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Membrane skeleton–bilayer interaction is not the major determinant of membrane phospholipid asymmetry in human erythrocytes *

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Transbilayer phospholipid distribution, membrane skeleton dissociation/association, and spectrin structure have been analysed in human erythrocytes after subjecting them to heating at 50 °C for 15 min. The membrane skeleton dissociation/association was determined by measuring the Tris-induced dissociation of Triton-insoluble membrane skeletons (Triton shells), the spectrin-actin extractability under low ionic conditions, and the binding of spectrin-actin with normal erythrocyte membrane inside-out vesicles (IOVs). The spectrin structure was ascertained by measuring the spectrin dimer-to-tetramer ratio as well as the spectrin tryptophan fluorescence. Both the Tris-induced Triton shell dissociation and the spectrin-actin extractability under low ionic conditions were considerably reduced by the heat treatment. Also, the binding of heated erythrocyte spectrin-actin to IOVs was significantly smaller than that observed with the normal cell spectrin-actin. Further, the quantity of spectrin dimers was appreciably increased in heat-treated erythrocytes as compared to the normal cells. This change in the spectrin dimer-to-tetramer ratio was accompanied by marked changes in the spectrin tryptophan fluorescence. Inspite of these heat-induced alterations in structure and bilayer interactions of the membrane skeleton, the inside-outside glycerophospholipid distribution remained virtually unaffected in the heat-treated cells, as judged by employing bee venom and pancreatic phospholipase A_2, fluorescamine and Merocyanine 540 as the external membrane probes. These results strongly indicate that membrane bilayer–skeleton interaction is not the major factor in determining the transbilayer phospholipid asymmetry in human erythrocyte membrane.

Introduction

Human erythrocyte membrane is composite of two structural units, viz. membrane skeleton (or cytoskeleton) and membrane bilayer. While the membrane bilayer is comprised of lipids and integral membrane proteins, membrane skeleton is a reticulate type of structure formed from three major (spectrin, actin and polypeptide 4.1) and several minor peripheral membrane proteins and is associated with the cytoplasmic face of the membrane bilayer through protein–protein and protein–phospholipid interactions [1]. The membrane bilayer-skeleton association controls not only the membrane mechanical stability and deformability but also the lateral mobility of the integral membrane proteins [2]. Besides, this association has been considered as the major factor in maintaining the asymmetric phospholipid distribution across the erythrocyte membrane bilayer [3].

Erythrocyte membrane phospholipids are asymmetrically distributed in the two halves of the membrane bilayer. The choline-containing phospholipids (PC and SM) are present mainly in the outer monolayer, whereas the aminophospholipids (PE and PS) are localized almost exclusively in the inner monolayer [4]. This typical transbilayer phospholipid asymmetry is disturbed in

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Abbreviations: RBC, red blood cells; PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PMSF, phenylmethylsulfonyl fluoride; GSH, glutathione; ATP, adenosine 5′-triphosphate; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecylsulfate; PBS, phosphate-buffered saline; Mc 540, Merocyanine 540; IOVs, inside-out vesicles; ROVs, right side-out vesicles; TLC, thin-layer chromatography.

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erythrocytes which contain defective membrane skeleton [5–8].

The major membrane skeletal protein, spectrin, is known to undergo irreversible structural changes by heating the erythrocyte ghosts [9,10] or the pure protein [11] at about 50 °C. But interestingly, heating of intact erythrocytes in identical conditions did not give rise to any change in the transbilayer phospholipid organization [12], questioning the exclusive role of membrane skeleton in maintaining the phospholipid asymmetry [3]. To further investigate this problem, we have analysed both membrane skeleton and transbilayer phospholipid organization in the heat-treated human erythrocytes.

Materials and Methods

Materials

Phospholipases A_2 from bee venom and hog pancreas, fluorescamine, PMSF, pepstatin A, leupeptin, GSH, ATP, adenosine and Triton X-100 were purchased from Sigma Chemical Company. Sepharose CL-4B and Con A-Sepharose were from Pharmacia. Mc 540 was procured from Serva Feinbiochemica. Sodium [^{125}I]-iodide was bought from Amersham. Precoated silica gel 60F-254 TLC plates (20 × 20 cm, 0.2 mm thickness) were obtained from E. Merck.

Erythrocyte isolation

Red cells from fresh human blood were isolated by removing the plasma and buffy coat. The cells were routinely passed through a Cellulose CF-11 column to ensure complete removal of leucocytes [13].

Heat treatment

Erythrocytes were diluted to 10% hematocrit with 10 mM glycylglycine containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM glucose and 1 mM adenosine (pH 7.4), and subjected to heating in a LKB Thermostatic Circulator (Model 2219 Multitemp II) at 50 ± 0.1 °C (or stated otherwise) under an atmosphere of humidified nitrogen for 15 min. The cells were harvested by centrifugation (370 × *g*), and washed at least four times to remove microvesicles formed during the incubation. The absence of microvesicles was confirmed by light microscopy and electron microscopy. Hemolysis during the heat treatment was never more than 0.2%.

Erythrocyte ATP and GSH level determination

ATP levels in red cells were determined by an enzymatic test employing Sigma Diagnostics ATP kit (procedure No. 366-UV). GSH levels were measured according to Beutler et al. [14].

Scanning electron microscopy

Specimens of both the normal and heated erythrocytes for electron microscopy were prepared according to Burns and Gratzer [15]. The cells were suspended to 10% hematocrit in PBS. Equal volumes of the cell suspension and 2.5% glutaraldehyde were mixed and incubated at 15–20 °C for 1 h. The cells were washed three times to remove the excess glutaraldehyde, and a very thin film of the washed cells spread on a cover slip. It was dried and then washed twice successively with 5-ml aliquots of increasing concentration of acetone in water (20, 40, 60, 80 and 100% (v/v)). The cells were then coated with palladium-gold alloy and examined in a Philips SEM 515 electron microscope at a magnification of 1550 × at a 30° tilt angle.

