



HOKKAIDO UNIVERSITY

Title	Canine reticulocyte exosomes : parallel and selective extrusion of Na,K-ATPase and stomatin during reticulocyte maturation in dogs with HK and LK red cell phenotypes
Author(s)	Komatsu, Tomohiko; 小松, 智彦
Degree Grantor	北海道大学
Degree Name	博士(獣医学)
Dissertation Number	甲第9656号
Issue Date	2010-06-30
DOI	https://doi.org/10.14943/doctoral.k9656
Doc URL	https://hdl.handle.net/2115/43146
Type	doctoral thesis
File Information	komatsu_thesis.pdf



**Canine Reticulocyte Exosomes: Parallel and Selective Extrusion
of Na,K-ATPase and Stomatin during Reticulocyte Maturation
in Dogs with HK and LK Red Cell Phenotypes**

(犬網状赤血球エクソゾーム：HK型ならびにLK型犬の網状赤血球成熟過程
におけるNa,K-ATPaseとstomatinの選択的同時排出機構)

Tomohiko Komatsu

*Laboratory of Molecular Medicine, Department of Veterinary Clinical Sciences
Graduate School of Veterinary Medicine, Hokkaido University*

Contents

Abbreviations

General Introduction	1
Chapter 1	4
Introduction	5
Materials and Methods	7
Results	11
Discussion	20
Summary	23
Chapter 2	24
Introduction	25
Materials and Methods	27
Results	30
Discussion	40
Summary	44
General Conclusion	45
References	47
Acknowledgements	52
Abstract in Japanese	53

Abbreviations

Abbreviations used in this study are as follows:

HK	high potassium (and low sodium)
LK	low potassium (and high sodium)
GLUT1	glucose transporter 1
Hsc70	heat shock protein cognate 70
MALDI-TOF MS....	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
OHSt	overhydrated stomatocytosis
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
TfR	transferrin receptor

In the present study, one-letter and three-letter abbreviations for amino acid residues are employed.

General Introduction

Reticulocyte maturation into erythrocytes is the final step in differentiation and maturation of erythroid cells. Reticulocyte maturation 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 (Haas, 1991) and/or by extrusion of vesicles known as exosomes (Johnstone, 2006; Johnstone *et al.*, 1987). Exosomes have been documented in reticulocytes (Blanc *et al.*, 2005; de Gassart *et al.*, 2003; Johnstone *et al.*, 1987), epithelial cells (van Niel *et al.*, 2006), and dendritic cells (Chaput *et al.*, 2006; Denzer *et al.*, 2000). 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 (Blanc *et al.*, 2005; Johnstone, 2006). 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 (Gémimnard *et al.*, 2001; Géminard *et al.*, 2004), leads to the complete loss of TfR in mature erythrocytes in which TfR is no longer required.

Dogs, as well as several other mammalian species including sheep and cow, are divided into two phenotypes based upon their erythrocyte cation concentrations (Chan *et al.*, 1964; Inaba, 2000; Maede *et al.*, 1983). One, the HK phenotype, is characterized by high potassium (K^+) and low sodium (Na^+) ion concentrations within the erythrocytes and the other, the LK phenotype, by low K^+ and high Na^+ concentrations. This phenotypic difference is attributed to the presence, or absence, of Na,K-ATPase in the erythrocyte membrane (Inaba

and Maede, 1986; Maede *et al.*, 1983). Na,K-ATPase content, and activity, in dog red cells are both markedly reduced during the maturation of reticulocytes into erythrocytes (Inaba and Maede, 1986; Maede and Inaba, 1985). HK dogs retain Na,K-ATPase in erythrocytes due to the high activity of the enzyme in precursor cells, whereas total loss of reticulocyte Na,K-ATPase occurs upon reticulocyte maturation in LK dogs (Inaba and Maede, 1986; Maede and Inaba, 1985). Since the HK phenotype is inherited in an autosomal recessive manner (Maede *et al.*, 1983) and, in turn, the LK phenotype is inherited in a dominant manner, the red cell HK and LK phenotype is controlled by the putative genotypes, *hk/hk* for HK dogs and *hk/lk* and *lk/lk* for LK dogs. However, the gene responsible for these genotypes/phenotypes is unknown. In addition, although proteolytic degradation (Inaba and Maede, 1986) and extrusion of exosomes (Johnstone *et al.*, 1987) are likely to be involved in the process of membrane remodeling during reticulocyte maturation in HK and LK dogs, the mechanisms for these processes remain unknown (Inaba, 2000).

A previous study has revealed that the exosomes released from human reticulocytes contain some lipid raft-associated proteins, including a 31-kDa protein stomatin (previously called protein 7.2) (de Gassart *et al.*, 2003). 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 (OHSt), a pathological condition characterized in these cells by increased permeability to Na⁺ and K⁺ and a stomatocytic shape change (An and Mohandas, 2008; Bruce *et al.*, 2009; Lande *et al.*, 1982; Stewart, 2004). Various studies have implicated stomatin in the regulation of ion transporters and the stretch- or pressure-sensitive system (Fricke *et al.*, 2003;

Martinez-Salgado *et al.*, 2007; Price *et al.*, 2004; Stewart *et al.*, 1992). Moreover, a recent study shows that stomatin also enhances the transport of dehydroascorbic acid by GLUT1 (glucose transporter 1) in erythrocytes (Montel-Hagen *et al.*, 2008). Interestingly, stomatin and other lipid raft-associated proteins are sorted into exosomes during reticulocyte maturation and induce phenotypic changes in erythrocytes (de Gassart *et al.*, 2003; Rabesandratana *et al.*, 1998).

These findings suggested that Na,K-ATPase is incorporated into exosomes and interacts with stomatin during reticulocyte maturation in dogs and that canine HK/LK red cell phenotypes are somehow related to the expression of stomatin in erythroid cells. The purpose of the present study was to verify these hypotheses. In Chapter 1, it was examined whether reticulocyte maturation in dogs involves extrusion of Na,K-ATPase in association with that of other membrane constituents including stomatin. Then, in Chapter 2, the levels of stomatin, Na,K-ATPase, and some other membrane proteins in erythrocyte membranes from HK and LK dogs were analyzed. Some parts of this thesis have been published as follows:

1. Komatsu, T., Arashiki, N., Otsuka, Y., Sato, K., and Inaba, M. (2010) Extrusion of the Na,K-ATPase and the transferrin receptor in different population of exosomes with lipid raft-associated proteins during reticulocyte maturation in dogs. *Jpn. J. Vet. Res.* **58**, in press
2. Komatsu, T., Sato, K., Otsuka, Y., Arashiki, N., Tanaka, K., Tamahara, S., Ono, K., and Inaba, M. (2010) Parallel reductions in stomatin and Na,K-ATPase through the exosomal pathway during reticulocyte maturation in dogs: stomatin as a genotypic and phenotypic marker of high K⁺ and low K⁺ red cells. *J. Vet. Med. Sci.* **72**, in press

Chapter 1

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

Introduction

Exosomes are small membrane vesicles 50–100 nm in diameter, corresponding to the internal vesicles contained in multivesicular bodies (Johnstone *et al.*, 1987; van Niel *et al.*, 2006). After multivesicular bodies fuse with the plasma membrane, the exosomes are released into the extracellular milieu. Exosomes have been found in reticulocytes (Blanc *et al.*, 2005; de Gassart *et al.*, 2003; Johnstone *et al.*, 1987), epithelial cells (van Niel *et al.*, 2006), and dendritic cells (Chaput *et al.*, 2006; Denzer *et al.*, 2000). While the chaperone protein Hsc70 and some lipid raft-associated proteins are common in exosomes from various sources, the specific exosomal protein composition reflects the cell of origin (Blanc *et al.*, 2005; Johnstone, 2006). 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 (Gémimnard *et al.*, 2001; Géminard *et al.*, 2004), 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 (Denzer *et al.*, 2000). 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 (Inaba and Maede, 1986; Maede and Inaba, 1985). 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 (Inaba and Maede, 1986), 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 (Blostein and Grafova, 1990; Johnstone *et al.*, 1987). However, the mechanism by which Na,K-ATPase is incorporated as a component of exosomes remains unknown.

A previous study has demonstrated that the exosomes released from human reticulocytes contain some lipid raft-associated proteins, including stomatin (de Gassart *et al.*, 2003). Stomatin is a ubiquitous lipid raft-associated membrane protein and is absent from the red blood cells of patients with OHSt (Bruce *et al.*, 2009; Lande *et al.*, 1982; Stewart, 2004). Various studies have implicated stomatin in the regulation of ion transporters and the stretch- or pressure-sensitive system (Fricke *et al.*, 2003; Martinez-Salgado *et al.*, 2007; Price *et al.*, 2004; Stewart *et al.*, 1992). Therefore, we hypothesized that stomatin interacts with Na,K-ATPase within the exosomal pathway during reticulocyte maturation in dogs.

The purpose of the studies in Chapter 1 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, reticulocytes and the vesicles released from reticulocytes in culture were collected and were characterized for their protein contents by immunoblotting and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and their changes during maturation into erythrocytes were also analyzed.

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 (Hieble-Dirschmied *et al.*, 1991), was a kind gift from Dr. Rainer 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. Masayuki 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 (Inaba and Maede, 1986; Inaba *et al.*, 1996). 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 (Maede and Inaba, 1985). 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 (Maede and Inaba, 1985) on the third day after the last bleeding.

Reticulocyte culture and separation of exosomes

Reticulocytes were cultured as described (Maede and Inaba, 1985). After 24 and 72 hours of incubation, culture supernatants were centrifuged at 18,000 x g for 20 minutes at 4°C to sediment cell debris. The resulting supernatants were then centrifuged at 100,000 x g for 1 hour 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 (de Gassart *et al.*, 2003) 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 x g for 20 hours, 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 $100,000 \times g$ for 1 hour and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Aliquots were slot blotted and assayed for ganglioside GM1 using a previously reported method (Salzer *et al.*, 2002).

Preparation of red blood cell membranes

Red blood cell membrane ghosts were prepared as described previously (Inaba *et al.*, 1996). 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) (Ito *et al.*, 2006). 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 (de Gassart *et al.*, 2003).

Mass spectrometry analysis

MALDI-TOF MS analyses were performed as described previously (Arashiki *et al.*, 2010). In

brief, the proteins were digested in gel slices with 10 µg/ml Trypsin Gold (Promega) for 16 hours 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

Selective extrusion of membrane proteins in canine reticulocyte exosomes

The small vesicles (P2 vesicles) were obtained 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 (Johnstone *et al.*, 1987) and reports that Hsc70 and TfR are characteristic to reticulocyte exosomes (Blanc *et al.*, 2005; Johnstone, 2006), 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 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 reticulocyte membranes after 72 hours of incubation, resulting in its total absence from mature erythrocyte membranes as previously reported (Inaba and Maede, 1986). 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 hours 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.

