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Citation	Japanese Journal of Veterinary Research, 58(1), 17-27
Issue Date	2010-05
DOI	10.14943/jjvr.58.1.17
Doc URL	http://hdl.handle.net/2115/43054
Type	bulletin (article)
File Information	JJVR58-1_p17-27.pdf

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Japanese Journal of Veterinary Research 58(1): 17–27, 2010

FULL PAPER

Extrusion of Na,K-ATPase and transferrin receptor with lipid raft-associated proteins in different populations of exosomes during reticulocyte maturation in dogs

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Received for publication, February 3, 2010; accepted, February 24, 2010

Abstract

The present study characterizes canine reticulocyte exosomes. Exosomes are small membrane vesicles involved in membrane remodeling that are released from reticulocytes during the final maturation step of red blood cells. The vesicles collected from reticulocyte culture supernatants by differential centrifugation contained major exosomal proteins including heat shock protein cognate 70 (Hsc70) and transferrin receptors (TfR), consistent with the definition of the exosome. In addition, the Na,K-ATPase α-subunit and stomatin, a lipid raft-associated protein, were extruded by the exosome pathway, possibly leading to the absence of these proteins in erythrocytes, while the major protein constituents of erythrocyte membranes, spectrin and band 3 were retained in reticulocytes and not expelled into exosomes. The Na,K-ATPase α-subunit, as well as TfR and about half of the stomatin contained in exosomes, was obtained in a detergent-soluble fraction that was distinct from the lipid raft microdomain. Moreover, Na,K-ATPase and a portion of stomatin were distributed differently to Hsc70, TfR, stomatin, and ganglioside G_{M1} in vesicles separated with sucrose density gradient centrifugation. These results demonstrate that a heterogeneous group of exosomes participates in the loss of Na,K-ATPase and membrane remodeling during reticulocyte maturation in dogs.

Keywords: dog, exosome, Na,K-ATPase, reticulocyte, stomatin

Introduction

Reticulocyte maturation into erythrocytes involves remodeling of the plasma membrane and protein contents of cells. Unwanted membrane proteins are removed from reticulocytes by protein degradation through the proteasome system[11] and/or by extrusion of vesicles known as exosomes[16,17]. Exosomes are small membrane vesicles 50–100 nm in diameter, corresponding to

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the internal vesicles contained in multivesicular bodies[17,27]. After multivesicular bodies fuse with the plasma membrane, the exosomes are released into the extracellular milieu.

Exosomes have been documented in reticulocytes[2,6,17], epithelial cells[27], and dendritic cells[5,7]. Proteins including the chaperone heat shock protein cognate 70 (Hsc70) and some lipid raft-associated proteins are common in exosomes from various sources, but the specific exosomal protein composition reflects the cell of origin[2,16]. The major protein characteristic to reticulocyte exosomes is the transferrin receptor (TfR). The association of TfR and Hsc70, and their extrusion from reticulocyte membranes into exosomes[9,10], leads to the complete loss of TfR in mature erythrocytes in which TfR is no longer required. In contrast, exosomes from antigen-presenting cells contain major histocompatibility complex II molecules, but not TfR[7]. These findings have suggested that the biogenesis of exosomes in various cells involves common mechanisms as well as a cell type-specific selection process for the proteins to be extruded.

Dogs are unique in their reticulocyte maturation, since this process of membrane remodeling is associated with the loss of the Na,K-ATPase[13,20]. Consequently, dogs usually possess low potassium (LK) erythrocytes characterized by high intracellular Na^+ and low intracellular K^+ concentrations. In addition to ATP-dependent proteolytic degradation, i.e., proteasomal degradation, which accounts for the removal of some Na,K-ATPase in canine reticulocytes[13], vesicular extrusion by exosomes is the most likely pathway involved in this process, as the exosomal expulsion of Na,K-ATPase has also been suggested in sheep[3,17]. However, the mechanism by which Na,K-ATPase is incorporated as a component of exosomes remains unknown.