Erythrocyte membrane phospholipid hydrolysis by phospholipase A_2

A 0.25 ml aliquot of packed erythrocytes was suspended in 5 ml of 10 mM glycylglycine buffer containing 100 mM KCl, 50 mM NaCl, 0.25 mM $MgCl_2$, 0.25 mM $CaCl_2$ and 44 mM sucrose (pH 7.4). To it was added 15 IU of bee venom phospholipase A_2 and the mixture incubated at 37 °C. The enzyme reaction was stopped by washing the cells with PBS (pH 7.4) containing 5 mM EDTA. The extent of hemolysis was determined at the end of each incubation prior to EDTA wash by comparing the hemoglobin content in the supernatant of each sample to that of a 100% hemolysed control. Treatments with porcine pancreatic phospholipase A_2 (20 IU/0.25 ml packed cells) were carried out at 37 °C in the above incubation medium, except that the concentration of $CaCl_2$ was 10 mM. Unsealed erythrocyte ghosts were also treated with both the enzymes essentially under the conditions described above for the intact cells.

Lipids were extracted from the enzyme-treated cells (without lysis) or ghosts according to Rose and Oklander [16]. Individual phospholipids were separated by two-dimensional TLC as described earlier [17]. Spots for different phospholipids were identified after staining the plate with iodine vapor followed by ninhydrin spray. These were removed and eluted with a mixture of methanol and chloroform (1 : 1, v/v) several times. Total phosphorous present in each spot was determined according to Ames and Dubin [18]. The recoveries of various phospholipids from silica gel were greater than 95%.

Erythrocyte labeling with Mc 540

Labeling of erythrocytes with Mc 540 was carried out following the published procedure [19]. Cells were suspended to a concentration of about $5 \cdot 10^7$ cells/ml incubation medium (10 mM Tris containing 0.25 M sucrose, 15 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$ (pH 7.4)) containing 3% AB^+ human serum and Mc 540 (40 μg/ml cell suspension). The mixture was incubated for 10 min at 37 °C in dark. The cells were then quickly washed three times with 10 mM Tris containing 140

mM NaCl, 10 mM KCl and 3 mM $MgCl_2$ (pH 7.4). Fluorescence microscopy was performed in a Leitz Diavert microscope.

Erythrocyte labeling with fluorescamine

Labeling of aminophospholipids with fluorescamine in intact cells as well as in unsealed ghosts was carried out at 37°C using varying fluorescamine concentrations, essentially according to Franck et al. [20]. The labeling did not increase by increasing the fluorescamine concentration beyond 6 μmol/μmol phospholipid. Lipids from cells were extracted [16], and separation between the labeled and unlabeled phospholipid affected by two-dimensional TLC [17].

Electrophoresis

Protein compositions in erythrocyte membranes, isolated membrane skeletons (Triton shells) and spectrin-actin extract were determined by SDS-polyacrylamide gel electrophoresis followed by densitometry of the Coomassie blue-stained gels. The erythrocyte membranes were prepared following the published procedure [21], except that PMSF (0.25–1.0 mM), pepstatin A (2 μg/ml) and leupeptin (2 μg/ml) were also included in the lysis buffer. Spectrin-actin was extracted from the erythrocyte membranes essentially according to Bennett and Branton [22]. Triton insoluble membrane skeletons from the erythrocyte membranes were prepared as described by Ohanian and Gratzer [23]. SDS-polyacrylamide gel electrophoreses were carried out essentially according to Hubbard and Lazarides [24] using 5% acrylamide as stacking and 10% acrylamide as running gels. The gels were stained with Coomassie brilliant blue R 250 and scanned on Shimadzu dual wavelength scanner CS-910 at 560 nm.

The spectrin dimer-to-tetramer ratio was analysed in the spectrin-actin 4°C extract by electrophoreses on agarose gels under nondenaturing conditions, as described earlier [25]. The protein compositions of the spectrin dimer and tetramer bands were determined by two-dimensional gel electrophoretic analysis [26].

Erythrocyte membrane vesicle preparation

IOVs from the erythrocyte membranes were prepared using the standard procedure [27]. Contamination of ROVs and unsealed membranes was removed by Con A-Sepharose treatment. The purity of the vesicles was assessed by measuring the acetyl choline esterase activity [28].

Spectrin-actin binding with IOVs

Spectrin-actin extracted from the erythrocyte membranes at 4°C was labeled with ^{125}I using the known method [29]. The binding of the ^{125}I-labeled spectrin-actin with IOVs was carried out as described earlier [27].

Spectrin isolation

Spectrin was isolated and purified by the published procedure [30]. The purity was established by SDS-polyacrylamide gel electrophoresis.

Spectrin tryptophan fluorescence measurements

Steady-state spectrin tryptophan fluorescence was measured on a Shimadzu RF-540 spectrofluorometer using excitation wavelength of 295 nm. The absorbance of all the protein solutions was 0.1 at 295 nm. Measurements were made in Tris-buffered saline (20 mM Tris containing 145 mM NaCl (pH 7.5)) at 22–24°C.

The quantum yields of tryptophan fluorescence were calculated using the following equation:

$$Q = Q_{st}[(\text{area})/(\text{area})_{st}][(\text{abs})_{st}/(\text{abs})]$$

where, (area) and $(\text{area})_{st}$ represent the areas of emission spectrum of the protein and the standard, *N*-acetyltryptophanamide, respectively. The (abs) and $(\text{abs})_{st}$ represent the absorbance at 295 nm of the protein and the standard respectively. Q_{st} is the quantum yield of the standard. The value, 0.13, reported by Chen [31] was used as the quantum yield of *N*-acetyltryptophanamide.

