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The lamprey (*Lampetra fluviatilis*) erythrocyte; morphology, ultrastructure, major plasma membrane proteins and phospholipids, and cytoskeletal organization

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Summary

The aim of this study was to characterize the erythrocyte of the lamprey (*Lampetra fluviatilis*), a primitive vertebrate. The lamprey erythrocyte predominantly has a non-axisymmetric stomatocyte-like shape. It has a nucleus and a haemoglobin-filled cytosol with a few organelles and vesicular structures. Surprisingly, there is no marginal band of microtubules. Sodium dodecylsulphate polyacrylamide gel electrophoresis followed by Coomassie blue staining of isolated plasma membranes revealed a single band at the level of the human spectrin doublet. Major bands also occurred at approximately 175 kDa and comigrating with human erythrocyte actin (~ 45 kDa). The presence of spectrin, actin and vimentin was shown by immunoblotting. Band 3 protein, the anion exchanger in higher vertebrates, seemed to be highly deficient or lacking, as was also the case with ankyrin. Confocal laser scanning microscopy combined with immunocytochemical methods showed spectrin, actin and vimentin mainly to be localized around the nucleus, from where actin- and vimentin-strands extended out into the cytoplasm. Actin also seemed to be present at the plasma membrane. Phospholipid analyses of plasma membrane preparations showed the presence of the same four major phospholipid groups as in the human erythrocyte, although with higher and lower amounts of phospatidylcholine and sphingomyelin, respectively. The low fluorescein isothiocyanate conjugated annexin V binding, as monitored by flow cytometry, indicated that phosphatidylserine is mainly confined to the inner membrane leaflet in the lamprey erythrocyte plasma membrane.

Keywords: erythrocyte (lamprey), morphology, ultrastructure, band 3, spectrin, phospholipid

Introduction

Lampreys belong to the most ancient group of vertebrates, the Agnathans (cyclostomata). The functional properties of the lamprey erythrocyte differ markedly from those of all other erythrocytes. For example, the lamprey erythrocyte plasma membrane is characterized by a very low permeability to chloride and acid-base equivalents (protons and bicarbonate ions) (Ohnishi and Asai 1985, Nikinmaa and Rail 1987, Tufts and Boutilier 1989, Brill *et al.* 1992), indicating the absence of an anion transporter protein. In higher vertebrates, the anion exchanger protein (Band 3) is the major integral plasma membrane protein of erythrocytes (Fairbanks *et al.* 1971, Steck 1978) and it is implicated in the maintenance of the cell shape by forming associations with the spectrin-actin based membrane skeleton network (Nakao 1990). If the low anion permeability is associated with a lack or a low number of the anion-exchanger protein in the lamprey erythrocyte plasma membrane, then the regulation and maintenance of erythrocyte shape may be different in lamprey erythrocytes than in erythrocytes of other vertebrate groups. However, up to the present, both the shape of the lamprey erythrocyte and its structural components have been poorly characterized. For this reason, the present study has been carried out on the erythrocyte of the river lamprey *Lampetra fluviatilis*. The human erythrocyte and the nucleated rainbow trout (*Oncorhynchus mykiss*) erythrocyte were used as references.

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Results

Morphology

Figure 1A shows the morphology of lamprey erythrocytes as visualized by interference contrast microscopy. Similar cell shapes occurred in unfixed blood samples and in blood from unanaesthetized animals (not shown). A scanning electron microscopy (SEM) micrograph is shown in figure 1B. The lamprey erythrocytes predominantly showed a highly variable non-axisymmetric shape. Usually, several invaginations occurred in the stomatocyte-like cell. The 'diameter' of cells was about 7.5 μm. The invaginated character of cells is possibly pronounced due to the sample preparation for SEM. Some discocyte-like cells occurred. Human (not shown) and trout (figure 2B) erythrocytes maintained their typical discoid and flattened oval shape, respectively, following the used fixation protocol.

Ultrastructure

Thin sections of lamprey erythrocytes are shown in figures 2A1 and 2A2 (higher magnification). The position of the nucleus cannot be well defined due to the stomatocytic cell shape. Eu- and heterochromatin, and nucleophores, are seen. Small vesicular structures (mitochondrion (M)) occurred in the cytosol. There is no marginal band of microtubules. For a comparison, a transmission electron microscopy (TEM) micrograph of trout erythrocytes is shown in figure 2B. A marginal band of microtubules (arrow and inset) is present in the flattened oval trout erythrocyte (see

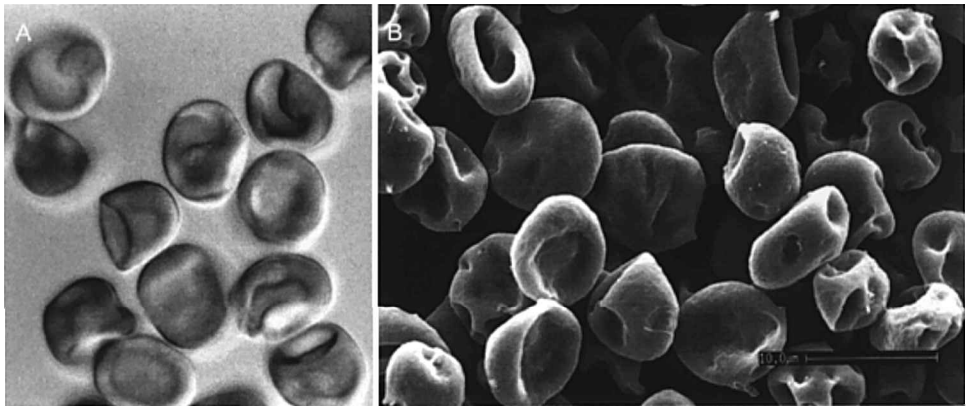

Figure 1. Lamprey erythrocyte morphology. (A) Interference contrast (Nomarski) micrograph. (B) SEM micrograph.