A previous study has revealed that the exosomes released from human reticulocytes contain some lipid raft-associated proteins, including stomatin[6]. Stomatin is a ubiquitous lipid raft-associated membrane protein, the founder of the stomatin protein family, and is absent from the red blood cells of patients with hereditary overhydrated stomatocytosis, a pathological condition characterized in these cells by increased permeability to Na^+ and K^+ and a stomatocytic shape change[4,19,25]. Various studies have implicated stomatin in the regulation of ion transporters and the stretch- or pressure-sensitive system[8,21,23,26]. Therefore, we hypothesized that stomatin interacts with Na,K-ATPase within the exosomal pathway during reticulocyte maturation in dogs.

The purpose of the present study was to examine whether membrane remodeling during reticulocyte maturation involves selective extrusion of Na,K-ATPase in association with that of other membrane constituents including stomatin. To do that, we collected reticulocytes and the vesicles released from reticulocytes in culture, characterized their protein contents by immunoblotting and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and analyzed their changes during maturation into erythrocytes.

Materials and Methods

Dogs: Three beagle dogs weighing approximately 10 kg were used. These dogs were clinically healthy, and the hematological parameters of their red blood cells were within reference ranges. All of the experimental procedures met with the approval of the Laboratory Animal Experimentation Committee, Graduate School of Veterinary Medicine, Hokkaido University.

Antibodies: Mouse anti-human stomatin monoclonal antibody, GARP-50[12], was a kind gift from Dr. R. Prohaska (Institute of Medical Biochemistry, University of Vienna, Austria). Rabbit antiserum directed against the C-terminal peptide of human glucose transporter

1 (GLUT1) was a kind gift from Dr. M. Saito (Hokkaido University, Japan). The rabbit anti-dog kidney Na,K-ATPase α-subunit antibody and rabbit anti-dog spectrin polyclonal antibody have been described previously[13,14]. The rabbit anti-dog band 3 antibody was raised in rabbits using the band 3 polypeptide purified from dog erythrocytes as antigen and purified on a Protein G-Sepharose 4FF column (Amersham Biosciences). Anti-human ESA/flotillin-2 (BD Transduction Laboratories) and anti-human transferrin receptor (Zymed Laboratories) antibodies were also used. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Rockland Immunochemicals) were used as secondary antibodies.

Experimental reticulocytosis in dogs and reticulocyte preparation: Experimental reticulocytosis was induced in dogs as previously described[20]. In brief, dogs were bled daily (approx. 200 ml) via the jugular vein for 3 consecutive days. The hematocrit value for each dog was reduced from 45% to 25%, on average, with reticulocyte counts of approximately 5% to 10%. Reticulocytes were separated from whole blood using Percoll (Amersham Biosciences) discontinuous gradient centrifugation[20] on the third day after the last bleeding.

Reticulocyte culture and separation of exosomes: Reticulocytes were cultured as described[20]. After 24 and 72 hr of incubation, culture supernatants were centrifuged at 18,000 × *g* for 20 min at 4°C to sediment cell debris. The resulting supernatants were then centrifuged at 100,000 × *g* for 1 hr at 4°C to obtain pellets containing vesicles (P2 vesicles). When the P2 vesicles were further analyzed by sucrose gradient centrifugation, they were collected on 2.3-M sucrose cushions.

Sucrose gradient analysis of P2 vesicles: The P2 vesicles were fractionated on a linear sucrose gradient as reported previously[6] with some modifications. Briefly, the P2 vesicles were suspended in 1.5 ml of 20 mM HEPES/NaOH, pH 7.4, and were layered on the top of a linear density gradient consisting of 10% to 35% sucrose in the same buffer. Gradients were centrifuged at 100,000 × *g* for 20 hr, after which 12 fractions were collected from the top of the tube. Each fraction was diluted 10-fold in 20 mM HEPES/NaOH, pH 7.4, and vesicles were pelleted by centrifugation at 100,000 × *g* for 1 hr and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Aliquots were slot blotted and assayed for ganglioside G_{M1} using a previously reported method[24].

Preparation of red blood cell membranes: Red blood cell membrane ghosts were prepared as described previously[14]. The protein concentrations of membranes were determined using a protein assay kit (Bio-Rad Laboratories) using bovine serum albumin as the standard.