Spectrin tryptophan fluorescence quenching measurements were made at 22–24°C essentially according to Lehrer [32] using iodide [KI] as the water-soluble quencher. The concentration of spectrin used was $4.3 \cdot 10^{-8}$ M. The data was analysed using the appropriate forms of equations proposed by Lehrer [32]. The basic equation is:

$$F_0/(F - F_0) = F_0/\Delta F = \left(\sum_1^n f_i K_i[Q]/(1 + K_i[Q])\right)^{-1} \quad (1)$$

where, F_0 and F are the fluorescence values in the absence and presence of a concentration [Q] of quencher (iodide), f_i is the fraction of total fluorescing sites (n) involved in quenching, and K_i is the quenching constant. When the distribution of fluorescent side chains in a protein molecule is heterogeneous, i.e., the side chains are under the influence of different environments such that one fraction is accessible (f_a) and another is inaccessible ($1 - f_a$) to the quencher, Eqn. 1 is reduced to:

$$F_0/\Delta F = (1/Kf_a[Q]) + 1/f_a \quad (2)$$

where, $f_a = \Sigma f_i$, the maximum accessible fraction of protein fluorescence. As $(1/[Q]) \rightarrow 0$ on a plot of $(F_0/\Delta F)$ vs. $(1/[Q])$, the intercept on the Y-axis yields $F_0/\Delta F_{max} = 1/f_a$. Thus the inverse value of the Y-intercept represents the accessible fraction, f_a. When the quencher has equal access to all the fluorescing sites of

the protein ($f_i = 1$ and all K terms are equal), Eqn. 1 simplifies to:

$$F_0/\Delta F = (1/K[Q]) + 1 \quad (3)$$

plots of $(F_0/\Delta F)$ vs. $(1/[Q])$ will be linear. Values of the effective quenching constant (K_{eff}) were obtained by dividing f_a by slope of the curve.

Results

Cell shape and ATP and GSH levels

Heating of the human erythrocytes at 50 °C for 15 min did not induce any significant change in the levels of intracellular ATP (normal RBC: 1.02 ± 0.16; heated RBC: 0.97 ± 0.09 mmol/l packed cells) and GSH (normal RBC: 158 ± 1.6; heated RBC: 162 ± 2.9 mg/100 ml packed cells). However, this treatment transformed the cell shape from discocytes to predominantly spherocytes and pyropoikilocytes (Fig. 1), which was consistent with the earlier studies [33].

Membrane phospholipid organization

Effect of the heat treatment on the transbilayer phospholipid organization in erythrocytes was analysed by means of bee venom and pancreatic phospholipases A_2, Mc 540 and fluorescamine as the external membrane probes. Table I shows that like the normal human erythrocytes, both bee venom and pancreatic phospholipases A_2 hydrolysed only PC and PE in the intact heated red cells. Also, the amounts of PE labeled by fluorescamine in the heated cells were similar to those observed in the normal erythrocytes (Normal RBC: $18.7 \pm 2.4\%$; heated RBC: $20.7 \pm 0.2\%$; unsealed ghosts: $70.2 \pm 0.1\%$). Furthermore, Mc 540 in the presence of 3% AB^+ human serum failed to stain the normal or heated cells (data not shown). These results demonstrate that heating of the human red cells at 50 °C for 15 min does not affect the transbilayer phospholipid distribution in their membranes. This is quite in agreement with the earlier observation [12].

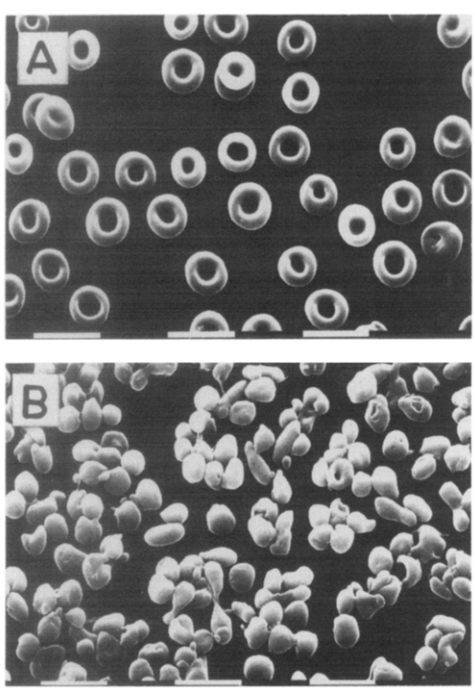

Fig. 1. Scanning electron micrographs of human erythrocytes. (A) Normal RBC; (B) RBC heated at 50 °C for 15 min. Bar, 10 μm.

TABLE I

Human erythrocyte phospholipid degradation by phospholipase A_2

Hemolysis during the enzyme reactions was never more than 3%. Values shown are means of three or four determinations ± S.D. No appreciable increase in the phospholipid hydrolysis by bee venom phospholipase A_2 was observed by further increasing the incubation time to 2 h.

Sample	Source of enzyme	Incubation time (min)	PC (%)	PE (%)	PS (%)
Normal cells	bee venom	10	36.0 ± 0.9	17.8 ± 0.5	0
		60	51.1 ± 1.6	20.0 ± 2.3	0
	hog pancreas	10	5.9 ± 0.5	0	0
Heated cells	bee venom	10	35.4 ± 0.2	17.3 ± 0.8	0
		60	54.7 ± 0.4	22.6 ± 1.2	0
	hog pancreas	10	8.7 ± 3.5	6.1 ± 0.4	0
Unsealed ghosts	bee venom	10	95.0 ± 0.8	94.8 ± 1.0	86.9 ± 2.3

Membrane protein composition

Fig. 2 shows that membrane protein composition in erythrocytes was altered upon heating the cells at > 46 °C; at least four major and several minor new protein bands appeared on the SDS-polyacrylamide gel electrophoretograms of the ghosts prepared from the heated erythrocytes. This change is caused by an increased tendency of cytosolic proteins to associate with the membrane during heating, since proteins corresponding to the new bands were present also in the normal erythrocyte cytosol (Fig. 2) and intensities of these bands progressively increased in the membrane with an increase in the cell heating temperature (Fig. 2), with a concomitant decrease in their intensities in the cytosol (data not shown).

