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Membrane skeletons in avian erythrocytes as revealed by the quick-freezing and deep-etching method

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Summary. Ultrastructure of chicken erythrocytes were examined by the quick-freezing and deep-etching (QF-DE) method. Some erythrocytes were fixed with paraformaldehyde and prepared with erythrocyte-splitting method or saponin treatment to remove soluble proteins before quick-freezing. Others were prepared in the cytosol buffer with the erythrocyte-splitting method to obtain natural state of cytoskeletons. Non-expanding membrane skeletons were highly condensed on the cytoplasmic side of lipid membrane in the para-formaldehyde-fixed specimens. Under unilateral extension of the specimens, long stretched filaments were connected alternately with condensed filamentous or granular structures under erythrocyte membranes. As the membrane skeletons got closer to the marginal bands, they become more dense network structures. Moreover, in the fresh unfixed specimens, dense networks of filaments were localized underlying erythrocyte membranes in a relatively intact state. Fine filaments connected the marginal microtubule bands to the cytoplasmic sides of erythrocyte membranes. The different distribution of each cytoskeletal component and the association of these structures may support the elliptocytic shape of chicken erythrocytes and resist the dynamic circumstance.

Key words: Erythrocyte, Membrane skeleton, Quick-freezing

Introduction

The shape and structural integrity of mammalian erythrocyte membranes are maintained by a subcortical cytoskeletal network. In the normal human erythrocyte, the membrane skeleton is organized in a spider-like unit, especially in a hexagonal lattice which is composed of spectrin tetramer filaments and medium-sized oligomers cross-linked by junctional complexes of short F-actin filaments (Tsukita et al., 1980; Shen et al., 1986; Liu et al., 1987). It was also reported that variable extension of the spectrin helix responding to the mechanical stress was attributed to the elastic property of erythrocytes (McGough and Josephs, 1990). Recently, an erythrocyte-splitting method has been developed for ultrastructural study on cytoplasmic aspects of erythrocyte membranes (Ohno, 1992; Ohno et al., 1993). It is now relatively accepted that the spectrin dimer exists only in an elongated molecular shape of approximately 100 nm length under tensile load or in low ionic strength buffers, whereas under physiological ionic strength and in vivo the dimer is shorter in length (Ursitti and Wade, 1993; Speicher and Ursitti, 1994). The previous ultrastructural study of fresh unfixed erythrocytes with the quick-freezing and deep-etching (QF-DE) method supported that fragile membrane skeletons underlying the human erythrocyte were easily modified by the specimen preparation steps (Ohno et al., 1994; Terada et al., 1996).

Avian elliptocytic erythrocytes also have sub-membranous spectrin-actin networks, proteins analogous to mammalian ankyrin and band 3 and the marginal band, as well as vimentin intermediate filament system (Granger and Lazarides, 1982). However, the organization of membrane skeletons under physiological ionic strength and in vivo has not yet been reported in detail. So we have examined ultrastructures of cyto-skeletons, especially the membrane skeleton in chicken erythrocytes, by our erythrocyte-splitting method combined with the QF-DE method.

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Materials and methods

A summarized flow chart of the present methods is illustrated in Fig. 1. Fresh blood was withdrawn from the wing veins of two large white Leghorn chickens, weighing 2 kg, with a syringe containing 0.1% heparin. The blood was centrifuged (1,200 rpm, 5 min) and washed three times with phosphate-buffered saline (PBS) containing Mg/EGTA (140mM NaCl/5mM $NaPO_4$/2mM $MgCl_2$/1mM EGTA, pH 7.4) and 10 μM

taxol (Sigma Chemical Co., St. Louis, MO, USA) to obtain erythrocyte pellets.

Some of the pellets were fixed with 2% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4, for 2 min and then washed in PB three times by centrifugation. The fixed erythrocytes were split open to remove soluble proteins from their cytoplasm by the cell-splitting method devised by one of the present authors (Ohno, 1985). Briefly, the erythrocytes were sandwiched between two silane-glutaraldehyde-coated coverslips, and split open under the phase contrast microscope. After being deprived of soluble proteins by a thorough washing with PBS, the split erythrocytes on the coverslips were fixed with 0.25% glutaraldehyde in PB for 15 min. Other paraformaldehyde-fixed erythrocytes were only attached to silane-glutaraldehyde-coated coverslips and then treated with 0.5% saponin in PBS to remove soluble proteins for 30 min, washed with PBS repeatedly and fixed with 0.25% glutaraldehyde in PB for 30 min.

Other unfixed erythrocyte pellets were mechanically split open in the same way as that of the fixed erythrocytes in another buffer solution (referred to as cytosol buffer) containing Mg/EGTA (100 mM KCl/2mM $MgCl_2$/1mM EGTA/20 mM $NaPO_4$, pH 7.2) and 10 μM taxol. They were washed in the same buffer to remove soluble proteins and then fixed with 2% paraformaldehyde in PB for 30 min and washed in PB.

