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Thermal Properties of Young Red Blood Cells Are Indicative of an Age-Dependent Regulation of Membrane-Skeleton Interaction

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The effects of red blood cell (RBC) age on membrane thermal properties have been investigated by using a 16-nitroxide stearic acid spin probe. We detected in unfractionated and most dense cells (2% fraction of circulating cells) a thermal transition at 40°C that in young cells (1% fraction) was lowered at 33–35°C. Spectrin seems to be directly involved in the transition detected in both young and unfractionated cells, as showed by the disappearance of the breaks after low salt extraction of spectrin. A further indication for a role of spectrin in this transition comes from its characteristic thermal unfolding above 40°C.

However, young cells did not show changes either in the thermal unfolding of spectrin or in the distribution of spectrin dimer, tetramer, and high oligomeric forms. These data rule out that spectrin of young RBC is modified in its thermal properties and indicate that young cells may have a different spectrin-membrane interaction.

Treatment of unfractionated ghosts with an antibody specific for a fragment of the 10K domain of protein 4.1, which is fully competent for the spectrin-actin binding, produced an evident lowering of the transition temperature. The same antibody did not affect the thermal transition of young ghosts. Our results suggest that spectrin-membrane interactions may be regulated during RBC lifespan.

Key words: spin labeling, red blood cell membrane thermal transitions, spectrin-membrane interaction, aging

The human red blood cell (RBC) has a life span of approximately 120 days, at the end of which it is removed from circulation by the cells of the reticuloendothelial system

Abbreviation used: RBC, red blood cell; 16NS, 2-(14-carboxytetradecyl)-2-ethyl-4, 4-dimethyl-3-oxazolidinyloxy; 4MSL, 4-maleimido 2,2,6,6-tetramethyl-1-piperidinyloxy; Anti-10K, antibody specific for the sequence 451–464 of domain 10K of protein 4.1; DFP, diisopropylfluorophosphate; PMSF, phenylmethylsulphonylfluoride; EDTA, ethylenediaminetetracetic acid; PBS, phosphate-buffered saline; CD, circular dichroism.

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[1,2]. It is well established that during its life span significant changes in the biochemical and biophysical properties of the membrane occur [3]. Old RBC showed decreased cell deformability, increased cell density, and increased membrane rigidity [4,5]. RBC mechanoelastic properties, which are crucial for cell crossing through small capillaries, are believed to be due to the skeleton that underlines the membrane. Spectrin tetramers and short actin filaments are the major components of this membranoskeleton. Spectrin linkage to intrinsic membrane glycoproteins (band 3 and glycophorins) is mediated by two anchoring proteins, namely, protein 2.1 and protein 4.1 [6]. The resulting supramolecular structure is thought to be elastic, flexible, and essential to maintain cell shape and deformability. It is conceivable that changes of membrane properties, observed during RBC aging, reflect alterations of skeletal proteins. At present, several changes in RBC membrane proteins have been reported to occur during the aging process, such as irreversible crosslinking between spectrin and hemoglobin [7], polymerization of membrane proteins [8], and oxidative damages [9].

Previous work in this laboratory [10,11] has shown that a spin-labeled stearic acid detected three characteristic thermal transitions in RBC at 8.0, 20.0, and 40.0°C. These thermal transitions were due to the interactions of skeletal proteins with the membrane [10,11]. The aim of this work was to study the effects of the aging process on RBC thermal properties. We found that, early in the RBC life span, changes occurred in a spectrin-dependent membrane thermal transition. These changes seem to be related to a modified interaction between the spectrin-actin network and the membrane.

MATERIALS AND METHODS

Materials

2-(14-Carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy (16 NS) and 4-maleimido 2,2,6,6-tetramethyl-1-piperidinyloxy (4MSL) spin labels were obtained from Syva (Palo Alto, CA). Diisopropylfluorophosphate (DFP) was obtained from Fluka (Buchs, Switzerland), phenylmethylsulphonylfluoride (PMSF) from Sigma (St. Louis, MO), and ethylenediaminetetraacetic acid (EDTA) from Carlo Erba (Milan, Italy).

