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Reduced transglutaminase-catalyzed cross-linking of exogenous amines to membrane proteins in sickle erythrocytes

Samir K. Ballas [a,*], Narla Mohandas [b,d], Margaret R. Clark [b,d], Stephen H. Embury [c], Eugene D. Smith [a], Laurence J. Marton [b,c] and Stephen B. Shohet [b,c,d]

[a] *Cardeza Foundation for Hematologic Research, Department of Medicine, Jefferson Medical College, Philadelphia, PA 19107 and Departments of* [b] *Laboratory Medicine and* [c] *Medicine,* [d] *MacMillan-Cargill Hematology Research Laboratory, and* [e] *Brain Tumor Research Center, University of California, San Francisco, CA 94143 (U.S.A.)*

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In order to determine the capacity of sickle cells to undergo transglutaminase-catalyzed cross-linking of membrane proteins, human normal and sickle erythrocytes were incubated with [*ring*-2-^{14}C]histamine in the presence of Ca^{2+} and ionophore A23187. The [^{14}C]histamine incorporation into membrane components was observed in freshly prepared erythrocytes. Incorporation of radioactivity into spectrin and Band 3 membrane components was significantly ($P < 0.001$) less in sickle erythrocytes than in normal cells. Transglutaminase deficiency was excluded by the finding of increased activity of this enzyme in sickle cells from patients with reticulocytosis. The incorporation of [^{3}H]spermine into red cell membranes was also less in sickle erythrocytes than in normal cells under the same conditions of incubation used for [*ring*-2-^{14}C]histamine. Sickle erythrocytes were more permeable to these amines than normal cells. It is proposed that the γ-glutamyl sites of membrane proteins in sickle erythrocytes are less accessible for transglutaminase-catalyzed cross-linking to histamine and polyamines in vitro, perhaps due to prior in vivo activatioan of this enzyme by the increased calcium in sickle cells and/or shielding secondary to altered membrane organization.

The accumulation of Ca^{2+} in intact human erythrocytes from normal donors leads to the formation of a new high-molecular weight membrane protein larger than spectrin [1]. The polymer has a heterogeneous size distribution and is rich in γ-glutamyl-ε-lysine cross-links [2]. It is now well established that the formation of these cross-links is mediated by a normally dormant, Ca^{2+}-dependent, endogenous red cell transglutaminase [1]. Once activated by calcium ions, the transglutaminase catalyzes the formation of covalent γ-glutamyl-ε-lysine cross-link by means of an acyl transfer reaction, in which the γ-carboxamide group of peptide-bound glutamine residues are the acyl acceptors [3]. Moreover, it has been recently shown that the acyl donors in this reaction need not be ε-amino groups of peptide-bound lysine but can be any of a variety of primary amines, including diamines, spermine and other polyamines [3,4]. The sickle erythrocyte provides a unique opportunity to investigate the consequences of an increased cellular calcium concentration in vivo. It is now well established that sickle erythrocytes contain more calcium than normal cells and that sickling enhances the ingress of calcium into the red cell [5]. Lorand and his colleagues [6] have

* To whom correspondence should be addressed at: Cardeza Foundation, 1015 Walnut Street, Philadelphia, PA 19107, U.S.A.

proposed that the activation of transglutaminase may lead to protein cross-links that have a detrimental effect on red cell properties. However, efforts to identify the expected high molecular weight, non-reducible protein polymer in sickle cells have been unsuccessful so far [7,8]. However, it has been shown that sickle erythrocytes contain a higher concentration of primary amines, especially spermine, than normal cells and that about 50% of total cellular spermine content in sickle cells is membrane-bound [9]. The obvious question, then, is whether activation of the endogenous transglutaminase in sickle cells leads to the cross-linking of endogenous cytoplasmic amines into membrane proteins. This study was designed to investigate this possibility.

Materials and Methods

Materials. Fresh heparinized human blood was obtained from patients with sickle cell anemia and from healthy donors at the Thomas Jefferson University Hospital Blood Bank. The calcium ionophore A23187 was a generous gift of Dr. Hamill of Lilly Research Laboratories. [^{14}C]Putrescine (96.36 mCi/mmol) and [^{3}H]spermine (23.5 Ci/mmol) were from New England Nuclear. [*ring*-2-^{14}C]Histamine (59.7 mCi/mmol) was from Amersham, Searle. Unlabeled histamine and spermine were from Sigma and putrescine from Eastman, Kodak. *N,N'*-Dimethylcasein was prepared by the procedure of Lin et al. [10], and was dissolved in 50 mM Tris-HCl (pH 7.5) to give a 2.0% protein solution.