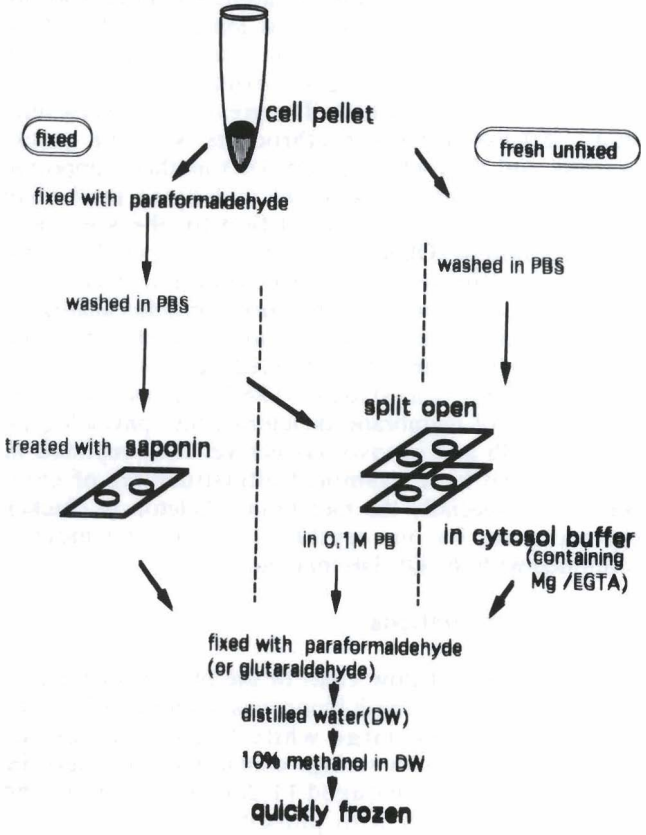

Fig. 1. Representative schema of preparation procedures.

The coverslips with the specimens were positioned on the specimen holder and quickly frozen in an isopentane-propane mixture (-193 ºC) cooled in liquid nitrogen (Ohno, 1992). The specimens on the coverslips were freeze-fractured in liquid nitrogen with a scalpel, transferred in an Eiko FD-3AS machine and deeply-etched under the vacuum condition of 1~4x10^{-7} Torr at -95ºC for 10-20 min. Replica membranes of the exposed cytoplasmic side of erythrocyte membranes were then prepared by evaporation of platinum at an angle of 25º and subsequently of carbon at an angle of 90º. The replica membranes were treated routinely in household bleach and put on Formvar-filmed copper grids (Ohno, 1992). They were observed in a Hitachi H-600 or H-8100 electron microscope.

Light microscopic immunostaining was carried out on the erythrocyte membranes attached to coverslips, which was split or saponin-treated. They were washed with PBS and treated with 0.1M lysine in PB for 30 min. Then they were immunostained, firstly with rabbit polyclonal anti-spectrin antibody (Transformation Res., MA, USA), polyclonal anti-actin antibody (Miles Scientific, IL, USA), and mouse monoclonal anti-α-tubulin antibody (Monosan, Uden, Netherlands). They were secondary immunostained with goat anti-rabbit IgG or anti-mouse IgG coupled to FITC (Cappel, NV, USA), respectively. Some saponin-treated erythrocytes were stained with phalloidin-FITC to identify actin filaments (Sigma Chemical Co., St. Louis, MO, USA). They were additionally washed in PBS, mounted on slide glasses and viewed by a fluorescent microscope.

Results

The prepared chicken erythrocytes maintained their elliptocytic morphology after paraformaldehyde fixation and saponin treatment, revealed by Wright-Giemsa staining and replica of erythrocytes (Fig. 2a,b). Immunofluorescence microscopy with polyclonal anti-spectrin antibody showed uniform staining along the entire surface membrane (Fig. 2c). When an erythrocyte was split open and the upper erythrocyte membrane was disrupted, the immunostaining of spectrin could be observed to be restricted to the erythrocyte membrane (Fig. 2d). The immunostaining with anti-actin antibody showed the same staining pattern as the spectrin (Fig. 2e), whereas phalloidin-FITC staining appeared to be weaker (Fig. 2f). The marginal band was located along the cell periphery, where the tubulin was linearly immunostained (Fig. 2g). The cooling treatment of the erythrocyte at 4 ºC for 20 min let the microtubules depolymerize, as reported previously (Miller and Solomon, 1984) (Fig. 2h).

Replica electron micrograph presented that a fixed and saponin-treated erythrocyte was freeze-fractured in the middle of cytoplasm including a nucleus, and that cytoplasmic structures and filamentous components could be seen (Fig. 3). The connecting filaments

appeared to be intermediate filaments, judging by their sizes (about 14 nm in diameter). The intermediate filaments spanned from the nuclear membrane to the plasma membrane and were attached to the plasma membrane in fashions of both lateral and endpoint associations. However, their attachment to the nuclear membranes was mostly in the way of end-on insertions. The membrane skeletons in split erythrocytes, as revealed by QF-DE method after fixation and saponin treatment, were forming condensed networks under the erythrocyte membrane (Fig. 4). The lower erythrocyte membrane was attached to the coverslips and underlying membrane skeletons were extended (Fig. 4a). During the unilateral extension, they underwent an uniaxial stretching force. The long filaments were in bundles and the condensed structures were in a bunch (Fig. 4b). The former structures were alternated with the latter structures. The upper cell membrane was curled up and mildly extended membrane skeletons could be observed (Fig. 4a,c). The intersections of filaments were shorter and the skeletons were more condensed than those on the lower membrane (Fig. 4c). Some of the intermediate filaments could be traced from the starting point of a nuclear membrane, via lateral attachment of the plasma membrane, and then to the end point of the nuclear membrane (Fig. 4a).