SDS-PAGE and immunoblotting: Proteins were separated by SDS-PAGE using 8% or 10% SDS-polyacrylamide gels followed by staining with Coomassie brilliant blue. Immunoblotting was performed as previously described using the ECL chemiluminescence detection system (Amersham Biosciences)[15]. Quantification of membrane proteins was performed by densitometric scanning using a GS-800 densitometer (Bio-Rad Laboratories). In some experiments, membrane proteins were analyzed after separation into Triton-soluble and -insoluble fractions as described previously[6].

Mass spectrometry analysis: MALDI-TOF MS analyses were performed as described previously[1]. In brief, the proteins were digested in gel slices with 10 μg/ml Trypsin Gold (Promega) for 16 hr at 37°C after reduction with 10 mM dithiothreitol and alkylation of cysteine residues with 55 mM iodoacetamide. Peptides were eluted from gels, desalted using C18 ZipTips (Millipore), and crystallized using

saturated α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics) as a matrix. Full-scan mass spectra of the tryptic peptides were collected using a Bruker autoflex MALDI-TOF mass spectrometer, and the peptide masses obtained were used to search the NCBI and Swiss-Prot sequence databases for protein identification using MASCOT software.

Results

We obtained small vesicles (P2 vesicles) from the culture supernatant of canine reticulocytes by differential centrifugation. Electron microscopy revealed that about 70% of the vesicles were 20 nm to 60 nm in diameter, while the rest of the population was in the range of 70–90 nm (data not shown). The P2 vesicles contained several major polypeptides that were readily seen by Coomassie brilliant blue staining with a profile totally different from that of reticulocyte and erythrocyte membranes (Fig. 1A). The MALDI-TOF MS analysis indicated that the most abundant polypeptides, with apparent molecular weights of 70 kDa (polypeptide *f* in Fig. 1A) and 96 kDa (polypeptide *b*), were Hsc70 and TfR, respectively (Table 1). This finding is consistent with observations of exosomes from sheep reticulocytes[17] and reports that Hsc70 and TfR are characteristic to reticulocyte exosomes[2,16], indicating that the P2 vesicles are principally composed of the exosomes shed from reticulocytes. The P2 vesicles also contained some related or truncated polypeptides relevant to TfR or transferrin (Fig. 1A and Table 1). Immunoblotting demonstrated that Hsc70 and TfR were found in reticulocyte membranes and P2 vesicles, but not in erythrocyte membranes (Fig. 1B), supporting the hypothesis that P2 vesicles extruded these obsolete proteins from reticulocytes to result in their total absence in erythrocytes.

Next, to assess the exosomal release of Na,K-ATPase and some lipid raft domain proteins, we analyzed the contents of these