Isolation of cells. Erythrocytes were washed at 4°C by repeated centrifugation (three times 1000 $\times g$ for 10 min) in 0.16 M NaCl and 5 mM Tris-HCl (pH 7.4) and were resuspended to 50% in the washing buffer. Paired sickle and control blood samples, both collected within a 20–30 min period were processed in parallel for each experiment, within 1 h of collection. The percentage of reticulocytes was determined on whole blood samples stained with brilliant cresyl blue.

[^{14}C]Putrescine assay for transglutaminase. The [^{14}C]putrescine assay for guinea pig transglutaminase [11] was modified for use in studying the red cell enzyme by observing the incorporation of putrescine into *N,N'*-dimethylcasein. Transglutaminase activity in hemolyzed cell suspensions was measured as follows: samples of red cell suspensions prepared as described above were frozen at −70°C, thawed at room temperature, and at the moment of complete thawing, 20 μl (about 2.5 mg of protein) aliquots were mixed with 20 μl of 16 mM unlabeled putrescine in 50 mM Tris-HCl (pH 7.5), 100 μl of 2.0% dimethylcasein, 10% of [^{14}C]putrescine (1.0 μCi) and 20 μl of 50 mM $CaCl_2$ in 50 mM Tris-HCl (pH 7.5). The volume was brought to 200 μl by the addition of 30 μl of 50 mM Tris-HCl (pH 7.5) so that the final concentrations of putrescine and $CaCl_2$ were 1.6 mM and 5 mM, respectively. The reaction was initiated by the final addition of calcium chloride and incubation at 37°C. The reaction was terminated by the addition of 4 ml of cold 5% trichloroacetic acid at specific time points. The precipitate was collected on Millipore filters and washed well with 5% trichloroacetic acid. The filter disks were dissolved in 0.2 ml of formic acid and 10 ml of naphthalenedioxane counting fluid [12,13] was added. Radioactivity was measured with a Packard liquid scintillation counter. The assay was linear to 30 min.

Incorporation of [ring-2-^{14}C]histamine into erythrocyte membrane proteins. This was determined by the analytical method of Siefring et al. [2] as follows: an incubation mixture was prepared to contain 0.5 ml of fresh red cell suspension, prepared as described above, 60 μl of 100 mM histamine in 0.16 M NaCl/5 mM Tris-HCl (pH 7.4) 50 μl of 0.6 mM A23187, 50 μl [*ring*-2-^{14}C]histamine (10.0 μCi) and 150 μl of 50 mM $CaCl_2$ in 0.16 M NaCl/5 mM Tris-HCl (pH 7.4). The volume of the reaction mixture was brought to 1.5 ml with 0.16 M NaCl/5 mM Tris-HCl (pH 7.4) so that the final concentration of histamine, A23187 and $CaCl_2$ were 4 mM, 20 μM and 5 mM, respectively. The A23187 ionophore was prepared as a 3 mM stock in dimethylsulfoxide and was diluted 5-fold in 0.16 M NaCl/5 mM Tris-HCl immediately before use. The reaction was initiated with a final addition of $CaCl_2$ and incubation at 37°C. In some experiments $MgCl_2$ was used instead of $CaCl_2$. Following incubation for 3–6 h, the cells were washed three times as described above and lysed in 5 mM phosphate buffer (pH

8.0). Membranes were separated by centrifugation (48 000 × *g* for 10 min) and washed repeatedly in 50 ml of the lysing buffer to remove as much hemoglobin as possible. Washed ghosts were either used immediately or stored at −70°C until further use.

Incorporation of [^{3}H]spermine into erythrocyte membrane proteins. The experimental procedure was similar to that described above except that spermine was used instead of histamine. 20 μl of [^{3}H]spermine (20 μCi) and 60 μl of 100 mM spermine in 0.16 M NaCl/5 mM Tris-HCl, were added to each reaction mixture so that the final concentration of spermine was 4 mM.

Membrane analysis. Total red cell membrane protein content was measured by the method of Lowry et al. [14] using bovine serum albumin as a standard. The amount of hemoglobin retained by the membranes was determined by the modified benzidine method using *o*-tolidine instead of benzidine [15] and was subtracted from the total protein determined by the Lowry method in order to estimate the true membrane protein content. Membranes were further analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS). Aliquots were dissolved in 2% SDS, 2% β-mercaptoethanol, held for 3 min at 100°C and electrophoresed on 5.6% polyacrylamide gels containing 0.1% SDS [16]. The gels were stained with Coomassie brilliant blue, R-250, and the bands were numbered according to the system of Fairbanks et al. [17] as modified by Steck [18]. The proportion of each band of the stained gels was assessed by densitometry, using a Gilford spectrophometer fitted with a Model 2410 linear transport accessory scanning through a 0.1 mm slit at 560 nm. Stained gels were sliced into 1.0-mm thick pieces, and the radioactivity in each slice was determined after solubilization in Soluene 350 overnight, followed by the addition of 10 ml of liquefluor-toluene scintillation solution. Radioactivity in the whole gel and in each band region was determined and expressed as cpm/mg protein (excluding hemoglobin).