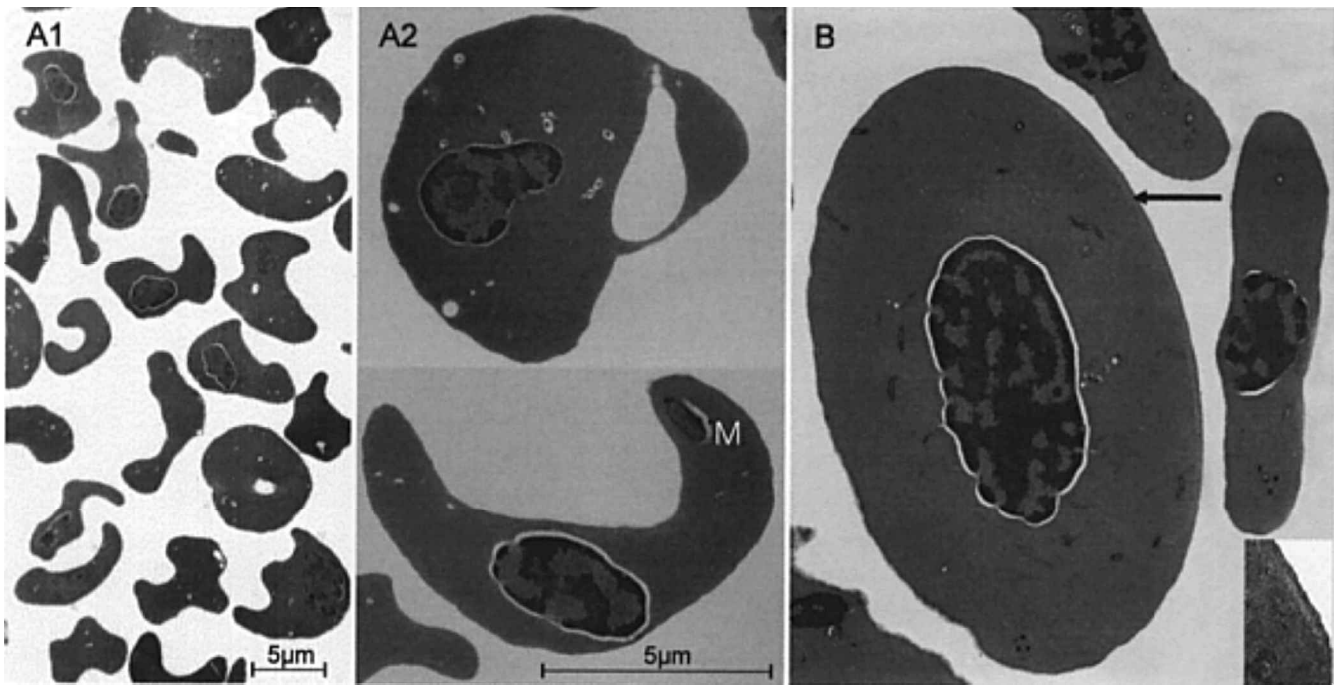

Figure 2. Lamprey erythrocyte ultrastructure (TEM). (A1) Lamprey erythrocytes. (A2) High magnification of lamprey erythrocytes. Mitochondrion (M). (B) Trout erythrocytes (reference). Marginal band of microtubules (arrow and in inset). Magnification as in A2.

also Sekhon and Beams 1969). Some organelles and vesicular structures are seen in the cytosol.

Plasma membrane protein composition

Lamprey erythrocyte ghost preparations (L) were subjected to sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining and their protein pattern was compared with that of human erythrocyte ghosts (H) (figure 3A). Starting from the top, one band comigrated with human erythrocyte α-spectrin and a second band appeared at approximately 175 kDa. No band comigrating with human erythrocyte β-spectrin was seen. A weak double/poly band is observed in the 95 kDa region at human band 3. There was also banding in the ~70 kDa region (at human band 4.2) and at ~60 kDa. One heavy band, comigrating with human band 5 (actin), occurs at ~45 kDa and one at ~33 kDa (comigrating with one of the major bands of lamprey erythrocyte cytosol, not shown). No high molecular weight bands occurred in lamprey erythrocyte cytosol preparations (not shown). Comparing the banding in these samples to samples prepared in the presence of iodoacetamide (Thomas *et al.* 1983) and phenylmethylsulphonylfluoride (PMSF), an additional band/double band in the ~125 kDa region occurred (figure 3B).