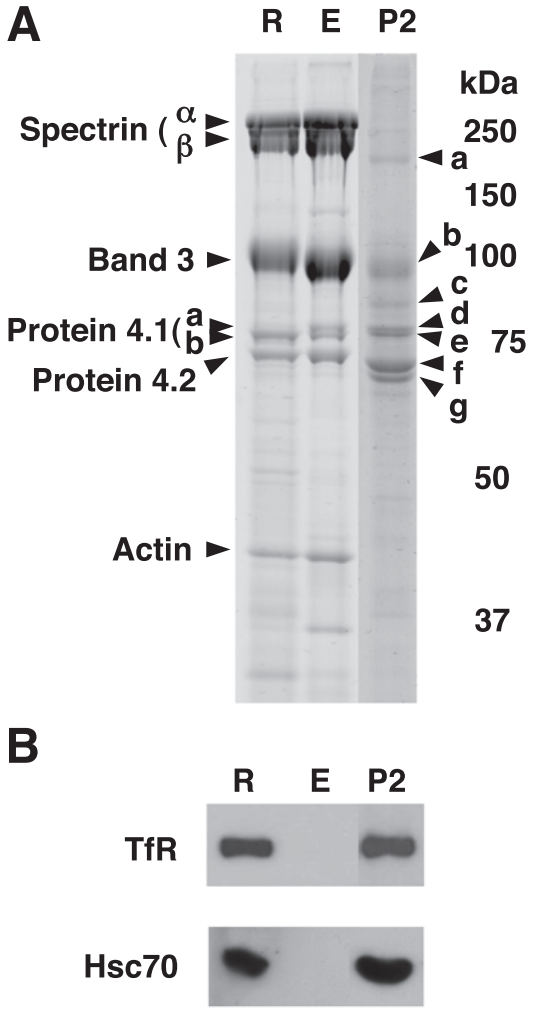

Fig. 1. Protein composition in the P2 vesicles released from canine reticulocytes. A. Proteins in canine reticulocyte (*R*) and erythrocyte (*E*) membranes, and the P2 vesicles collected from the reticulocyte culture supernatants (*P2*), were separated by SDS-PAGE followed by Coomassie brilliant blue staining. Major polypeptides shown as *a* through to *g* were subjected to MALDI-TOF mass analysis (see Table 1). Major red blood cell membrane proteins are indicated. The migration positions of size marker proteins are also shown (kDa). B. TfR (95 kDa) and Hsc70 (70 kDa) were detected in reticulocyte (*R*) and erythrocyte (*E*) membranes and P2 vesicles (*P2*) by immunoblotting.

proteins in P2 vesicles and residual reticulocyte membranes during reticulocyte maturation *in vitro* (Fig. 2). In this experiment, using a reticulocyte preparation with a high reticulocyte count of 98%, the Na,K-ATPase α-subunit was decreased to about 10% of its original content in

Table 1. Summary of MALDI-TOF mass spectrometric analysis of the major polypeptides in P2 vesicles.

Polypeptides in Fig. 1A	Protein identified	NCBI accession number	Theoretical molecular mass (kDa)	Number of matched peptides*	Sequence coverage (%)†
a	α2-macroglobulin§	NM001109795	167.5	12	9
b	TfR	NM001003111	86.6	8	12
c	TfR, truncated form	NM001003111	86.6	9	19
d	Transferrin isoform 2	XM859371	79.1	6	9
e	Transferrin isoform 8	XM859475	77.7	6	9
f	Hsc70	XP848246	67.8	8	16
g	Serum albumin§	NM180992	69.3	8	16

*Number of tryptic peptides possessing masses matched with those expected for the identified protein.
†The percentage of amino acid sequences covered by the matched peptides.
§These two proteins were bovine proteins derived from the fetal calf serum added to the culture medium.