Statistical significance was determined by Student's or the paired *t*-test whenever applicable.

Red cell membrane permeability to amines. Freshly drawn erythrocytes were washed and resuspended in 0.16 M NaCl and 5 mM Tris-HCl (pH 7.4) and incubated at 37°C for 5 min. Permeability to amines was determined by measuring the intracellular accumulation of [*ring*-2-^{14}C]histamine or [^{3}H]spermine. Histamine (4 mM) containing 10 μCi [^{14}C]histamine or 4 mM spermine containing 20 μCi [^{3}H]spermine, both in 0.16 M NaCl and 5 mM Tris-HCl (pH 7.4) were added to washed erythrocyte suspensions. Cells were incubated at 15% hematocrit in a final volume of 1.5 ml in the presence of 20 μM A23187 and 5 mM $CaCl_2$. At 0 and 3 h after addition of the radioactivity, aliquots of cell suspension were removed and washed three times in 10 ml ice-cold, Tris-$MgCl_2$ (pH 7.4). The washed cells were decolorized with 0.5 ml H_2O_2 (30%) and radioactivity was determined in a liquid scintillation counter. Samples of whole cell suspensions were also obtained immediately after addition of the radioactive amine for determination of the specific activity of the external medium. Erythrocyte permeability to polyamines was assessed from the ratio of the cpm in 1 μl of external medium [19].

Determination of polyamine levels in red cell membranes. Red cell membranes were prepared by the following two methods for the determination of their polyamine content. In the first method hemoglobin-free membranes were prepared by extensive washing in 5 mM phosphate buffer (pH 8.0). Aliquots of membranes were hydrolyzed in 6 N HCl at 106°C for 18 h. The acid hydrolysates were dried under nitrogen and redissolved in 250 μl water. 50-μl aliquots were taken for polyamine determination. This method gave an estimate of total membrane polyamine content, including both the covalently and noncovalently bound moieties. The second method of membrane preparation was designed to determine only the polyamines that were covalently bound to membrane proteins. Washed, hemoglobin-free membranes from normal and sickle erythrocytes were reduced in 2% SDS and 2% β-mercaptoethanol at 90°C for 5 min. Membrane proteins were then precipitated with 20% trichloroacetic acid and washed extensively in 5% trichloroacetic acid and diethyl ether, respectively. Washed membranes were redissolved in 0.2 M *N*-ethylmorpholine acetate buffer (pH 8.1) containing 50 μg of trypsin [20,21] and were hydrolyzed in 6 N HCl as in the first method. 50-μl aliquots of each acid hydrolysate were

analyzed for polyamine content using a Dionex Instruments Inc. (Sunny Vale, CA) D-500 aminoacid analyzer as previously described [22].

Results

Incorporation of labeled amines into erythrocyte membrane proteins

Fig. 1 shows the membrane protein pattern and the intrinsic labeling of membrane components by [*ring*-2-^{14}C]histamine in intact normal and sickle erythrocytes. Both membrane protein patterns depict the previously described effects of calcium, namely, the appearance of polymer X on top of the gel, the increase in polypeptide 2.3 (M_r 174 000) and the disappearance of band 4.1 [19]. Band 4.3 was relatively increased in membranes from sickle erythrocytes. Radioactivity incorporated into spectrin (bands 1 and 2) and into band 3 was less in sickle erythrocytes than in normal. The extent of reaction of [^{14}C]histamine with polymer X and the two major proteins of red cell membranes, spectrin and band 3, was estimated by summing the radioactivity in the gel slices corresponding to these proteins. These data are summarized in Table I. The total incorporation of [^{14}C]histamine into membranes was significantly lower ($P < 0.001$) in sickle erythrocytes than control cells. This was a consistent finding in all samples studied, irrespective of the reticulocyte count. It must be emphasized that the cpm shown in Table I represent the sum total of the radioactivity in the gel slices. Since membrane samples were reduced in 2% SDS and 2% β-mercaptoethanol prior to electrophoresis, and since both the gel and the running buffer contain SDS, the incorporated radioactivity is most likely due to covalent binding of the tracer amine. Analysis of the incorporation of radioactivity into various membrane components shows that the amount of radioactivity incorporated into polymer X was the same in all samples. Incorporation of radioactivity into spectrin and band 3, however, was significantly lower in membranes from sickle erythrocytes than control cells. Results were the same whether incubations were carried out for 3 or 6 h. There was no significant incorporation of radioactivity when $CaCl_2$ was omitted from the incubation mixture or replaced by $MgCl_2$. In order to determine whether the decreased incorporation into membrane proteins of sickle erythrocytes could have been related to a high proportion of reticulocytes, we conducted similar