There was no heat-induced degradation of major membrane proteins, as relative intensities of these proteins in the heated erythrocyte ghosts appeared similar to those observed in the normal cell ghosts (Fig. 2). This was further supported by our finding that quantities of these proteins in intact erythrocytes (without lysis) re-

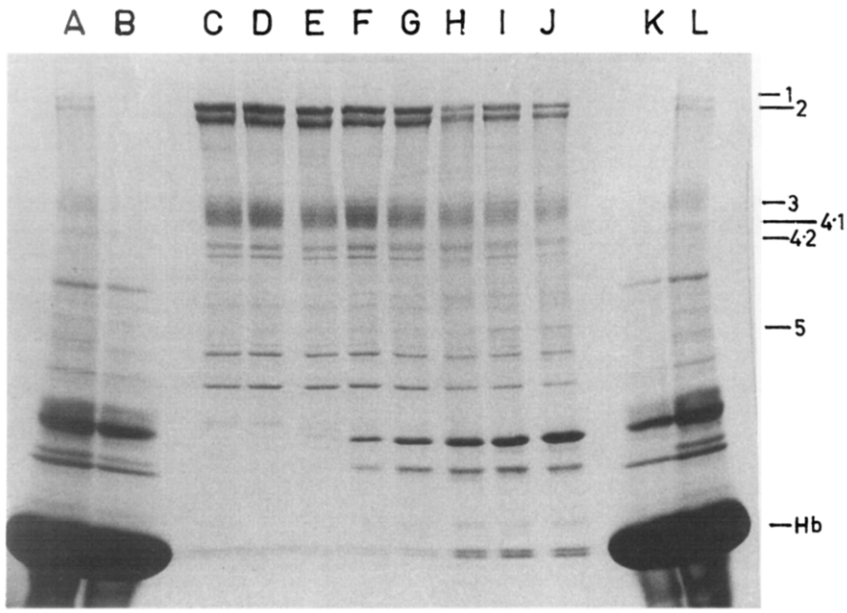

Fig. 2. Protein composition in human erythrocytes. (A) Normal intact RBC; (B) normal RBC cytosol; (C) normal RBC membrane; (D–J) membranes derived from RBC after heating at 42° C (D), 44° C (E), 46° C (F), 48° C (G), 50° C (H), 52° C (I), 54° C (J); (K) cytosol derived from RBC after heating at 50° C; (L) intact RBC heated at 50° C. Heating was carried out for 15 min. The proteins were quantitated by scanning the gels, as given in Materials and Methods. The amounts of protein that could not enter into the gels were negligible in case of both normal and heated erythrocyte ghosts.

mained unchanged after heating. Most of the new protein bands seen in the electrophoretograms of the heated erythrocyte ghosts may therefore be considered to result from the heat-induced association of the cytosolic proteins to the membrane rather than the membrane protein degradation. However, the possibility that some of the new minor protein bands could arise from some membrane protein degradation can not be ruled out completely.

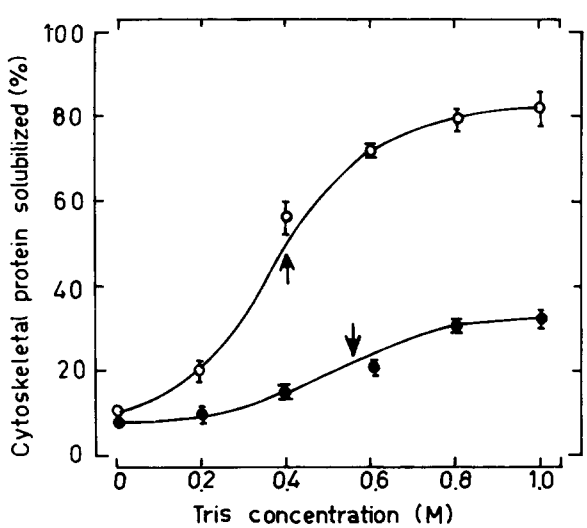

Fig. 3. Tris-induced dissociation of Triton-insoluble membrane skeletons (Triton shells). Open circles, normal human RBC; closed circles, human RBC heated at 50° C for 15 min. Values are means of three determinations ± S.D. Arrows indicate Tris concentrations required to affect the half-maximal dissociation.

Membrane skeleton dissociation / association

Effect of heating on the intermolecular associations within the erythrocyte membrane skeleton was assessed by studying the Tris-induced dissociation of the Triton

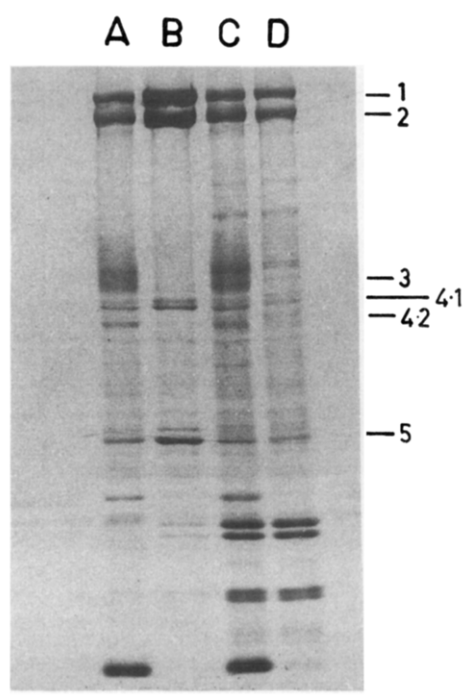

Fig. 4. Protein composition in Triton shells. (A) Normal human RBC membrane; (B) Triton shells prepared from normal RBC membrane; (C) membranes derived from human RBC after heating at 50° C for 15 min; (D) Triton shells prepared from the heated RBC membrane.

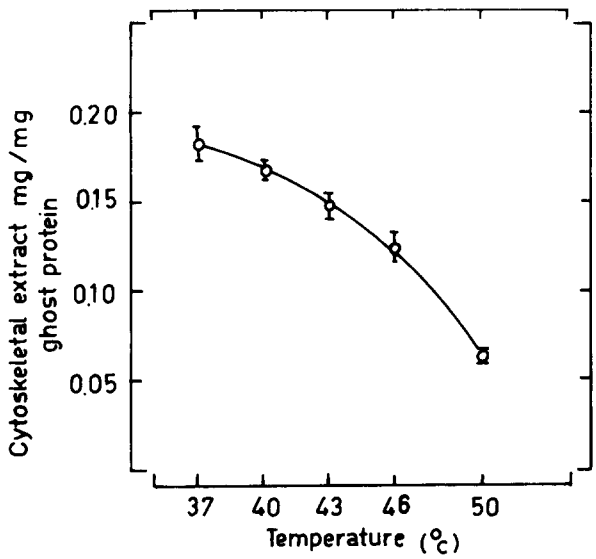

Fig. 5. Effect of erythrocyte heating (15 min) on the membrane skeleton extractability under low ionic conditions. Values shown are means of three determinations ± S.D.