Immunoblotting

Immunoblotting indicated the presence of actin, vimentin and spectrin in the lamprey erythrocyte plasma membrane preparations (figure 4A–E). An anti-human actin antibody strongly cross-reacted with a band comigrating with human actin (figure 4A). A chicken anti-baby hamster kidney (BHK) vimentin antibody crossreacted with a band at ~60 kDa (figure 4B). A monoclonal anti-vimentin antibody cross-reacted with a band of a similar molecular weight (not shown). With the use of an anti-chicken/turkey spectrin antibody, spectrin was localized to a band comigrating with human α-spectrin and to a band at ~175 kDa (figure 4C). This band is apparently equivalent to the band observed in coomassie stained protein profiles (figure 3A and B). There were also a band at ~ 125 kDa and several bands of lower molecular weight, which apparently are proteolytic spectrin fragments. A polyclonal (figure 4D) and a monoclonal (not shown) anti-human band 3 antibody, as well as a polyclonal anti-ankyrin antibody (figure 4E), showed no cross-reaction with membrane preparations from lamprey erythrocytes. The protease inhibitors iodoacetamide and PMSF were present during sample preparation. All controls with secondary horseradish peroxidase (HRP) antibodies were essentially blank (not shown).

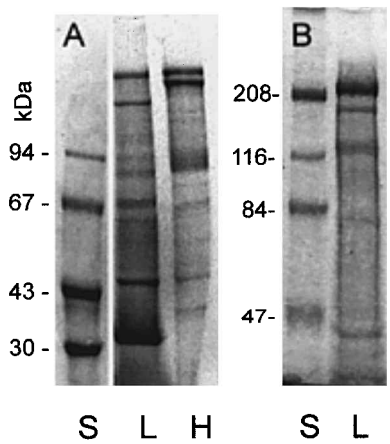

Figure 3. Lamprey erythrocyte plasma membrane proteins as identified by coomassie blue staining of SDS-PAGEs. Dodge-ghosts were prepared in the absence (A) and presence (B) of iodoacetamide (10 mM) and PMSF (0.2 mM). Standard (S); Lamprey erythrocyte ghosts (L); Human erythrocyte ghosts (H). A was run on a gradient (4–15% polyacrylamide) gel and B on a continuous (7.5% polyacrylamide) gel.

Localization of proteins in situ

The localization of cytoskeletal components in the lamprey erythrocyte was examined by confocal laser scanning microscopy (CLSM) using the trout erythrocyte as a reference (figure 5). A specific reaction of cellular structures occurred with antibodies against spectrin, vimentin and actin, in contrast to incubation with secondary fluorescent antibody controls only. An anti-chicken/turkey spectrin antibody mainly bound to the nucleus of both lamprey and trout erythrocytes (figure 5A and B). The plasma membrane was not, or very weakly, stained. The staining pattern was not Ca^{2+} dependent (not shown). A staining of the marginal band of microtubules region was observed in trout erythrocytes. In lamprey erythrocytes, staining with tetramethylrhodamine-isothiocyanate (TRITC) phalloidin, a specific actin staining molecule, was found to be extensive around the nucleus, with treads extending out from the nucleus (figure 5C). A weak staining of the plasma membrane was also seen. In trout erythrocytes, staining of the marginal band of micro-

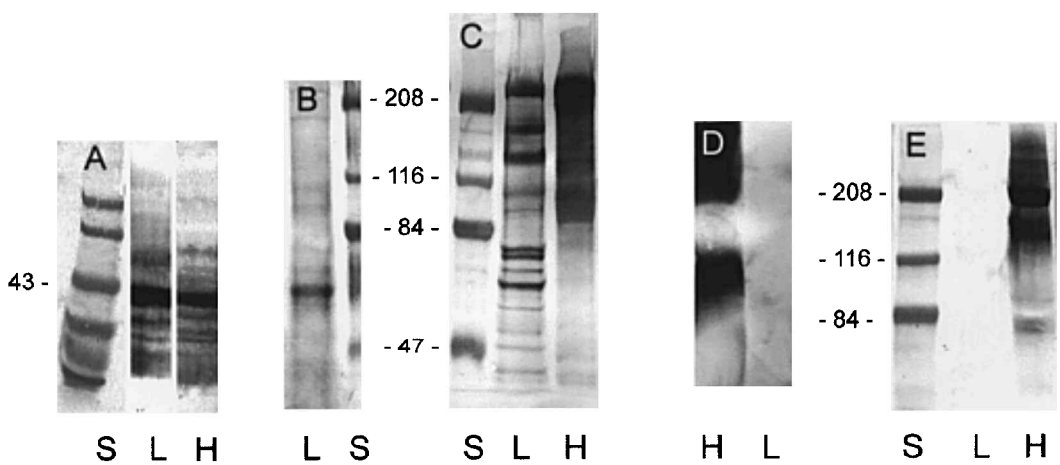

Figure 4. Lamprey erythrocyte plasma membrane proteins as identified by immunoblotting of SDS-PAGEs. (A) Antibody against actin. (B) Antibody against vimentin. (C) Antibody against spectrin. (D) Antibody against band 3 protein. (E) Antibody against ankyrin. Standard (S); Lamprey erythrocyte ghosts (L); Human erythrocyte ghosts (H). Samples were prepared in the presence of iodoacetamide (10 mM) and PMSF (0.2 mM). A was run on a gradient (4–15% polyacrylamide) gel and B–E on continuous (7.5% polyacrylamide) gels.

tubules region occurred (figure 5D). Furthermore, the plasma membrane seemed to be weakly stained, while staining of the nucleus did not occur. In lamprey erythrocytes, vimentin seemed to be mainly localized around the nucleus (figure 5E). Occasionally, strands of vimentin extended out into the cytoplasm. In trout erythrocytes, vimentin was located to the cytoplasm (figure 5F). The anti-tubulin antibody did not crossreact with the lamprey erythrocyte (not shown) but cross reacted well with the trout erythrocyte, where a characteristic marginal band of microtubules was visualized (figure 5G).