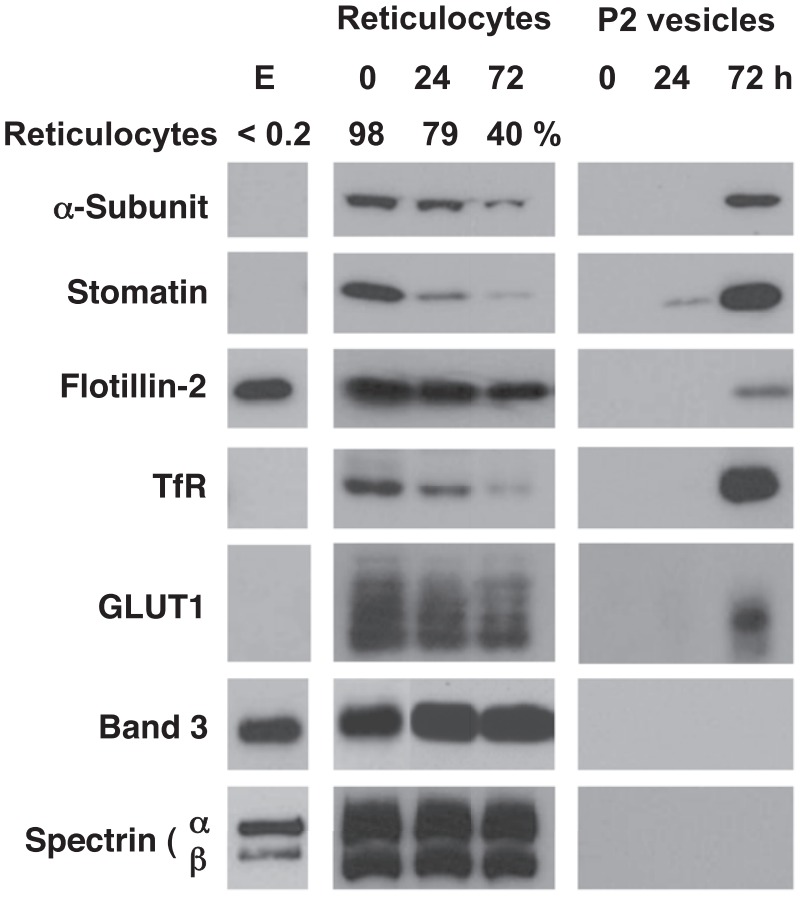

Fig. 2. Selective extrusion of membrane proteins via the exosome system during reticulocyte maturation *in vitro*. Cell membranes and P2 vesicles were prepared from reticulocyte cultures after the indicated incubation times (0, 24, and 72 hr). Reticulocyte membrane proteins (*Reticulocytes*, 10 μg/lane) and the proteins in P2 vesicles (*P2 vesicles*) derived from equivalent volumes of reticulocytes were separated by SDS-PAGE and analyzed by immunoblotting for the α-subunit of Na,K-ATPase, stomatin, flotillin-2, TfR, GLUT1, band 3 (anion exchanger 1), and spectrin. Erythrocyte membrane proteins (*E*) were also analyzed (1 μg/lane for band 3 and spectrin, 10 μg/lane for others).

reticulocyte membranes after 72 hr of incubation, resulting in its total absence from mature erythrocyte membranes as previously reported[13]. Similar reductions were observed in the contents of TfR, the glucose transporter GLUT1, and the lipid raft-associated protein stomatin, all of which were also absent from erythrocytes. These proteins were observed at high levels in P2 vesicles after 72 hr of incubation, indicating their exosomal release. Another lipid raft-associated protein, flotillin-2, also showed extrusion in exosomes associated with a reduction in reticulocytes; however, the reduction of flotillin-2 was less pronounced than that of Na,K-ATPase, TfR, or stomatin, and approximately 80% of flotillin-2 was retained in erythrocytes. In contrast, no significant decrease in reticulocytes or extrusion in exosomes was observed for band 3 or α- and β-spectrin, the major components of erythrocyte membranes. These findings indicate that certain membrane proteins of canine reticulocytes were selectively extruded from cell membranes through the exosome pathway.

A previous study demonstrated the presence of lipid rafts in exosomes and suggested that these membrane microdomains participate in exosomal protein sorting[6]. We therefore examined the distribution of canine reticulocyte exosome proteins in lipid rafts obtained as insoluble membrane fractions upon solubilization with the detergent Triton X-100. The vast majority of TfR and Hsc70 in P2 vesicles were solubilized in an isotonic solution of 1% Triton X-100 (Fig. 3A). Immunoblotting demonstrated that the Na,K-ATPase α-subunit and TfR were