Canine exosomes consist of heterogeneous populations

A previous study demonstrated the presence of lipid rafts in exosomes and suggested that these membrane microdomains participate in exosomal protein sorting (de Gassart *et al.*, 2003). Therefore, the distribution of canine reticulocyte exosome proteins were examined 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 readily and totally solubilized with the detergent (Fig. 3B). In contrast, GLUT1 was obtained in the Triton-insoluble fraction, in agreement with previous observation (Wilkinson *et al.*, 2008). Interestingly, stomatin, which has been shown to interact with GLUT1 (Montel-Hagen *et al.*, 2007; Zhang *et al.*, 2001), 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). Considering the previously reported association between TfR and Hsc70 (Géminard *et al.*, 2001; Géminard *et al.*, 2004) and interaction between stomatin and GLUT1 (Montel-Hagen *et al.*, 2007; Zhang *et al.*, 2001), 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 \approx 1.090 g/ml), as shown in the Coomassie brilliant blue-stained gel. An abundance of a lipid raft marker GM1 was observed in fractions 7 and 8. GM1 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.

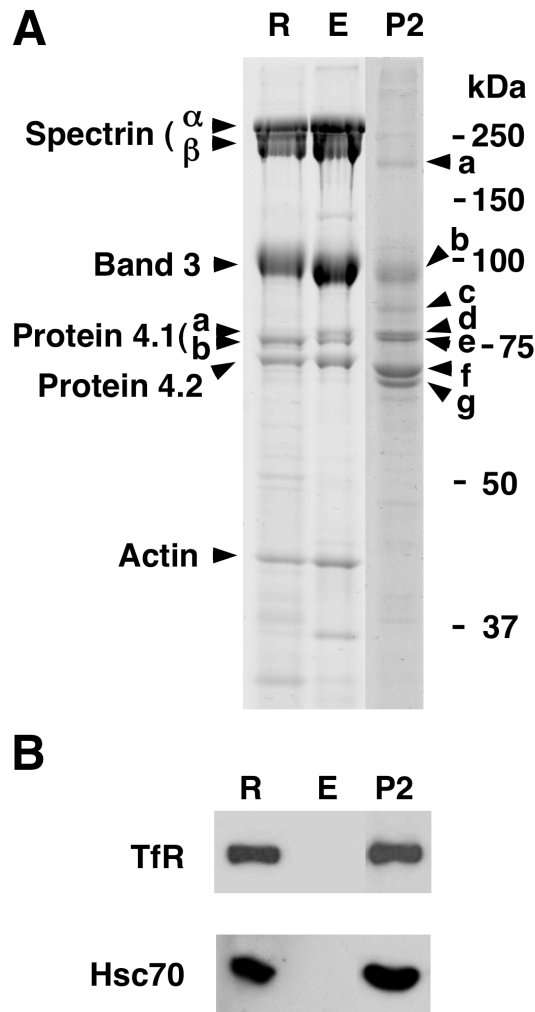


Figure 1. Protein composition in the P2 vesicles released from canine reticulocytes.

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.

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	α 2-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.

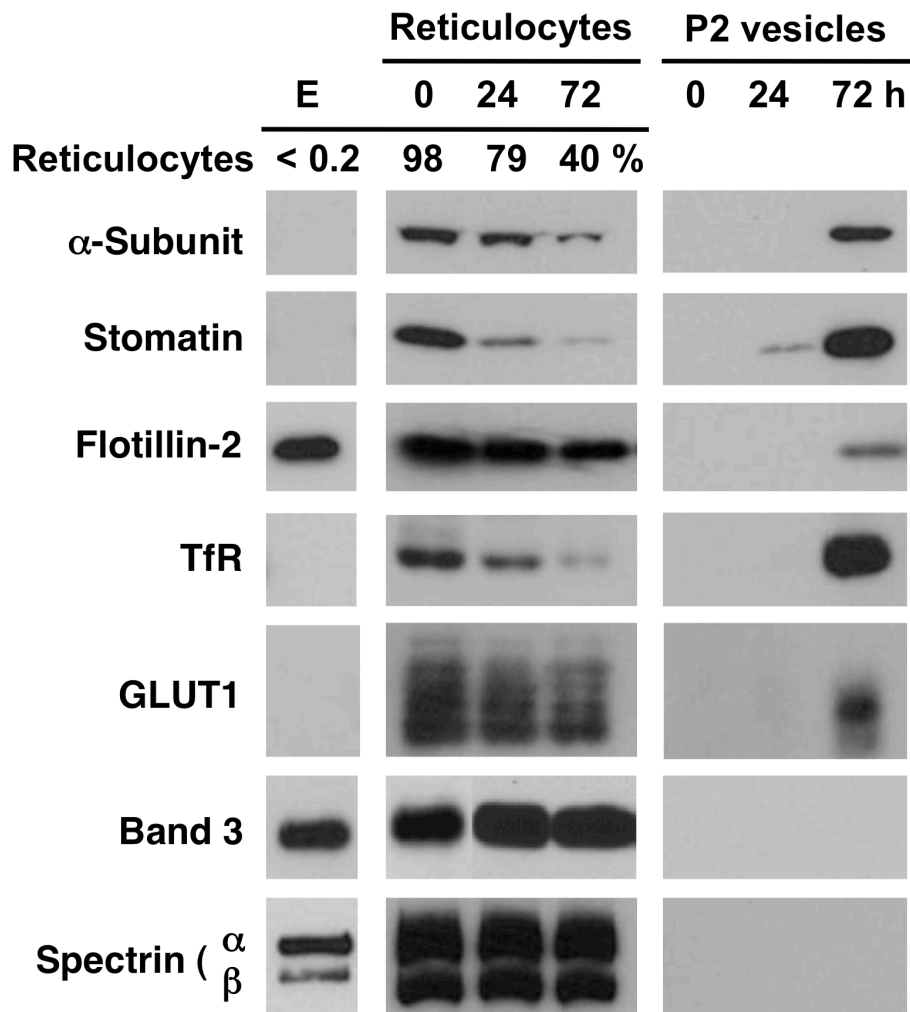


Figure 2. Selective extrusion of membrane proteins via the exosome system during reticulocyte maturation *in vitro*.

Cell membranes and P2 vesicles were prepared from reticulocyte cultures after the indicated incubation times (0, 24, and 72 h). Reticulocyte membrane proteins (*Reticulocytes*, 10 μ g/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 μ g/lane for band 3 and spectrin, 10 μ g/lane for others).

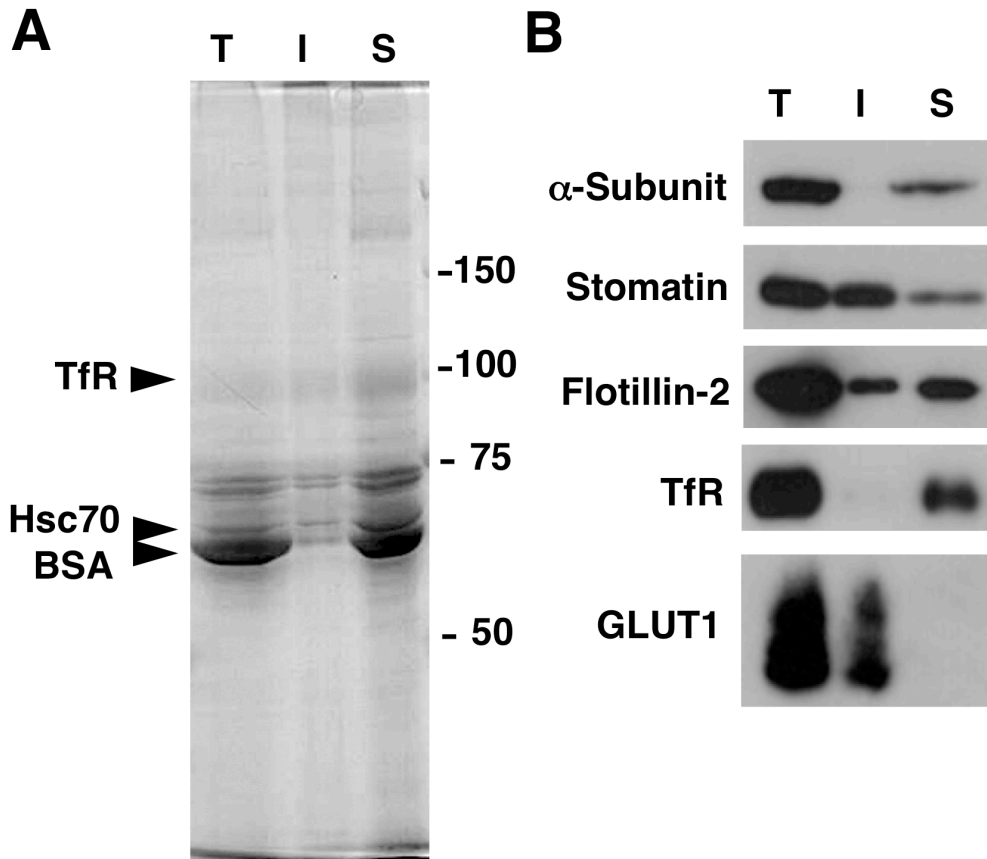


Figure 3. Triton X-100 solubility of the proteins in P2 vesicles.

A. The proteins in P2 vesicles were solubilized in 1% Triton X-100 isotonic buffer and divided into detergent-soluble and -insoluble fractions (de Gassart *et al.*, 2003). 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.