Eosin-5-maleimide, reported to stain band 3 protein of human erythrocytes (Chiba *et al.* 1990, Cobb and Beth 1990), stained human (figure 6B) and trout (figure 6C) erythrocytes, but not lamprey erythrocytes (figure 6B). This further indicates the absence of band 3 protein in lamprey erythrocytes.

Phospholipid composition

The lamprey erythrocyte plasma membrane preparations had a similar qualitative phospholipid composition (phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine and sphingomyeline) as the human erythrocyte membrane. However, the lamprey erythrocyte membrane preparations contained 35% more phosphatidylcholine and 42% less sphingomyelin than the human erythrocyte (table 1).

Phospholipid asymmetry

Similar to human erythrocytes (Hägerstrand *et al.* 1998), very few ($1.5 \pm 0.4\%$, $n=3$) lamprey (and trout (not shown)) erythrocytes showed a fluorescein-isothiocyanate (FITC)

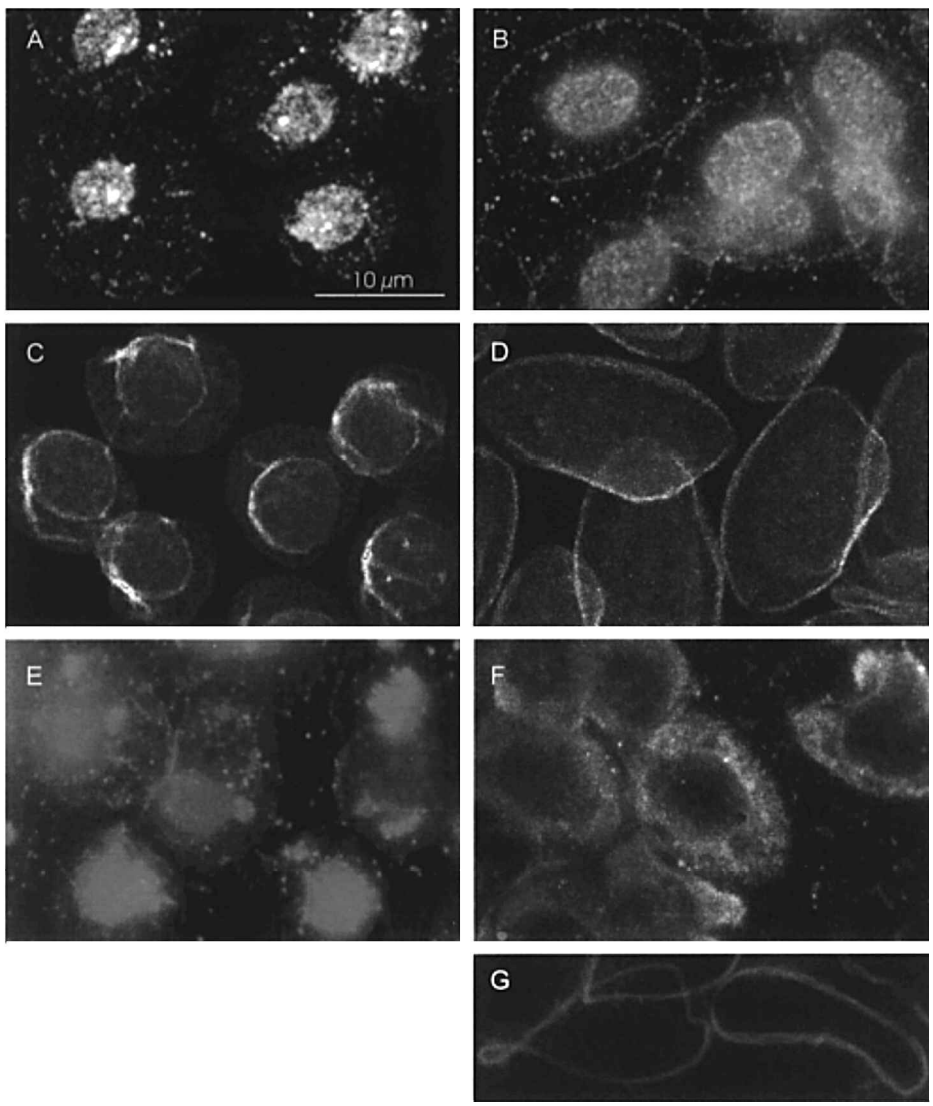

Figure 5. *In situ* immunolocalization of proteins (CLSM). Staining of lamprey erythrocytes with antibodies against (A) spectrin, (C) actin and (E) vimentin, and of trout erythrocytes (reference) (B) spectrin, (D) actin, (F) vimentin and (G) tubulin.