Young RBC Preparation and In Vitro Aging

One unit (400–450 ml) of whole blood from normal donors was collected in citrate-phosphate-dextrose-adenine and subjected to leukocyte filtration with the Erypur system (Organon Teknika, The Netherlands). RBC were fractionated according to their density by the IBM 2991 Cell Processor [12]. Young cells were collected in a fraction corresponding to about 30% of the blood unit, and old cells, in the remaining 70%. With respect to the life span, the 30% fraction of RBC was defined "young" because it showed a mean increase in survival of 41% compared to the old fraction [12]. These two fractions were further fractionated by centrifugation in 150 mM NaCl, 5 mM sodium phosphate, pH 7.8 (PBS), for 1 h at 30,000*g* [13], and the less dense (top) and the most dense (bottom) fractions were taken. Fractionated RBC were analyzed for enzyme activities, reticulocyte count, and hemoglobin level as previously described [14].

The ratio of band a:b of protein 4.1 could be considered another useful index of cell aging. Triton X-100 membrane skeletons were prepared from RBC according to Mueller et al. [15] and the ratio of band a:b peak-area of protein 4.1 evaluated by densitometric analysis (LKB Ultroscan II, Bromma, Sweden) of Coomassie Blue-

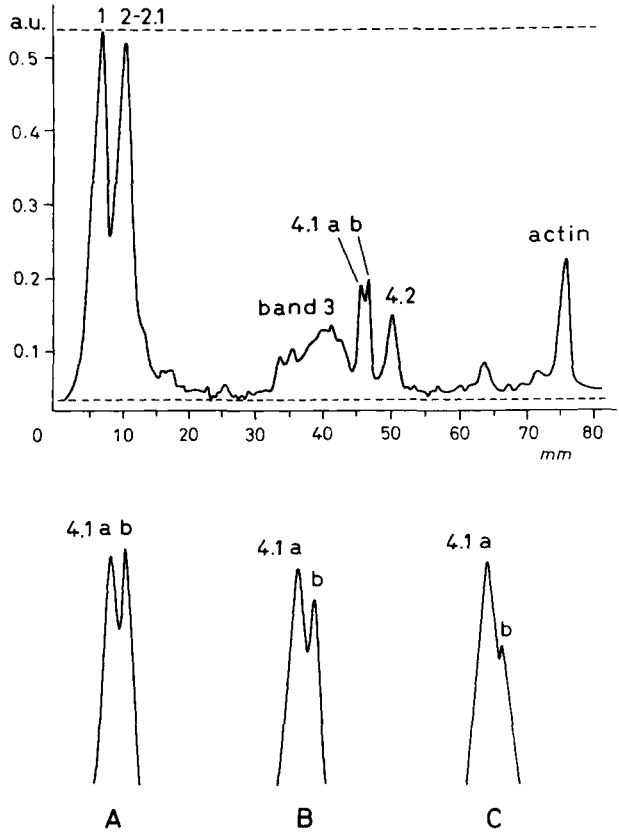

Fig. 1. Densitometric profiles of RBC membrane skeletal proteins (Triton X-100 residues). Full densitometric profile of young 1% RBC (upper) and particular of band 4.1 a:b ratio of density-separated cells (lower). Young 1% RBC (**A**); unfractionated RBC (**B**); and most dense cells (about 2% of circulating RBC) (**C**). Triton X-100 insoluble proteins from density-separated RBC were boiled in gel sample buffer and electrophoresed on a 6–15% SDS gradient gel. The ratio of **band a** and **b** of protein 4.1 was 1.38 in A, 2.05 in B, and 3.80 in C.

stained sodium dodecyl sulfate polyacrylamide gels. Figure 1 shows that band a:b ratio of protein 4.1 in young cells was lower than that of unfractionated cells and increased in the most dense cells. In vitro aging of young RBC was performed at 10% hematocrit in a buffer containing 130 mM NaCl, 20 mM KCl, 1 mM $MgCl_2$, 10 mM $NaHCO_3$, 1% BSA, penicillin-streptomycin 100 u/ml, pH 7.4. After 4 and 18 h of incubation at 37°C, cells were pelletted and washed with PBS.