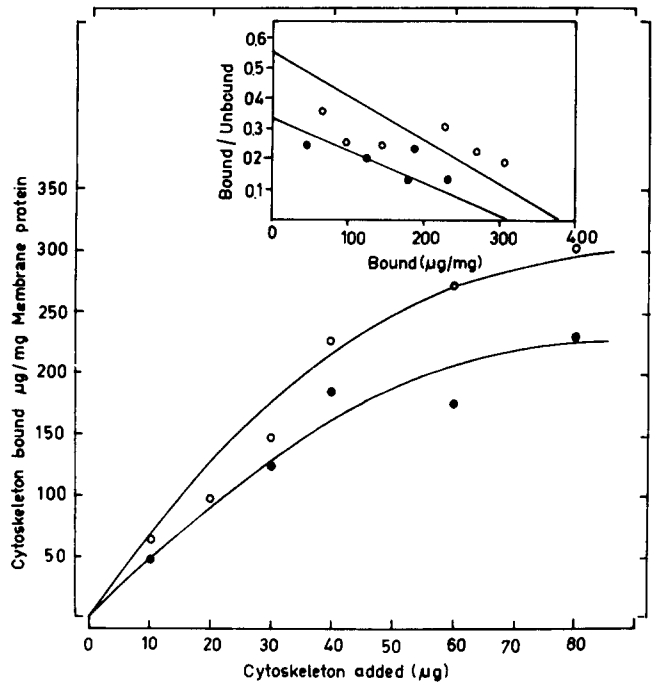

Fig. 7. Spectrin-actin binding with normal erythrocyte membrane inside-out vesicles. Open circles, normal RBC spectrin-actin; closed circles, heated RBC spectrin-actin. Inset: Scatchard analysis of the binding data.

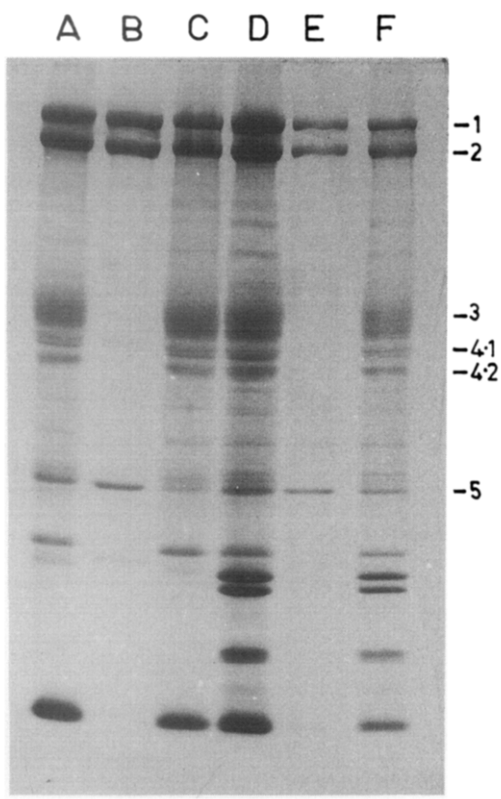

Fig. 6. Protein composition of membrane skeleton extracts obtained from the human erythrocyte membrane under low ionic conditions. (A) Normal RBC membrane; (B) skeletal extract from normal RBC membrane; (C) residual normal RBC membrane pellet; (D) membranes derived from RBC after heating at 50 °C for 15 min; (E) skeletal extract from heated RBC membrane; (F) residual heated RBC membrane pellet.

shells isolated from both normal and heated erythrocytes. Fig. 3 shows that over 80% of the total normal erythrocyte Triton shell proteins were dissociated by 1 M Tris. However, under identical conditions, only about 30% of the total heated erythrocyte Triton shell proteins were solubilized by the Tris (1 M) treatment. Also, the Tris concentration required for the half-maximal dissociation was increased from about 0.40 M to 0.55 M by subjecting the cells to heating at 50 °C. Analysis of the protein composition in the shells (Fig. 4) revealed that the quantities of the band 3 protein in the membrane skeletons, thus prepared, were not appreciably increased by the heat treatment. It further showed that most of the heated erythrocyte membrane-associated cytosolic proteins were present in the Triton-insoluble, rather than the Triton-soluble, fraction (Fig. 4). Further experiments were done to analyse the protein composition in the Tris-soluble fraction. Results of these experiments (not shown) showed that the Triton shell-associated cytosolic proteins were not dissociated by treatment with varying Tris concentration upto 1 M. From these observations, we infer that the heat treatment does not probably enhance the membrane bilayer-skeleton interactions, but it does increase the protein-protein associations within the skeleton.

To further establish that the membrane skeleton dissociation is affected in the heated erythrocytes, we measured the spectrin-actin extractability after treating the membranes with low ionic strength buffer. Fig. 5

shows that the extractability decreased with the heating temperature, which was consistent with the earlier observation [34]. The amounts of extracted proteins from the ghosts prepared from the 50 °C heated erythrocytes were about one-third of that observed with the normal erythrocyte membrane. Analysis of the protein composition in the extracts by SDS-polyacrylamide gel electrophoresis revealed that the new proteins that were observed in the membranes of heated cells were only poorly extracted with spectrin-actin (Fig. 6), under our experimental conditions.

In order to ascertain whether the membrane bilayer-skeleton associations remain unaffected after the heat treatment, we have measured the binding of spectrin-actin, extracted from both normal and heated cells, with IOVs prepared from the normal erythrocytes. Results of these studies (Fig. 7) indicate that association of the heated erythrocyte spectrin-actin with the normal erythrocyte membrane bilayer is reduced (K_a: 0.33) as compared to that of spectrin-actin isolated from the normal cells (K_a: 0.55).