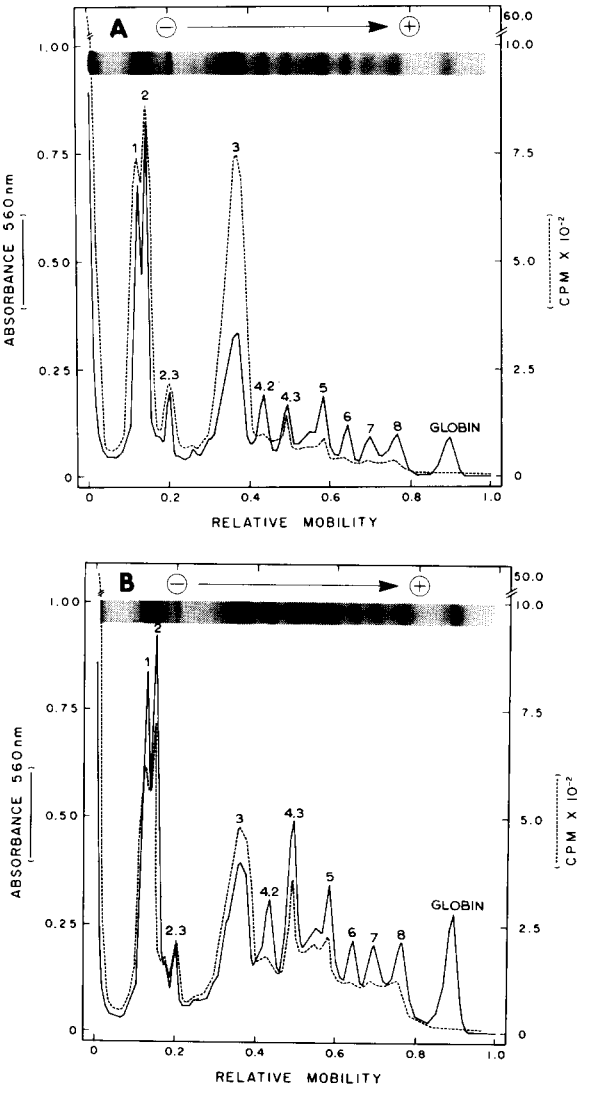

Fig. 1. Intrinsic labeling of normal (A) and sickle (B) membrane proteins by [^{14}C]histamine in intact erythrocytes. Fresh cells were incubated with ionophore, $CaCl_2$ and [^{14}C]histamine for 6 h at 37°C as described in the text. Following incubation membranes were isolated and analyzed by SDS-polyacrylamide gel electrophoresis. Gel A contains 94 μg and B contains 102 μg of membrane proteins. Gels were stained by Coomassie brilliant blue, R-250. Radioactivity incorporated into spectrin and band 3 is less in sickle erythrocytes (B) than in normal cells. No significant radioactivity could be found in the gels in the absence of Ca^{2+}.

TABLE I

INCORPORATION OF [^{14}C]HISTAMINE INTO MEMBRANE PROTEINS OF NORMAL AND SICKLE ERYTHROCYTES

Washed erythrocytes were incubated with [*ring*-2-^{14}C]histamine, 20 μM ionophore and 5 mM $CaCl_2$ in 0.16 M NaCl/5 mM Tris-HCl (pH 7.4) for 3 h at 37°C. Radioactivity incorporated into membrane proteins excluding hemoglobin and into individual polypeptides was determined as described in the text and Fig. 1. In the absence of Ca^{2+} no radioactivity was incorporated. Values are mean ± S.D. Number of determinations is indicated in parentheses.