To see the difference between the fixed ultrastructure and non-fixed one of membrane skeletons, the erythrocytes were split in the cytosol buffer and quickly frozen (Fig. 5a-d). When the pressing strength over sandwiched coverslips was weak, only the upper lipid membrane was stripped off (Fig. 5a). The edge of remained membranes was curled up naturally, and the underlying networks could be seen. The filamentous components with a relatively uniform thickness of 10 to 12 nm were seen to have a tortuous course and to join to each other to form networks. Several filamentous components converged into a junction point with a spot-like configuration, showing a spider-like unit (Fig. 5a). When the upper cell membrane was completely removed by the cell-splitting method, the cytoplasmic side of the lower membrane was easily observed (Fig. 5b-d). In comparison with Figure 5a, there were many globular structures among the filaments. The unfixed erythrocyte

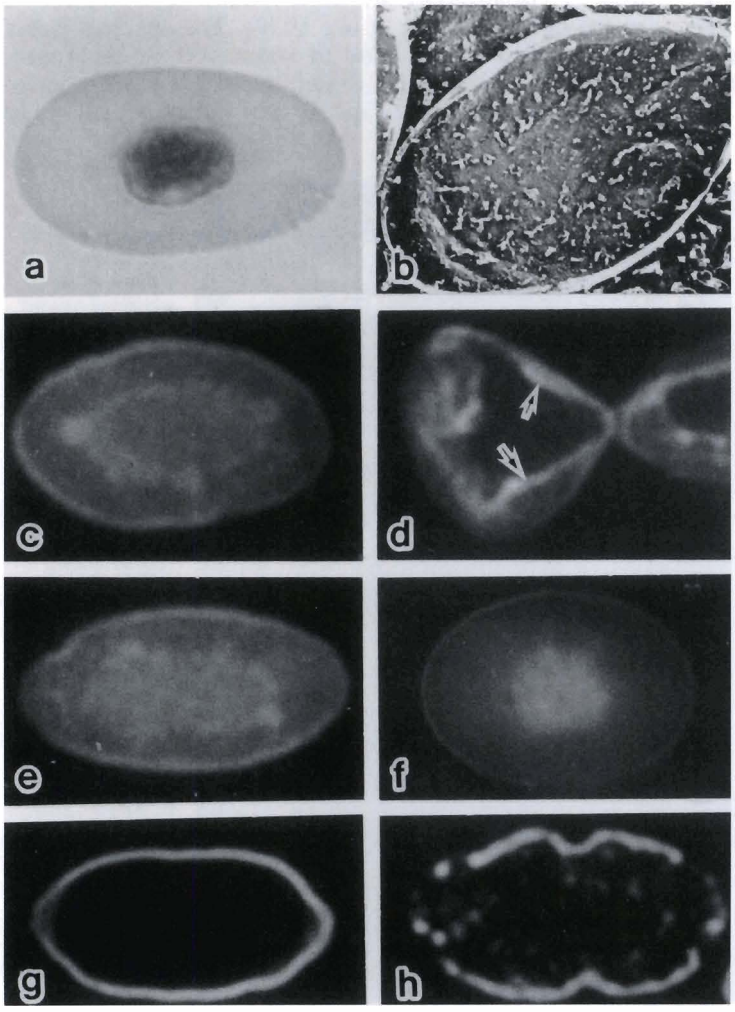

Fig. 2. Light and electron micrographs of paraformaldehyde-fixed erythrocytes. **a.** Wright-Giemsa staining. x 3,600. **b.** Saponin-treated and QF-DE replica. x 4,400. **c.** Saponin-treated and incubated with anti-spectrin antibody. x 3,600. **d.** Split and incubated with anti-spectrin antibody. Arrows are the line of residual membrane by splitting, indicating positive immuno-reactions. x 3,600. **e.** Saponin-treated and incubated with anti-actin antibody. x 3,600. **f.** Phalloidin-FITC. x 3,600. **g.** Anti-tubulin antibody. x 3,600. **h.** Fresh erythrocytes kept at 4 ºC for 20 min are fixed, saponin-treated and incubated with anti-tubulin antibody. x 3,600

in the cytosol buffer presented many intermediate filaments on the membrane skeletons, and fine filaments of 7-10 nm in diameter were anchored to the intermediate filaments and associated with membrane skeletons (Fig. 5b,c). Fine networks were clearly observed in some areas.

Several microtubules were seen under the plasma membrane and were organized into bundles designated as marginal bands (Fig. 6a, inset). In QF-DE preparations, the microtubule bundles could be seen three-dimensionally (Fig. 6). The marginal band was labile, especially at cold temperature, and the taxol in cell preparations was used to inhibit depolymerization. In the fixed and saponin-treated erythrocytes, the microtubules were located at their periphery and microtubule-associated filaments could be observed (Fig. 6a,c). As the membrane skeletons got closer to the marginal bands, they became denser networks (Fig. 6a). When the membrane skeletons were disrupted in non-fixed preparations because of the complete stripping of lipid membrane detached microtubules always remained, but their lines were disordered (Fig. 6b). When the lipid membrane layer was partially removed, underlying membrane skeletons and marginal bands could be easily observed (Fig. 6c). Fine filaments, 7 to 10 nm in diameter, connected the microtubules to other membrane skeletons. These fine filaments had different structures from those of membrane skeletons.

