

Association of Decreased Membrane Protein Phosphorylation With Red Blood Cell Spherocytosis

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A close association between sphering of human red cells and deficient phosphorylation of their membrane proteins has been documented in three separate situations. Red cells rendered spheroidal by exposure to: (1) elevated temperatures; (2) two sulfhydryl inhibitors (*N*-ethylmaleimide or paramercuribenzoate); or (3) in the genetic situation of hereditary spherocytosis—all manifest

deficient phosphorylation of endogenous membrane proteins by ATP. In the two former cases, we have noted an exact association between the onset of red cell sphering (e.g., as temperatures rose above 48°C or *N*-ethylmaleimide concentrations exceeded 2 μmoles/ml RBC) and the development of deficient ghost protein phosphorylation.

HUMAN RED BLOOD CELLS can undergo reversible shape transformations, particularly from discs to spheres and back to discs.¹ These changes, reflecting cellular ATP concentrations, suggest that relatively minor and reversible chemical alterations in certain membrane constituents may regulate red cell shape. The documented involvement of ATP, and the recent findings from several laboratories²⁻⁵ that red cell membrane proteins can be phosphorylated by ATP and dephosphorylated by membrane phosphatases, has led us to explore whether such phosphorylation/dephosphorylation reactions may be correlated with red cell shape. Since membrane phosphorylation in other tissues frequently reflects protein kinase reactions, which, in turn, are often modulated by cyclic 3'5' AMP (cAMP),⁶⁻¹² and since we have recently reported that cAMP may affect RBC shape,¹³ we have examined ghost phosphorylation in the presence and absence of this nucleotide. In studies published elsewhere,^{14,15} we have previously demonstrated that vinblastine in concentrations that induce red cell sphering and cupping¹⁶ concomitantly inhibits phosphorylation of ghosts by ³²P-γ-labeled ATP. In the present studies, we expand these data with the demonstration that membrane phosphorylation declines exactly simultaneously with red cell sphering induced by elevated temperatures¹⁷ or by the membrane thiol inhibitors, *N*-ethylmaleimide (NEM) or paramercuribenzoate.¹⁸ Moreover, the importance of membrane protein phosphorylation in regulating red cell shape is still further supported by data to be presented (and data also collected by Greenquist and Shohet¹⁹) that such phosphorylation is consistently diminished in erythrocyte membranes in hereditary spherocytosis (HS).

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MATERIALS AND METHODS

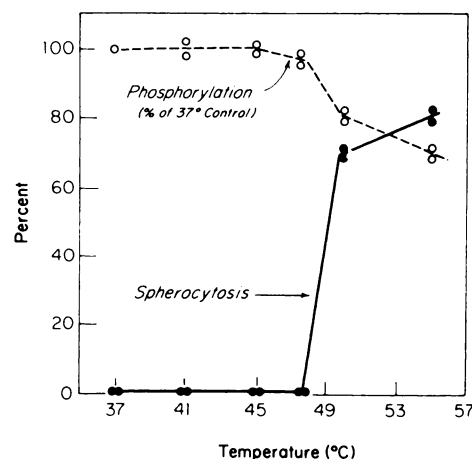
Fresh heparinized blood from normal or HS patients was centrifuged, and buffy coats were removed. The derived erythrocytes were washed three times in phosphate-buffered isotonic saline (pH 7.4; $[\text{PO}_4] = 12 \text{ mM}$; $[\text{glucose}] = 14 \text{ mM}$) with further buffy coat removals. Ghosts were prepared by the method of Dodge et al.²⁰ in which the initial two washes of ghosts were performed in 20 mosm sodium phosphate (pH 7.4) and the final wash in 20-mosm sodium phosphate (pH 6.5). Phosphorylation of ghosts by ^{32}P - γ -labeled ATP (19.8 Ci/mmole) (New England Nuclear, Boston, Mass.), both with and without added cAMP (10^{-6} M), was determined by the method of Guthrow et al.⁴ after freeze-thawing the ghosts three times, but with slight modifications. Thus, ghost protein was adjusted to 200–250 μg as determined by the Lowry technique²¹ after correction for bound hemoglobin analyzed by the pyridine hemochromogen technique.²⁰ The standard assay mixtures contained EGTA (0.3 mM) and Mg^{2+} (10 mM), and were incubated in duplicate at 37°C for 10 min; the reaction was stopped at 10 min, at which time phosphorylation was still virtually linear,⁴ by adding aliquots to ice-cold trichloroacetic acid (7.5%), redissolving the precipitate in 1 N NaOH, reprecipitating in trichloroacetic acid, and repeating this cycle for a total of three times. The final precipitate was dissolved in 10 ml of a mixture of toluene-Bio-Solv (Beckman Chemicals, Fullerton, Calif.) and Liquiflor (New England Nuclear, Boston, Mass.) scintillation fluid; radioactivity was determined by liquid scintillation spectrometry utilizing appropriate internal standardization. Alternatively, the final TCA precipitate was dissolved in 1% sodium dodecyl sulfate (SDS) and subjected to polyacrylamide gel electrophoresis for assay of zone labeling by the method of Guthrow et al.⁴ As demonstrated by others,⁴ and confirmed by us, significant (10%–20%) label resided in phospholipids in these experiments. Therefore, we utilized SDS gel electrophoresis to document that observed differences in membrane phosphorylation in our various manipulations represented alterations in labeling of phosphoproteins and not in phospholipids. This analysis was rendered simple in that phospholipids migrated rapidly (almost with the tracking dye) in the electrophoresis system utilized;²² the major phosphorylated proteins, on the other hand, migrated as high molecular weight entities at the opposite end of the gels.