	Source of erythrocytes		
	Normal	Sickle cell anemia	
		High reticulocyte	Low reticulocyte
Total radioactivity incorporated into membranes			
1. cpm/mg membrane protein	(10) 47087 ± 7100	(10) 26227 ± 3982	(5) 26838 ± 3261
2. % of control value	100	55.5	59.0
3. *P* value (compared to normal)	–	< 0.001	< 0.001
Total radioactivity in polymer X			
1. cpm/mg membrane protein	(10) 158759 ± 28103	(10) 140106 ± 38753	(5) 152504 ± 37381
2. % of control value	100	88.3	96.1
3. *P* value (compared to normal)	–	> 0.05	> 0.05
Total radioactivity in spectrin			
1. cpm/mg membrane protein	(10) 35125 ± 7781	(10) 20270 ± 4809	(5) 20435 ± 4304
2. % of control value	100	57.7	58.2
3. *P* value (compared to normal)	–	< 0.001	< 0.001
Total radioactivity in band 3			
1. cpm/mg membrane protein	(10) 34663 ± 8378	(10) 16447 ± 5135	(5) 17406 ± 2271
2. % of control value	100	47.5	50.2
3. *P* value (compared to normal)	–	< 0.001	< 0.001

experiments on blood obtained from two patients with autoimmune hemolytic anemia and reticulocytosis. In contrast to sickle cells, the total radioactivity incorporated into red cell membrane proteins was 149% of the control value in the first patient and 142.3% in the second. Incorporation into spectrin and band 3 was similarly and proportinately increased in the reticulocyte-rich samples. Since the patients with autoimmune hemolytic anemia studied had hemoglobin and reticulocyte levels comparable to those of sickle patients, the decreased incorporation of radioactivity in membrane proteins of sickle cells does not seem to be due to the anemia or the high reticulocyte count.

The total incorporation of [^{3}H]spermine into intact red cell membranes was much lower than that of histamine, but sickle cells incorporated 65.6% of the total radioactivity incorporated by normal erythrocytes ($P < 0.025$). Thus, in five experiments performed in parallel, the mean incorporation of [^{3}H]spermine was 2871 ± 507 cpm and 1884 ± 466 cpm in normal and sickle erythrocytes, respectively. The amount of total radioactivity was too low to permit measurement of the extent of spermine binding to individual membrane components.

Transglutaminase activity in normal and sickle erythrocytes

That the decreased incorporation of radioactive amines into membrane proteins of sickle red cells was not due to decreased transglutaminase activity was indicated by measuring the activity of this enzyme in normal and sickle erythrocytes during 60 min of incubation. The activity of this enzyme was found to be 213.8 ± 68.98% of that in normal cells in 15 determinations ($P < 0.001$). Fig. 2 shows the kinetics of transglutaminase-mediated incorporation of [^{14}C]putrescine into dimethylcaseine

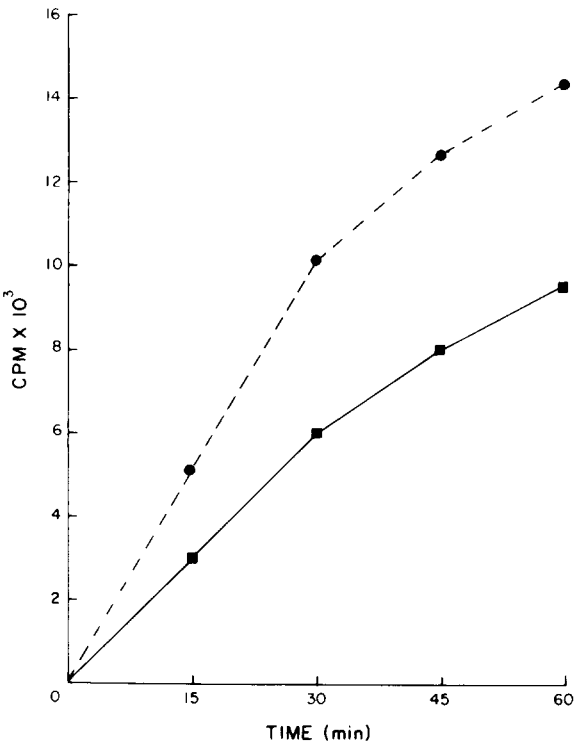

Fig. 2. Red cell transglutaminase activity determined by the incorporation of [^{14}C]putrescine into dimethylcasein in the presence of control (■———■) and sickle (●- - - - -●) erythrocyte lysates. Both reaction mixtures contained the same amount of lysate. There is greater incorporation of radioactivity in the presence of sickle cells at all points. Radioactivity covalently bound to proteins by the action of Ca^{2+}-stimulated transglutaminase was measured as described in Materials and Methods and in Table I.

induced by hemolysates of both sickle and normal cells in one representative experiment. The incorporation caused by sickle lysate is higher than that due to control lysate throughout the incubation period. There was no incorporation of radioactivity in either cell preparation when $CaCl_2$ was omitted from the incubation mixture.