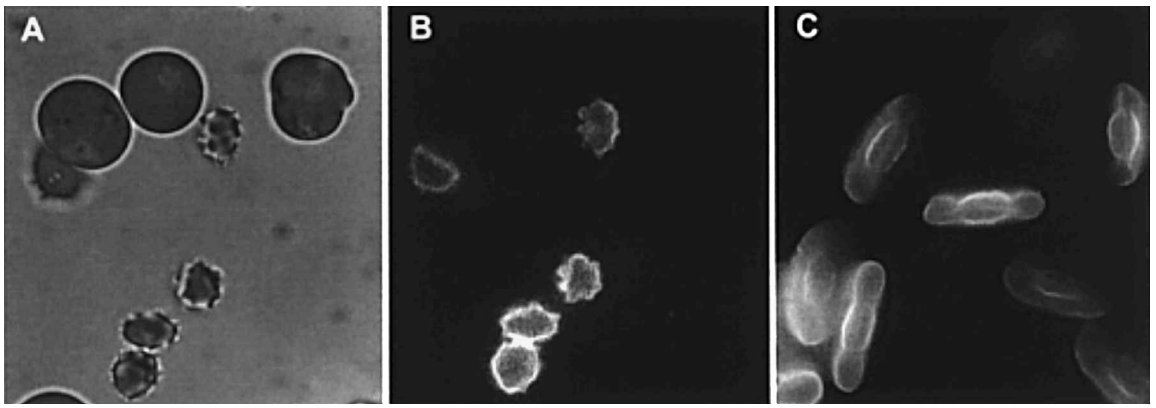

Figure 6. Erythrocytes incubated with eosin-5-maleimide for detection of band 3 protein. The same field of a mixed sample of human and lamprey erythrocytes as observed by light microscopy (A) and fluorescence microscopy (B). (C) trout erythrocytes in fluorescence microscopy.

Table 1. Phospholid composition (%)

	Lamprey	Human
Phosphatidylethanolamine	36+4.2	34+6.5
Phosphatidylserine	18+2.7	18+2.3
Phosphatidylcholine	31+3.1*	23+3.7*
Sphingomyelin	14+3.8**	24+5.0**

Ghost preparation of lamprey and human erythrocytes were compared (n=5). Means of phosphatidylcholine (*) and sphingomyeline (**) content were significantly ($p < 0.01$, students t-test) different. The method of phospholipid detection (densitometric scanning) is not strictly quantitative.

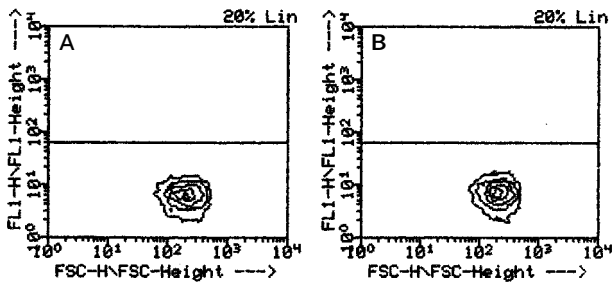

Figure 7. Flow cytometry analysis of lamprey erythrocytes. The figure shows a separate experiment where erythrocytes had been incubated (A) without and (B) with FITC-annexin V. Per cent cells showing a fluorescence above the threshold was determined.

annexin V-binding above background (cells incubated without FITC-annexin V) (figure 7).

Discussion

Cell shape

Although some functional properties of the lamprey erythrocyte are known, as for example the very low permeability of the plasma membrane to chloride and acid-base equivalents (protons and bicarbonate ions) (Ohnishi and Asai 1985, Nikinmaa and Railo 1987, Tufts and Boutilier 1989, Brill *et al.* 1992), the structural properties of the lamprey erythrocyte are poorly characterized. Concerning the normal shape of lamprey erythrocytes, both a discoid and an invaginated shape have been suggested (Potter *et al.* 1974). As shown in figure 1, the lamprey erythrocyte may show a variety of shapes within the stomatocytic shape class (see also Carter *et al.* 1972). The surface area and the volume of the lamprey erythrocyte have been estimated to 284 μm^2 and 288 μm^3, respectively (Virkki and Nikinmaa 1994), giving a relative cell volume (Deuling and Helfrich 1976) of 0.64.

The findings presented in the present study may provide some explanations for the irregular stomatocytic shape of the lamprey erythrocyte. Apparently, the absence of a marginal band of microtubules is an essential property of the lamprey erythrocyte allowing its varying non-axisymmetric poly-invaginated shape. As shown in different species, mature erythrocytes having a marginal band of microtubules generally possess a smooth cell surface (Sekhon and Beams 1969, Sekhon and Maxwell 1970, Cohen *et al.* 1982).

An important property in erythrocytes affecting erythrocyte shape and plasma membrane stability is the character of the interactions between the plasma membrane and the membrane skeleton (Cohen *et al.* 1982, Lange *et al.* 1982, Iglic *et al.* 1995, Iglic 1997). Band 3 protein, the anion exchanger, is the major integral protein linking the plasma membrane to the membrane skeleton in human erythrocytes (Nakao 1990). In the present study, it was observed that the Coomassie blue staining of SDS-PAGE was very weak in the assumed band 3 region (~95 kDa) in comparison to that in human erythrocyte samples (figure 3A). Furthermore, antibodies against human erythrocyte band 3 were negative in lamprey erythrocytes, and eosin-5-maleimide, which binds to human erythrocyte band 3 protein (Chiba *et al.* 1990, Cobb and Beth 1990), did not stain the lamprey erythrocyte plasma membrane. These results are interpreted to mean that the lamprey erythrocyte plasma membrane lacks or is deficient in band 3 protein. This interpretation is supported by previous protein profile (Ohnishi and Asai 1985) and anion

transport studies (Nikinmaa and Railo 1987) in lamprey erythrocytes. Also, ankyrin, important for the band 3 protein-spectrin interactions in human erythrocytes (Van Dort *et al.* 1998), seems to be absent in lamprey erythrocytes, as indicated by the absence of cross-reactivity with an anti-human ankyrin antibody (figure 3E).

