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Membrane Perturbations of Erythrocyte Ghosts by Spectrin Release

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The cytoskeleton plays an important role in the stability and function of the membrane. Spectrin release from erythrocyte ghosts makes the membrane more fragile. However, the detail of membrane fragility has remained unclear. In the present study, the effects of incubation temperatures and polyamines on the membrane structure of ghosts under hypotonic conditions have been examined. Upon exposure of ghosts to a hypotonic buffer at 0–37°C, reduction of ghost volume, spectrin release and decrease of band 3-cytoskeleton interactions were clearly observed above 30°C. However, such changes were completely inhibited by spermine and spermidine. Interestingly, conformational changes of spectrin induced at 37°C or 49°C were not suppressed by both polyamines. Flow cytometry of fluorescein isothiocyanate-labelled ghosts exposed to 37°C demonstrated the two peaks corresponding to ghosts with normal spectrin content and decreased one. Taken together, these results indicate that the degree of spectrin release from the membrane under hypotonic conditions is not same in all ghosts, and that polyamines inhibit the spectrin release followed by changes in the membrane structure, but not conformational changes of spectrin.

Key words: band 3, erythrocyte, flow cytometry, polyamine, spectrin.

Abbreviations: $C_{12}E_8$, octaethylene glycol mono-*n*-dodecyl ether; DNDS, 4,4′-dinitrostilbene-2, 2′-disulphonate; FITC, fluorescein isothiocyanate; NPIA, *N*-(1-pyrenyl) iodoacetamide; PBS, phosphate-buffered saline, 10 mM sodium phosphate, 150 mM NaCl, pH 7.4; 5P8, 5 mM sodium phosphate, pH 8.

Cytoskeletal proteins such as spectrin and actin in erythrocytes underlie lipid bilayers and play an important role in the stability and function of membranes (*1*, *2*). Particularly, spectrin is a key protein of cytoskeleton and bound to transmembrane proteins such as band 3 and glycophorin C via ankyrin and protein 4.1, respectively (*3*, *4*). Spectrin comprises two large subunits, α (280 kDa) and β (246 kDa), which are aligned side-to-side and in an antiparallel orientation to form heterodimer αβ (Fig. 1) (*1*, *2*). Heterodimers associate head-to-head to form tetramers and oligomers (*1*, *2*). A spectrin tetramer–dimer equilibrium is dependent on temperature and ionic strength (*5*). For instance, the tetramer dissociates into the dimers above 30°C under hypotonic conditions (*5*). In spectrin dimers and tetramers, the amino (N) terminal region of α subunit interacts with the carboxy (C) terminal region of β subunit (*6*). The mutations of amino acid residues in these regions produce insufficient interactions between α subunit and β subunit, and result in morphologically abnormal erythrocytes as seen in diseases with hereditary elliptocytosis (*7*).

There are many studies about the role of spectrin concerning membrane stability of erythrocytes. Upon denaturation of spectrin at 49°C, vesiculation of erythrocytes occurs (*8*). Chemical modifications of spectrin with SH-reactive reagents such as *N*-ethylmaleimide make the membrane more fragile at temperatures lower than 49°C (*9*, *10*). Membrane deformability decreases upon transition from spectrin tetramer to dimers (*11*). Upon disruption of the cytoskeletal network by spectrin release, the ability to reseal the membrane is lost (*12*). However, the details of membrane structural changes induced by spectrin release have not yet been determined.

Polyamines are present in various kinds of cells and have multiple biological functions. For instance, they can stabilize the double helical structure of DNA by bridging between strands (*13*). The regulated intracellular levels of polyamines are required for DNA replication, and their reduced levels affect cell growth and apoptosis (*14*, *15*). The level of polyamines in the erythrocyte is 10–15 μM (*16*) and they are known to stabilize the membrane skeleton (*17*). The release of spectrin from the membrane under hypotonic conditions is inhibited by polyamines (*18*). Spermine decreases the membrane deformability and stabilizes the membrane skeleton against the fragmentation induced by high shear stress (*17*). Haemoglobin and basic proteins such as cytochrome *c* and ribonuclease also stabilize spectrin (*19*). Thus, it seems likely that the membrane skeleton is stabilized by electrostatic interactions with polyamines.

In this study, the effects of polyamines on membrane structural changes in ghosts exposed to a hypotonic buffer have been examined. Under hypotonic conditions, spectrin is released from the membrane.