Erythrocyte membrane permeability to histamine and spermine

To determine whether the decreased incorporation of amines into intact sickle cells could be explained by reduced permeation of the amine into the cell interior, we measured the uptake of histamine and spermine into sickle and normal cells. Sickle erythrocytes were more permeable to the amines studied. The mean uptake of [^{14}C]histamine and [^{3}H]spermine into sickle cells was 25 and 35%, respectively, higher than that into control cells. Thus, when cells were incubated in 4 mM [*ring*-2-^{14}C]histamine $31.7 \pm 6.80\%$ ($n = 12$) of the total radioactivity accumulated within sickle erythrocytes in 3 h and $25.5 \pm 7.33\%$ ($n = 12$) in normal erythrocytes. In the case of 4 mM [^{3}H]spermine, 3.5 ± 1.87 ($n = 9$) and $2.6 \pm 1.24\%$ ($n = 9$) of the radioactivity accumulated in sickle and normal erythrocytes, respectively, after 3 h of incubation. The decreased incorporation of radioactive amine into membrane proteins of sickle erythrocytes, therefore, cannot be due to decreased membrane permeability. It must be emphasized that our measurement of amine uptake do not actually measure the permeability of the membrane to amines. Since sickle and normal erythrocytes bind different amounts of amine internally, the intracellular pool sizes of the amines are different in each cell and this would affect transport rates. Our measurements of permeability only reflect the differential overall uptake of amine into the different cell types.

TABLE II

TOTAL POLYAMINE (COVALENTLY AND NON-COVALENTLY BOUND) LEVELS IN RED CELL MEMBRANES

Washed hemoglobin-free membranes were isolated from normal and sickle erythrocytes and their polyamine content was determined after acid hydrolysis, as described in Materials and Methods. n.d., not detectable at high level of sensitivity.

Erythrocyte	Exp. No.	Polyamine (pmol/mg membranes protein)		
		Putrescine	Spermidine	Spermine
Normal	1	110	276	331
	2	n.d.	151	454
	3	55	109	164
	4	146	97	146
Sickle cell	1	400	2267	2444
	2	n.d.	3498	2153
	3	170	5277	3949
	4	n.d.	4267	2987
	5	88	3033	4835
	6	n.d.	9068	13515
	7	47	2212	2071
	8	n.d.	4408	4228
	9	251	3039	2761

Polyamine levels in normal and sickle red cell membranes

An alternative explanation for reduced amine incorporation by sickle cells during in vitro incubation could be that binding sites for the amines were already occupied by amines previously bound in vivo. To test this possibility we determined the amounts of native polyamines associated with unincubated sickle and normal cells (Table II). Differences in total polyamine (covalently plus non-covalently bound) were most pronounced in the spermidine and spermine contents, which, on average, were at least 10-times higher in sickle membranes than in control preparations (Table II). The proportions of covalently bound polyamines were estimated by analysis of membranes subjected to reduction in SDS and β-mercaptoethanol and extensive washing in trichloroacetic acid and then diethyl ether, a treatment that removes most, but not all, non-covalently bound polyamines. As shown in Table III, most of the polyamine content was non-covalently bound, but the residual covalently bound polyamines were 2–3-times higher in sickle mebranes than in normal membranes. It should be emphasized that, depending on how strongly polyamines are bound to the membrane, the amount recovered will be affected by the extent of washing. Recovery of membrane-bound polyamines, in this sense, may, therefore, be arbitrary. We like to stress, however, that the experiments described in Tables II and III were performed on normal and sickle membranes which were prepared in parallel and washed to the same extent under the same conditions.

Transglutaminase-catalyzed cross-linking of isolated membranes

If membrane proteins from sickle erythrocytes have an altered structure or if they are biochemically modified, isolated membranes may demonstrate altered cross-linking patterns. In order to test this hypothesis, membranes from normal and sickle erythrocytes were isolated and their ability to bind polyamines was assayed as follows: 0.3 mg of membrane proteins were incubated with 10 μCi [^{3}H]spermine, 5 mM $CaCl_2$ (final concentration) and 50 μl of fresh hemolysate prepared from normal red cells as a source of the transglutaminase. The mixture, 200 μl final volume, was incubated at 37°C for 1 h and the radioactivity incorporated into membranes was determined on Millipore filters as described in Materials and Methods. Isolated sickle membranes exhibited decreased cross-linking (69% of control) of exogenous spermine compared to control membranes: 461 ± 103 cpm ($n = 11$) vs. 711 ± 115 cpm ($n = 10$), respectively. The extent of incorporation of [^{3}H]spermine into sickle membranes was similar (about 65% of control values) whether incubations started with intact cells or isolated membranes as mentioned above. This is further indication that membrane proteins from sickle erythrocytes may have an intrinsic abnormality responsible for their reduced ability to cross-link exogenous amines.

