Exosomes are small vesicles containing membrane protein. Exosomes are formed by membrane budding into the lumen of an endocytic compartment that leads to the formation of multivesicular intracellular structures. Fusion of these multivesicular structures with the plasma membrane leads to the release of internal vesicles, termed exosomes, into the medium^{15, 25}. It is known that many enzyme activities in reticulocytes are rapidly reduced as the cells mature to erythrocytes²⁰. During the maturation of reticulocytes a population of exosomes is formed which contains a variety of membrane proteins lost from the cells during maturation, indicating that exosome formation is an important route for removal of specific membrane proteins⁵. In the results presented here, an increase of Hsp70 in reticulocyte membranes was observed in proportion to the increase of the protein in vesicles until 48 hours after the incubation of reticulocytes *in vitro*. At 96 hours after the incubation, however, only a trace amount of Hsp70 was detected in the membrane, whereas a large amount of the protein was present in the vesicles. These results indicate that Hsp70 might play a central role in exosome formation in canine reticulocytes, resulting in maturation of

the cells.

Hsp70 has been detected in exosomes from mammalian reticulocytes and avian immature red cells^{10, 11, 12, 22, 24}. In reticulocytes from sheep and rats, Hsp70 was closely associated with the similar transferrin receptor (TfR) which was released in exosomes during their maturation to erythrocytes, and TfR in exosomes was preferentially bound to Hsp70²¹. It has been demonstrated that Hsp70 binds to exosomal TfR with the characteristics expected of a chaperone/peptide interaction. TfR is known to promote efficient iron uptake and to disappear from the reticulocyte surface during maturation into erythrocytes. In canine reticulocytes, however, it is unclear whether Hsp70 is associated with TfR or other proteins in exosomes. As described elsewhere, canine erythrocytes lack the membrane-bound enzyme Na,K-ATPase. Although canine reticulocytes have considerable amounts of the enzyme, the cells lose it rapidly and completely during maturation into erythrocytes¹⁴. In this phenomenon, it may be possible to consider that Hsp70 is closely associated with the Na,K-ATPase in exosomes in canine reticulocytes, and that Na,K-ATPase may be removed from the reticulocyte membrane as TfR is lost from maturing reticulocytes via exosome formation. This possibility, however, remains to be clarified.

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