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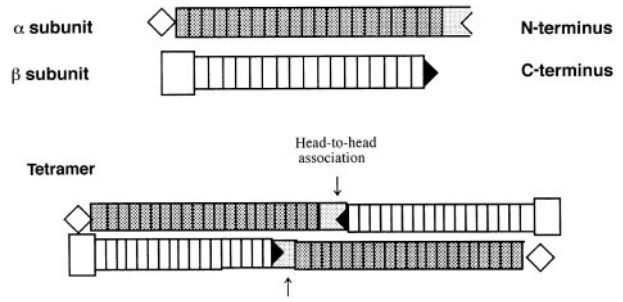

Fig. 1. **Model of spectrin tetramer formation by head-to-head association of α- and β-subunits.** The α subunit consists of N-terminal, multiple 106-residue repeat (rectangles), and C-terminal (diamond) domains. The β subunit is comprised of N-terminal actin binding (large rectangle), 17 homologous (106-residue) repeat (rectangles), and C-terminal domains. Arrow shows the head-to-head self association site of αβ dimers.

Therefore, the results obtained with the membranes prepared under our artificial conditions may contribute to the understanding of pathological molecular mechanism of hereditary spherocytosis characterized by spectrin deficiency (*20*). We demonstrate that the spectrin release from ghosts followed by the reduction of membrane volume can be monitored by flow cytometry, and that polyamines inhibit the spectrin release, but not conformational changes of spectrin.

MATERIALS AND METHODS

Materials—Compounds were obtained from the following sources: 4,4′-dinitrostilbene-2,2′-disulphonate (DNDS), Tokyo Kasei; eosin-5-maleimide and *N*-(1-pyrenyl) iodoacetamide (NPIA), Molecular Probes (Oregon, USA); fluorescein isothiocyanate isomer I (FITC), spermine and spermidine, Sigma; octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$), Nikko Chemicals; Triton X-100, Nacalai Tesque. All other chemicals were of reagent grade.

Ghost Volume—Human blood was obtained from the Fukuoka Red Cross Blood Center. The blood was centrifuged for 10 min at $1000 \times g$ and 4°C. The plasma and buffy coat were carefully removed. The erythrocytes were washed three times with phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, pH 7.4). For ghost preparation, the erythrocytes were haemolyzed at 0°C using 10 volumes of 5 mM sodium phosphate, pH 8 (5P8). The haemolyzates were centrifuged at $10,000 \times g$ for 10 min at 4°C. The pellets were washed three or four times with 5P8 to get white ghosts. These open ghosts were used in all experiments mentioned subsequently. Ghosts (30 μl) were suspended in 5P8 (270 μl, 10% haematocrit), and then incubated for 30 min at 0–37°C. For polyamine treatment, ghosts were incubated for 30 min at 37°C in 5P8 containing 2 mM spermine or 2 mM spermidine. After incubation, ghost suspensions were centrifuged for 10 min at $11,000 \times g$ and 4°C. The supernatants were removed carefully and the pellets were suspended in 30 μl of 5P8. Parts of the suspensions were drawn into a microhaematocrit tube (diameter of 0.6 mm, length of 35 mm), and then centrifuged for 10 min at $6000 \times g$ and 4°C. The ghost volume was estimated by measuring the lengths of ghost phase and 5P8 phase in a microhaematocrit tube.

Release of Membrane Proteins from Ghosts Exposed to Hypotonic Buffer—To examine the protein release from the membrane, ghosts suspended at 10% haematocrit in 5P8 were incubated for 30 min at 0–37°C. For the effect of polyamines on release of membrane proteins, ghosts were similarly incubated in 5P8 containing 2 mM spermine or 2 mM spermidine. After incubation, the ghost suspensions were centrifuged at $15,000 \times g$ for 20 min at 4°C. The protein concentrations in the supernatants were determined by the method of Lowry *et al.* (*21*). The residual pellets were used for SDS–PAGE on a slab gel containing 2.5% (stacking gel) and 8% (separation gel) acrylamide, according to the method of Laemmli (*22*). The gels were stained with Coomassie blue.

Flow Cytometry—Ghosts (20% haematocrit) were incubated with 0.5 mM FITC in 5P8 for about 15 h at 0°C, and then centrifuged for 10 min at $10,000 \times g$ and 4°C. The pellets were washed three times with 5P8. The detection of the membrane proteins labelled with FITC was performed using SDS–PAGE as mentioned earlier. The bands were detected by UV irradiation. FITC-labelled ghosts (5 μl) suspended in 45 μl of 5P8 were incubated for 30 min at 0–37°C. For polyamine treatment, FITC-labelled ghosts were incubated for 30 min at 37°C in 5P8 containing 2 mM spermine or 2 mM spermidine. After incubation, the suspensions (2 μl) were added into 2 ml of 5P8 at 0°C for the measurement of flow cytometry. Flow cytometric data were obtained using an EPICS XL-MCL flow cytometer (Coulter, Hileah, FL). For each measurement, 50,000 particles were examined.