TABLE III

COVALENTLY-BOUND POLYAMINE LEVELS IN RED CELL MEMBRANES

Washed hemoglobin-free membranes wer isolated from normal and sickle erythrocytes and reduced with 2% SDS and 2% β-mercaptoethanol at 90°C for 5 min. Membrane proteins were then precipitated with 20% trichloroacetic acid and washed extensively in 5% trichloroacetic acid and diethyl ether, respectively. Washed membranes were redissolved in 0.2 M *N*-ethylmorpholine citrate buffer (pH 8.1), containing 50 μg of trypsin and hydrolyzed in 6 M HCl at 110°C, for 24 h for polyamine determination. n.d., not detectable at high level of sensitivity.

Erythrocyte	Expt. No.	Polyamine (pmol/mg membrane protein)		
		Putrescine	Spermidine	Spermine
Normal	1	23	n.d.	87
	2	n.d.	n.d.	45
Sickle cell	1	n.d.	108	155
	2	n.d.	189	171
	3	n.d.	210	133
	4	24	115	163

Discussion

In this study, we have looked for evidence concerning the possibility that Ca^{2+} accumulation by sickle cells may cause in vivo activation of the normally dormant transglutaminase enzyme. Our studies have yielded several indirect observations which are consistent with this possibility. First, during in vitro incubation with [^{14}C]histamine or [^{3}H]spermine, membranes from sickle erythrocytes incorporated only 55–65% as much amine as did

normal membranes. Moreover, the greatest part of this deficit was localized specifically to spectrin and band 3. Separate determinations of amine influx into whole cells showed that this difference could not be explained on the basis of decreased membrane permeability in the sikcle cells. In fact, the sickle cells actually took up more of the labeled amine, but then did not incorporate as much into the membrane. In addition, a [^{14}C]putrescine assay for transglutaminase activity using hemolysates and exogenous protein substrate showed that the level of enzyme activity was actually higher in sickle cell preparations than in normal. This finding rules out a loss of transglutaminase activity as the cause for reduced in vitro incorporation of spermine and histamine into sickle cell membranes. The observed differences between normal and sickle erythrocytes are unlikely to be due to reduced ionophore-mediated entry of calcium into the cells, since a number of well-established studies have demonstrated that sickle erythrocytes can be loaded with calcium using the divalent cation ionophore, A23187 [5,23,24].

Since sickle cells had both higher levels of transglutaminase activity and somewhat greater permeability to the amine, it seemed likely that the other substrate for the cross-linking reaction, the free γ-glutamyl acyl group, was reduced in sickle cells. In fact, previous, limited studies by others have shown that sickle cells contained more membrane-associated polyamines than normal cells [9], which could then block further reaction of the membrane with amines in vitro. In the present study, we measured the amount of membrane-associated polyamine in native membranes and, in confirmation of the previous studies, found substantially higher levels of spermine and spermidine associated with sickle cell membranes. Most of the total polyamine content, especially in sickle cells, was non-covalently bound. However, sickle membranes contained more than twice as much covalently bound polyamine as normal cells. Certainly, this covalently bound polyamine would be expected to block some acyl donor groups from further reaction. It is also possible that the non-covalent association of polyamines with membrane components could indirectly render some of the normal transglutaminase reaction sites inaccessible in sickle cells.

Other chemical probes have suggested an alteration of membrane organization that sometimes results in altered reactivity of certain membrane components [25,26]. For example, nearest-neighbor analysis of membrane proteins using protein-protein cross-linking agents has suggested alterations in membrane organization, particularly in irreversibly sickled cells [25]. Other studies [27] have shown that methylation of erythrocyte membrane protein is reduced in sickle cell anemia, possibly because of altered membrane conformation. Finally, studies of membrane lipid organization have shown that deoxy sickled cells have a transient change, and that irreversibly sickled cells retain a permanent change in the distribution of anionic and neutral phospholipids. Since other observations suggest that the characteristic asymmetry of red cells membrane phospholipids is mediated by membrane-associated proteins [26,28], this partial loss of lipid asymmetry has also been taken as an indication of altered membrane conformation in sickle cells. The precise nature of these alterations in membrane organization has not been defined. Polyamines have been found to stabilize erythrocyte membranes [29], perhaps because the polyvalent polyamine cations are capable of bridging carboxyl groups of proteins to phospholipids via non-covalent interactions. Such interactions may perturb the normal conformation and reacativity of membrane components, and could conceivably cause steric hindrance of the sites involved in the transglutaminase reaction.