Membrane Preparation, Skeletal Protein Extraction, and Treatment With Antibodies

Unsealed ghosts were prepared from fresh human RBC with 5 mM sodium phosphate pH 8 buffer, at 0°C. To avoid proteolytic degradation, 0.1 mM PMSF was added. Skeletal proteins (mainly spectrin and actin) were extracted at 37°C for 30 min with 0.3 mM sodium phosphate, 0.1 mM EDTA, 0.1 mM PMSF, pH 9.4 [16]. Formation of spectrin oligomers was studied by electrophoresis of extracted spectrin on 2–4% gradient nondenaturing polyacrylamide slab gels at 4°C [17]. Gels were electro-

phoresed at 50 V for 48 h in a buffer containing 40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA, pH 7.4, according to Morrow and Marchesi [16].

Preparation and affinity purification of anti-10K polyclonal antibody specific for the sequence 451–464 from the N-terminal of protein 4.1 was performed as previously described [18]. The specificity and the ability of this antibody to decrease the association of protein 4.1 with spectrin and F-actin has been described by Correas et al. [18]. The antibody was dissolved in 5 mM sodium phosphate, pH 8 buffer, at 0.5–1 mg/ml and added to unsealed ghosts at a 1:1 molar ratio with respect to protein 4.1. After 20 min of incubation at 0°C, to allow diffusion of the antibody inside the membrane, ghosts were resealed at 37°C with a buffer containing 150 mM KCl, 5 mM sodium phosphate, 0.25 mM ATP, 0.25 mM $MgCl_2$, 1 mM DFP, and 0.1 mM PMSF, pH 7.5. Membranes were checked for permeability to macromolecules before and after the resealing with a fluorescent dextran as previously described [19].

Spin Labeling and Spectra Analysis

Before spin labeling, intact RBC, resealed ghosts, and spectrin-depleted vesicles were washed with PBS and concentrated by centrifugation to 50% v/v. 16NS spin probe has been used to study thermal properties of RBC. To 3.8 μg of probe, evaporated under a nitrogen stream, an amount of cells corresponding to 0.5 mg of lipid was added. After few minutes at room temperature, the spin label was found completely associated with membranes. Membranes, packed by centrifugation, were injected into a sealed capillary (50 μl capillette, Boehringer Mannheim GmbH, West Germany). Spectra were recorded on a Varian E-4 spectrometer equipped with a variable temperature accessory) (Varian Associated Inc., Palo Alto, CA) and operated at 9.12 GHz, 3,260 G field set, 100 kHz field modulation, 1.25 G modulation amplitude, and 18 mW microwave. Temperature was monitored by a digital thermometer set above the cavity. At each temperature, three spectra were recorded and the average value of peak heights used for computer analysis. Changes in the freedom of motion of 16NS with temperature were analyzed by the empirical parameter log (h0/h − 1) as described elsewhere [20].

Spin labeling of ghost proteins with the maleimide analog 4MSL was performed as previously described [11]. Spectra of 4MSL-ghosts were analyzed by measuring the amplitude ratio of weakly (w) and strongly (s) immobilized components, an index frequently used to study protein conformational changes [21,22].

Circular Dichroism

Circular dichroism measurements of purified spectrin were made by a Jasco J-41 A spectrometer (Tokyo, Japan). Ellipticity in the region of peptide cotton effect was measured using thermostatted cells of 10 or 1 mm pathlength. Heating rate was 0.33°C/min and controlled by a temperature programmer Haake PG-20 (Berlin, FRG).

RESULTS

Thermal Properties of Density-Separated RBC and In Vitro Aging Experiments

As shown in Figure 2, unfractionated RBC showed three thermal breaks (also referred to as thermal transitions) at 8.0, 20.0, and 40.0 ± 1.0°C, whereas young RBC separated by the IBM 2991 Cell Processor showed a reproducible decrease at 37.0°C of