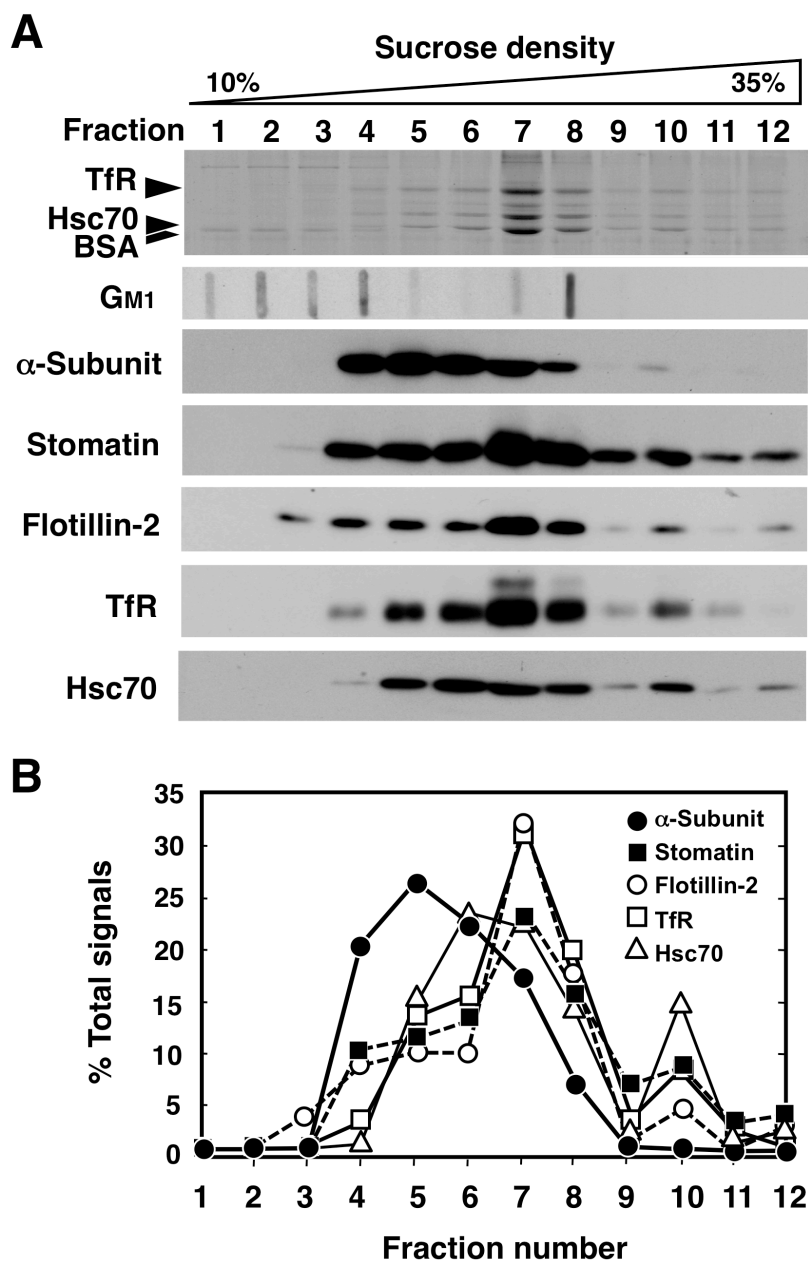


Figure 4. Distribution of P2 vesicles containing Na,K-ATPase, stomatin, and other proteins on a sucrose density gradient.

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.

Discussion

Although canine reticulocytes contain considerable levels of Na,K-ATPase, this protein is rapidly lost during maturation into erythrocytes (Inaba and Maede, 1986; Maede and Inaba, 1985). The present study demonstrates that the exosome pathway is involved in this process, consistent with previous studies of sheep reticulocytes conducted by other groups (Blostein and Grafova, 1990; Johnstone *et al.*, 1987). 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 (Blanc *et al.*, 2005; de Gassart *et al.*, 2003; Johnstone *et al.*, 1989), 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 hours 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 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 (Inaba and Maede, 1986).

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 (Blanc *et al.*, 2005) and are consistent with reports that lipid raft-associated proteins are major components of exosomes and important for exosome formation (de Gassart *et al.*, 2003).

Previous studies have demonstrated that exosomal release of TfR in rat reticulocytes and several other cell lines involved association with Hsc70 through a YXX Φ motif (where Φ is a bulky hydrophobic amino acid residue and X represents any amino acid) within the TfR cytoplasmic domain (Géminard *et al.*, 2001; Géminard *et al.*, 2004). 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 YXX Φ motif (Tyr⁴⁷²-Ala-Lys-Ile⁴⁷⁵) 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 (Fricke *et al.*, 2003; Martinez-Salgado *et al.*, 2007; Price *et al.*, 2004; Stewart *et al.*, 1992).

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. This study has further demonstrated that canine reticulocyte exosomes are heterogeneous in their protein and lipid compositions.

Summary

In Chapter 1, canine reticulocyte exosomes were identified and characterized. The small vesicles collected from reticulocyte culture supernatants by differential centrifugation contained major exosomal proteins including Hsc70 and 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 GM1 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.

Chapter 2

Parallel reductions in stomatin and Na,K-ATPase through the exosomal pathway during reticulocyte maturation in dogs: stomatin as a genotypic and phenotypic marker of high K^+ and low K^+ red cells

Introduction

Dogs are divided into two phenotypes based upon their erythrocyte cation concentrations (Inaba, 2000; Maede *et al.*, 1983), namely HK and LK red cell phenotypes. As described in the General Introduction, the HK and LK phenotype is controlled by the putative genotypes, *hk/hk* for HK dogs and *hk/lk* and *lk/lk* for LK dogs, although the gene responsible for these genotypes/phenotypes is unknown.

Dominant inheritance of high Na⁺ and low K⁺ concentrations in LK dogs (Maede and Inaba, 1985) is a condition similar to that seen in OHSt in humans (An and Mohandas, 2008; Stewart, 2004), although dogs usually have no abnormality in erythrocyte shape. OHSt is a heterogeneous group of disorders characterized by massively increased permeability of the affected red cells to monovalent cations. This results in overhydration, hemolytic anemia with stomatocytosis, an elevated mean corpuscular volume, a reduced mean corpuscular hemoglobin concentration, and increased glycolysis to fuel the Na,K-ATPase activity (Bruce *et al.*, 2009; Stewart, 2004). Interestingly, HK erythrocytes also display some of the characteristics of OHSt; HK cells have a shortened lifespan, increased osmotic fragility, increased mean corpuscular volume and a reduced mean corpuscular hemoglobin concentration, all suggesting an increase in cell water content (Maede *et al.*, 1983). HK erythrocytes also show an increase in glycolytic activity, which provides ATP, and persistence of immature enzymes. This enables the maintenance of increased Na,K-ATPase activity and a high intracellular K⁺ concentration (Inaba and Maede, 1989; Maede and Inaba, 1987). Thus, HK and LK erythrocytes in dogs have both shared and different characteristics to those in the

OHSt phenotype.

In human OHSt, stomatin is deficient in the erythrocyte membrane (Fricke *et al.*, 2003; Lande *et al.*, 1982). Stomatin is a ubiquitous lipid raft-associated membrane protein present in both erythrocytes and epithelial cells (Morrow and Parton, 2005; Salzer and Prohaska, 2001; Stewart, 2004) with a unique hairpin-loop topology (Hieble-Dirschmied *et al.*, 1991), and may function to regulate both cation transport and a stretch- or pressure-sensitive system (Fricke *et al.*, 2003; Martinez-Salgado *et al.*, 2007; Price *et al.*, 2004; Stewart *et al.*, 1992). A recent study shows that stomatin also enhances the transport of dehydroascorbic acid by GLUT1 (glucose transporter 1) in erythrocytes (Montel-Hagen *et al.*, 2008). Interestingly, stomatin and other lipid raft-associated proteins are sorted into exosomes during reticulocyte maturation and induce phenotypic changes in erythrocytes (de Gassart *et al.*, 2003; Rabesandratana *et al.*, 1998), and it occurs also in canine reticulocytes as demonstrated in Chapter 1. These findings suggested that canine HK/LK red cell phenotypes are somehow related to the expression of stomatin in erythroid cells.

In Chapter 2, several analyses were done to examine this hypothesis. We analyzed the levels of stomatin, Na,K-ATPase, and some other membrane proteins in erythrocyte membranes from HK and LK dogs from two independent families and from genetically independent control LK dogs, and found a marked difference in stomatin content between the *hk/hk*, *hk/lk*, and *lk/lk* genotypes.

Materials and Methods

Dogs

The dogs used were from two families of Japanese mongrel (a mixed breed of Japanese Shiba; Fig. 5, dog numbers 1–8). Some Beagle dogs were also used as controls (dog numbers 9–12). These dogs, except for one HK dog (number 2) that had mild anemia due to chronic babesiosis, were clinically healthy. Hematological parameters for their red cells were all within reference ranges, although the HK red cells had a mean corpuscular volume slightly larger than that of the LK red cells, as demonstrated previously (Maede *et al.*, 1983). All experimental procedures met with the approval of the Laboratory Animal Experimentation Committee, Graduate School of Veterinary Medicine, Hokkaido University.

Antibodies

The antibodies used were described in the Chapter 1.

Preparation of red cell membranes and analyses of proteins

Preparation of red cell membrane ghosts, SDS-PAGE, and immunoblotting were done as described in Chapter 1. The cholesterol concentration of the membranes was also determined using the cholesterol E test Wako kit (Wako Pure Chemical Industries).

Experimental reticulocytosis in dogs, separation and culture of reticulocytes

These were performed basically as described in the previous chapter. Briefly, reticulocytosis

was induced in LK and HK dogs, weighing 8–10 kg. Reticulocytes were separated and cultured as described. At the indicated times, cells were collected and the membranes and vesicles released from the reticulocytes (exosomes) were obtained by differential centrifugation.

Cloning of stomatin cDNAs from HK and LK dogs

Canine stomatin cDNA was isolated and cloned from bone marrow cell cDNAs obtained from a Beagle dog (LK erythrocytes), and those from a HK dog. In brief, the partial cDNA fragment was isolated by PCR amplification using the following primer pair: hStmp1 (forward: 5'-GGTCGCATTTTACAAGGAGGAG-3' for nt 285 → 306) and hStmp2 (reverse: 5'-CTTCTGCAGCCATAGCTCTCTG-3' for nt 690 ← 711) designed from the human stomatin cDNA sequence (GenBank accession number NM_004099) as described previously (Ito *et al.*, 2006). Using gene-specific primers (cStmp1 (forward), 5'-CGTGCACCGACAGCTTCATCAAG-3' for nt 327 → 349; cStmp2 (reverse), 5'-TCCAGGGTGCACTGCATGTTATG-3' for nt 584 ← 606) synthesized according to the sequence obtained for the fragment, 5'- and 3'-rapid amplification of cDNA ends reactions were performed to determine the sequences of the 5'- and 3'-stretched canine stomatin cDNA. Consequently, a cDNA clone of 2,732 bp encoding a canine stomatin polypeptide consisting of 284 amino acid residues with a theoretical molecular weight of 31,207 Da was obtained (GenBank accession number AB467286). For further comparison of the coding region sequences from several HK and LK dogs, cDNAs were obtained from total RNA isolated from peripheral blood reticulocytes, and amplified by PCR using the following primer pair:

cStmp3 (forward: 5'-GGATTCCTCGCGGCAGGGCT-3' for nt 7 → 26) and cStmp4 (reverse: 5'-AACCTTTGTTGCTAGGGTCG-3' for nt 1,082 ← 1,101). These cDNAs were then sequenced using a CEQ DTCS Quick start kit (Beckman Coulter Inc.) on a CEQ 8000 DNA sequencer (Beckman Coulter Inc.).