Sphering of red cells was induced by (1) heating washed red cells for 30 min in glucose-containing, phosphate-buffered saline at various elevated temperatures in a water-bath capable of maintaining temperatures at $\pm 0.2^\circ\text{C}$, or (2) incubating washed red cells for 1 hr at 37°C with various concentrations of *N*-ethylmaleimide or paramercuribenzoate, obtained and dissolved as previously described.¹⁸ One aliquot of incubated cells was assayed for osmotic fragility and cell K^+ ,¹⁸ while another was used to prepare ghosts and assay phosphorylation by AT^{32}P as described above.

RESULTS

Heating red cells at 50°C is well known to produce both sphering of red cells and their splenic sequestration upon reinjection; in fact, clinically such cells have been widely used for splenic scanning.¹⁷ When analyzed more rigorously, a very sharp transition temperature is apparent, above which red cells become osmotically fragile spheres (Fig. 1, solid line); i.e., osmotic fragility (as measured by hemolysis occurring in 0.50% NaCl solution) suddenly increases between 48°C and 50°C. At this same temperature interval, but not below, the AT^{32}P phosphorylation of ghosts prepared from these heated red cells is inhibited (Fig. 1, dashed line). In the studies depicted (Fig. 1), red cells have been heated for only 3 min prior to ghost preparation; after this relatively short period, SDS gel electrophoresis of the solubilized ghosts demonstrates no loss of spectrin (bands 1 and 2)²³ or accumulation of extraneous, cytoplasmic proteins. Moreover, assay of labeling in gel fractions from three experiments has revealed that the bulk of the decrement in phosphorylation of heated red cell ghosts occurs in high molecular weight proteins (roughly 50% decrease in spectrin and 35% decrease in band 3 radioactivity). If red cells are heated for

Fig. 1. Close association of heat-induced red cell sphering and inhibition of membrane phosphorylation. Intact red cells heated for 3 min at various temperatures manifest an abrupt increase in hemolysis in 0.50% NaCl (spherocytosis) above 48°C (solid line). A simultaneous decrease in radiolabeled ATP-phosphorylation of ghosts prepared from these fragile cells occurs in parallel (dashed line). Phosphorylation per milligram of (nonhemoglobin) membrane protein is depicted as a percentage of that in control cells kept at 37°C.



more prolonged periods (30 min), phosphorylation of their membranes is inhibited by more than 90% (not shown).

One of us has previously shown that the sulfhydryl inhibitor, *N*-ethylmaleimide (NEM), causes red cells to sphere, lose K^+ , and be sequestered in the spleen when reinjected.¹⁸ These effects occur only after red cell glutathione has been completely titrated by the NEM (which requires approximately 2 μ moles NEM/ml packed RBC). Above this concentration, red cells sphere and other membrane properties such as cation permeability suddenly change (as exemplified by K^+ loss in Fig. 2, solid line). Exactly paralleling this change, decreased $AT^{32}P$ -phosphorylation of ghosts prepared from the NEM-treated red cells becomes evident (Fig. 2, dashed line). When analyzed by SDS gel electrophoresis, decreased phosphorylation occurs mainly in high molecular weight proteins, as with heated red cells (e.g., spectrin and band 3 radioactivity decreasing 35%–50%). Total spectrin content remains stable during the course of these experiments. Analogous parallel alterations in membrane permeability and ghost