Discussion

The organization of cytoskeletons in the chicken erythrocyte has been demonstrated by the QF-DE method. The interaction between intermediate filaments and the nuclear membrane could be clearly seen in Figs. 3 to 4. The intermediate filaments appeared to bind to membrane skeletons via both lateral and endpoint associations. It has been reported that spectrin promotes lateral associations of the intermediate filaments with lipid cell membrane and hence forms their lateral associations (Langley and Cohen, 1987). Ankyrin, however, was reported to bind only to the amino-terminal end of unpolymerized vimentin (Georgatos and Marchesi, 1985). So vimentin might be tightly associated with the nuclear membrane, possibly providing mechanical support and keeping the nucleus in a central place of the cytoplasm.

The mature chicken erythrocyte has been reported to possess most of the human erythrocyte membrane components, consisting of the proteins, such as spectrin, actin, ankyrin, and band 4.1 (Granger et al., 1982). The purified human spectrin dimers or tetramers were directly visualized by low angle shadowing with electron microscopy (Shotton et al., 1979). The spectrin dimer appears as a flexible rod of about 100 nm in length. Moreover two spectrin dimers can bind each other to form a tetramer rod of nearly 200 nm. A recent study

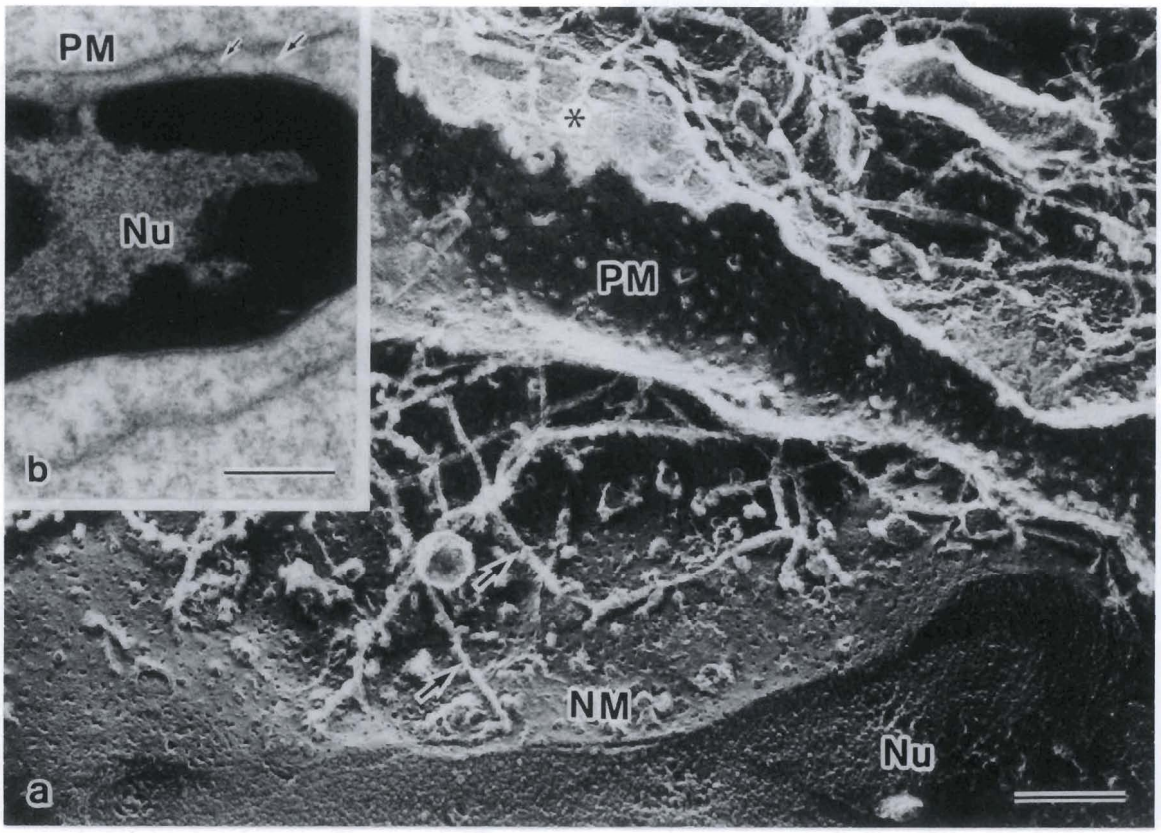

Fig. 3. a. Freeze-fractured erythrocytes, which are treated with saponin after paraformaldehyde fixation. Intermediate filaments in the cytoplasm are spanning (arrows) from the nuclear membrane (NM) to the cytoplasmic side of plasma membrane (PM). The cytoplasmic side of another erythrocyte is also observed (asterisk). Nu: nucleus. Bar: 500 nm; x 32,000. **b.** Ultrathin section of a saponin-treated erythrocyte. Some intermediate filaments are observed (arrow) Bar: 500 nm; x 28,000

demonstrated the spreading appearance of the human erythrocyte, as revealed by the negative staining method after air-drying, changing ionic strength and Triton treatment (Derick et al., 1992). The erythrocyte shape is attributable to the properties of membrane skeletons that are mainly composed of spectrin tetramers and medium-sized oligomers cross-linked by a short F-actin filament. However, the present study of fresh chicken erythrocytes supported the hypothesis that the chicken spectrin in the native membrane skeleton might not be organized in an elongated form, but is condensed, presumably through intramolecular and/or intermolecular interactions. These structures resemble the model of «the intact skeletal network» presented by Ursitti et al. (Ursitti et al., 1991;