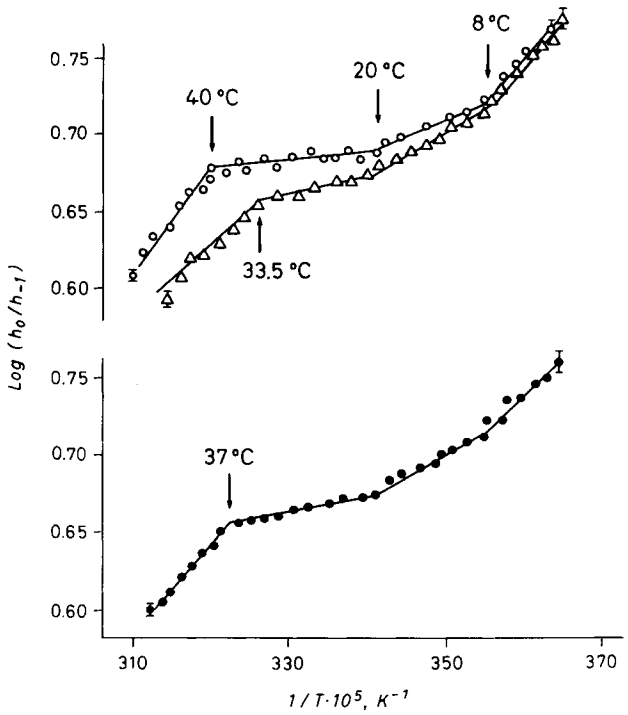

Fig. 2. Thermal properties of density-separated RBC labeled with 16NS. Unfractionated RBC (○), young 1% RBC (△), and young cells separated by the IBM 2991 Cell Processor (●). Intact RBC were labeled with 16NS spin probe, and electron spin resonance spectra were recorded as described under Materials and Methods. Points are average values of three spectra; typical ± SEM are indicated. Break temperatures were determined by computer analysis as described elsewhere [20].

the highest transition temperature. The other two transitions at 8 and 20°C were not modified in young cells. The lowering of the highest transition temperature in young RBC was not within the experimental errors, because the breaks were evaluated with an accuracy of ±1.0°C. The 37.0°C transition was observed in a population of young cells corresponding to about 1/3 of circulating RBC and could not be simply attributed to a contamination of white cells. In fact, the contamination of white cells of this fraction (filtered with the Erypur system) was comparable to that of unfractionated RBC. Further, the spin label eventually bound to white cells was rapidly reduced to unparamagnetic hydroxylamine and could not contribute significantly to 16NS-RBC spectra (M. Minetti, unpublished observations).

Young (30% of the blood unit) and old cells (70% of the blood unit) obtained from the IBM 2991 Cell Processor were further fractionated by centrifugation (see Materials and Methods) and the less dense (top) and the most dense (bottom) fractions were investigated. The less dense fraction accounting for 1% of the blood unit is hereafter referred to as young 1% RBC. The transition temperature of young 1% RBC was lower than that of the 30% fraction, and, according to different preparations ($n = 5$) was between 33 and 35°C (a typical result is shown in Fig. 2). The most dense cells (2% of the blood unit) did not differ significantly in their membrane transition temperature from the 70% old fraction as well as from unfractionated cells (data not shown). However, with

respect to the most dense cells, there are conflicting reports concerning the use of density techniques for generating truly senescent cells (for a comprehensive discussion, see [3]).

Storage of human RBC in vitro without an energy source has been shown to produce biophysical and biochemical changes that are similar to those observed in in vivo aged cells [23]. As shown in Figure 3, incubation at 37°C for 4 h of young 1% RBC produced an increase of the transition temperature from 34.5 to 37°C. Long incubation times further increased the transition temperature (after 18 h, the transition temperature was observed at 42.7°C, results not shown).

We have reported in a previous work [11] that the 40.0°C transition of unfractionated cells was due to spectrin unfolding and could be eliminated by low salt extraction of ghosts. As shown in Figure 4, low salt extraction of ghosts produced the disappearance of the high transition temperature in both young and unfractionated membranes. Taken together, these results (Figs. 2, 4) were indicative that young cells have a spectrin-dependent thermal transition at a lower temperature.

Thermal Properties of Spectrin From Young RBC

One possible explanation for the lowering of the transition temperature observed in young RBC could be that spectrin of these cells has a modified thermal behavior. We analyzed spectrin thermal properties by using 1) circular dichroism (CD) and 2) protein spin labeling with a maleimide analog (4MSL). Spectrin has an high α-helix content (70–80%), and the intensity of CD spectra at 222 nm in the 20–70°C temperature range detect a thermal transition due to the loss of its α-helix structure [11,24]. As shown in Figure 5a, thermal properties of spectrin isolated from young 1% RBC did not differ significantly from those of unfractionated cells. Ghosts were also spin labeled with 4MSL that alkylates mainly the sulphydryl groups of proteins. Spectra of 4MSL-ghosts as a function of temperature detect thermal changes that arise from fixed sites of proteins neighboring -SH groups labeled by the probe [21]. Previous studies [11,21] showed that although this spin label binds both intrinsic and peripheral proteins the thermal change detected above 40°C was due only to the unfolding of membrane-bound spectrin. Figure 5b shows that thermal properties of 4MSL-ghosts in young and unfractionated RBC were not different.

