Phosphorylation in Erythrocyte Membranes From Abnormally Shaped Cells

By Alfred C. Greenquist and Stephen Shohet

Erythrocyte protein phosphorylation was examined in membrane preparations of 25 patients with hereditary spherocytosis (HS). Reduced phosphorylation in substrate polypeptides was observed in 22 HS erythrocyte membranes for both splenectomized and nonsplenectomized patients. A reduction in labeling was also observed in a polypeptide which was labeled only in the presence of cAMP. No reduction was observed in membranes from immunologically acquired spherocytosis cells or from cells from patients with hereditary elliptocytosis. Heating membranes to 45°C caused negligible inhibition of membrane protein phosphorylation, while heating to 50°C extensively inhibited phosphorylation. Dephosphorylation of membrane protein that occurred in isolated membranes was not dependent upon cAMP.

The factors that control shape and deformability appear to be intrinsic to the erythrocyte membrane, since isolated membranes can retain the biconcave shape and exhibit deformability properties similar to the intact cells. A number of mechanical models have been presented to rationalize the formation of the disc shape. Under conditions of metabolic depletion with the loss of ATP, the red cell undergoes a disc to sphere transformation. This transformation is readily reversible by reincubation of cells with glucose. Preceding the shape transition, the cell or the corresponding isolated membrane becomes less deformable. This loss in deformability is also reversible with adenosine.

In addition to shape changes, erythrocytes from patients with hereditary spherocytosis exhibit numerous other abnormalities that have been reviewed by Jacob. Several of these properties appear to demonstrate the involvement of abnormal membrane behavior. Both the cell and the isolated membranes are less deformable than normal, and the addition of adenosine can reverse the reduced deformability; the cells show increased passive permeability to sodium ions, membrane (Na⁺ + K⁺) adenosinetriphosphatase activity (ATPase) is enhanced; the membranes are unstable during in vitro incubation and will release lipid by a budding process, and membrane protein extracts show reduced precipitability with increasing ionic strength or divalent metal concentration. It is reasonable to anticipate that determination of the underlying biochemical defect in this cell may help to identify factors that control cell shape and deformability, which are also likely to be a function of the membrane.

Several groups have described protein kinases in human erythrocyte mem-
branes that catalyze the phosphorylation of membrane polypeptides. A protein phosphatase which causes membrane protein dephosphorylation has also been described. The functions of these reactions are not known. However, altered phosphorylation of erythrocyte membrane protein has been described in myotonic and Duchenne muscular dystrophies. We have previously reported on the reduced phosphorylation activity in six patients with hereditary spherocytosis. The studies presented here extend these observations and further describe studies on hereditary elliptocytes and acquired spherocytes. From these and our previously described observations, we present a tentative hypothesis that the phosphorylated state of membrane protein may influence normal cell shape and that the cellular defects in hereditary spherocytosis may be due to a phosphorylation defect in that cell.

MATERIALS AND METHODS

Membrane isolation and electrophoresis. Membranes from control and pathologic red cells from heparinized blood were obtained according to the Dodge procedure, substituting 0.01 M Tris-Cl, (prepared from 0.2 M Tris buffer stock, pH 7.4 at 23°C), for the hemolyzing buffer. Protein concentration was determined from the absorbance at 280 nm in 1% sodium dodecyl sulfate (SDS) or by Lowry protein assays of membrane protein, using bovine serum albumin as a standard. Hemoglobin content was determined by the pyridine hemochromogen assay.

Membrane preparations were incubated in 1% SDS, 50 mM dithiothreitol, 0.01 M sodium phosphate, pH 7.0, 1 mM EDTA for 1 hr at 37°C prior to electrophoresis. Electrophoresis was performed on either 3% or 5% polyacrylamide gels (PAGE) containing 0.1% SDS and a 0.05 M sodium phosphate buffer as described by Weber and Osborn. Some 3% gels were run overnight to resolve spectrin band 1 from band 2 more efficiently. When required, gels were fixed and stained in the first staining solution described by Fairbanks et al. followed by destaining in 10% acetic acid. Gels containing radiolabeled polypeptides were sliced in 3-mm sections; the gel segments were transferred to scintillation vials and dissolved in 0.2 ml of 30% H2O2. Counting was performed by Cerenkov radiation in a Packard Model 3341 liquid scintillation spectrophotometer with an efficiency of 28%.