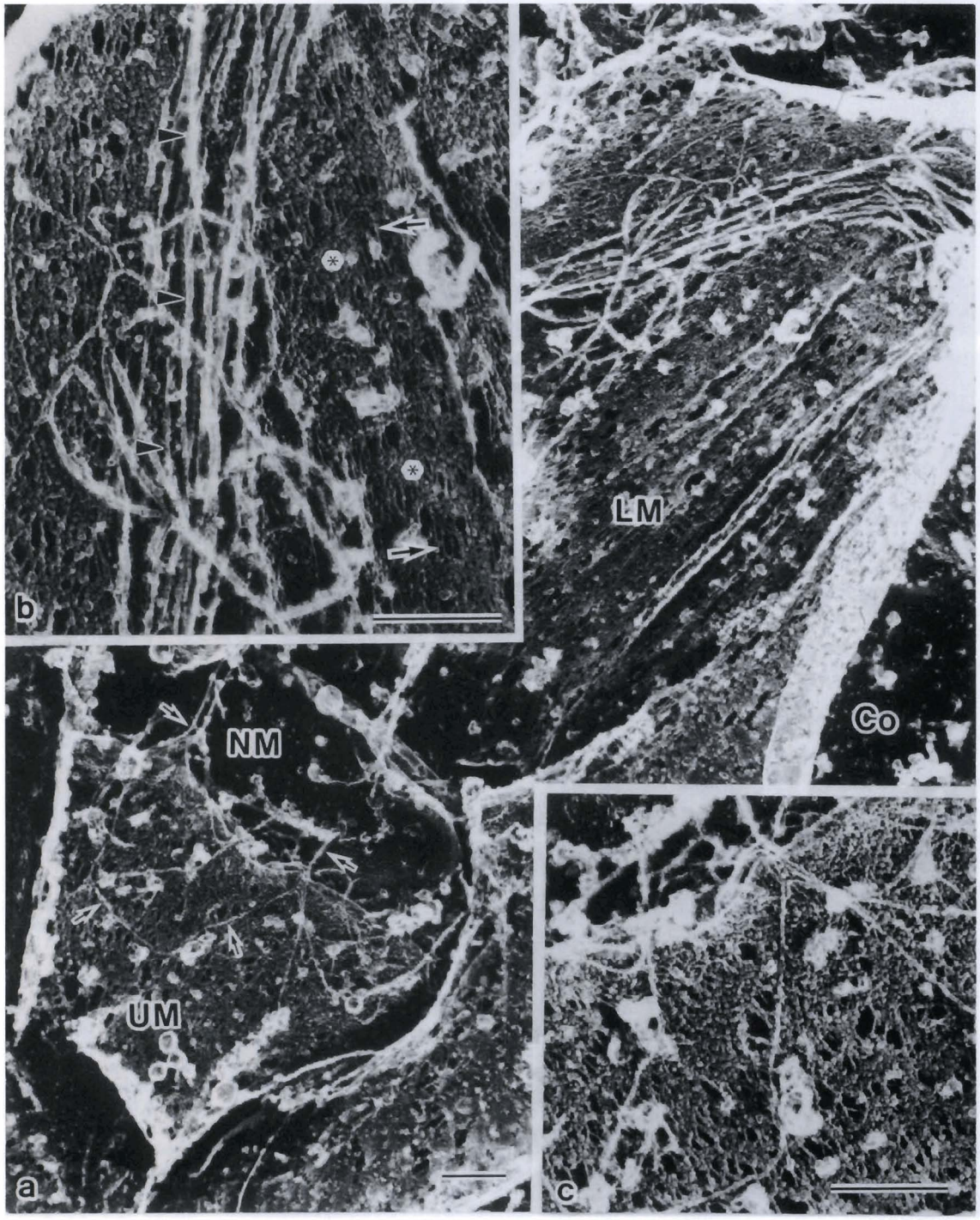

Fig. 4. a. Replica electron micrographs showing the erythrocyte membrane on a coverslip (Co), prepared by the cell-splitting method after paraformaldehyde fixation. The lower erythrocyte membrane (LM) is severely stretched, whereas the upper one (UM) is mildly stretched. Some intermediate filaments (small arrows) are turning from the plasma membrane to the nuclear membrane (NM). Bar: 500 nm; x 18,800. **b.** Higher magnified view of the lower membrane skeletons shown in a. During one directional extension, they undergo the uniaxial stretching. The long filaments (small arrows) are decorated with aggregated granular structures (asterisks). Marginal bands in the peripheral areas are also seen (arrowheads). Bar: 500 nm; x 35,500. **c.** Higher magnified view of the upper membrane skeletons shown in a. Note the more condensed membrane skeletons with granular structures. Bar: 500 nm; x 39,000