Results

Stomatin levels in HK and LK dog erythrocytes

The canine stomatin polypeptide deduced from the isolated cDNA sequence (GenBank accession number AB467286) consists of 284 amino acid residues and has very high amino acid sequence similarity to human stomatin. Two possible palmitoylation sites (Cys³⁰ and Cys⁸⁷) and a proline residue in the hydrophobic domain of the N-terminal region are conserved, suggesting that it has a hairpin-loop topology, with the N- and C-terminal sequences being cytoplasmic (Fig. 6), as suggested for human stomatin (Hieble-Dirschmied *et al.*, 1991; Kadurin *et al.*, 2009). Sequence analysis of the cDNA fragments corresponding to the coding region of stomatin from several dogs (Fig. 5) showed the presence of a polymorphism, with nucleotide substitutions at codons 60, 242, and 255, corresponding to Tyr⁶⁰, Thr²⁴², and Thr²⁵⁵ (GenBank accession number AB467287). However, these substitutions were silent and showed no linkage with the HK/LK red cell phenotype. Based on these results, we quantified the stomatin content of HK and LK dog erythrocytes using a monoclonal antibody to human stomatin (Hieble-Dirschmied *et al.*, 1991).

Erythrocyte membranes from HK dogs contained higher levels of the Na,K-ATPase α -subunit than human erythrocytes, but those from LK dogs with the *hk/lk* and *lk/lk* genotypes showed no detectable signals (Fig. 7A, and Inaba and Maede, 1986). Immunoblot analysis showed that the GARP-50 monoclonal antibody reacted with the 31-kDa stomatin polypeptide in erythrocyte membranes from both HK dogs and humans, and that the stomatin content of HK erythrocytes was comparable with that in human erythrocytes.

In contrast, LK erythrocytes, particularly in LK cells from dogs with the *lk/lk* genotype, exhibited a remarkable reduction in stomatin content. Quantification of the relative abundance of stomatin by densitometry scanning within the linear range of the immunoblot signals (Fig. 7B) demonstrated that the stomatin levels of the *lk/lk* and *hk/lk* erythrocytes were less than 2% and $12\% \pm 9.2\%$, respectively, of the mean value of those of the *hk/hk* cells (Fig. 7C). We can rule out the possibility that this observation was the result of decreased immunorecognition by the antibody, caused by a mutation within the stomatin from LK dogs, since no difference was found in the amino acid sequences. Several other LK dogs also gave results that suggested a lack of both stomatin and the Na,K-ATPase α -subunit in their erythrocytes (data not shown).

Since stomatin is a lipid raft-associated protein (Rabesandratana *et al.*, 1998; Salzer and Prohaska, 2001), we also analyzed several other markers of lipid rafts in the erythrocytes, including flotillin-1 and -2, both of which are lipid raft-associated proteins (Morrow and Parton, 2005), and cholesterol. We found no significant difference in the levels of these molecules between the different genotypes (Figs. 7A and 7C).

These data demonstrate that the reduced stomatin levels in the LK erythrocytes are not related to the lipid raft content, and that the stomatin content of erythrocyte membranes is a suitable genotypic marker for red cell HK/LK phenotypes.

Reduction in stomatin content during reticulocyte maturation in dogs

Previous studies have shown that the maturation of reticulocytes into erythrocytes is associated with major alterations in membrane structure and function, including changes in

Na,K-ATPase (Inaba and Maede, 1986; Maede and Inaba, 1985) and TfR levels (Johnstone *et al.*, 1989; Pan and Johnstone, 1983). It is also known that, in humans, stomatin and some lipid raft-associated proteins are extruded by exosomes during reticulocyte maturation and that this induces the phenotypic changes seen in erythrocytes (de Gassart *et al.*, 2003; Rabesandratana *et al.*, 1998). To test if this is the case for reticulocyte maturation in dogs, we examined for changes in stomatin content, as well as for alterations in the content of other membrane proteins in HK (*hk/hk*) and LK (*lk/lk*) dogs.

Figure 8 shows a typical result obtained after immunoblot analysis of reticulocyte and erythrocyte membranes from the same dog. LK dog reticulocytes (with a reticulocyte count of 98%) contained the Na,K-ATPase α -subunit at readily detectable levels, although it comprised only about 20% of that seen in HK erythrocytes (Figs. 8A and 8B). The α -subunit content of HK dog reticulocytes (reticulocyte count of 55%) was about twice that of the HK erythrocytes, and would be expected to be even greater if we had obtained a reticulocyte preparation with higher purity. These data are consistent with our previous estimations (Inaba and Maede, 1986). A very similar profile was obtained for the stomatin content of HK and LK reticulocytes. The stomatin content of LK reticulocytes was about 14% of that of HK erythrocytes, and HK reticulocytes had a higher stomatin content than erythrocytes, suggesting a reduction in stomatin content during reticulocyte maturation (Figs. 8A and 8B). These are consistent with observations in Chapter 1. Immunoblotting also showed that GLUT1, which is known to interact with stomatin in human erythrocytes (Montel-Hagen *et al.*, 2008), was present in the reticulocytes of both HK and LK dogs, whereas it was absent in their erythrocytes. As seen for stomatin and the Na,K-ATPase α -subunit, GLUT1 levels were

higher in HK cells.

In contrast, TfR levels in HK reticulocytes were compatible with those in LK reticulocytes (taking into account the different reticulocyte counts mentioned above), but TfR was completely absent from the erythrocytes of both phenotypes (Fig. 8). In addition, the flotillin-2 content was comparable in both HK and LK reticulocytes, as observed for HK and LK erythrocytes (Fig. 7). Similar differences between reticulocytes and erythrocytes were seen for all the membrane proteins analyzed in several other reticulocyte preparations from both HK and LK dogs, though they had lower reticulocyte counts (data not shown).

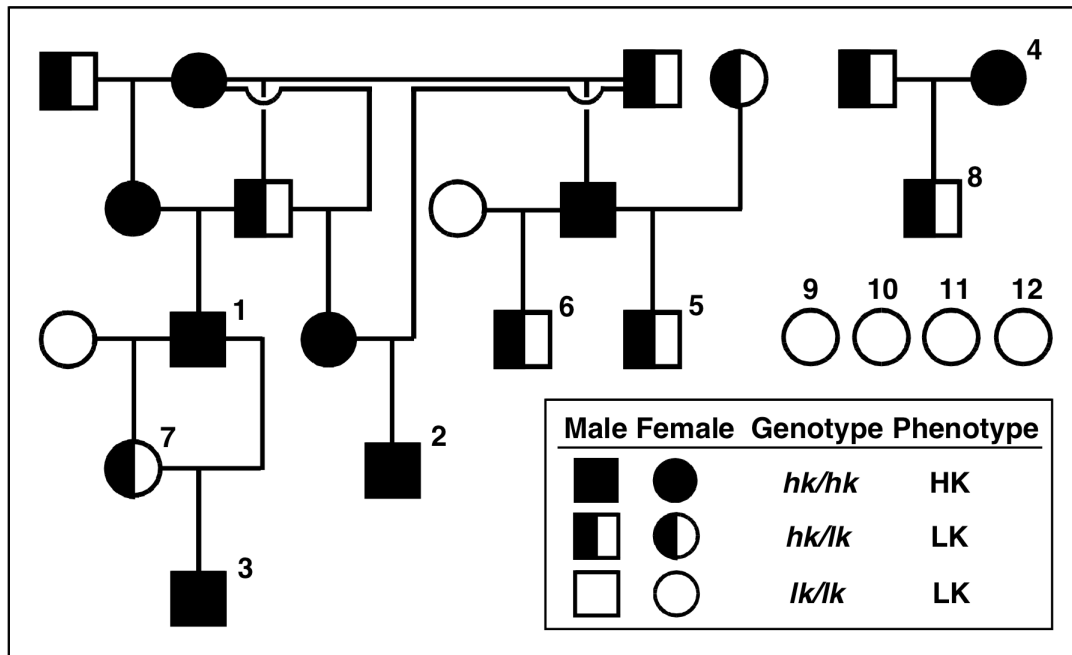


Figure 5. Pedigree of dogs including HK and LK dogs.

Two families consisting of several generations of Japanese mongrel dogs used in this study are shown. Dogs possessing HK erythrocytes with the *hk/hk* genotype (numbers 1–4) and LK erythrocytes with the *hk/lk* genotype (numbers 5–8) were studied. Beagles with LK erythrocytes due to the *lk/lk* genotype (numbers 9–12) were used as controls. The phenotypes and genotypes for erythrocyte Na⁺ and K⁺ concentrations are indicated in the inset.