Band 3-Cytoskeleton Interactions—To label band 3 with eosin-5-maleimide, erythrocytes (20% haematocrit) in PBS were pre-treated with 0.5 mM DNDS at 37°C for 10 min and treated with eosin-5-maleimide (0.1 mg/ml) for 15 min at room temperature. After incubation, the erythrocytes were washed three times with PBS, once with PBS containing 0.5% (w/v) bovine serum albumin, and then three times with PBS. Ghosts were prepared from eosin-5-maleimide-labelled erythrocytes using 5P8, incubated in 5P8 at 0–37°C for 30 min, and then solubilized by 0.5% (w/v) $C_{12}E_8$ for 10 min at 0°C. Solubilized samples were centrifuged at $20,400 \times g$ for 20 min at 4°C. The proportion of band 3 interacted with cytoskeleton was estimated from fluorescence intensities of eosin-5-maleimide in supernatants and pellets.

Pyrene Labelling of Membrane Proteins—Ghosts in 5P8 were pre-incubated for 30 min at 0, 23, 37 or 49°C, and then treated with NPIA (10 μg/ml) for about 10 h at 0°C. The NPIA-labelled ghosts were washed several times with 5P8, and then solubilized by 1% (v/v) Triton X-100 at 0°C. The solubilized samples were centrifuged at $20,400 \times g$ for 20 min at 4°C. The pellets (Triton shells) were washed once with 5P8 and suspended in 5P8. Fluorescence spectra of NPIA were measured using a model FP-750 spectrometer (JASCO, Japan) with excitation at 345 nm (slit width, 5 nm). To assess the difference

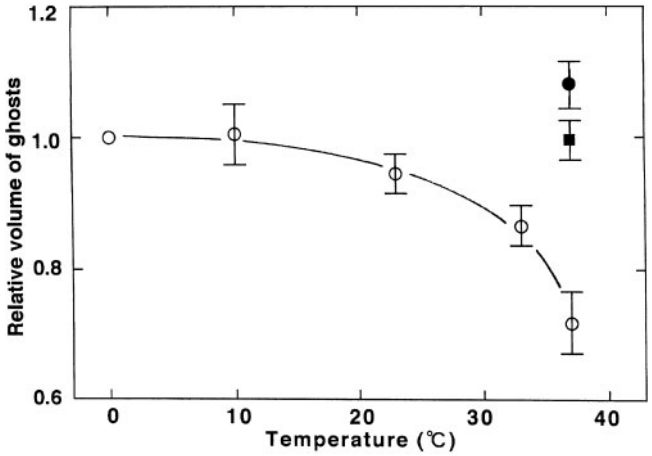

Fig. 2. **Temperature dependence of ghost membrane volume.** Ghosts suspended in 5P8 were incubated in the absence (open circle) and presence of spermine (filled circle) or spermidine (filled square) for 30 min at 0–37°C. After incubation, the suspensions were drawn into microhaematocrit tubes and centrifuged. The volume of ghost membranes at each temperature is normalized to that at 0°C. Values are means ± SD for three experiments.

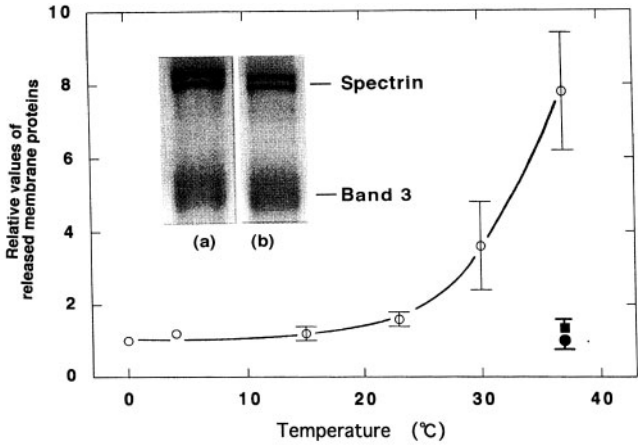

Fig. 3. **Temperature dependence of membrane proteins released from ghosts in hypotonic buffer.** Ghosts suspended in 5P8 were incubated in the absence (open circle) and presence of spermine (filled circle) or spermidine (filled square) for 30 min at 0–37°C, and centrifuged. The protein concentrations of supernatants were determined, and normalized to that at 0°C. The inset is SDS–PAGE of pellets obtained from ghosts exposed to 0 (a) or 37°C (b) in the hypotonic buffer. A part of gel is shown. Values are means ± SD for three experiments.

in fluorescence intensity, a Student's *t*-test for paired data was used.