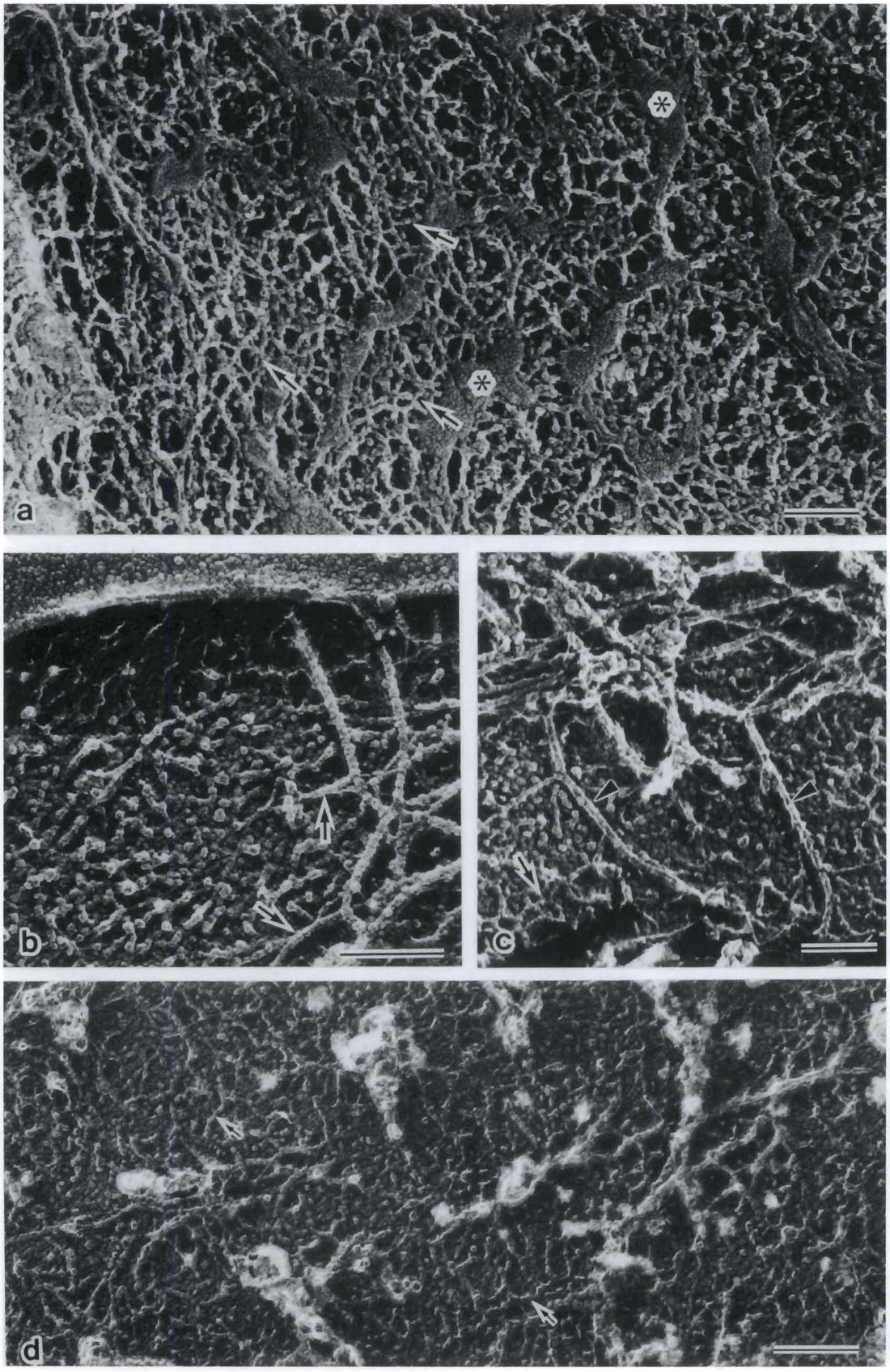

Fig. 5. a. Replica electron micrograph showing the fresh erythrocyte membrane skeletons. Fine networks underlying the lipid membrane are viewed from extracellular space (arrows). In some parts, the lipid membranes still remain (asterisks). Bar: 200 nm; x 56,000. **b. and c.** Erythrocytes are split open and the cytoplasmic side of their membrane is exposed. Intermediate filaments (arrowheads) are associated with fine filaments and attached to the membrane skeletons (small arrows). Fine networks and many globular structures compose the membrane skeletons. PM: plasma membrane. Bar: 200 nm; b, x 80,000; c, x 60,200. **d.** Most of the intermediate filaments are detached from the membrane and fine networks of membrane skeletons (arrows) are observed. Bar: 200 nm; x 66,000