Spectrin structure

Heat-induced structural changes in spectrin were ascertained by analysing the spectrin dimer-to-tetramer ratio in the crude 4 °C spectrin-actin extracts, and the spectrin folding by fluorescence spectroscopy in the pure spectrin samples isolated from the extracts. Fig. 8 shows that spectrin in the normal erythrocyte membrane 4 °C spectrin-actin extracts existed mainly as tetramer and oligomer, and to a small extent as dimer. Unlike this observation, considerable fraction of spectrin was present as dimer in the crude spectrin-actin, extracted at 4 °C from the membranes of heated cells. Also, the spectrin tetramer-to-dimer conversion at 37 °C was higher in the heated erythrocyte spectrin-actin as compared to that observed in the normal erythrocyte spectrin-actin. These results strongly suggest that spectrin is structurally altered by heating the human red cells at 50 °C.

To further examine the validity of the above suggestion, we analysed the spectrin structure by measuring the spectrin tryptophan fluorescence in the absence as well as in the presence of a quencher. The normal spectrin exhibited an emission maxima at 342 nm, which was lower than that observed for the heated erythrocyte spectrin (346 nm). Also, the spectrin tryptophan fluorescence quantum yield (normal: 0.086; heated: 0.128) was significantly increased when the cells were heated at 50 °C for 15 min. These findings strongly indicate that spectrin folding is affected in the heated erythrocytes. To further establish that this indeed is the case, we carried out quenching experiments using iodide as the water-soluble tryptophan fluorescence quencher. The results (not shown) indicated that the tryptophan residues in both normal and heated erythrocyte spectrins

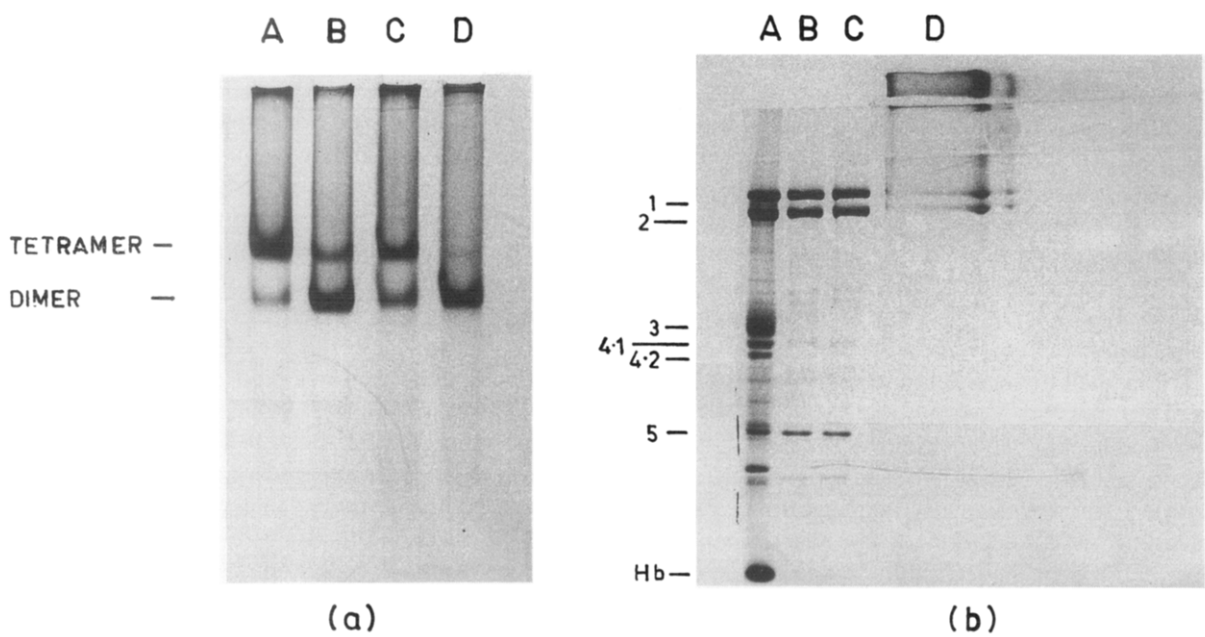

Fig. 8. (a) Analysis of the spectrin dimer-to-tetramer ratio in membrane skeleton extracts obtained under low ionic conditions. A, Normal RBC membrane 4 °C extract; B, normal RBC membrane 4 °C extract incubated at 37 °C (15 min); C, heated RBC membrane 4 °C extract; D, heated RBC membrane 4 °C extract incubated at 37 °C (15 min). (b) Analysis of protein composition in spectrin dimer-tetramer bands by two-dimensional gel electrophoresis. The first dimension was run on agarose gels under nondenaturing conditions, whereas the second dimension was on agarose-polyacrylamide gels under denaturing conditions. A, Normal RBC membrane; B and C, normal RBC membrane 4 °C extract; D, strip from nondenaturing gel showing spectrin dimer and tetramer bands. It may be noted that the bands corresponding to spectrin dimers and tetramers contain spectrin only.

were present in more than one environment. Plotting of the data according to the modified Stern-Volmer relationship revealed that about 70% of the total fluorescence of tryptophan residues in normal erythrocyte spectrin were readily accessible to the quencher, as apposed to about 83% in the heated erythrocyte spectrin.

Discussion

This study shows that heating of the human red cells at 50 °C besides affecting the membrane skeletal dynamics and interactions with the membrane bilayer, also affects the spectrin dimer-tetramer equilibrium and folding. Also, it demonstrates that the membrane protein composition in the heated cells is altered mainly due to the strong association between membrane skeletal and cytosolic proteins. However, the present data is not sufficient to ascertain whether this association is promoted by the observed structural changes in spectrin and/or the possible heat-induced denaturation of the cytosolic proteins, but it is likely that some of these proteins might have interacted with the membrane skeleton for providing protection to its protein components against the heat-induced structural damage [35,36].