RESULTS

Temperature Effects on Ghost Volume in the Hypotonic Buffer—The ghost volume in the hypotonic buffer was examined as a function of the incubation temperatures (0–37°C). Here, the ghost volume corresponds to that of membrane lipid bilayer itself. The volumes of ghost membranes remained almost constant at 0 and 10°C, but decreased abruptly at higher temperatures (Fig. 2).

It is well known that cytoskeletal network in ghost membranes is perturbed in hypotonic buffers, but its perturbation is suppressed by polyamines such as spermine and spermidine (*17*, *18*). So, the effect of polyamines on ghost volume was examined. The reduction of ghost volume at 37°C in the hypotonic buffer was completely inhibited by spermine and spermidine.

Release of Cytoskeletons from Ghost Membranes—Figure 3 shows the temperature dependence of membrane proteins released from ghosts in the hypotonic buffer. In the range of 0–23°C, the amounts of released proteins were small. However, membrane proteins were readily released from ghosts above 30°C. The SDS–PAGE analysis of ghosts incubated at 0°C and 37°C in the hypotonic buffer showed that spectrin molecules are partially released from the membrane at 37°C. The release of such membrane proteins at 37°C was completely suppressed by polyamines such as spermine and spermidine (Fig. 3).

SDS–PAGE and Flow Cytometry of FITC-Labelled Ghosts—Ghosts in the hypotonic buffer were treated with 0.5 mM FITC at 0°C. The SDS–PAGE analysis of FITC-treated ghosts demonstrated that spectrin among membrane proteins is mainly labelled with FITC (Fig. 4A). Ghosts labelled with FITC were incubated at 0–37°C in the hypotonic buffer with or without polyamines, and then used for the flow cytometric analysis (Fig. 4B). The linewidth at half-height of peak represents the distribution of particles with different fluorescence intensities. Therefore, one peak with the narrow linewidth at 0°C shows that spectrin contents in all ghosts are almost the same. Similar peak was also seen at 23°C. On the other hand, one peak with broad linewidth was observed at 33°C, suggesting the existence of ghosts with various contents of spectrin due to its release. Interestingly, the two peaks appeared in the case of 37°C. However, no such peaks appeared in the presence of spermine (Fig. 4B, bottom panel) and spermidine, or under isotonic conditions (data not shown). The position of peak with the strong fluorescence intensity at 37°C was identical with that observed in the presence of spermine (Fig. 4B, bottom panel). These results suggest that there are ghosts with normal spectrin contents and decreased ones upon exposure to hypotonic buffer at 37°C. To examine the effect of FITC labelling on the membrane structure, ghosts were preincubated at 37°C in the hypotonic buffer, and then labelled with FITC at 0°C. Ghosts labelled thus with FITC also showed the two peaks as seen at 37°C of Fig. 4B (data not shown). Previously, we showed that open ghosts and sealed particles such as vesicles suspended in buffer are discriminated by the flow cytometry (*8*, *23*). From forward scatter versus side scatter dot plots of samples used in Fig. 4B, open ghosts were separated from vesicles as shown in Fig. 4C. The formation of vesicles from ghosts obviously increased at 37°C, but was suppressed by spermine.

Band 3-Cytoskeleton Interactions—When intact erythrocytes are treated with eosin-5-maleimide, anion exchanger proteins (band 3) are specifically labelled with eosin molecules (*24*). Eosin-labelled ghosts in the