To test if a different spectrin oligomer distribution was responsible for the lowered thermal transition in young cells, we analyzed spectrin extracted from density-separated

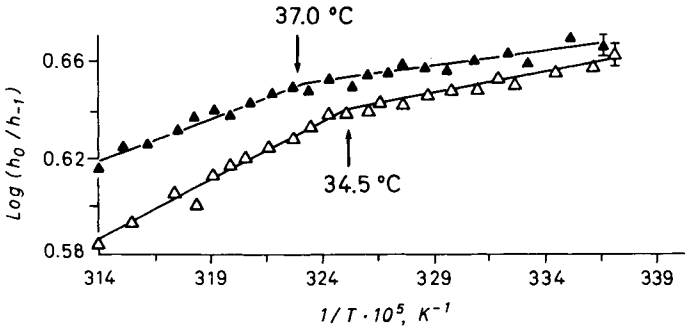

Fig. 3. Effects of in vitro aging on the thermal properties of 16NS-labeled RBC. Young 1% RBC before (△), and after (▲) storage at 37°C for 4 h without an energy source. Data analysis was as in Figure 2.

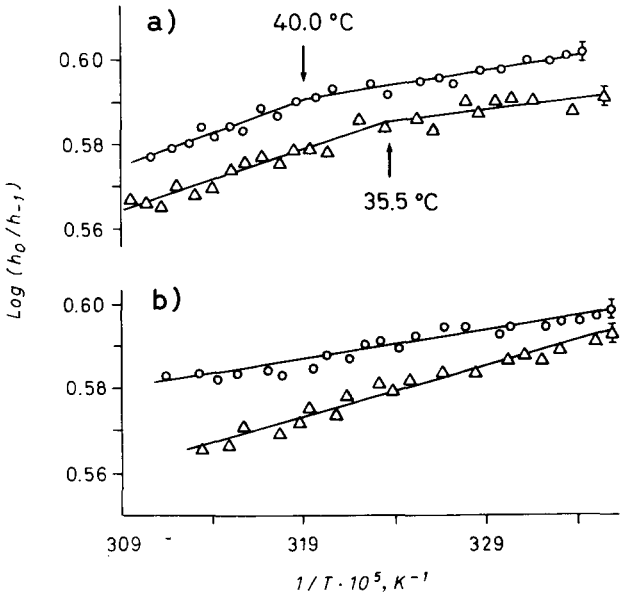

Fig. 4. Effects of spectrin-actin extraction on the thermal properties of 16NS-labeled membranes. **a:** Control ghosts from unfractionated RBC (○) and from young 1% RBC (△). **b:** Effects of low salt extraction on unfractionated ghosts (○); and on young 1% RBC ghosts (△). Low salt extraction of ghosts selectively removed spectrin an actin [16]. Data analysis was as in Figure 2.

cells by nondenaturing polyacrylamide gel electrophoresis. The predominant forms of spectrin, prepared by low ionic strength extraction at 37°C or at 0°C, were dimers and tetramers, respectively [25]. The relative amount of dimers, tetramers, and high oligomers were not different between young 1% and unfractionated RBC (results not shown). However, in agreement with previous results [25], a 2.5-fold increase of high oligomeric forms was observed in the most dense RBC fraction (results not shown).