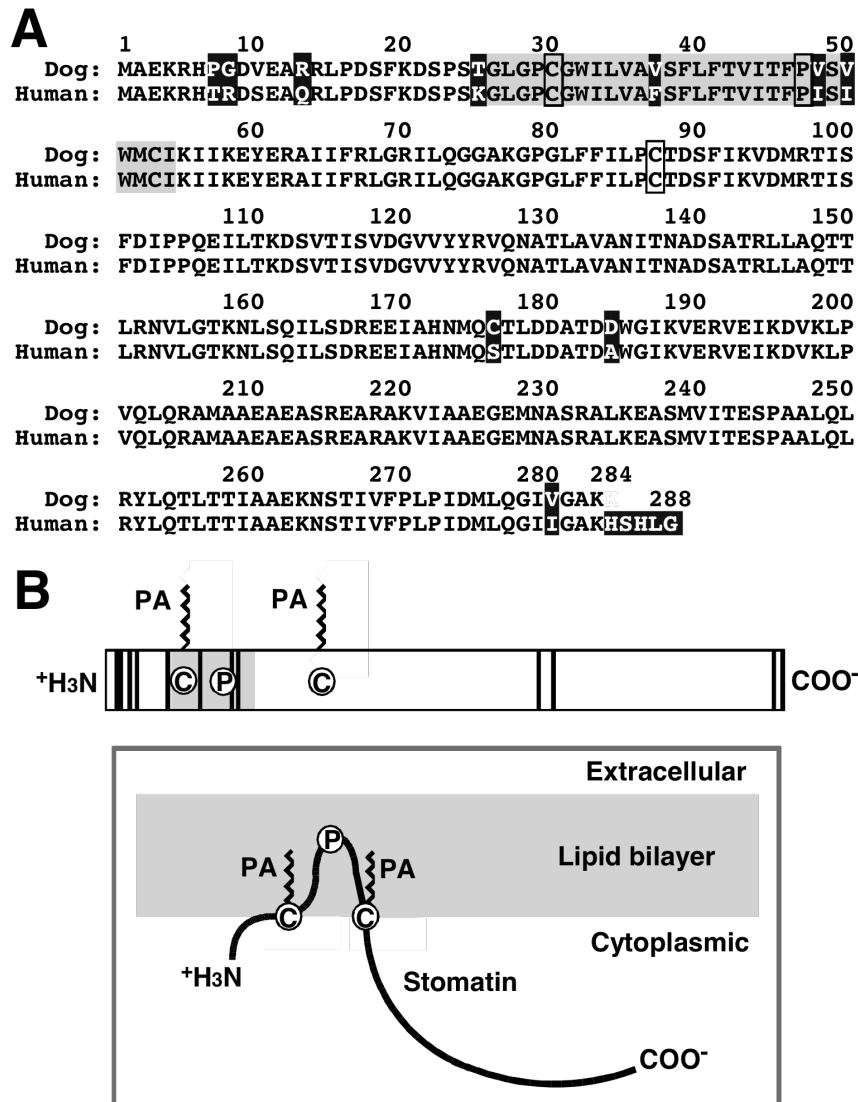


Figure 6. Deduced amino acid sequence and proposed membrane topology of canine stomatin.

A. Canine stomatin cDNAs were isolated from the bone marrow cells of LK and HK dogs. The amino acid sequence of canine stomatin was deduced from the obtained cDNA sequence (GenBank accession number AB467286) and is shown aligned with the amino acid sequence of human stomatin (GenBank accession number NM_004099). Non-identical amino acids are highlighted. Possible conserved palmitoylation sites (Cys³⁰ and Cys⁸⁷) and the conserved Pro⁴⁷ are boxed, and a hydrophobic segment (amino acids 26–54) is shaded. B. Linear schematic (top) and possible membrane topology based on previous studies (Hieble-Dirschmied et al., 1991; Kadurin et al., 2009) (bottom) of canine stomatin, illustrating the hairpin-loop structure (see text). PA associated with C = Cys residues accessible to palmitoylation; P = conserved Pro residue.

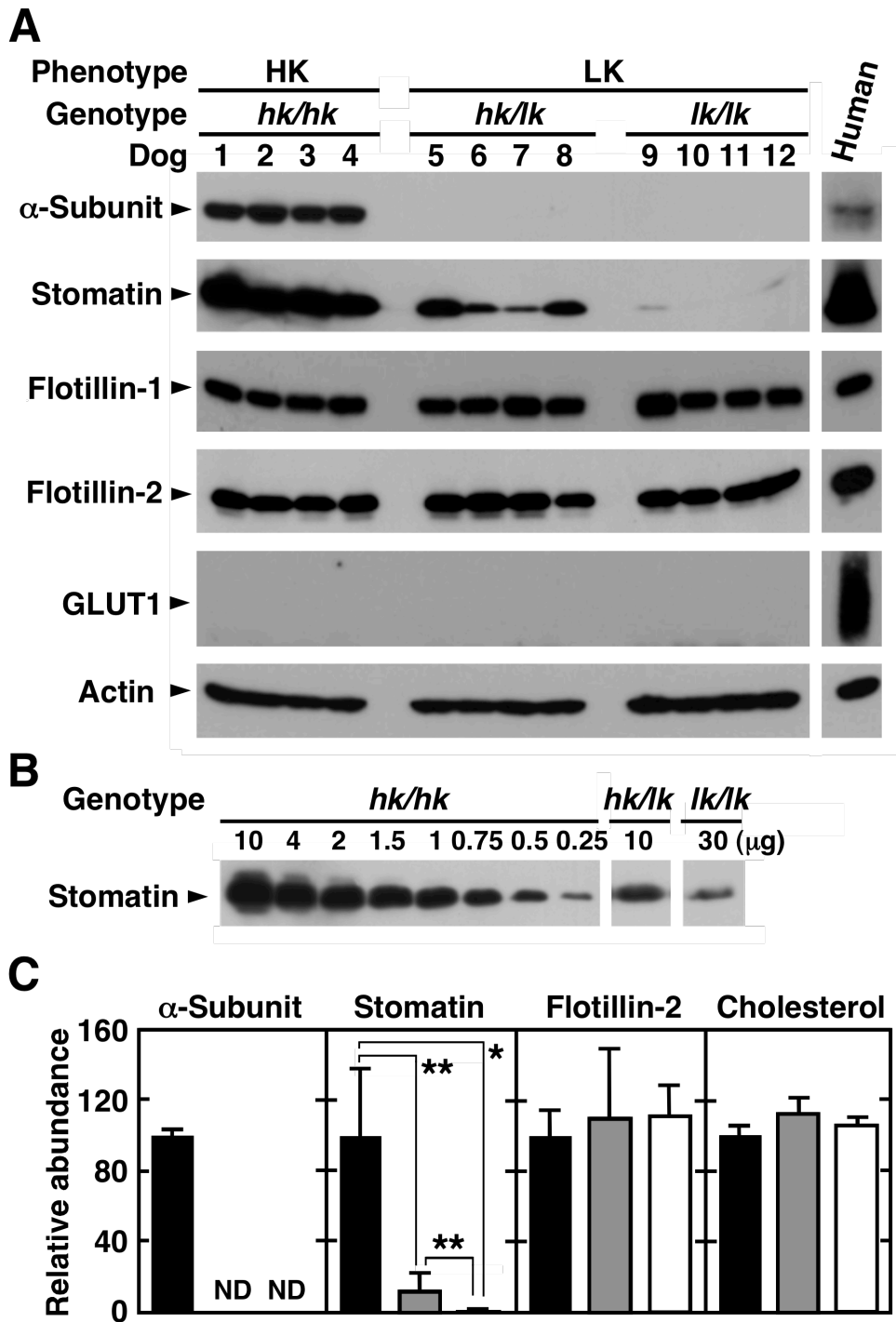


Figure 7. Content of stomatin, and several other proteins, in HK and LK erythrocytes.

Continued to the following page.

Figure 7. Content of stomatin, and several other proteins, in HK and LK erythrocytes.

A. Typical immunoblots of stomatin (31 kDa), the Na,K-ATPase α -subunit (96 kDa), flotillin-1 (49 kDa), flotillin-2 (41 kDa), and actin (43 kDa) in erythrocyte membranes from HK (genotype *hk/hk*, numbers 1–4 in Fig. 1) and LK (genotypes *hk/lk* and *lk/lk*, numbers 5–8 and 9–12, respectively) dogs as well as human erythrocytes are shown. **B.** Typical immunoblots for stomatin quantification in which the erythrocyte membrane proteins from dog numbers 1 (*hk/hk*), 7 (*hk/lk*), and 12 (*lk/lk*) were analyzed. **C.** Erythrocyte levels of the indicated proteins, plus the actin control, were obtained by densitometric scanning of the immunoblots. The relative abundance of these proteins, and cholesterol, in erythrocyte membranes was normalized against actin and expressed as percentages compared with the mean values for HK dogs. Data represent the mean \pm S.D. (n = 4, **p* < 0.005, ***p* < 0.05). Actual cholesterol contents in erythrocyte membranes (mg/mg protein) were 0.43 ± 0.022 , 0.47 ± 0.038 , and 0.44 ± 0.015 (the mean \pm S.D., n = 4 for each) for *hk/hk*, *hk/lk*, and *lk/lk* dogs, respectively.

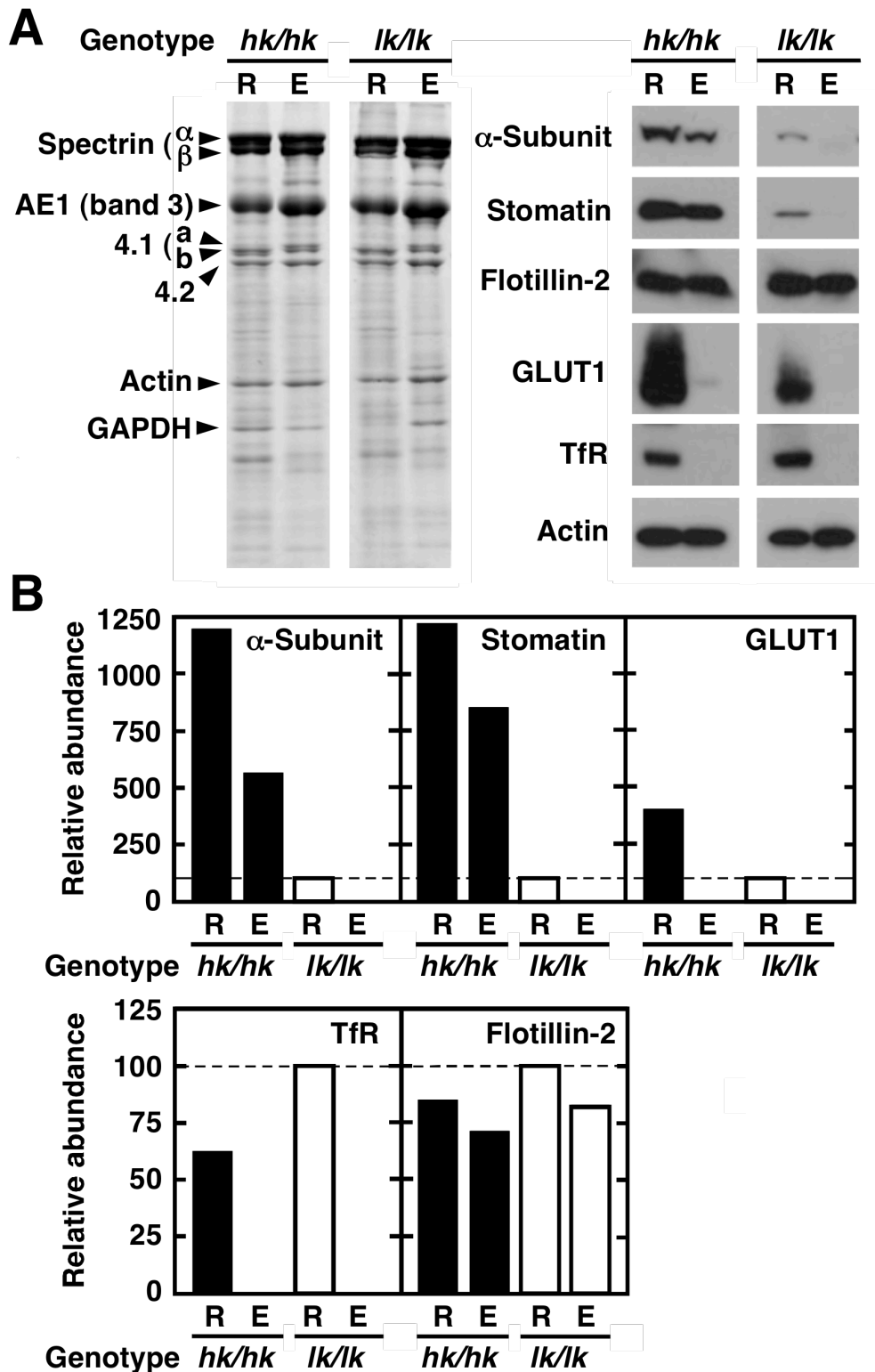


Figure 8. Comparison of stomatin and other membrane proteins in the reticulocytes and erythrocytes of HK and LK dogs.