Human erythrocyte membrane undergoes the first thermal transition at about 50 °C, which has been shown to correspond with the partial unfolding of spectrin in the membrane [9,10]. This is in accordance with the present study which shows that spectrin, at least in the extractable portion of spectrin-actin, is unfolded by subjecting the intact erythrocytes to heating at 50 °C. Further, although we could not directly determine the heat-induced structural changes in the total erythrocyte spectrin, we strongly believe that the portion of spectrin which could not be removed from the heated erythrocyte membrane might have also been structurally altered by the heat treatment. This belief is based primarily on our experimental observations on the spectrin-actin extractability (Figs. 5 and 6) as well as the Tris-induced Triton shell dissociation patterns (Figs. 3 and 4).

Recent studies by Yoshino and Minari [11] have revealed that heating of an isolated sample of human erythrocyte spectrin at 50 °C leads to an extensive denaturation and consequently aggregation of β-spectrin, resulting in dissociation of spectrin dimers into monomers. Little or no effect on α-spectrin was observed by these workers under their experimental conditions. Unlike these findings, we did not observe any such dissociation of spectrin dimers when the intact erythrocytes were heated at 50 °C. It may therefore be inferred that spectrin in an isolated sample experiences much greater heat effects than in the intact cells. This seems logical, since spectrin in erythrocytes is present in a highly organised form [37,38].

Poor extraction of spectrin-actin from the heated erythrocyte membrane at low ionic strength, could result from an altered association between the membrane skeletal proteins and/or of membrane skeleton with the overlying membrane bilayer. However, based on our analysis of the protein composition in the Triton shells (Fig. 4), we believe that membrane skeleton association with band 3 protein did not increase after the heat treatment. Also, the possibility of an increased interaction between the heated erythrocyte spectrin and membrane phospholipids may be ruled out, since heat denatured spectrin has earlier been shown to weakly interact with phospholipids as compared to normal spectrin [39]. It would therefore seem that the observed decrease in the spectrin-actin extractability is probably due to the increased intermolecular associations within the membrane skeleton of the heated erythrocytes. This is in complete agreement with our observations on Tris-induced dissociation of the Triton shell proteins (Fig. 3).

It appears that heating of the human erythrocytes at 50 °C produces structural defects, at least, in the membrane skeletal proteins which in turn could affect the skeleton dynamics and binding with the overlying membrane bilayer. This is well supported by our findings that the Tris concentration required for the half-maximal dissociation of Triton shell proteins was increased from about 0.40 M to 0.55 M upon subjecting the erythrocytes to the heat treatment (Fig. 3). Also, the spectrin tetramer-dimer equilibrium was altered in the heated erythrocytes (Fig. 8). Besides, the binding of spectrin-actin, isolated from the heat-treated erythrocytes, with the normal erythrocyte membrane IOVs was significantly reduced as compared to that observed with the normal erythrocyte membrane spectrin-actin (Fig. 7).

Earlier studies have shown that structural abnormalities in erythrocyte spectrin are often accompanied by the enhanced accessibility of membrane aminophospholipids to phospholipases and amino-group labeling reagents in intact erythrocytes [5–8]. Also, preferential interactions of spectrin and polypeptide 4.1 with PS have been demonstrated in vitro [39–45]. Based on these findings it has been believed that the asymmetric transbilayer phospholipid distribution is maintained primarily by the inner layer phospholipid-skeletal protein interactions in erythrocytes [3,7,46,47]. But our present results do not seem to support this belief, as we did not observe any change in the erythrocyte membrane phospholipid hydrolysis by phospholipase A_2 or the aminophospholipid labeling by fluorescamine after heating the cells at 50 °C for 15 min, though under these conditions the membrane skeleton structure and interactions were significantly altered. We, therefore, conclude that the skeletal protein–aminophospholipid interaction, alone, can not be responsible for the asym-

metric phospholipid distribution across the human erythrocyte membrane, and that besides the membrane skeleton, there must be another factor that plays a major role in maintaining the phospholipid asymmetry. This is quite in agreement with the recent study which showed that inspite of the altered membrane skeleton, the phospholipid asymmetry remains unaltered in the diamide-treated human red cells, and that this asymmetry in these cells is maintained primarily by the ATP-dependent aminophospholipid pump, together with the membrane skeleton [48].

Several studies have shown that the erythrocyte membrane contains an ATP-dependent pump that translocates PE and PS from the outer to the inner monolayer [49–52]. It has been suggested that this phospholipid pump, rather than the membrane skeleton, is the main factor that determines the transbilayer phospholipid asymmetry in the native erythrocyte membrane [53,54]. Since the putative phospholipid translocase is not much affected by heating the human erythrocytes at 50 °C for 15 min [55], our observation that the membrane phospholipid asymmetry remains unaltered by the heat treatment seems consistent with the above suggestion.

The present study demonstrates that the membrane skeleton–bilayer interaction, alone, is not sufficient for maintaining the phospholipid asymmetry in human erythrocytes. This is quite in accordance with the earlier studies which have shown that interactions of skeletal proteins with PE and PS are not strong enough to account for the preferential distribution of these phospholipids in the inner surface of the erythrocyte membrane bilayer [56,57]. However, our results do not exclude the possibility that membrane skeleton could still play an important role in maintaining the asymmetry by stabilizing the aminophospholipid distribution in the inner monolayer [48], due to its interactions with PS [39–45].