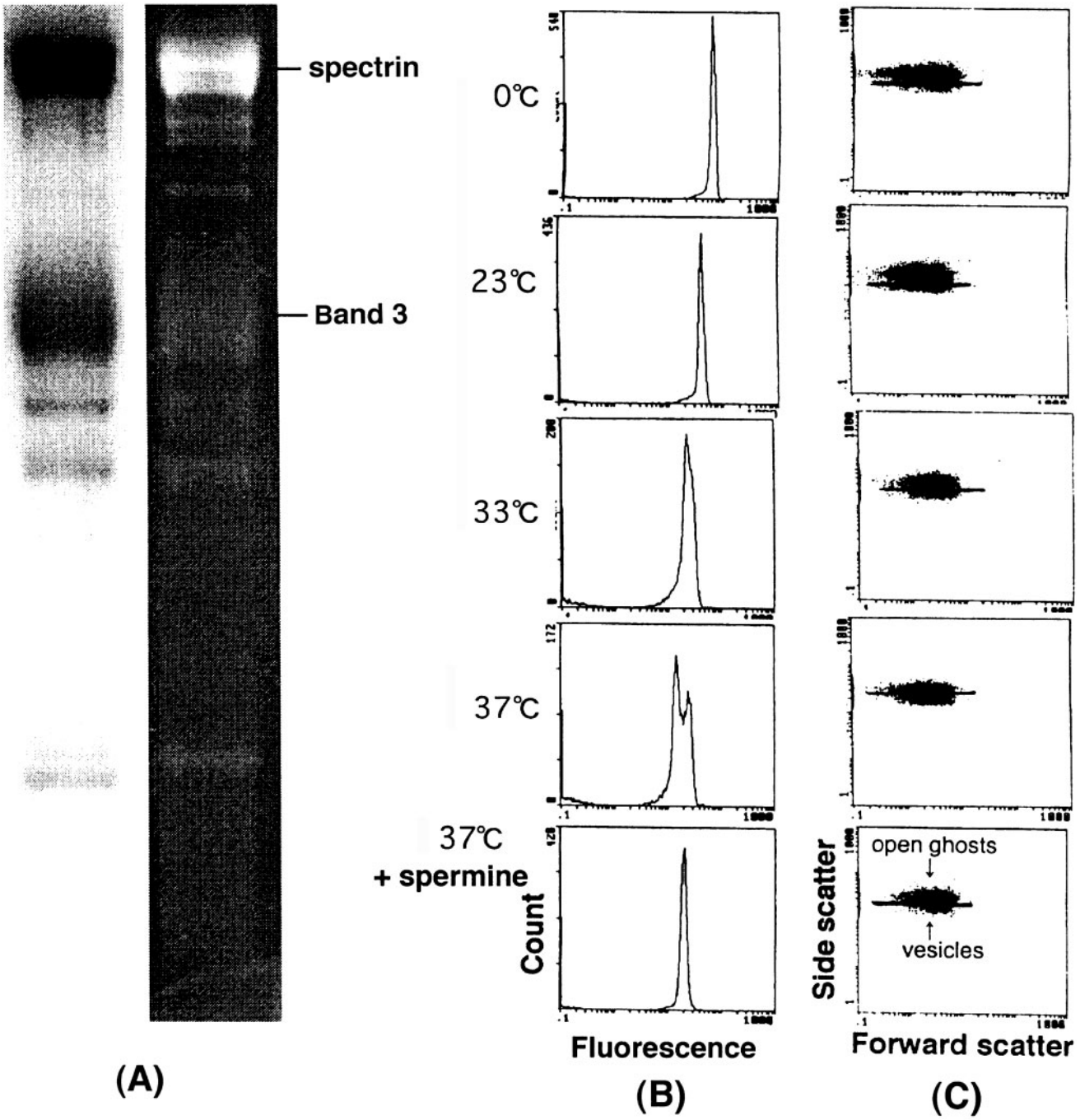

Fig. 4. **SDS–PAGE and flow cytometry of FITC-labelled ghosts.** (A) SDS–PAGE of FITC-labelled ghosts. Left lane, Coomassie blue staining; right lane, UV irradiation. (B) Flow cytometry of FITC-labelled ghost suspensions incubated for 30 min at 0–37°C in the hypotonic buffer with or without 2 mM spermine. (C) Dot plots of forward scatter against side scatter of samples used in (B). Regions containing open ghosts and vesicles are separated by a horizontal line. For each measurement, 50,000 particles were examined.

hypotonic buffer were incubated at 0–37°C, and then solubilized at 0°C by non-ionic detergent, $C_{12}E_8$. Band 3-cytoskeleton interactions remain stable against the solubilization of membrane proteins by non-ionic detergents such as $C_{12}E_8$ and Triton X-100 (*25*). So, only band 3 interacted with cytoskeleton is pelleted with spectrin by centrifugation, whereas free band 3 is observed in the supernatant (*25*, *26*). The ratio of band 3 associated with cytoskeleton was estimated from fluorescence intensities of band 3 in pellets and supernatants. The proportion of band 3 interacted with cytoskeleton was ~40% in ghosts incubated at 0–23°C in the hypotonic buffer, and then decreased up to a few percents at 37°C (Fig. 5). However, such breakdown in linkage of band 3 with cytoskeleton at

37°C was completely inhibited by spermine and spermidine.

Conformational Changes of Spectrin Exposed to Hypotonic Buffer at 0, 23 or 37°C—The fluorescence of pyrene is useful to detect conformational changes of proteins. For instance, excimer fluorescence of pyrene is observed in denatured spectrin (*8*, *27*). Ghosts were exposed to 0, 23 or 37°C in the hypotonic buffer, and then labelled with NPIA at 0°C. The fluorescence spectrum of pyrene in Triton shells, which contain spectrin, is shown in Fig. 6A. The ratio (I'/I) of fluorescence intensities at 386 and 462 nm was used as a measure of conformational changes of spectrin (*27*). The value of I'/I at 0°C was almost the same as that at 23°C (Fig. 6B). However, its value at 37°C was significantly increased, compared with those at 0 and 23°C. Interestingly, the conformational changes of spectrin at 37°C were not suppressed by polyamines. It is well known that spectrin is denatured at 49°C, and the excimer fluorescence appears in NPIA-labelled spectrin (*8*, *27*). So, the effect of polyamines on the excimer fluorescence was examined using ghosts. Excimer fluorescence of pyrene was observed in ghosts exposed to 49°C in the presence of polyamines (Fig. 6C). In ghosts exposed to 49°C in the absence of polyamines, most of spectrin was released from the membrane. Therefore, the appearance of the excimer fluorescence was not clear.