A deficiency in band 3 protein (and in ankyrin), in lamprey erythrocytes may result in weak membrane skeleton-plasma membrane interactions, and thereby in the non-axisymmetrical stomatocytic equilibrium shape. Namely, since the lateral mobility of the membrane embedded proteins is normally limited by the skeleton network (Sheetz 1983), a deficiency in the skeleton-bilayer interactions may lead to non-discocytic equilibrium shapes due to a non-uniform lateral distribution of membrane proteins (Kralj-Iglic *et al.* 1996, Svetina *et al.* 1996). In addition, it has been stressed that also alterations in the abundance of membrane embedded proteins, and in their effective molecular shape (Israelachvili *et al.* 1980), may shift the equilibrium discocyte shape towards non-axisymmetric shapes (Khodadad and Weinstein 1983, Palek 1987, Liu *et al.* 1991, Gimsa and Ried 1995, Kralj-Iglic *et al.* 1996).

Additional support for the assumption that band 3 protein deficiency in lamprey erythrocytes affects the cell shape comes from a recent study showing that bovine erythrocytes completely deficient in band 3 protein have a similar poly-invaginated shape as lamprey erythrocytes (Inaba *et al.* 1996). It was suggested that the shape and the unstability of these cells are due either to deficient membrane skeleton-plasma membrane interactions or to a fragmented membrane skeleton, caused by the band 3 protein deficiency. However, it was recently suggested that membrane skeleton organization does not depend on the presence of band 3 protein (Peters *et al.* 1996). These authors showed that mouse erythrocytes lacking band 3 protein were unstable, but showed a nearly normal membrane skeleton. It was suggested that band 3 protein is necessary for plasma membrane stability because of its interaction with plasma membrane lipids.

It should be mentioned that the existence of a protein that is structurally, immunologically and physiologically related to band 3 protein in the lamprey erythrocyte plasma membrane has been demonstrated (Kay *et al.* 1995, Cameron *et al.* 1996), however, its function is still unclear. An unusual composition of the spectrin based membrane skeleton in lamprey erythrocytes, as indicated by the occurrence of a single high molecular weight spectrin band in coomassie stained SDS-PAGEs (figure 3A), may also be reflected in the membrane skeleton-plasma membrane interactions.

The present study supports previous studies (Gimsa and Ried 1995, Bobrowska-Hägerstrand *et al.* 1998, Seifert 1998), indicating that the ratio between the outer and the inner plasma membrane leaflet areas (Sheetz and Singer 1974, Svetina and Zeks 1989) can not account for all observed phenomena in erythrocyte shape transformations. It can be concluded that skeleton-bilayer interactions, skeleton shear elasticity as well as a non-homogenous lateral distribution of membrane components may play important roles in the determination of the equilibrium erythrocyte shape (Sheetz 1983, Kralj-Iglic *et al.* 1996, Iglic *et al.* 1998a, b).

Plasma membrane protein composition

A comparison of lamprey and human erythrocyte ghost preparations indicates several striking similarities in the main protein composition, but also some obvious differences. Actin and vimentin seem to have a similar molecular size in lamprey and human erythrocytes. However, only a single high molecular weight spectrin band occurred in lamprey erythrocytes, compared to the spectrin doublet in human erythrocytes. Since lamprey erythrocyte spectrin was shown to be sensitive to proteolytic degradation it may be argued that either α- or β-spectrin is proteolytically fragmented. A Ca^{2+}-sensitive proteolysis of spectrin into fragments of the observed molecular sizes has been previously reported (Speicher *et al.* 1980, Thomas *et al.* 1983, Gaczynska and Bartosz 1989). However, no indications for another protein of a molecular weight higher than ~175 kDa, except that comigrating with human α-spectrin, was observed. The possibility that α- and β-spectrin have a similar molecular weight in lamprey erythrocytes should be regarded. A single spectrin band has previously been reported in a marine invertebrate (Pinder *et al.* 1978). It may be relevant to note that a Ca^{2+}-induced proteolytic breakdown of α-spectrin, but not of β-spectrin, has been reported in chicken erythrocytes (Thomas *et al.* 1983) and that avian spectrin isoforms are reported to have a varying tissue specific β-subunit, but a common α-subunit (Coleman *et al.* 1989).