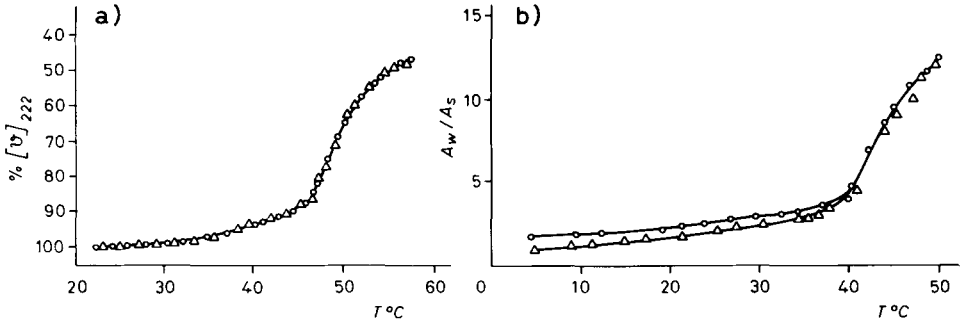

Fig. 5. Thermal behavior of spectrin in unfractionated RBC and young 1% RBC. **a:** Circular dichroism spectra of spectrin purified from unfractionated (△) and young 1% RBC (○). ϑ = percentage of residual ellipticity at 222 nm. Heating rate: 0.33°C/min. **b:** Aw/As ratio of 4MSL-labeled membrane proteins obtained from unfractionated (△) and young 1% RBC (○). Conformational changes of 4MSL-labeled membrane proteins were analyzed by measuring the amplitude ratio of weakly (w) and strongly (s) immobilized components of the low field line. This ratio is a sensitive index of protein structure in the neighboring of -SH groups labeled by the probe [21].

Effects of Antibodies Specific for Protein 4.1

A second possible explanation for the changes in young RBC thermal properties could be that young cells may have a modified spectrin-membrane interaction. We reported in a previous paper [11] that not only anti-spectrin but also anti-protein 4.1 antibodies affected the 40°C transition. In particular, the anti-protein 4.1 antibody effaced not only the thermal transition at 8°C, which is dependent on protein 4.1 [10], but also lowered the temperature of the 40°C transition. For this reason, we analyzed the effects of a particular polyclonal antibody (referred to as anti-10K) obtained against a synthetic peptide representing the sequence 451–464 from the N-terminal of protein 4.1 [26]. This sequence is located within the 10K region of protein 4.1, which had been previously shown to be fully competent for spectrin-actin binding [18,27]. Interestingly, treatment of unfractionated ghosts with the anti-10K antibody at 1:1 molar ratio with respect to protein 4.1 did not affect the 8 and 20°C thermal transitions (data not shown), but produced an evident lowering of the transition temperature from 40.0 to 35.5°C (Fig. 6). In contrast, both the 35.5°C thermal transition (Fig. 6b) and the 8 and 20°C transitions (data not shown) of the young cells were not affected by the anti-10K antibody. These results showed that the anti-10K antibody treatment of control ghosts produced a membrane with thermal transitions similar to that of young RBC (compare Fig. 4a with Fig. 6).

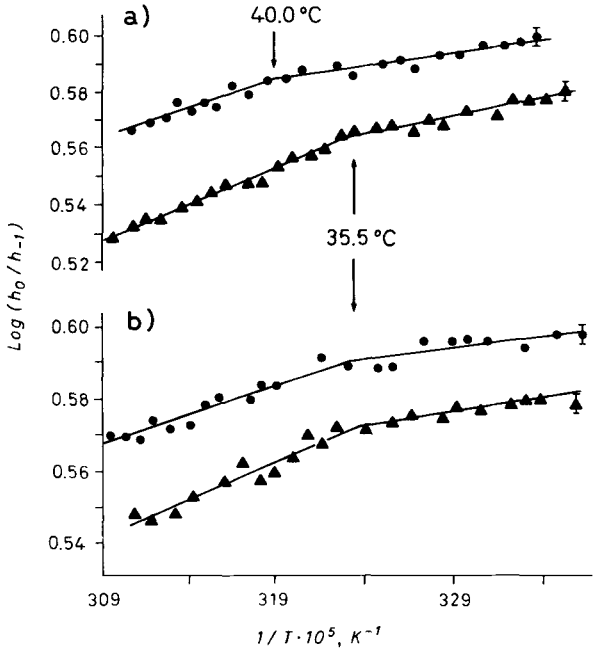

Fig. 6. Effects of the anti-10K polyclonal antibody on the thermal properties of 16NS-labeled ghosts. This antibody was specific for a fragment of the domain 10K of protein 4.1. The antibody was allowed to diffuse inside unsealed ghosts as described in Materials and Methods. Ghosts resealed with the IgG fraction of non-immunized rabbit serum (●) or with anti-10K antibody (▲). Ghosts were obtained from (**a**) unfractionated RBC and (**b**) young 1% RBC. Data analysis was as in Figure 2.