The presence of increased covalently linked polyamines in sickle cells can be rationalized on the basis of the observed elevation of Ca^{2+}, which could lead to in vivo activation of the transglutaminase enzyme. Other investigators have looked for non-reducible cross-linking of membrane proteins in sickle cells as an indication of in vivo activation of the enzyme, but have not observed any of the expected high molecular weight aggregates. It may be that the levels of Ca^{2+} attained by circulating sickle cells, while high enough (100–300 μM [5]) to activate the endogenous transglutaminase [6], do not lead to detectable protein aggregates, since changes in SDS-polyacrylamide gel electrophoresis patterns require a higher level of Ca^{2+} (> 0.5 mM) [30,31]. The observation of increased covalently bound poly-

amine does suggest that in vivo transglutaminase activation may be triggered at a more subtle level by the accumulation of Ca^{2+} in sickle cells. While even Ca^{2+}-independent association of much larger amounts of polyamines with normal red cell membranes has profound effects on membrane material properties [29], the physiologic effect of these much smaller amounts found in circulating sickle cells remains to be defined.

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References

1 Lorand, L., Weismann, L.B., Epel, D.L. and Bruner-Lorand, J. (1976) Proc. Natl. Acad. Sci. USA 73, 4479–4481
2 Siefring, G.E., Apostol, A.B., Velasca, P.T. and Lorand, L. (1978) Biochemistry 17, 2598–2604
3 Folk, J.E. and Finlayson, J.S. (1977) Adv. Protein Chem. 31, 1–133
4 Schrode, J. and Folk, J.E. (1978) J. Biol. Chem. 253, 4837–4840
5 Eaton, J.W., Skelton, T.D., Swofford, H.S., Koplin, C.E. and Jacob, H.S. (1973) Nature 246, 105–106
6 Lorand, L., Siefring, G.E., Jr. and Lowe-Krentz, L. (1978) J. Supramol. Struct. 9, 427–440
7 Palek, J. and Lux, S.E. (1983) Semin. Hematol. 20, 189–224
8 Ballas, S.K. and Burka, E.R. (1980) Br. J. Haematol. 46, 627–629
9 Natta, C.L., Motyczka, A.A. and Kremzner, L.T. (1980) Biochem. Med. 23, 144–149
10 Lin, Y., Means, G.E. and Feeney, R.E. (1969) J. Biol. Chem. 244, 789–793
11 Fesus, L. and Laki, K. (1977) Biochemistry 16, 4061–4066
12 Bray, G.A. (1960) Anal. Biochem. 1, 279–285
13 Folk, J.E. and Cole, P.W. (1966) Biochim. Biophys. Acta 122, 244–264
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
15 Crosby, L.M. and Furth, F.W. (1956) Blood 11, 380–383
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
17 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
18 Steck, T.L. (1972) J. Mol. Biol. 66, 295–305
19 Clark, M.R., Morrison, C.E. and Shohet, S.B. (1978) J. Clin. Invest. 21, 329–337
20 Folk, J.E., Park, M.H., Chung, S., Schrode, J., Lester, E.P. and Cooper, H.L. (!980) J. Biol. Chem. 255, 3695–3700
21 Rice, R.H. and Green, H. (1977) Cell 11, 417–422
22 Feindenfeld, J. and Marton, L.J. (1980) Cancer Res. 40, 1961–1966
23 Eaton, J.W., Berger, E., White, J.G. and Jacob, H.S. (1978) Br. J. Haematol. 38, 57–62
24 Clark, M.R., Greenquist, A.C. and Shohet, S.B. (1976) Blood 48, 899–909
25 Rubin, R.W., Milikowski, C. and Wise, G.E. (1980) Biochim. Biophys. Acta 595, 1–8
26 Lubin, B., Chiu, D., Bastalky, J., Roelofsen, B. and Van Deenen, L.L.M. (1981) J. Clin. Invest. 67, 1643–1649
27 Ro, J.Y., Neilan, B., Magee, P.N., Paik, W.K. and Kim, S. (1981) J. BIol. Chem. 256, 10572–10576
28 Rossi, M.E., Mohandas, N., Bernstein, S.E., Mentzer, W.C., Clark, M.R. and Shohet, S.B. (1982) Blood 60 (Suppl. 1), 26a
29 Ballas, S.K., Mohandas, N., Marton, L.J. and Shohet, S.B. (1983) Proc. Natl. Acad. Sci. USA 80, 1942–1946
30 Anderson, D.R., Davis, J.L. and Carraway, K.L. (1977) J. Biol. Chem. 252, 6617–6623
31 Palek, J. and Liu, S.C. (1979) J. Supramol. Struct. 10, 79–96