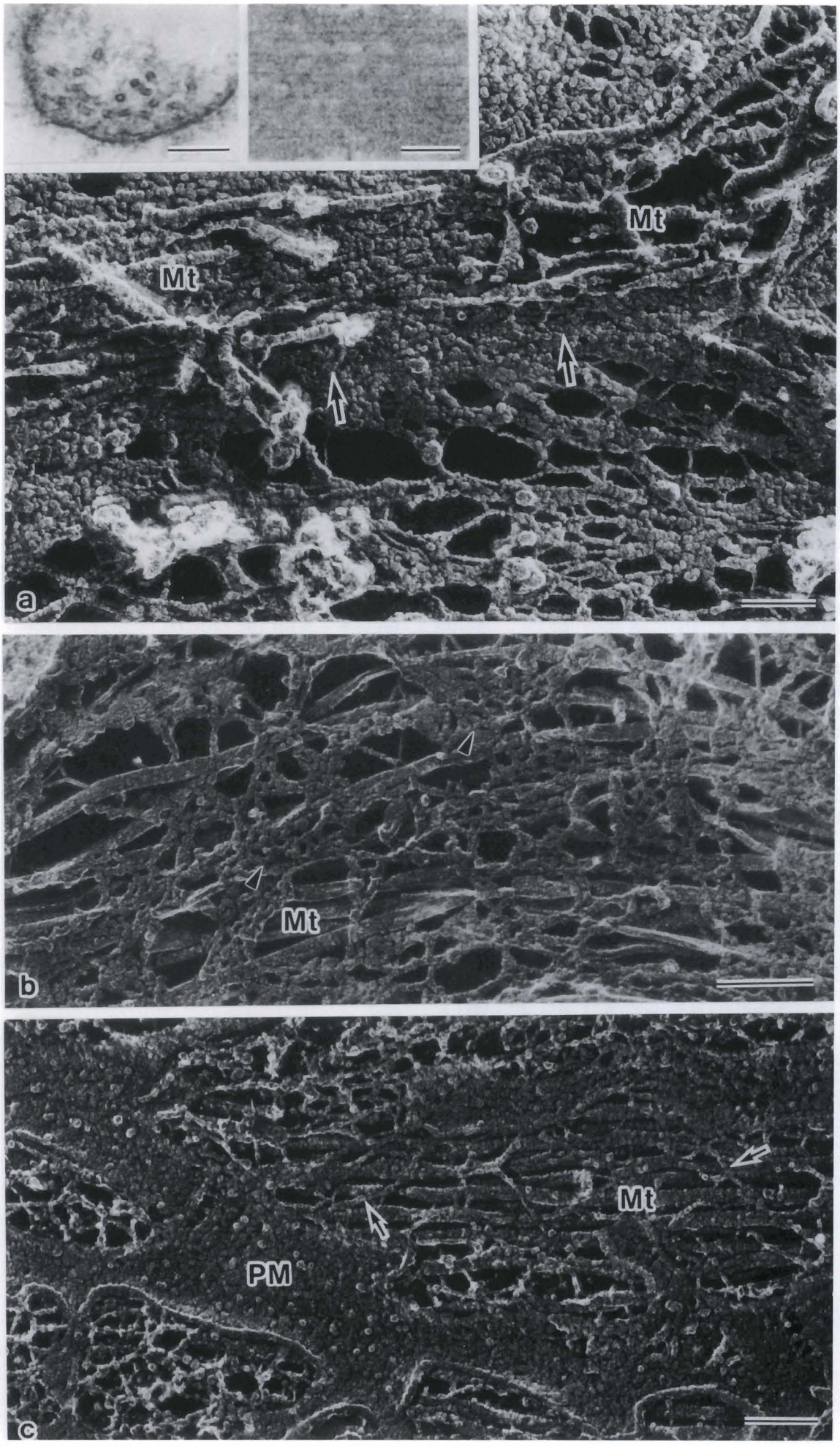

Fig. 6. a. Replica electron micrographs showing the marginal band and membrane skeletons of an erythrocyte, which are split open after paraformaldehyde fixation. The erythrocyte membrane along the marginal band is more granular and condensed (arrows) in contrast to other membrane skeletal regions (asterisk). Bar: 200 nm; x 66,000. **Inset:** cytoskeletal organization, such as marginal bands, in conventional cross section (left) and longitudinal section (right), prepared after the saponin treatment. Bar: 200 nm; left, x 55,000; right, x 51,000). **b.** Ultrastructure of the marginal bands in fresh erythrocytes prepared by cell-splitting method. When the membrane skeletons are partially removed and disrupted (arrowheads), the marginal bands are also identified. Bar: 200 nm; x 82,000. **c.** Ultra-structure of the marginal bands in fresh erythrocytes prepared by cell-splitting method. Fine filaments (arrows) span between the marginal bands and the membrane skeletons. The surface membrane layer is mechanically removed, and underlying membrane skeletons and marginal bands can be observed. Mt: Microtubule. PM: plasma membrane layer. Bar: 200 nm; x 64,000

Ursitti and Wade, 1993). Moreover, we recently examined the en face cytoplasmic sides of human fresh erythrocyte membranes by the QF-DE method with the erythrocyte-splitting method, which improved the resolution in direct views of the native cytoplasmic aspects (Ohno et al., 1994). Some specimens in the present study were fixed with paraformaldehyde, and the membrane skeletons underwent an isometric uniaxial stretching to a long rectangle during the extension, as shown in Figure 4. The alternate structure with the bundled filaments and the bunched granules may be representative of the high deformability of erythrocytes. The granular components underlying the membrane skeleton may imply other additional proteins, such as band 4.1, ankyrin, tropomyosin, tropomodulin, adducin and dematin (Fowler, 1986). Further examination to identify these proteins is now undertaken by the immunocytochemical method.