Continued to the following page.

Figure 8. Comparison of stomatin and other membrane proteins in the reticulocytes and erythrocytes of HK and LK dogs.

A. Reticulocyte and erythrocyte membranes from HK (genotype *hk/hk*, number 4 in Fig. 5) and LK (genotype *lk/lk*, number 12) dogs were analyzed by SDS-PAGE followed by Coomassie brilliant blue staining (left) and immunoblotting (right) for stomatin, the Na,K-ATPase α -subunit, flotillin-2, GLUT1 (50–60 kDa), TfR (95 kDa), and actin. The major membrane proteins are indicated by arrowheads in the left panel. GAPDH = glyceraldehyde 3-phosphate dehydrogenase. **B.** The amounts of the various proteins were quantified using densitometry, and normalized relative to actin as described in the legend for Fig. 7. Levels are expressed as percentages relative to the content of *lk/lk* reticulocytes.

Discussion

Dogs, in general, have erythrocytes of the LK phenotype, characterized by high Na^+ and low K^+ concentrations (Chan *et al.*, 1964) due to the loss of Na,K-ATPase during reticulocyte maturation (Inaba and Maede, 1986; Maede and Inaba, 1985). HK dogs retain Na,K-ATPase in their erythrocytes due to the high activity of the enzyme. One of the major new findings of the present study is that, in dogs, stomatin levels in the reticulocyte plasma membrane show a marked reduction during maturation that paralleled the decrease in Na,K-ATPase levels (Fig. 8). Consequently, the LK erythrocytes of *lk/lk* dogs have significantly reduced levels of stomatin compared with HK erythrocytes (Fig. 7). Decreases in stomatin levels during reticulocyte maturation appear to occur in both HK (genotype *hk/hk*) and LK (genotype *hk/lk*) dogs, but their erythrocytes display different stomatin levels. The stomatin level in erythrocytes is most likely dependent on that in the precursor cells. Taking this into account, the amount of stomatin in LK dog reticulocytes with the *lk/lk* genotype was thus much less than that in HK dog reticulocytes (Fig. 8). Hence, the relative abundance of stomatin in erythrocytes is a characteristic of the genotype for the HK/LK red cell phenotype and, therefore, is a suitable marker for genotyping LK dogs.

The role of the exosomal pathway in reticulocyte maturation is to discard obsolete proteins such as TfR, as they are not required by erythrocytes, and to aid the extensive remodeling of the plasma membrane needed to acquire the biconcave shape characteristic of erythrocytes (Blanc *et al.*, 2006; Johnstone *et al.*, 1987). However, the molecular basis of protein sorting during exosome formation, and the role, if any, of lipid raft-associated proteins,

including stomatin, is still unclear.

Stomatin is the founder of the stomatin protein family in ubiquitous cells and is absent from the red cells of patients with OHSt, a pathological condition characterized by increased permeability of the cells to Na⁺ and K⁺ and a stomatocytic morphology (Lande *et al.*, 1982; Stewart, 2004). Several interactions of stomatin with membrane proteins, notably with the acid sensing ion channels (Price *et al.*, 2004), GLUT1 (Zhang *et al.*, 2001), and other various findings have implicated stomatin in the regulation of ion/solute transporters and the stretch- or pressure-sensitive system (Fricke *et al.*, 2003; Martinez-Salgado *et al.*, 2007; Price *et al.*, 2004; Stewart *et al.*, 1992). Together with the apparent relationship between the HK/LK genotypes and erythrocyte stomatin content described above, it is of interest to suppose that stomatin interacts with the Na,K-ATPase and cooperates with the enzyme during the maturation process of canine reticulocytes through the exosomal pathway as discussed in the previous chapter. Our preliminary study by immunoprecipitation of stomatin in Triton-soluble fraction from reticulocyte membranes using the anti-stomatin antibody showed no α -subunit of Na,K-ATPase in the immunoprecipitate. However, this result itself does not rule out a possibility of the interaction. This idea requires proof, since the regulation of stomatin expression itself, or some other relevant protein, might affect the HK phenotype representing immature erythroid precursor cells with an autosomal recessive inheritance (Inaba, 2000).

Considering that the relative abundance of erythrocyte stomatin in HK dogs is comparable with that in humans (Fig. 7), and that the red cells in OHSt have 2%–5% of the normal amount of stomatin (Fricke *et al.*, 2003), the reduction of stomatin in LK erythrocytes with the *lk/lk* genotype appears compatible with the situation in human OHSt. However, these

LK erythrocytes, i.e., normal dog erythrocytes, do not show overhydration and consequent stomatocytic change, since the cells do not suffer from a profuse membrane leakage of Na⁺ and K⁺ that is characteristic to all OHSt cases with stomatin deficiency (Fricke *et al.*, 2003). Also, the volume of dog erythrocytes is regulated mainly by coordination between the shrinkage-induced Na/H exchange and the swelling-induced K-Cl cotransporter (Adragna *et al.*, 2004; Parker *et al.*, 1990). These data confirm that the high intracellular Na⁺ and low intracellular K⁺ contents in LK dog erythrocytes are caused by a common mechanism, i.e., the lack of Na,K-ATPase, and that the mechanism is different from the mechanism that elicits high Na⁺ and low K⁺ concentrations in OHSt red cells. The data also suggests that stomatin deficiency plays no direct role in the etiology of OHSt (Fricke *et al.*, 2004; Zhu *et al.*, 1999). In this context, a recent study has identified amino acid substitutions in the Rhesus (Rh)-associated glycoprotein as the cause for the monovalent cation leak in OHSt (Bruce *et al.*, 2009). In addition, it is noted that the reduction profile observed for stomatin, Na,K-ATPase, and GLUT1 during reticulocyte maturation is basically the same in both *hk/hk* and *lk/lk* dogs, while TfR showed different features, as described above (Fig. 8). This discrepancy may indicate that the immature red cell phenotype in HK dogs appears in the very early stages of erythroid differentiation, but not in the erythroblast stage (Fig. 9 in General Conclusion), since a recent study shows that little change in the surface expression of TfR (CD71) is observed between early- and late-stage erythroblasts (Chen *et al.*, 2009).

In conclusion, this study shows that the maturation of canine reticulocytes is associated with a parallel reduction in stomatin and Na,K-ATPase levels. Thus, the erythrocyte content of these two proteins is a characteristic of particular HK/LK genotypes

and phenotypes. Stomatin, in particular, is a suitable marker for the genotyping of LK dogs.

Summary

Dogs can be divided into two genetic groups (a minor HK phenotype and a major LK phenotype) based on erythrocyte monovalent cation concentrations, which are controlled by the putative *hk* and *lk* allelic genes. HK dogs retain Na,K-ATPase in their erythrocytes due to the high activity of the enzyme in their precursor cells, whereas total loss of reticulocyte Na,K-ATPase occurs in LK dogs. The studies in this chapter report that the levels of the lipid raft-associated membrane protein stomatin decrease in parallel with those of Na,K-ATPase during reticulocyte maturation due to its extrusion in exosomes. The stomatin content of HK reticulocytes is higher than that of LK reticulocytes, and remains in the erythrocytes at levels compatible with that in human erythrocytes. However, it is almost absent from LK erythrocytes with the *lk/lk* genotype; similar to the deficiency seen in human red cells with overhydrated stomatocytosis. LK erythrocytes from *hk/lk* genotype dogs show reduced, but not negligible, levels of stomatin. These results indicate that the erythrocyte stomatin level is a suitable genotypic marker for the HK/LK red cell phenotype, and suggests a functional association between stomatin and Na,K-ATPase. The absence of morphological abnormalities in the erythrocytes of stomatin-deficient LK dogs also confirms that stomatin deficiency and stomatocytic shape change are independent from each other.

General Conclusion

Reticulocyte maturation in dogs is associated with marked changes in contents of various membrane proteins. The present study showed that this membrane remodeling process in dogs involves the selective extrusion of Na,K-ATPase and a lipid raft-associated protein stomatin via the exosome pathway, as well as several other proteins incorporated into reticulocyte exosomes such as Hsc70 and TfR (Fig. 9). In exosomes, the Na,K-ATPase, TfR, and about half of the stomatin were found in detergent-soluble fraction that was distinct from the lipid raft-derived fraction. Moreover, Na,K-ATPase and a portion of stomatin were distributed differentially to Hsc70, TfR, and ganglioside GM1 in vesicles separated by sucrose density gradient centrifugation. This study also showed that the levels of stomatin decrease in parallel with those of Na,K-ATPase during reticulocyte maturation. The stomatin content of HK reticulocytes is higher than that of LK reticulocytes, and remains in the erythrocytes at levels compatible with that in human erythrocytes. However, it is almost absent from LK erythrocytes with the *lk/lk* genotype; similar to the deficiency seen in human red cells with overhydrated stomatocytosis. LK erythrocytes from *hk/lk* genotype dogs show reduced, but not negligible, levels of stomatin.