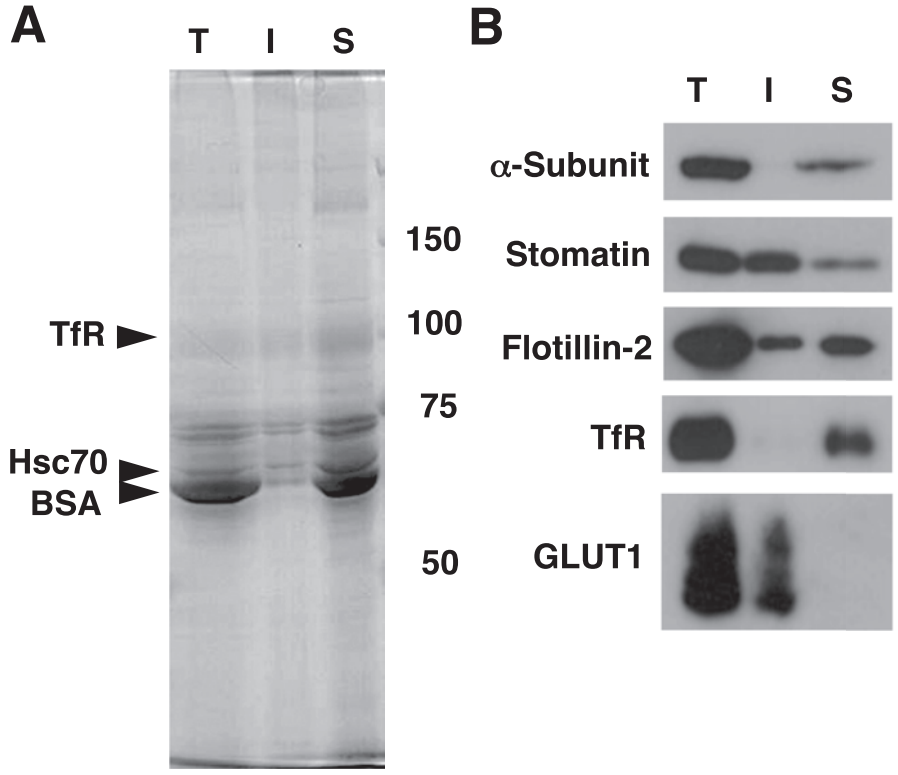

Fig. 3. Triton X-100 solubility of the proteins in P2 vesicles. A. The proteins in P2 vesicles were solubilized in 1% Triton X-100 isotonic buffer and divided into detergent-soluble and -insoluble fractions[6]. Total proteins in the original membranes (*T*) and Triton-soluble (*S*) or -insoluble (*I*) fractions corresponding to the equivalent volume of the original membranes were separated by SDS-PAGE and labeled with Coomassie brilliant blue. The migration positions of major proteins including TfR, Hsc70, and bovine serum albumin (*BSA*) are indicated. B. The distributions of the α-subunit, stomatin, flotillin-2, TfR, and GLUT1 were analyzed by immunoblotting for each fraction described above.

readily and totally solubilized with the detergent (Fig. 3B). In contrast, GLUT1 was obtained in the Triton-insoluble fraction, in agreement with previous observation[28]. Interestingly, stomatin, which has been shown to interact with GLUT1[22,29], and flotillin-2 were observed in both Triton-soluble and -insoluble fractions. These proteins demonstrated a similar distribution in reticulocyte membranes with the exception of flotillin-2, which was exclusively detected in the detergent-insoluble fraction (data not shown). After immunoprecipitation of the Triton-soluble fraction of reticulocyte membranes using an anti-stomatin antibody, stomatin and GLUT1, but not the α-subunit of Na,K-ATPase, were observed in the immunoprecipitate (data not shown). Considering the previously reported association between TfR and Hsc70[9,10] and interaction between stomatin and GLUT1[22,29], the coexistence of these pairs of proteins in the detergent-soluble and -insoluble fractions, respectively, is likely indicative of distinct mechanisms for protein sorting in exosomes and suggests an association between Na,K-ATPase and other exosomal proteins including detergent-soluble stomatin distributed in non-lipid raft environments.

To examine this hypothesis, we fractionated P2 vesicles based on their gravity by sucrose density gradient centrifugation and compared their protein compositions (Fig. 4). The vesicles distributed in fractions 4–12 had a gravity range of 1.059 g/ml to 1.144 g/ml, with the highest protein contents in fraction 7 (gravity ≈ 1.090 g/ml), as shown in the Coomassie brilliant blue-stained gel. An abundance of a lipid raft marker G_{M1} was observed in fractions 7 and 8. G_{M1} signal in fractions 1–4 was caused by the presence of bovine serum albumin in the culture medium. Immunoblotting confirmed the predominance of TfR and Hsc70 in fractions 5–8, particularly in fraction 7. The profiles of stomatin and flotillin-2 were similar to those of TfR and Hsc70. In contrast, the α-subunit of Na,K-ATPase was predominantly found in fractions 4–6 (gravity, 1.059–1.079 g/ml), in which major exosomal proteins such as Hsc70 and TfR appeared to be minor components, and was less abundant in denser fractions. Similar levels of stomatin and flotillin-2 were also observed in fractions 4, 5, and 6. These results indicate that canine reticulocyte exosomes (P2 vesicles) are comprised of heterogeneous vesicles characterized by diverse protein components.