Phosphorylation of membrane protein. The phosphorylation conditions were similar to those previously described, using a 0.5-ml reaction mixture containing 0.25 mg of freeze-thawed membrane protein, 0.15 mM γ-32P ATP (20-150 cpm/pmole), 2.5 mM Mg2+, and 0.01 M Tris-Cl at pH 7.4. Cyclic AMP was added at 1 μM when required. Incubations were at 37°C for 1 hr; the reaction was stopped with 2.0 ml of a cold solution of 0.01 M Tris-Cl, 0.05 M NaCl, and 1 mM EDTA at pH 7.5. Incubations for 1 hr approached a limiting value for phosphate labeling of protein. The suspension was centrifuged at 20,000 g for 10 min and the membranes washed twice prior to analysis by SDS PAGE. Alternatively, samples were resuspended directly after initial centrifugation in 1% SDS, 50 mM dithiothreitol and applied to polyacrylamide gels.

Breakdown of membrane phosphoprotein. Membrane polypeptides were phosphorylated as described above, and the labeled membranes were washed and then reincubated in a 0.5-ml reaction mixture containing 0.01 M Tris-Cl and further additions as noted in Table 3. Alternatively, after incubation for 1 hr, 5 mM unlabeled ATP and 5 mM Mg2+ were added to prevent any further significant 32P incorporation, and the membranes were then reincubated for 15 or 30 min at 37°C.

RESULTS

Labeling of Protein in Normal and Hereditary Spherocytosis Erythrocyte Membranes

The SDS PAGE polypeptide patterns obtained from normal HS membranes are shown in Fig. 1. HS and control membrane polypeptide patterns were the same except occasionally for the enhanced retention of small amounts of
polypeptide, which migrated to the position observed for globin polypeptide. Hereditary elliptocytosis (HE) membranes were identical to controls (not shown). The numbers used for designation of the bands are according to Steck.28 Labeling of individual bands in the “spectrin area” of the gel (bands 1, 2, and 2.1) was resolved after electrophoresis in 3% gels overnight. The principal site of labeling was in band 2, as reported by others.14,16,17 Significant labeling in band 1 was not observed, and our previous measurement of band 1 labeling may have been the result of ineffective resolution on a 5% gel or the use of outdated blood in the identification of labeled polypeptide substrates. A second site of labeling was found in the band 3 region of the gel. An additional band with an approximate MW of 50,000 was labeled in the presence of cAMP. As previously reported,14 labeling in this position was negligible in the absence of cAMP. Low molecular weight protein is also reported to be labeled in the membrane.51 If present under the conditions used here, it moves as a very mobile species22 and has not been further characterized. The average level of labeling after 1 hr incubation at 37°C was 338 pmol per milligram of membrane protein in band 2 and 316 pmol per milligram of membrane protein in band 3. However, the absolute level of phosphorylation was highly variable between experiments on the same preparations. This factor necessitated establishing the variation in normal samples by simultaneous preparation and analysis of multiple controls. Simultaneous analysis of nine controls yielded phosphorylation levels which had an SD of ±10% of the average labeling value in band 2 and ±13% in band 3 (for 12 controls).

In Fig. 2, the level of polypeptide phosphorylation is compared to control stroma (controls for HS were from laboratory personnel and exhibited labeling...
Fig. 2. Phosphorylation of hereditary spherocytosis membrane proteins in isolated membrane preparations. Membranes were incubated for 1 hr at 37°C with 0.15 mM γ-32P-ATP. Incorporation of label into membrane polypeptides was measured in polypeptides separated by SDS polyacrylamide gel electrophoresis. Band designations are shown in Fig. 1. Values were determined in quadruplicate for each sample with a SE of less than ±10% of the average. Values of HS membranes were determined as the per cent of control membranes run simultaneously. Column a, the distribution of values in spectrin for a group of controls prepared and run simultaneously, which gave a standard deviation of ±10% of the average labeling; Column b, labeling of spectrin in HS membranes without cAMP; Column c, labeling of spectrin in HS membranes with 1 μM cAMP added (simultaneous analysis of nine controls in the presence of cAMP gave an SD of ±7.4% of the absolute labeling value in spectrin, not shown); Column d, the distribution of values in band 3 for a group of controls from two experiments which gave an SD of ±13% of the average labeling; Column e, labeling of band 3 of HS membranes without cAMP; Column f, labeling of band 3 of HS membranes with 1 μM cAMP added (simultaneous analysis of nine controls in the presence of cAMP gave an SD of ±9.6% of the absolute labeling value in band 3, not shown). Labeling data for spectrin in the presence of cAMP may also include, in addition to band 2, incorporations into bands 1 and 2.1, which were not resolved in these analyses.