These findings demonstrate that a heterogeneous group of exosomes participates in membrane remodeling during reticulocyte maturation in dogs and suggest that Na,K-ATPase and stomatin can associate together in non-lipid raft segments of the membrane in reticulocytes and subsequently in some population of exosomes, leading to their parallel reduction during reticulocyte maturation (Fig. 9). The findings of the present study also

indicate that the erythrocyte stomatin level is a suitable genotypic marker for the HK/LK red cell phenotype, and suggests that again a functional association between stomatin and Na,K-ATPase. The absence of morphological abnormalities in the erythrocytes of stomatin-deficient LK dogs also confirms that stomatin deficiency and stomatocytic shape change are independent from each other. The mechanisms remain to be solved include those for selective incorporation of Na,K-ATPase and stomatin and for HK/LK phenotypic diversity and its causative gene regulation in dogs.

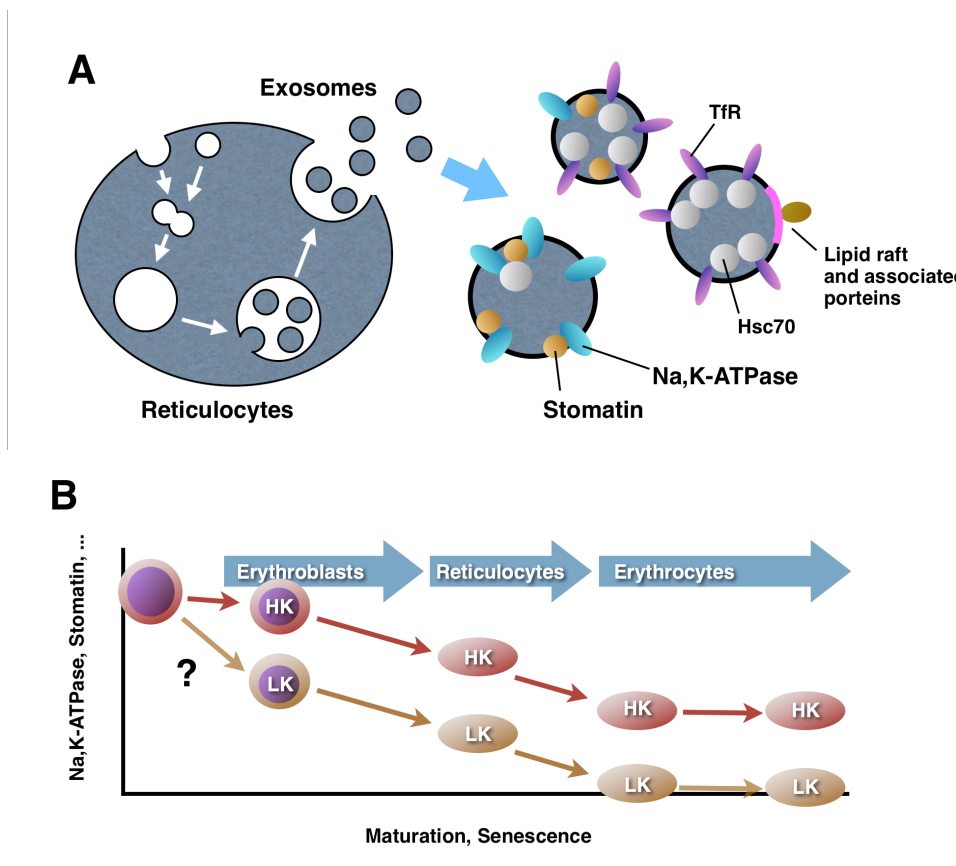


Figure 9. Summary for the findings of the present study

A. Canine reticulocyte exosomes consist of heterogeneous vesicles with different protein and lipid components. **B.** Changes in membrane protein contents, including Na,K-ATPase and stomatin, during erythroid cell maturation in HK and LK dogs.

References

- Adragna, N. C., Di Fulvio, M. and Lauf, P. K. (2004) Regulation of K-Cl cotransport: from function to genes. *J. Membrane Biol.* **201**, 109-137.
- An, X. and Mohandas, N. (2008) Disorders of red cell membrane. *Br. J. Haematol.* **141**, 367-375.
- Arashiki, N., Otsuka, Y., Ito, D., Yang, M., Komatsu, T., Sato, K. and Inaba, M. (2010) The covalent modification of spectrin in red cell membranes by the lipid peroxidation product 4-hydroxynonenal. *Biochem. Biophys. Res. Commun.* **391**, 1543-1547
- Blanc, L., de Gassart, A., Géminard, C., Bette-Bobillo, P. and Vidal, M. (2005) Exosome release by reticulocytes. An integral part of the red blood cell differentiation system. *Blood Cells Mol. Dis.* **35**, 21-26.
- Blostein, R. and Grafova, E. (1990) Decrease in Na⁺-K⁺-ATPase associated with maturation of sheep reticulocytes. *Am. J. Physiol.* **259**, C241-C250.
- Bruce L. J., Guizouarn, H., Burton, N. M., Gabillat, N., Poole, J., Flatt, J. F., Brady, R. L., Borgese, F., Delaunay, J. and Stewart, G. W. (2009) The monovalent cation leak in overhydrated stomatocytic red blood cells results from amino acid substitution in the Rh-associated glycoprotein. *Blood* **113**, 1350-1357.
- Chan, P. C., Calabrese, V. and Theil, L. S. (1964) Species differences in the effect of sodium and potassium ions on the ATPase of erythrocyte membranes. *Biochim. Biophys. Acta* **79**, 424-426.
- Chaput, N., Flament, C., Viaud, S., Taieb, J., Roux, S., Spatz, A., André, F., LePecq, J. B., Boussac, M., Garin, J., Amigorena, S., Théry, C. and Zitvogel, L. (2006) Dendritic cell derived-exosomes: biology and clinical implementations. *J. Leukoc. Biol.* **80**, 471-478.
- Chen, K., Liu, J., Heck, S., Chasis, J. A., An, X. and Mohandas, N. (2009) Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 17413-17418.
- de Gassart, A., Géminard, C., Février, B., Raposo, G. and Vidal, M. (2003) Lipid raft-associated protein sorting in exosomes. *Blood* **102**, 4336-4344.

- Denzer, K., van Eijk, M., Kleijmeer, M. J., Jakobson, E., de Groot, C. and Geuze, H. J. (2000) Follicular dendritic cells carry MHC class II-expressing microvesicles at their surface. *J. Immunol.* **165**, 1259-1265.
- Fricke, B., Argent, A. C., Chetty, M. C., Pizzery, A. R., Turner, E. J., Ho, M. M., Iolascon, A., von Düring, M. and Stewart, G. W. (2003) The “stomatin” gene and protein in overhydrated hereditary stomatocytosis. *Blood* **102**, 2268-2277.
- Fricke, B., Jarvis, H. G., Reid, C. D. L., Aguilar-Martinez, P., Robert, A., Quittet, P., Chetty, M., Pizzery, A., Cynober, T., Lande, W. F., Mentzer, W. C., von Düring, M., Winter, S., Delaunay, J. and Stewart, G. W. (2004) Four new cases of stomatin-deficient hereditary stomatocytosis syndrome: association of the stomatin-deficient cryohydrocytosis variant with neurological dysfunction. *Br. J. Haematol.* **125**, 796-803.
- Géminard, C., Nault, F., Johnstone, R. M. and Vidal, M. (2001) Characteristics of the interaction between Hsc70 and the transferrin receptor in exosomes released from reticulocyte maturation. *J. Biol. Chem.* **276**, 9910-9916.
- Géminard, C., de Gassart, A., Blanc, L. and Vidal, M. (2004) Degradation of AP2 during reticulocyte maturation enhances binding of hsc70 and Alix to a common site on TFR for sorting into exosomes. *Traffic* **5**, 181-193.
- Haas, A. L. (1991) Ubiquitin-mediated processes in erythroid cell maturation. *Adv. Exp. Med. Biol.* **307**, 191-205.
- Hieble-Dirschmied, C. M., Entler, B., Glotzmann, C., Maurer-Fogy, I., Stratowa, C. and Prohaska, R. (1991) Cloning and nucleotide sequence of cDNA encoding human erythrocyte band 7 integral membrane protein. *Biochim. Biophys. Acta* **1090**, 123-124.
- Inaba, M. (2000) Red blood cell membrane defects. pp. 1012-1019. *In: Schalm's Veterinary Hematology*, 5th ed. (Feldman, B. F., Zinkl, J. G. and Jain, N. C. eds.), Lippincott Williams & Wilkins, Philadelphia.
- Inaba, M. and Maede, Y. (1986) Na,K-ATPase in dog red cells. Immunological identification and maturation-associated degradation by the proteolytic system. *J. Biol. Chem.* **261**, 16099-16105.
- Inaba, M. and Maede, Y. (1989) Inherited persistence of immature type pyruvate kinase and hexokinase isozymes in dog erythrocytes. *Comp. Biochem. Physiol.* **92B**, 151-156.
- Inaba, M., Yawata, A., Koshino, I., Sato, K., Takeuchi, M., Takakuwa, Y., Manno, S., Yawata,

- Y., Kanzaki, A., Sakai, J., Ban, A., Ono, K. and Maede, Y. (1996) Defective anion transport and marked spherocytosis with membrane instability caused by hereditary total deficiency of red cell band 3 in cattle due to a nonsense mutation. *J. Clin. Invest.* **97**, 1804-1817.
- Ito, D., Koshino, I., Arashiki, N., Adachi, H., Tomihari, M., Tamahara, S., Kurogi, K., Amano, T., Ono, K. and Inaba, M. (2006) Ubiquitylation-independent ER-associated degradation of an AE1 mutant associated with dominant hereditary spherocytosis in cattle. *J. Cell Sci.* **119**, 3602-3612.
- Johnstone, R. M. (2006) Exosomes biological significance: A concise review. *Blood Cells Mol. Dis.* **36**, 315-321.
- Johnstone, R. M., Asam, M., Hammond, J. R., Orr, L. and Turbide, C. (1987) Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J. Biol. Chem.* **262**, 9412-9420.
- Johnstone, R. M., Bianchini, A. and Teng, K. (1989) Reticulocyte maturation and exosome release: transferrin receptor containing exosomes shows multiple plasma membrane functions. *Blood* **74**, 1844-1851.
- Kadurin, I., Huber, S. and Gründer, S. (2009) A single conserved proline residue determines the membrane topology of stomatin. *Biochem. J.* **418**, 587-594.
- Lande, W. M., Thiemann, P. W. and Mentzer, W. M. (1982) Missing band 7 membrane protein in two patients with high Na, low K erythrocytes. *J. Clin. Invest.* **70**, 1273-1280.
- Maede, Y. and Inaba, M. (1985) (Na,K)-ATPase and ouabain binding in reticulocytes from dogs with high K and low K erythrocytes and their changes during maturation. *J. Biol. Chem.* **260**, 3337-3343.
- Maede, Y. and Inaba, M. (1987) Energy metabolism in canine erythrocytes associated with inherited high Na⁺- and K⁺-stimulated adenosine triphosphatase activity. *Am. J. Vet. Res.* **48**, 114-118.
- Maede, Y., Inaba, M. and Taniguchi, N. (1983) Increase of Na-K-ATPase activity, glutamate, and aspartate uptake in dog erythrocytes associated with hereditary high accumulation of GSH, glutamate, glutamine, and aspartate. *Blood* **61**, 493-499.
- Martinez-Salgado, C., Benckendorff, A. G., Chiang, L.-Y., Wang, R., Milenkovic, N., Wetzel, C., Hu, J., Stucky, C. L., Parra, M. G., Mohandas, N. and Lewin, G. R. (2007) Stomatin