Cytoskeletal organization

Immunocytochemical staining combined with CLSM localized lamprey erythrocyte spectrin, actin and vimentin to the area around the nuclear envelope and, in the case of actin and vimentin, also to branches reaching out into the cytoplasm (figure 5A, C and E). In the case of actin, a weak staining was also observed at the plasma membrane, probably representing the membrane skeleton. The absence of plasma membrane staining with the spectrin antibody remains obscure. A perinuclear location or a nuclear anchoring of intermediate filaments like vimentin (Virtanen *et al.* 1979, Woodcock 1980, Centozne *et al.* 1986), spectrin (Zagon *et al.* 1986) and proteins with the molecular masses of actin and spectrin (Chen *et al.* 1996) is, however, in accordance with previous findings in nucleated erythrocytes. In avian erythrocytes, vimentin has been suggested to connect the nucleus with the plasma membrane by spanning from the membrane skeletons to the nuclear envelope (Granger and Lazarides 1982, Centozne *et al.* 1986, Lazarides 1987), thereby keeping the nucleus in its central position (Centozne *et al.* 1986, Repasky and Gregorio 1991). No specific staining with tubulin antibodies was observed in lamprey erythrocytes (not shown). The absence of a marginal band of microtubules was also revealed by the TEM studies (figure 2). Interestingly, a marginal band of microtubules is present in hagfish erythrocytes (Sekhon and Maxwell 1970, Mattison and Fänge 1977) which, based on recent molecular evidence, separated from the general vertebrate lineage long before the separation of lampreys from the other vertebrates (Rasmussen *et al.* 1998). The presence of a marginal band of microtubules can be

considered to be an ancient feature, which is secondarily lost both in lampreys and in mammals. Thus, lack of the marginal band of microtubules is now demonstrated for both nucleated (lampreys) and non-nucleated (mammalian) erythrocytes. In trout erythrocytes, which were used as a reference in this study, spectrin was located in the marginal band of microtubules region, as was actin, but also in the area around the nucleus (figure 5B and D). Vimentin was located in the cytoplasm (figure 5F). It should be noted that the preparation of cells for CLSM studies may interfere with the native location of the studied structural components.

Plasma membrane phospholipids

Phospholipid analyses of lamprey cell membrane preparations demonstrated that the major phospholipids were similar to those in the human erythrocyte. However, the amount of phospatidylcholine was higher and the amount of sphingomyelin lower compared to the human erythrocyte. A contribution to the phospholipid profile of remnant nuclear envelopes in the ghost preparations is possible. In hen erythrocytes, the nuclear envelope has been estimated to be rich in phosphatidylcholine and poor in sphingomyelin, compared to the plasma membrane (Allan and Raval 1987).

The low FITC-annexin V binding, as detected by flow cytometry, indicated that phosphatidylserine in the lamprey erythrocyte is, as in the human erythrocyte (Hägerstrand *et al.* 1998), mainly confined to the inner membrane leaflet.

It should, finally, be stressed that a study of the lamprey erythrocyte plasma membrane composition is complicated by the necessity to denucleate cells. The nucleus should be discarded because the nuclear envelope may significantly contribute to the overall phospholipid and membrane protein pool of the cell. Nuclear components may also, besides their gelating effect, interact with the membrane components under study. Furthermore, depletion of nuclear material from the cell, in this case by transferring cells to ghosts, may result in some cytoplasmic proteins anchored to the nuclear envelope being partly lost together with the nucleus.

In conclusion, the unique non-axisymmetric stomatocytic equilibrium shape of lamprey erythrocytes may be related to the absence of membrane skeleton anchoring band 3 protein, resulting in deficient plasma membrane – membrane skeleton interactions and in a non-uniform lateral distribution of membrane embedded proteins.

Experimental procedures

Antibodies and chemicals

Rabbit anti-chicken spectrin (S1390), mouse monoclonal anti-band 3 (B9277), mouse monoclonal anti-vimentin (V5255) and tetramethylrhodamine-isothiocyanate conjugated phalloidin (P5157) were obtained from Sigma (St. Louis, MO, USA), mouse monoclonal anti-tubulin (N356) from Amersham (Buckinghamshire, UK). Chicken anti-BHK vimentin and mouse anti-human actin were kind gifts from John Eriksson (Turku Center of Biotechnology, Åbo/Turku), and rabbit anti-human band 3 and rabbit anti-human ankyrin kind gifts from Ismo Virtanen (Institute of Pathology, Helsinki University). Fluorescence-conjugated secondary antibodies were obtained from Zymed (San Francisco, CA, USA) and horse radish peroxidase-conjugated secondary antibodies from BioRad (Richmond, CA, USA). Iodoacetamide and phenylmethylsulphonylfluoride were obtained from Sigma and eosin-5-maleimide from Molecular Probes.

Isolation of erythrocytes

River lampreys (*Lampetra fluviatilis*) were caught from the rivers Aura å and Kymmene älv in Finland during their spawn run and were kept in the laboratory in well water at ~8°C. Isolation of erythrocytes was carried out at room temperature (RT) 22°C. Lampreys were anaesthetized (3-aminobenzoic acid ethyl ester, Sigma A5040, 1 g/l medium), wherafter blood was drawn (needle diameter 0.6 mm) by dorso-ventral vein punctation into a syringe containing a few drops of heparin (25000 IE/ml). The cells were washed five times with the Hepes buffer (Hepes 10 mM, NaCl 128 mM, KCl 3 mM, $CaCl_2 \times 2H_2O$ 1.5 mM, $Mg_2Cl_2 \times 6H_2O$ 1.5 mM, pH 7.6), which corresponds to the *in vivo* plasma composition of the lamprey erythrocyte (Nikinmaa and Railo 1987). The uppermost layer of the erythrocyte suspension (buffy coat) was discarded at every wash. The cells were finally suspended in the buffer at a cell density of $\sim 0.8 \times 10^8$ cells/ml. Cells were usually used the day they were drawn.