DISCUSSION

Our results showed that, during RBC life span, changes occurred in a membrane thermal transition. About 1% of circulating RBC, with both the enzymatic and the protein 4.1 a:b ratio characteristics of young cells, showed a transition temperature at 33–35°C, whereas unfractionated and the most dense RBC showed this transition at 40.0°C. On the other hand, a consistent population of circulating RBC (less dense cells representing about 30% of total RBC) still showed a transition temperature lowered at 37.0°C, thus suggesting a "maturation" of the thermal properties during RBC life span. This hypothesis was supported by the finding that young 1% RBC after in vitro aging showed an increase in the transition temperature.

Spectrin was involved in the thermal transition of both young and unfractionated cells as shown by the effects of selective protein extraction. Spectrin around 40°C undergoes conformational changes detectable both in situ or in the isolated molecule, and we suggest that these conformational changes are in some way reflected in the properties of the lipid bilayer and may account for the membrane thermal transition detected by 16NS [11]. Nevertheless, we found with spin labeling and CD techniques that thermal properties of spectrin from young 1% and from unfractionated RBC were virtually identical. Calvert et al. [24] reported that dimer-oligomer equilibrium was a temperature-dependent phenomenon preceding massive spectrin unfolding [24]. However, the oligomer distribution of spectrin extracted from young 1% RBC was found similar to that of unfractionated RBC. These results rule out the hypothesis that thermal properties of young cell membranes were due to a change in the thermal properties of the whole spectrin molecule.

The other possible explanation for the thermal properties of young cells could be that some changes may occur in specialized protein domains involved in the binding of spectrin network to the membrane. These domains are thought crucial in the transfer to the bilayer of spectrin thermal motion. The binding of spectrin-actin network to the intrinsic membrane glycoproteins is mediated by at least two proteins referred to as protein 2.1 and protein 4.1 [6]. Especially, protein 4.1 has been shown to play a relevant role in skeleton-membrane stability [28] and membrane thermal properties [10,11]. This skeletal protein not only binds to glycophorins [29,30], but also forms a ternary complex with spectrin and actin and stabilizes their association [31]. Recently, an electron microscopy study of Liu et al. [32] of spread RBC membrane skeletons showed clear images of a protein network organized into a regular hexagonal lattice. Junctional complexes of short actin filaments and protein 4.1 represent the center and the corner of each hexagon and are linked to the adjacent complexes by filaments of spectrin. These electron microscopy micrographs of RBC membrane skeletons suggest that protein 4.1 is localized in a strategic position to regulate membrane events in response to spectrin changes (including spectrin changes induced by temperature). In this regard, it is interesting that the reticulocytes of acetylphenylhydrazine-treated rats have junctional complexes larger than do mature RBC (80–140 vs. 38–50 μm) [32], thus suggesting a possible remodeling and size reduction of these sites during reticulocyte-erythrocyte maturation.

We found that in unfractionated RBC the treatment with a polyclonal antibody, specific for a fragment of the 10K domain of protein 4.1 [27], mimicked a young membrane as far as the highest thermal transition was concerned by lowering that from

40.0 to 35.5°C without affecting the other two transitions. The same antibody did not affect the thermal properties of young membranes, thus suggesting that its membranoskeleton organization may be comparable to that of anti-10K-treated control membranes, at least for the thermal properties. According to Correas et al. [18], the effects of this antibody could be to decrease in situ the association of protein 4.1 with spectrin and F-actin. In conclusion, our results suggest that 1) changes in the thermal properties of young RBC membranes could be due to a different interaction between skeletal proteins and the membrane and 2) spectrin-protein 4.1 binding regions seem to be modified or "processed" during the RBC life span. Although the nature of this processing event remains speculative, at least two age-related post-translational modifications of protein 4.1 have been described. First, the aging process has been shown to decrease protein 4.1 phosphorylation and, on the other hand, protein 4.1 phosphorylation *in vitro* has been shown to reduce its binding affinity to spectrin [33,34]. Second, the band a:b ratio of protein 4.1 has been reported to increase with cell age and to be a relatively early event of the aging process [15].

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