- and sensory neuron mechanotransduction. *J. Neurophysiol.* **98**, 3802-3808.
- Montel-Hagen, A., Kinet, S., Manel, N., Mongellaz, C., Prohaska, R., Battini, J. L., Delaunay, J., Sitbon, M. and Taylor, N. (2008) Erythrocyte Glut1 triggers dehydroascorbic acid uptake in mammals unable to synthesize vitamin C. *Cell* **132**, 1039-1048.
- Morrow, I. C. and Parton, R. G. (2005) Flotillins and the PHB domain protein family: rafts, worms and anaesthetics. *Traffic* **6**, 725-740.
- Pan, B.-T. and Johnstone, R. M. (1983) Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell*, **33**, 967-977.
- Parker, J. C., McManus, T. J., Starke, L. C. and Gitelman, H. J. (1990) Coordinated regulation of Na/H exchange and [K-Cl] cotransport in dog red cells. *J. Gen. Physiol.* **96**, 1141-1152.
- Price, M. P., Thompson, R. J., Eshcol, J. O., Wemmie, J. A. and Benson, C. J. (2004) Stomatin modulates gating of acid-sensing ion channels. *J. Biol. Chem.* **279**, 53886-53891.
- Rabesandratana, H., Toutant, J.-P., Reggio, H. and Vidal, M. (1998) Decay-accelerating factor (CD59) are released within exosomes during in vitro maturation of reticulocytes. *Blood* **91**, 2573-2580.
- Salzer, U., Hinterdorfer, P., Hunger, U., Borcken, C. and Prohaska, R. (2002) Ca⁺⁺-dependent vesicle release from erythrocytes involves stomatin-specific lipid rafts, synexin (annexin VII), and sorcin. *Blood* **99**, 2569-2577.
- Salzer, U. and Prohaska, R. (2001) Stomatin, flotillin-1, and flotillin-2 are major integral proteins of erythrocyte lipid rafts. *Blood* **97**, 1141-1143.
- Stewart, G. W. (2004) Hemolytic disease due to membrane ion channel disorders. *Curr. Opin. Hematol.* **11**, 244-250.
- Stewart, G. W., Hepworth-Jones, B. E., Keen, J. N., Dash, B. C. J., Argent, A. C. and Casimir, C. M. (1992) Isolation of cDNA coding for an ubiquitous membrane protein deficient in high Na⁺, low K⁺ stomatocytic erythrocytes. *Blood* **79**, 1593-1601.
- van Niel, G., Porto-Carreiro, I., Simoes, S. and Raposo, G. (2006) Exosomes: a common pathway for a specialized function. *J. Biochem.* **140**, 13-21.
- Wilkinson, D. K., Turner, E. J., Parkin, E. T., Garner, A. E., Harrison, P. J., Crawford, M., Stewart, G. W. and Hooper, N. M. (2008) Membrane raft actin deficiency and altered Ca²⁺-induced vesiculation in stomatin-deficient overhydrated hereditary stomatocytosis.

Biochim. Biophys. Acta **1778**, 125-132.

Zhang, J.-Z., Abbud, W., Prohaska, R. and Ismail-Beigi, F. (2001) Overexpression of stomatin depresses GLUT-1 glucose transporter activity. *Am. J. Physiol. Cell Physiol.* **280**, 1277-1283.

Zhu, B. Y., Paszty, C., Turetsky, T., Tsai, S., Kuypers, F. A., Lee, G., Cooper, P., Gallagher, P. G., Stevens, M. E., Rubin, E., Mohandas, N. and Mentzer, W. C. (1999) Stomatocytosis is absent in “stomatin”-deficient murine red blood cells. *Blood* **93**, 2404-2410.

Acknowledgements

I express my sincere gratitude to Professor Mutsumi Inaba (Laboratory of Molecular Medicine, Graduate School of Veterinary Medicine) for his excellent supervision throughout this work.

I am also grateful to Professors Kazuhiro Kimura (Laboratory of Biochemistry), Associate Professor Tohru Yamamori (Laboratory of Radiation Biology), and Associate Professor Kota Sato (Laboratory of Molecular Medicine), all from Graduate School of Veterinary Medicine, for helpful discussion and critical reading the manuscript, Professor emeritus Yoshimitsu Maede and Associate Professor Masahiro Yamasaki (both from Laboratory of Internal Medicine) for discussion and encouragements, Professor Yasuhiro Kon, Associate Professor Yoshiharu Hashimoto, and Assistant Professor Osamu Ichii (all from Laboratory of Anatomy) for technical help in electron microscopy. I also thank Dr. Rainer Prohaska (University of Vienna) and Professor emeritus Masayuki Saito (Hokkaido University) for kindly providing GARP-50 and anti-GLUT1 antibodies, respectively, Drs. Yayoi Otsuka, Nobuto Arashiki, and Hirokazu Adachi and all members of the Laboratory of Molecular Medicine for their encouragements and excellent technical assistance.

Abstract in Japanese (要 旨)

Canine Reticulocyte Exosomes: Parallel and Selective Extrusion of Na,K-ATPase and Stomatin during Reticulocyte Maturation in Dogs with HK and LK Red Cell Phenotypes

(犬網状赤血球エクソゾーム：HK型ならびにLK型犬の網状赤血球成熟過程におけるNa,K-ATPaseとstomatinの選択的同時排出機構)

網状赤血球の成熟は、赤芽球系細胞分化・成熟の最終段階であり、成熟赤血球が必要とする膜の特性を獲得するために、トランスフェリンレセプター(TfR)をはじめとする過剰/不要な細胞膜成分を排除する過程である。この過程には、タンパク質分解、ならびにエクソゾーム(exosome)と呼ばれる微小小胞の放出が関わりとされる。犬網状赤血球にはNa,K-ATPaseが存在するが、これは成熟赤血球に至る過程で消失し、結果として、犬赤血球は一般に高Na⁺、低K⁺のLK型赤血球となる。一方で、柴犬や珍道犬には遺伝的にNa,K-ATPaseを保持したHK型赤血球をもつ個体が存在する。本研究の目的は、犬網状赤血球成熟過程におけるNa,K-ATPase消失におけるエクソゾームの役割と、そのHK/LK赤血球表現型との関連の解明である。

第1章では、犬網状赤血球の成熟過程で放出される小胞の性状を解析した。LK型犬から得た網状赤血球を孵置し、24、72時間後に上清を分別遠心すると微小小胞が得られた。この小胞は直径20~60nmで、Hsc70、TfRなど、既報エクソゾームの指標となるタンパク質を主要成分として含むことから、エクソゾームと判断した。膜タンパク質の解析から、犬の網状赤血球膜に含まれるが赤血球膜には存在しないNa,K-ATPase α サブユニット、Hsc70、TfR、GLUT1、ならびに脂質ラフトタンパク質のひとつstomatinは、いずれもこのエクソゾームに存在した。一方で、赤血球膜の主要タンパク質、バンド3、スペクトリンは検出されなかった。また、Na,K-ATPase、Hsc70、TfRはTriton X-100可溶性画分に、一方、GLUT1は不溶性画分に、GLUT1と相互作用するstomatinは両画分に含まれていた。さらに、このエクソゾームをショ糖密度勾配遠心で分画したところ、小胞は比重1.06 g/ml~1.14 g/ml(画分4~12)の範囲に広く分布し、Hsc70、TfR、stomatinは画分5~8、特にラフトマーカーのGM1と同じ画分7と8に多く含まれていた。一方、Na,K-ATPaseは、より比重の小さい

画分 4~6 に主に認められた。これらの結果から、網状赤血球エクソゾームの組成は均一ではなく、タンパク質構成の異なるポピュレーションから成ることが明らかになるとともに、Na,K-ATPase と一部の stomatin、Hsc70 が同一ポピュレーションに存在することが示唆された。

そこで第 2 章では、overhydrated stomatocytosis (OHSt)患者における欠損を発端に見出され様々なトランスポーターとの相互作用が提唱されている stomatin に焦点をあて、遺伝子型 *hk/hk* の HK 型、*hk/lk* と *lk/lk* 由来の LK 型各犬の網状赤血球と成熟赤血球における stomatin の含量を検討した。HK 型(*hk/hk*)の赤血球膜における stomatin 含量がヒト赤血球と同程度であるのに比べ、LK 型(*lk/lk*)はその 2%以下で OHSt 患者と同様の低値を示した。また、遺伝子型 *hk/lk* の LK 型赤血球の stomatin 含量は HK 型の 10%程度で、*hk/hk* 型、ならびに *lk/lk* 型と明確な差異を示した。上述のように LK 型(*lk/lk*)網状赤血球には stomatin が存在し、これに対する HK 型の赤血球、網状赤血球の stomatin 含量はそれぞれ 8 倍、12.5 倍であった。Na,K-ATPase と GLUT1 にも類似した量的関係が認められた一方で、TfR 含量は HK 型、LK 型の網状赤血球で同程度であった。これらの成績から、HK 型と LK 型は、いずれも網状赤血球の成熟にともなった stomatin と Na,K-ATPase の減少を同様に生じること、成熟赤血球の HK/LK 表現型が両型の網状赤血球における発現量の差違に基づくことが明らかになった。

以上のように、本研究は、複数のポピュレーションを含む犬網状赤血球エクソゾームが Na,K-ATPase や stomatin をはじめとする膜タンパク質の同時、かつ選択的な除去に寄与すること、ならびに stomatin の膜含量が HK/LK 遺伝子型の指標となることを明らかにした。加えて、これらの知見は、HK/LK 赤血球表現型を生じる本質的要因が TfR を発現するよりも前の前駆細胞の段階にあることを示唆するものである。