values within 10% of each other) prepared and run simultaneously with hereditary spherocytosis samples. Incorporation into spectrin was reduced in membranes from 22 patients with hereditary spherocytosis, with values ranging from 11% to 82% of control levels (average 50%). Similarly, labeling was reduced in a band-3 polypeptide with values from 26% to 90% of controls (average of values 59%). The addition of cAMP stimulated labeling of spectrin and band 3 about 1.2-fold. (Band 1 and band 2.1 labeling was not resolved in these experiments from band 2.) The addition of cAMP to HS membranes similarly stimulated incorporation, but the deficiency with respect to the corresponding control membranes persisted in both bands (Fig. 2). Three cases of hereditary spherocytosis have been examined which did not show reduction in membrane polypeptide labeling. In two of these cases, incorporation was actually elevated about 30% in bands 2 and 3. These cases have not as yet been available for reexamination.
Table 1. Labeling of a Membrane Polypeptide Requiring cAMP in Hereditary Spherocytosis

<table>
<thead>
<tr>
<th>Patient</th>
<th>32P Incorporation (% Normal)</th>
</tr>
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<tbody>
<tr>
<td>F.T.</td>
<td>23</td>
</tr>
<tr>
<td>J.S.</td>
<td>27</td>
</tr>
<tr>
<td>C.A.</td>
<td>29</td>
</tr>
<tr>
<td>M.B.</td>
<td>54</td>
</tr>
<tr>
<td>K.B.</td>
<td>63</td>
</tr>
</tbody>
</table>

Incorporation of 32P label into band 4.5 (apparent MW 50,000) was measured in the presence of 1 μM cAMP. Negligible incorporation into this polypeptide occurred in normal or HS membranes in the absence of cAMP. Values were determined in quadruplicate and the standard error of the mean for absolute values was less than ±10%.

Incorporation was also measured into a MW 50,000 polypeptide (band 4.5), which was labeled only in the per cent of cAMP (Table 1). Labeling of this polypeptide was deficient in the five preparations of HS membrane examined (average 39% of control).

The phosphorylation reaction was also examined in membranes from three cases of hereditary elliptocytosis from patients with active hemolysis, one case of acquired elliptocytosis (in β-thalassemia), and three cases of acquired spherocytosis (Coombs'-positive hemolytic anemia), as shown in Table 2. The incorporation in all of these cases was either normal or elevated.

One pair of preparations of control and HS membranes was extensively disrupted by sonication at 60 W for 1 min prior to labeling. This procedure resulted in a 1.2-fold overall increase of labeling in both the control and HS membranes, but did not alter the relative labeling deficiency in the HS membranes.

Thermal Stability of the Phosphorylation Reaction

Red cell membranes were incubated for 10 min at the temperature indicated in Fig. 3, then cooled on ice prior to initiation of the reaction. As shown in Fig. 3, the phosphorylation of spectrin was marginally affected at 45°C, but...
Fig. 3. Thermal stability of the phosphorylation reaction of spectrin. Membranes were incubated for 10 min at the specified temperature, then cooled on ice prior to initiation of the phosphorylation reaction at 37°C. Samples were applied directly to SDS-PAGE without prior washing of the membranes. Values were determined in quadruplicate, and the SEM for absolute values was less than ±10%.

extensively inhibited after exposure to 50°C. This temperature was near the first protein-dependent thermal transition observed by Jackson et al.30 Heating intact cells to 50°C is also known to produce spherocytes.30,31

Breakdown of the Phosphorylated Protein

The release of label from polypeptides was studied in membrane preparations from outdated blood. Samples were labeled by a preincubation for 1 hr, washed, and then reincubated in buffer at 37°C for 1/2 hr. The release of label from membrane polypeptides is shown in Table 3. Release rates from separate preparations showed considerable variation ranging from 40 to 160 pm/mg/hr. The presence of EDTA prevented the release of label from the phosphoprotein, which occurred in buffer alone. However, breakdown in phosphoprotein was not affected by the addition of 0.1 or 2.5 mM Ca²⁺. As previously shown,19 the addition of 2.5 mM Mg²⁺ enhanced the release of label in both the spectrin and band 3 area of the gel. Increase in Mg²⁺ to 20 mM did not further stimulate the release of label. Moreover, the addition of cAMP did not stimulate the dephosphorylation.