Light microscopy

The morphology of glutaraldehyde (1%) fixed isolated erythrocytes was studied in a hanging drop by phase contrast microscopy. For photography (interference contrast microscopy) erythrocytes were fixed with 1% glutaraldehyde and applied between object and cover glass. Earlier observations have indicated that the cell shape of fixed cells is not different from freshly drawn, untreated cells, but that the shape remains unchanged for longer periods of time.

Transmission electron microscopy

Erythrocytes were suspension-fixed in 1% glutaraldehyde in the buffer for 30 min at RT, postfixed in 1% OsO_4 in 0.9% NaCl for 30 min at RT, dehydrated in a graded series of acetone/water (50–100%, v/v) and finally embedded in Epon. The sections were stained with lead acetate and post-stained with uranyl acetate before examination in a JOEL 100SX electron microscope.

Scanning electron microscopy

Erythrocytes, fixed, post-fixed and dehydrated as above, were critical-point dried, gold-sputtered (Hägerstrand and Isomaa 1992), and finally examined in a Cambridge Instruments S360 microscope.

Isolation of plasma membranes

Cells were washed several times with the nominally Ca^{2+} and Mg^{2+} free buffer in the presence or absence of 10 mM iodoacetamide and 0.2 mM PMSF. Ghosts from lamprey and human erythrocytes were essentially prepared according to the method of Dodge *et al.* (1963), with the modification that the lysate was turned by hand for about 10 min after the first lysis, whereafter the aggregated DNA in the lamprey samples was discarded. 10 mM iodoacetamide and 0.2 mM PMSF were added to the lysis buffer (5 mM NaH_2PO_4, 1 mM EDTA (pH 7.4)) as indicated. It can be mentioned that the described method can not be applied for depleting trout erythrocytes from nucleus/chromatin because the whole sample became a gel.

Sodium dodecylsulphate polyacrylamide gel electrophoresis and immunoblot analysis

SDS-PAGE was performed according to Laemmli (1970) in a Bio-Rad Minigel apparatus using either gradient 4–15% TRIS-Glycine (Bio Rad 161-0902) or 7.5% Tris-HLC (Bio Rad 161-1100) gels. Samples were prepared by adding Laemmli sample buffer to packed isolated plasma membrane. Polypeptide bands were visualized

either with Coomassie brilliant blue staining or with immunodetection according to the method of Towbin *et al.* (1979) as previously described by Lindqvist *et al.* (1993). Either high range (BioRad 161-0309) or low molecular weight calibration kit (Pharmacia) standards were used.

Staining in situ

Coverslips were covered with polylysine (0.1 mg/ml) for 5 min and then washed and dried. Cells (hematokrit ~0.2%) were let to settle at coverslips for 10 min, whereafter the coverslips were gently washed with Allen buffer (145 mM NaCl, 5 mM KCl, 4 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.4) or microtubuli stabilizing buffer (0.1 M Hepes, 2 mM EGTA, 1 mM $MgSO_4$, 2 M glycerol, pH 6.9). Cells were permeabilized with 0.1% Triton X-100 (trout erythrocytes 0.2%) in respective buffer for 3 min at RT and then washed. Cells were fixed with 3.7% paraformaldehyde (PFA) for 30 min at RT. Following fixation, cells were washed with Allens buffer and then incubated for 30 min with the buffer containing 2% bovine serum albumin (BSA). After washing with Allens buffer cells were incubated for 60 min with the primary antibody in the presence of 2% BSA. After washing with the buffer, cells were stained with fluorescence conjugated secondary antibodies in the buffer containing 2% BSA. After extensive washing, the remaining buffer was suctioned from the coverslips, which were then put on a drop of Mowiol 40-88 (Aldrich) (containing 10% 1,4-diazabicyclo-[2,2,2]-octane (Sigma)) on the slides. On the next day, samples were sealed with nail furnish. For detection of band 3 protein, erythrocytes were incubated with 50 μg/ml eosin-5-maleimide for 30 min at RT and washed three times.

Confocal laser scanning microscopy

Samples were studied (100×/1.4 aperture immersion oil objective, 10×ocular) and scanned (~0.05 μm between scanned sections) using a Leica confocal laser scanning microscope. Scans were combined to images giving maximum projection. Best three-dimensional information was obtained by rotation of the three-dimensional images. Images were handled with the aid of Adobe software program and printed using Coreldraw software program.

Extraction of lipids

Lipid extraction from cells in chloroform/methanol/buffer (8 : 4 : 3, v/v) was performed as previously described (Hägerstrand and Isomaa 1994).

TLC and quantification of bands

Phospholipids were separated on Kieselgel 60 DC-fertigplatten (Merck 5626, Darmstadt, Germany) in chloroform/metanol/acetic acid/water (25 : 15 : 4 : 2, v/v). The plates were then dried and run in chloroform/diethyl ether/acetic acid (130 : 30 : 2, v/v) to avoid disturbances of front components during the densitometry. Plates were stained with 3% cupric acetate in 8% orthophosphoric acid and burned, whereafter major phospholipid classes were quantified by densitometric scanning using a National Institute of Health (USA) Image processing and analysis program. The method is not strictly quantitative.

Flow cytometry

Flow cytometry was performed as previously described (Hägerstrand *et al.* 1998). In short, 1 μl FITC-annexin V solution (Nexins Research B.V., Hoeven, The Netherlands) was added to 100 μl cell suspension (~10^8 cells/ml) and the sample was incubated on ice for 10 min. The sample was then suspended in 400 μl Hepes buffer, whereafter cells were analysed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

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