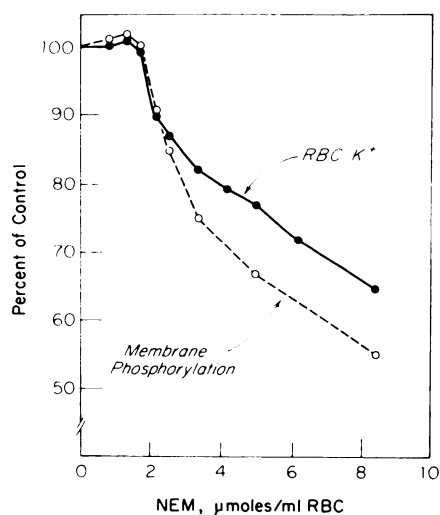


Fig. 2. Close association between NEM-induced K^+ leak and inhibition of membrane phosphorylation. Intact red cells treated with increasing concentrations of NEM abruptly lose K^+ and sphere at NEM concentrations above 2 μ moles/ml RBC (solid line). Phosphorylation by radiolabeled ATP (measured as in Fig. 1) diminishes in exact parallel (dashed line).

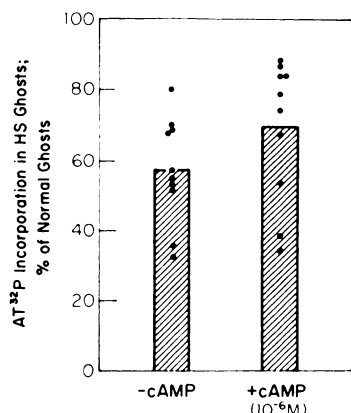


Fig. 3. Deficient phosphorylation of red cell membrane ghosts in hereditary spherocytosis. Phosphorylation by radiolabeled ATP of ghosts from ten HS patients is consistently less than that of normal ghosts incubated in parallel (= 100 on the ordinate). Phosphorylation with (right bar) or without (left bar) added cAMP is similarly defective.

phosphorylation are also obtained when spheroidogenic concentrations of another membrane thiol inhibitor, parahydroxymercuribenzoate, are added to red cells (not shown).

In addition to these data in artificially spheroided red cells, AT³²P phosphorylation of red cell ghosts is decreased in hereditary spherocytosis as well. As also previously reported by Greenquist and Shohet¹⁹ in ghosts from four such patients, we find a consistently deficient phosphorylation of HS ghosts (particularly of spectrin and band 3) in ten further patients from six unrelated families. This deficiency, which averages about 60% of that observed in normal ghosts incubated in parallel, is observed with or without added cAMP (Fig. 3). It is also not influenced by the splenectomy state (and thus the reticulocyte counts) of the patients. Moreover, phosphorylation is particularly depressed in ghosts prepared from red cells that have been drained from the splenic pulp during splenectomy of three of our HS patients (Table 1). Phosphorylation of ghosts from two patients with immune hemolytic anemia, elevated reticulocytes, and prominent spherocytosis, has not been significantly different from normal (not shown).

DISCUSSION

These results demonstrate a close association between spherocytosis of human red cells and deficient phosphorylation by AT³²P of their membrane proteins. This association has been documented in three separate situations in

Table 1. Further Depression of HS Ghost Phosphorylation in RBCs Drained From Splenic Pulp

Patient	ATP- γ^{32} P Incorporated:Control RBC* (%)	
	Peripheral RBC	Splenic RBC
D.W.	55.1	32.4
J.J.	87.1	52.4
T.H.	68.6	55.4

*Ghosts prepared from peripheral blood or from red cells drained through several slashes in spleens removed from three HS patients were utilized in the "protein kinase" assay described in the text. In each situation, ghosts prepared from normal peripheral blood were incubated under identical conditions in parallel. No ghosts from "normal" splenic red cells were available.