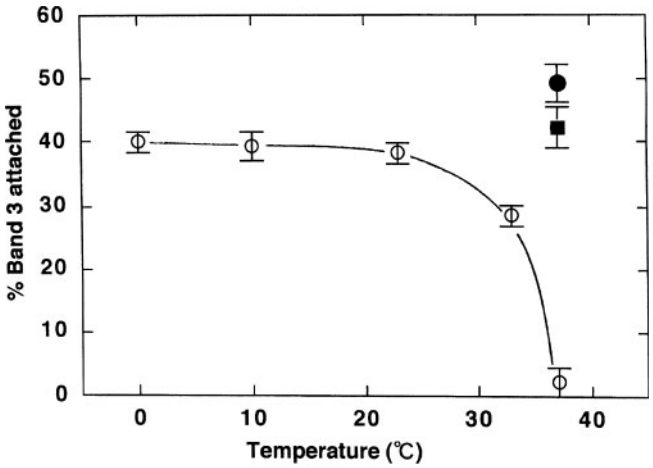

Fig. 5. **Temperature effects on band 3-cytoskeleton interactions in ghosts exposed to hypotonic buffer.** Eosin-labelled ghosts in 5P8 were incubated for 30 min at 0–37°C in the absence (open circle) and presence of spermine (filled circle) or spermidine (filled square). These ghosts were added to 5P8 containing 0.5% $C_{12}E_8$, and solubilized at 0°C. Solubilized solutions were centrifuged, and fluorescence of supernatant and pellet was measured. Values are means ± SD for three experiments.

DISCUSSION

In the present work, we have demonstrated membrane structural changes in ghosts exposed to the hypotonic buffer. Upon release of spectrin, the cytoskeletal network is disrupted so that the interactions of spectrin with band 3 via ankyrin are greatly perturbed. Under such