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References

1 Bennett, V. (1985) Annu. Rev. Biochem. 54, 273–304.
2 Sheetz, M.P. (1983) Semin. Hemat. 20, 175–188.
3 Haest, C.W.M. (1982) Biochim. Biophys. Acta 694, 331–352.
4 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71.
5 Haest, C.W.M., Plasa, G., Kamp, P. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32.
6 Lubin, B., Chiu, B., Bastacky, J., Roelofsen, B. and Van Deenen, L.L.M. (1981) J. Clin. Invest. 67, 1643–1649.
7 Williamson, P., Bateman, J., Kozarsky, K., Mattocks, K., Hermanowics, N., Choe, H.-R. and Schlegel, R.A. (1982) Cell 30, 725–733.
8 Kumar, A. and Gupta, C.M. (1983) Nature 303, 632–633.
9 Brandts, J.F., Erickson, L., Lysko, K., Schwartz, A.T. and Taverna, R.D. (1977) Biochemistry 16, 3450–3454.
10 Lysko, K.A., Carlson, R., Taverna, R., Snow, J. and Brandts, J.F. (1981) Biochemistry 20, 5570–5576.
11 Yoshino, H. and Minari, O. (1987) Biochim. Biophys. Acta 905, 100–108.
12 Dressler, V., Haest, C.W.M., Plasa, G., Deuticke, B. and Erusalimsky, J.D. (1984) Biochim. Biophys. Acta 775, 189–196.
13 Brovelli, A., Seppi, C., Bardoni, A., Balduini, A. and Lutz, H.U. (1987) Biochem. J. 242, 115–121.
14 Beutler, E., Duron, O. and Kelly, B.M. (1963) J. Lab. Clin. Med. 61, 882–888.
15 Burns, N.-R. and Gratzer, W.B. (1985) Biochemistry 24, 3070–3074.
16 Rose, H.G. and Oklander, M. (1965) J. Lipid Res. 6, 428–431.
17 Gupta, C.M. and Mishra, G.C. (1981) Science 212, 1047–1049.
18 Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769–775.
19 Schlegel, R.A., Phelps, B.M., Waggoner, A., Terada, L. and Williamson, P. (1980) Cell 20, 321–328.
20 Franck, P.F.H., Op den Kamp, J.A.F., Roelofsen, B. and Van Deenen, L.L.M. (1986) Biochim. Biophys. Acta 857, 127–130.
21 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617.
22 Bennett, V. and Branton, D. (1977) J. Biol. Chem. 252, 2753–2763.
23 Ohanian, V. and Gratzer, W.B. (1984) Eur. J. Biochem. 144, 375–379.
24 Hubbard, B.D. and Lazarides, E. (1979) J. Cell Biol. 80, 166–182.
25 Liu, S.-C. and Palek, J. (1980) Nature 285, 586–588.
26 Morrow, J.S., Speicher, D.W., Knowles, W.J., Hsu, C.J. and Marchesi, V.T. (1980) Proc. Natl. Acad. Sci. USA 77, 6592–6596.
27 Bennett, V. (1983) Methods Enzymol. 96, 313–324.
28 Kant, J.A. and Steck, T.L. (1973) Biochem. Biophys. Res. Commun. 54, 116–122.
29 Morrison, M., Mueller, T.J. and Huber, C.T. (1974) J. Biol. Chem. 249, 2658–2660.
30 Ungewickell, E. and Gratzer, W.B. (1978) Eur. J. Biochem. 88, 379–385.
31 Chen, R.F. (1967) Anal. Lett. 1, 35–42.
32 Lehrer, S.S. (1971) Biochemistry 10, 3254–3263.
33 Herrmann, A., Lentzsch, P., Lassmann, G., Ladhoff, A.-M. and Donath, E. (1985) Biochim. Biophys. Acta 812, 277–285.
34 Lux, S.E., John, K.M. and Ukena, T.E. (1978) J. Clin. Invest. 61, 815–827.
35 Napolitano, E.W., Pachter, J.S. and Liem, R.K.H. (1987) J. Biol. Chem. 262, 1493–1504.
36 Pelham, H. (1988) Nature 332, 776–777.
37 Chasis, J.A. and Shohet, S.B. (1987) Annu. Rev. Physiol. 49, 237–248.
38 Bennett, V. (1989) Biochim. Biophys. Acta 988, 107–121.
39 Mombers, C., De Gier, J., Demel, R.A. and Van Deenen, L.L.M. (1980) Biochim. Biophys. Acta 603, 52–62.
40 Sato, S.B. and Ohnishi, S.-I. (1983) Eur. J. Biochem. 130, 19–25.
41 Bonnet, D. and Begard, E. (1984) Biochem. Biophys. Res. Commun. 120, 344–350.
42 Cohen, A.M., Liu, S.-C., Derick, L.H. and Palek, J. (1986) Blood 68, 920–926.
43 Cohen, A.M., Liu, S.-C., Lawler, J., Derick, L. and Palek, J. (1988) Biochemistry 27, 614–619.
44 Shiffer, K.A., Goerke, J., Düzgüneş, N., Fedor, J. and Shohet, S.B. (1988) Biochim. Biophys. Acta 937, 269–280.
45 Rybicki, A.C., Heath, R., Lubin, B. and Schwartz, R.S. (1988) J. Clin. Invest. 81, 255–260.

46 Schwartz, R.S., Chiu, D.T.-Y. and Lubin, B. (1984) in Erythrocyte Membranes, Vol. 3: Recent Clinical and Experimental Advances, pp. 89–122, Alan R. Liss Inc., New York.
47 Kumar, A. and Gupta, C.M. (1984) Biochim. Biophys. Acta 769, 419–428.
48 Middelkoop, E., Vander Hoek, E.E., Bevers, E.M., Comfurius, P., Slotboom, A.J., Op den Kamp, J.A.F., Lubin, B.H., Zwaal, R.F.A. and Roelofsen, B. (1989) Biochim. Biophys. Acta 981, 151–160.
49 Seigneuret, M. and Devaux, P.F. (1984) Proc. Natl. Acad. Sci. USA 81, 3751–3755.
50 Daleke, D.L. and Huestis, W.H. (1985) Biochemistry 24, 5406–5416.
51 Tilley, L., Cribier, S., Roelofsen, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1986) FEBS Lett. 194, 21–27.
52 Connor, J. and Schroit, A.J. (1988) Biochemistry 27, 848–851.
53 Bitbol, M., Dempsey, C., Watts, A. and Devaux, P.F. (1989) FEBS Lett. 244, 217–222.
54 Devaux, P.F. (1989) FEBS Lett. 234, 8–12.
55 Calvez, J.-Y., Zochowski, A., Herrmann, A., Morrot, G. and Devaux, P.F. (1988) Biochemistry 27, 5666–5670.
56 Farmer, II, B.T., Harmon, T.M. and Butterfield, D.A. (1985) Biochim. Biophys. Acta 821, 420–430.
57 Maksymiw, R., Sui, S.-f., Gaub, H. and Sackmann, E. (1987) Biochemistry 26, 2983–2990.