which red cells are spheroidal; i.e., (1) heating to 50°C; (2) sulfhydryl-inhibitor exposure; and (3) the genetic error of hereditary spherocytosis. In addition, a similar relation has previously been noted in vinblastine-induced spherocytes.¹⁵ The exact correlation between onset of sphering and inhibition of membrane phosphorylation by red cells treated by heat (Fig. 1) and NEM (Fig. 2) support a linkage between the two phenomena that may or may not be fortuitous. We would emphasize that our data do not provide rigorous evidence that membrane phosphorylation is critical to red cell shape, and certainly in no way do we wish to suggest that a causal relationship between the two has been demonstrated. For instance, coincident, but independent, deleterious effects of heat upon protein kinases, which phosphorylate membrane proteins, on the one hand, and on fibrous proteins responsible for cell shape, on the other, might produce the data in Fig. 1. In addition, one of us¹⁸ has previously demonstrated that NEM has no detectable effect upon red cell membranes until all cytoplasmic glutathione has been titrated. Thereafter, the inhibitor is presumably free to react with all other thiols of the cell. That protein kinase thiols are evidently affected, with resulting diminution of their activity is, thus, not unexpected. An unrelated thiol-dependent system required for maintenance of cell shape might be altered simultaneously to give the seemingly close association of shape and membrane phosphorylation depicted in Fig. 2. On the other hand, the observation that membrane phosphorylation in unmanipulated hereditary spherocytes is diminished would seem to strengthen an interpretation that cell shape and membrane phosphorylation are more than fortuitously linked phenomena. Further studies are required, however, before such an interpretation can be made with certainty.

Our results also agree closely with those preliminarily reported by Greenquist and Shohet^{19,24} (although not with those of Zail and van den Hoek²⁵), who utilized somewhat different techniques. These workers also noted deficient ghost protein phosphorylation in hereditary spherocytosis (and in one case of hereditary stomatocytosis) and with thiol blockade, but made their measurements after more prolonged incubation, 1 hr, compared to 10 min in our studies. Moreover, they analyzed dephosphorylation kinetics as well. We have considered the possibility that excessive dephosphorylation, rather than sluggish phosphorylation, might underlie our results. However, Greenquist and Shohet noted similarly decreased membrane phosphorylation at the longer interval, at which time labeling equilibrium had occurred,²⁴ making such a premise unlikely. It also denies an explanation that deficient phosphorylation reflects excessive "endogenous" phosphorylation with unlabeled phosphate of membrane proteins. We have also considered the possibility that decreased labeling of phosphorylated entities other than proteins (e.g., lipids) might represent an artifact in our results. We have excluded this possibility by utilizing SDS gel electrophoresis to document that decreased protein labeling per se occurs in the various spherocytic situations studied. In fact, decreased labeling was observed mainly in high molecular weight protein zones 1 and 2 (spectrin) and 3.

How phosphorylation of certain membrane proteins may modulate the shape of red cells is unclear. We believe it likely, however, that spectrin is somehow involved in this modulation—a suggestion supported by at least three observa-

tions: (1) spectrin "melts" in calorimeters at 49°–50°C^{15,26}—a temperature range exactly one which produces red cell spherizing (Fig. 1); (2) vinblastine precipitates spectrin from solutions of membrane proteins and in similar concentrations induces spherizing of intact red cells;¹⁶ and (3) spectrin is virtually absent from spherocytes in a homozygous colony of mice with severe spherocytic hemolytic anemia.²⁷ In relation to the present studies, red cell membrane phosphorylation by AT³²P is also strikingly deficient in these animals as well.²⁷ Of further interest is the fact that spectrin, or a slightly smaller molecule, which runs as a leading shoulder of the spectrin peak in SDS polyacrylamide gels,²³ is the protein of the red cell membrane most readily phosphorylated by AT³²P.^{3,4,15}

In addition to the possibility that spectrin phosphorylation is involved in regulation of red cell shape, a most provocative observation is that a protein termed 4.2 (from its location in SDS polyacrylamide gel electrophoresis) is the most avid cAMP-binding protein of the red cell membrane.²⁸ Such binding by this protein suggests that it may be the major red cell protein kinase. Intriguingly, this identical protein is missing from some, but not all, red cell membrane protein extracts from Japanese patients with typical hereditary spherocytosis.²⁹

Whether, in fact, protein kinase is critically involved in regulating red cell shape remains to be proven, as does the manner in which a proper (e.g., phosphorylated) conformation of spectrin or other membrane proteins might produce the biconcave shape of normal, metabolically replete red cells. For the moment, we would speculate that various errors in phosphorylation of structural membrane proteins might lead to spherocytosis, as exemplified in the hereditary spherocytosis syndrome. Thus abnormalities in: (1) structural proteins, perhaps spectrin (as in mouse spherocytosis) or actin;³⁰ (2) protein kinase (as perhaps in the Japanese patients); or (3) ATP or cAMP production would all seem reasonable areas for future studies of spherocytosis.

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