Discussion

Although canine reticulocytes contain considerable levels of Na,K-ATPase, this protein is rapidly lost during maturation into erythrocytes[13,20]. The present study demonstrates that the exosome pathway is involved in this process, consistent with previous studies of sheep reticulocytes conducted by other groups[3,17]. Our data show that canine reticulocyte exosomes (P2 vesicles) have a protein composition very similar to that reported in exosomes released from sheep, rat, and human reticulocytes[2,6,18], and selectively extrude Hsc70, TfR, and some lipid raft-associated proteins including stomatin in addition to Na,K-ATPase.

The extent to which the exosome system alone can cause such remarkable reductions or loss of these membrane proteins is of considerable interest. We estimated the total amount of each protein in P2 vesicles and cell membranes after 72 hr of reticulocyte maturation *in vitro*, and compared those values with the quantification of immunoblot signals from immature reticulocyte membranes shown in Fig. 2. The amounts of Na,K-ATPase α-subunit, stomatin, TfR, and GLUT1 observed after incubation were 36%, 38%, 54%, and 58% of those estimated in the reticulocyte membranes before incubation, while as much as 95% of flotillin-2 was recovered under the same conditions. Thus, while substantial amounts of the former proteins disappeared over 3 days of incubation, exosomal extrusion appears to participate differently in the removal of each