The distribution of the membrane skeletal proteins has often been examined up to now. In the present study, more densely granular areas underlay the bundled microtubules, though some microtubules became discontinuous during the cell preparation, as observed in Figure 6 (Swan and Solomon, 1984). Therefore, the granular areas resembled the «track» of microtubules as previously suggested, which also lost the microtubule bundles during sonication under depolymerization-inhibitory conditions (Joseph-Silverstein and Cohen, 1984). When the overlying lipid membrane was stripped off, fine filaments between marginal bands and lipid cell membranes were identified. Three patterns in the association of the marginal microtubules with membrane skeletons were supported by in vitro experiments. First, the protein 4.1 binds to tubulin at a molar ratio similar to that described for brain microtubule-associated proteins (Correas and Avila, 1988). Second, the ankyrin can bind to tubulin as well as band 3 (Lux et al., 1990). The third participant is the microtubule-associated protein (MAP) like neuron MAP2, which links the microtubules to fodrin (brain spectrin) (Levene and Willard, 1981). A protein with apparent molecular weight of 280,000 (syncolin), which was immunoreactive with the antibody against hog brain MAP2, was purified from chicken erythrocytes (Feick et al., 1991). Further examinations are needed to identify these globular and filamentous proteins. They may clarify the mild stiffness of chicken erythrocyte membranes and maintain their shape.

In summary, the *in situ* erythrocyte shape and deformability, possibly supported by the membrane skeletons, are visualized by the erythrocyte-splitting method combined with the QF-DE method. Stable structures near the microtubule bundles in comparison with flexible spectrin-based membrane skeletons as illustrated in Figure 7. The membrane-associated erythrocyte skeleton visualized by the QF-DE method was densely organized. Moreover, the organization of

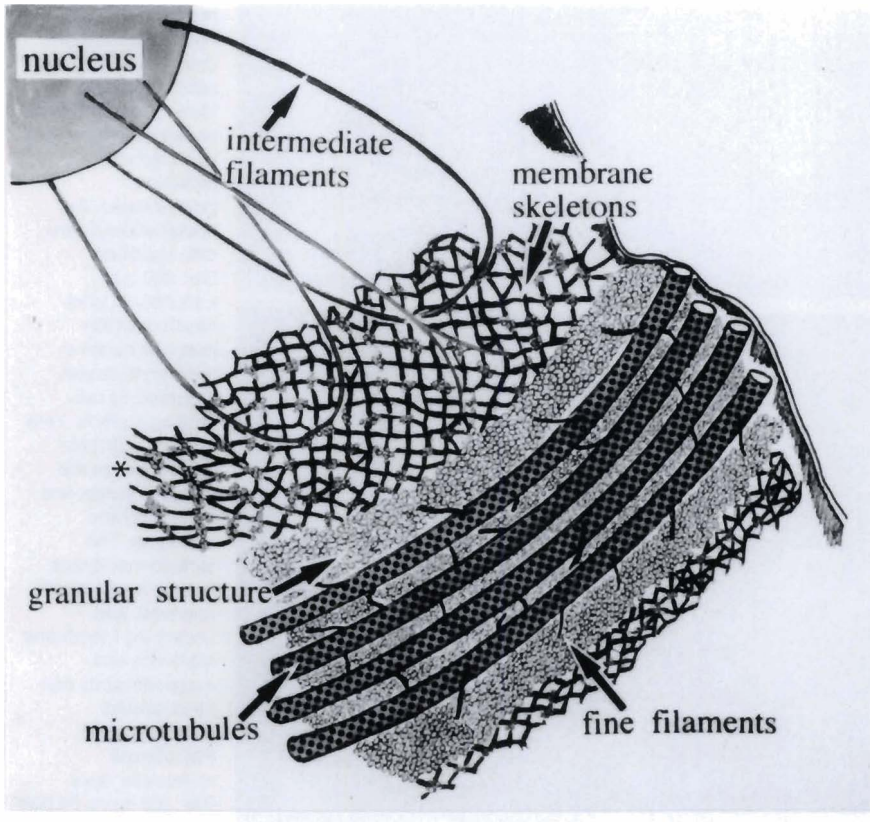

Fig. 7. Schematic representation of chicken erythrocyte cytoskeletons, obtained by the quick-freezing and deep-etching method. Membrane skeletons under marginal bands, which are connected by fine filaments, are very dense structures. Intermediate filaments span between a nucleus and membrane skeletons. Most membrane skeletons are in condensed form, whereas some are stretched during erythrocyte preparations (asterisk).

membrane skeleton near the microtubule was compact compared with its other region. The different distributions of each component are visualized in the present study. During erythrocyte preparations, the combination of paraformaldehyde fixation and non-fixation was needed before the erythrocyte-splitting and saponin treatment, in order to examine the membrane skeletons from the viewpoint of relatively dynamic states.

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