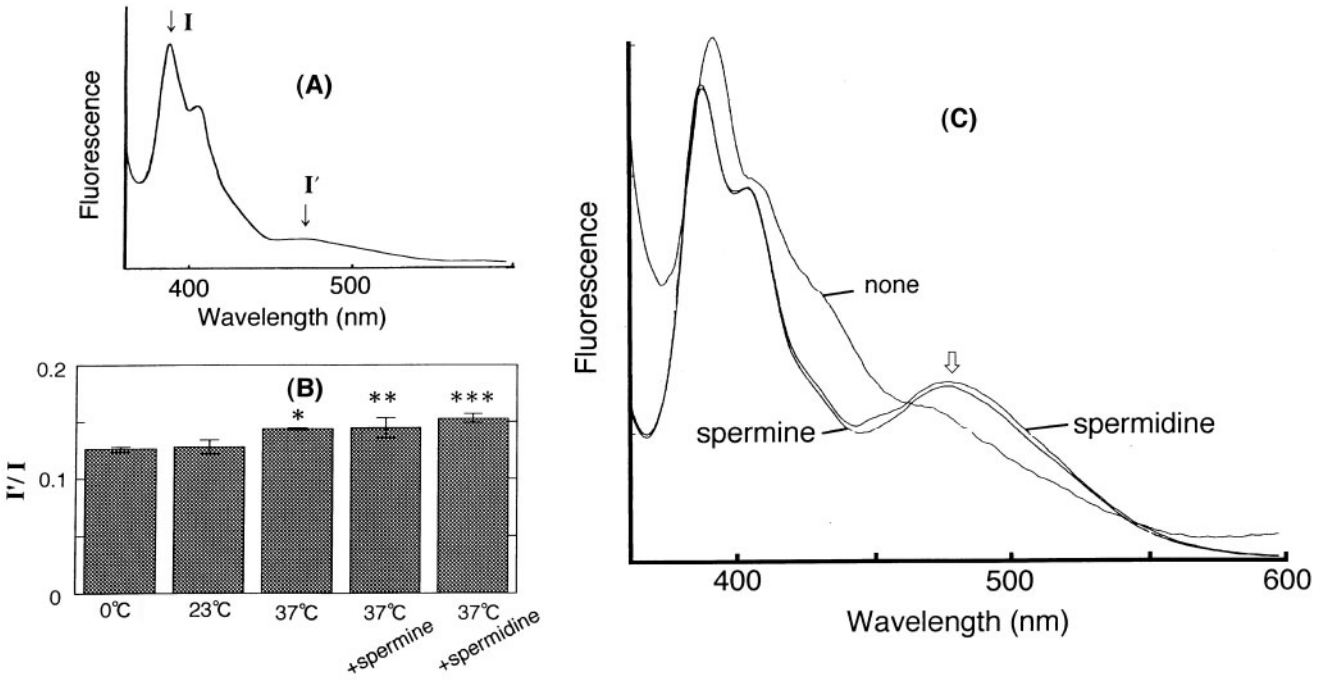

Fig. 6. **Fluorescence spectra of pyrene-labelled ghosts.** (A) Ghosts incubated in 5P8 at 0°C, and then labelled with NPIA at 0°C. In the fluorescence spectrum of Triton shells in 5P8, I and I' show the fluorescence intensities at 386 and 462 nm, respectively. (B) Effects of temperatures and polyamines on values of I'/I. Ghosts in 5P8 were exposed to 0, 23 and 37°C for 30 min in the presence or absence of polyamines, and then labelled with NPIA at 0°C. Triton shells were prepared from these ghosts. Values are means ± SD for three experiments. *, **, *** $P < 0.02$, 0.05, 0.01 versus 0°C, respectively. (C) Ghosts incubated in 5P8 with or without polyamines at 49°C, and then labelled with NPIA at 0°C. Arrow shows excimer fluorescence.

conditions, the volume of ghost membranes is reduced by vesiculation. However, no such changes occur upon inhibition of spectrin release by polyamines. These results reveal the important role of spectrin in the membrane stability. Generally, no spectrin is released from the membrane under isotonic conditions. However, spectrin-deficient erythrocytes are known as hereditary spherocytosis (*20*). Although such erythrocytes are under isotonic conditions, the shape of cells become spherocytic upon vesiculation induced by membrane instability due to spectrin deficiency. In spite of the spectrin release under our artificial conditions, therefore, the present data are expected to be useful to understand the properties of the membrane structure in hereditary spherocytosis.

When ghosts are incubated in a hypotonic buffer, cytoskeletal proteins such as spectrin and actin are released from the membrane (*5*, *28*). The amount of released proteins increases with increasing incubation temperatures or decreasing ionic strength of hypotonic buffer. The release of spectrin from ghost membranes in the hypotonic buffer is largely enhanced above 30°C, at which the transition of spectrin tetramers to dimers occurs (*5*). Such a release is completely suppressed by polyamines such as spermine and spermidine (*18*). The dissociation of spectrin tetramer to dimers is also suppressed by polyamines (*29*). These facts suggest that the affinity of spectrin to the membrane decreases upon dissociation to dimers. Here, when we consider the effect of polyamines, it is crucial to examine the binding mode of two spectrin αβ heterodimers to form tetramers $(\alpha\beta)_2$. For the formation of spectrin tetramer, α subunit in the αβ dimer associates head-to-head with β subunit in other αβ dimer (Fig. 1) (*30*). Using proteolytic footprinting, Speicher *et al*. (*31*) demonstrated that the N-terminal region (residues 7–45) of α subunit associates with the C-terminal region of β subunit. A helical wheel projection of this N-terminal region shows good amphipathic properties (*31*). Particularly, glutamic acids are rich in the hydrophilic region. It seems likely that the effects of the negative charge due to glutamic acids become dominant upon decreasing ionic strength of buffer, and the transition to spectrin dimers facilitates the spectrin release from ghosts. This idea is supported by the fact that the transition from spectrin tetramer to dimers under hypotonic conditions and the spectrin release from ghosts are greatly suppressed by polyamines such as spermine and spermidine with positive charges. Further, the phosphorylation sites of β subunit are located near the C-terminus (*32*). Thus, the binding sites of polyamines on spectrin assume to be the association region of the N-terminus of α subunit with the C-terminus of β subunit.