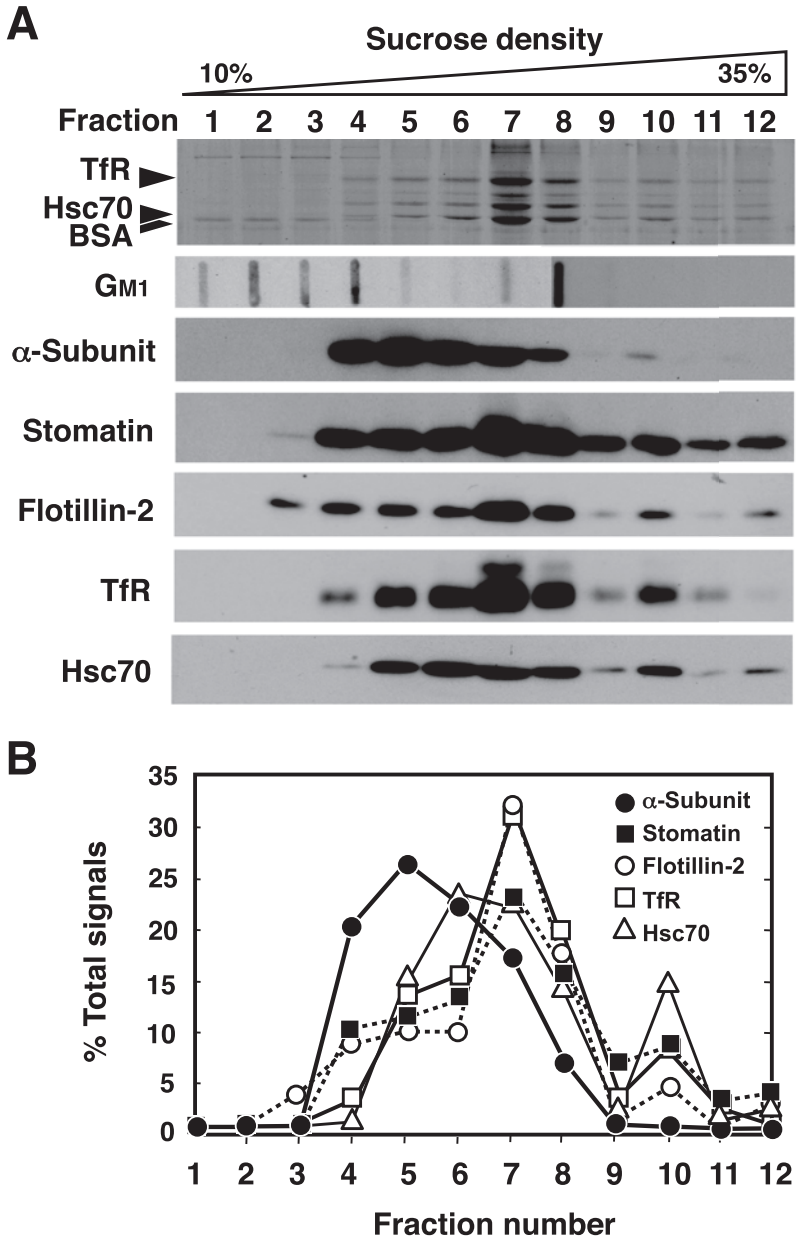

Fig. 4. Distribution of P2 vesicles containing Na,K-ATPase, stomatin, and other proteins on a sucrose density gradient. A. P2 vesicles were separated into 12 fractions on a sucrose linear density gradient consisting of 10% to 35% sucrose with gravity of 1.005 g/ml to 1.144 g/ml. Proteins in each fraction were analyzed by SDS-PAGE with Coomassie brilliant blue staining (*top panel*) and by immunoblotting for the α-subunit, stomatin, flotillin-2, TfR, and Hsc70, respectively. In the top panel, the positions of TfR, Hsc70, and BSA are indicated. The contents of GM1 were also examined; signals for GM1 in fractions 1–4 are mainly attributed to the culture medium. B. Signal intensities of each protein in the immunoblots (*A*) were measured by densitometric scanning and are shown as the percentage of the protein in each fraction. The inset shows the designation of symbols.

protein over the course of reticulocyte maturation. Although artificial loss due to sample preparation cannot be ruled out, other mechanisms are likely to also be involved in the reduction of these proteins, particularly Na,K-ATPase and stomatin, such as the ATP-dependent proteolytic degradation of Na,K-ATPase described in an earlier study[13].

The most noteworthy finding of the present study is that exosomes are not homogeneous,

but instead consist of heterogeneous populations with different protein and lipid compositions. The distribution pattern of Na,K-ATPase clearly differed from those of major exosomal proteins, including Hsc70, TfR, and ganglioside GM1, on a sucrose density gradient (Fig. 4), indicating that the vesicles enriched with Na,K-ATPase comprise a population of reticulocyte exosomes distinct from the major population, although either population may contain Hsc70, TfR, stomatin, and other proteins as minor or major constituents. The characteristics of these heterogeneous vesicles fit the criteria of exosomes[2] and are consistent with reports that lipid raft-associated proteins are major components of exosomes and important for exosome formation[6].

Previous studies have demonstrated that exosomal release of TfR in rat reticulocytes and several other cell lines involved association with Hsc70 through a YXXΦ motif (where Φ is a bulky hydrophobic amino acid residue and X represents any amino acid) within the TfR cytoplasmic domain[9,10]. Similar interactions may be involved in the exosomal release of Na,K-ATPase. Na,K-ATPase and Hsc70 coexist in populations of exosomes (Fig. 4, fractions 5–8), and the α-subunit of canine Na,K-ATPase possesses a YXXΦ motif (Tyr^{472}-Ala-Lys-Ile^{475}) in its large cytoplasmic domain (UniProtKB/Swiss-Prot accession number, P50997). Likewise, the distribution of stomatin and Na,K-ATPase in the same density gradient fractions, and the observation that Na,K-ATPase and stomatin were found in the detergent-soluble membrane fraction in P2 vesicles (Fig. 3) and in density fractions with lower GM1 content (Fig. 4), suggest that Na,K-ATPase and stomatin may also interact, as supposed for the regulation of ion transporters and the stretch- or pressure-sensitive system[8,21,23,26].

In conclusion, exosomal extrusion participates in the loss of Na,K-ATPase and stomatin, as well as several other proteins from canine reticulocytes, in addition to membrane remodeling over the course of reticulocyte maturation. Our study has further demonstrated that canine reticulocyte exosomes are heterogeneous in their protein and lipid compositions.

Acknowledgements

We thank Dr. R. Prohaska (University of Vienna) and Dr. M. Saito (Hokkaido University) for kindly providing GARP-50 and anti-GLUT1 antibodies, respectively.

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