When ghosts are treated with FITC, spectrin molecules are mainly labelled with this fluorophore. Spectrin release from ghost membranes is monitored using flow cytometry. When FITC-labelled ghosts are exposed to 37°C in the hypotonic buffer, two peaks are observed. In the presence of polyamines, however, only one peak appears because of the inhibition of spectrin release, and its position is identical with that of peak with the strong fluorescence intensity. Furthermore, in the case of ghosts prepared from FITC-labelled erythrocytes, where spectrin is labelled at low level, only one peak is observed despite spectrin release (Yamaguchi, T., *et al*. unpublished observations). This may be ascribed to a poor sensitivity to spectrin release due to low labelling of spectrin with FITC. Thus, the two peaks, one with the strong fluorescence intensity and one with the weak fluorescence intensity, are considered to correspond to no spectrin-released ghosts and spectrin-released ones, respectively. It is well known that spectrin-released membranes result in inside-out vesicles and right-side-out ones (*33*, *34*). Our results on dot plots also indicate the formation of vesicles from ghosts exposed to 37°C. From the light scattering measurements, it was found that spectrin-released ghosts result in large vesicles of diameter ~3 μm (Yamaguchi, T., *et al*. unpublished observations). Therefore, the peak with weak fluorescence intensity is considered to correspond to the large vesicles. Further, such two peaks appear upon FITC labelling at 0°C of ghosts pre-incubated at 37°C in the hypotonic buffer. This excludes a possibility that the appearance of the two peaks is due to the membrane perturbation induced by FITC labelling. Therefore, the two peaks indicate that the ghosts used are different in spectrin release from the membrane. Here, it is important to notice that the ghosts are prepared from erythrocytes which are heterogeneous on cell aging. The degrees of both spectrin oxidation and phosphorylation of protein 4.1 and ankyrin seem to be different in cell aging (*35*). The spectrin-membrane interactions are modulated by oxidation and phosphorylation of these proteins (*4*, *36*). Thus, it seems likely that the spectrin release from the membrane is also modulated by cell aging. In this stage, however, we cannot say what is the most dominant factor for spectrin release. If ghosts in the hypotonic buffer were incubated for longer times at 37°C, one peak with weak fluorescence intensity might be observed by the release of spectrin from all ghosts. The present work indicates that the spectrin content in individual ghost but not total ones is estimated from the flow cytometric measurement of FITC-labelled ghosts.

Spectrin molecules are attached to band 3 via ankyrin (*1*, *2*) and to glycophorin C via protein 4.1 (*3*, *4*). The numbers of copies of band 3 and glycophorin C per erythrocyte are 1.2×10^6 and $5\text{-}14 \times 10^4$, respectively (*37*). In addition, spectrin binds to ankyrin or protein 4.1 with the dissociation constant of 5×10^{-8} or 1×10^{-7} M, respectively (*38*, *39*). Therefore, the interaction of spectrin with band 3 via ankyrin is more essential for maintaining cell shape and mechanical strength. Ankyrin exhibits high affinity for spectrin tetramer than dimer (*36*). So, it seems likely that the interactions of spectrin with band 3 become weak upon transition from spectrin tetramer to dimers. To evaluate the band 3-cytoskeleton interactions in ghosts, we used an eosin labelling of band 3 (*24*). In intact membranes, about 30–40% of total band 3 are thought to be associated with cytoskeleton (*40*, *41*). Using our method, similar results were obtained in ghosts exposed to 0 or 10°C in 5P8. Upon disruption of cytoskeletal network above 30°C, band 3-cytoskeleton interactions are largely perturbed so that erythrocyte membranes become extremely fragile. The importance of membrane-cytoskeleton interactions

in the membrane stability is clear from the fact that band 3-deficient cells, although they bear normal levels of cytoskeleton, induce fragmented particles and vesicles (*42*). In ghosts exposed at 37°C to hypotonic buffers containing polyamines, the interactions between the membrane and cytoskeleton remain at normal level. Furthermore, the reduction of ghost volume is also inhibited by polyamines. Therefore, the abrupt reduction of membrane volume above 23°C in Fig. 2 may be ascribed to the vesiculation of ghosts.

Part of spectrin is released from the membrane at 37°C in hypotonic buffers. So, the conformational changes of spectrin were examined using pyrene. When spectrin thermally denatured is labelled with NPIA, excimer fluorescence of pyrene is observed (*8*). This suggests that mercapto groups in denatured spectrin are located within 2.4 Å each other. As previously indicated, I′/I values in Fig. 6B are utilized to monitor an appearance of excimer (*27*). The present data show a significant increment of I′/I values at 37°C, indicating irreversible conformational changes of spectrin at 37°C under hypotonic conditions. Interestingly, such conformational changes of spectrin is not suppressed by spermine and spermidine although the spectrin release from the membrane is completely inhibited by both polyamines. As mentioned earlier, N-terminal region of spectrin α subunit can associate head-to-head or side-to-side with C-terminal region of β subunit in tetramers or dimers, respectively. However, there is no cysteine in these association regions (*31*). Provided that polyamines bind to these association regions, the tetramer–dimer equilibrium of spectrin is affected by polyamines but the excimer fluorescence of pyrene is unaffected by ones. Each subunit in spectrin αβ dimers comprises of multiple 106-residue repeats, flanked by N- and C-terminal regions. Each repeat contains 0–3 cysteins (*43*, *44*). Thus, it is of interest to examine the excimer fluorescence on the basis of conformational changes of these repeats.

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