The release of label from membrane polypeptides was also examined by an alternative method in which labeled membranes were reincubated after the addition of 5 mM unlabeled ATP and Mg²⁺. This dilution of the label effec-

<table>
<thead>
<tr>
<th>Additions to Reincubation Mixture</th>
<th>³²P Release (pm/mg/30 min)</th>
<th>Spectrin</th>
<th>Band 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA, 2.5 mM</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>46</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Ca²⁺, 0.1 mM</td>
<td>47</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Ca²⁺, 2.5 mM</td>
<td>44</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺, 2.5 mM</td>
<td>75</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺, 2.5 mM + cAMP</td>
<td>73</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺, 20 mM</td>
<td>77</td>
<td>66</td>
<td></td>
</tr>
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</table>

Experiments were performed on membrane preparations from outdated blood, preincubated at 0.7 mg/ml for 1 hr with γ-³²P-ATP, washed, and reincubated for 30 min at 37°C with the additions noted. ³²P release was calculated from the difference between initial cpm in unreincubated control samples and cpm after reincubation. Cyclic AMP was utilized at 1 μM. Values are averages of triplicate determinations with an SEM of less than ±13%.
tively prevented further significant incorporation of $^{32}$P without otherwise manipulating the membranes or modifying the conditions. Again, results indicating a turnover of the spectrin and band-3 polypeptide phosphate were obtained (data not shown).

**DISCUSSION**

The results presented here demonstrate that erythrocyte membranes from patients with hereditary spherocytosis have reduced incorporation of phosphate from $\gamma$-$^{32}$P labeled ATP into membrane proteins. Recently, Jacob et al.\textsuperscript{32,33} have supported these observations on phosphorylation into erythrocyte membranes from patients with hereditary spherocytosis. This effect was in contrast to normal or elevated phosphorylation, which we found in membranes from acquired spherocytosis and in membranes from hereditary elliptocytes.

The principal polypeptides labeled in this reaction in normal cells were spectrin band 2 and at least one of the several “band-3” proteins.\textsuperscript{20,34} In the current studies of HS cells, incorporation into each of these proteins was reduced. In addition, labeling was also reduced in HS into a polypeptide which is labeled only in the presence of cAMP. At present, the involvement of cAMP in membrane properties of human erythrocytes is highly uncertain. The site of cAMP binding is intracellular,\textsuperscript{35} but the mechanism for generation of intracellular cAMP in human erythrocytes appears to be limited.\textsuperscript{36,37} Cyclic nucleotides at very high concentrations have been reported to prevent vinblastine-induced shape changes,\textsuperscript{33} but the reason for this is not known. We have not observed any inhibitory effect of vinblastine on levels of protein phosphorylation,\textsuperscript{19} suggesting that some other shape perturbing site is modified by vinblastine.

Recently, Zail et al.\textsuperscript{38} reported normal labeling in HS membranes from four patients. Although labeling for shorter incubation times might affect results, reduced labeling was found in HS membranes by Jacob et al.\textsuperscript{33} for short incubation times. The effect of buffer selection, specific ionic composition, and Mg\textsuperscript{2+} concentration may be significant, since the results from several laboratories\textsuperscript{14,16,17,39,40} have demonstrated that labeling patterns can be appreciably altered, depending upon the reaction condition selected. We have found three HS membrane preparations which failed to exhibit reduced labeling. This observation may be indicative of a second variant in the syndrome which has been considered previously.\textsuperscript{7}

The origin of reduced phosphorylation of membrane protein substrates in hereditary spherocytosis remains to be determined. The effect could arise from several mechanisms that include: (1) inaccessibility of ATP to the membrane interior; (2) altered substrate concentration or accessibility of substrate to the kinase; (3) altered rates of dephosphorylation by protein phosphatase; or (4) altered kinetic parameters or concentrations of the protein kinase. Inaccessibility to ATP is unlikely, since internal proteins of incubated ghosts from control and HS membranes are accessible to trypsin.\textsuperscript{22} Moreover, sonication, which enhances incorporation slightly, does so equally for both control and HS membranes. A more cryptic location of protein kinase in HS membranes cannot be excluded, however. An alteration in the content of substrate proteins could also affect phosphorylation. However, the SDS PAGE patterns obtained from
hereditary spherocyte, and elliptocyte membranes appear to be equivalent to normal membranes. Recently, two groups\textsuperscript{41,42} have reported that "band IV," (i.e., band 4.2) is deficient or missing in HS, but this finding was not observed among the preparations of HS examined in the present study.

A change in the conformation of substrate proteins could alter the level of phosphorylation. It is unlikely that such a change would involve a simultaneous alteration in the primary structure of two or more separate substrate polypeptides. However, cooperative changes in membrane organization or perhaps cell shape by these or other proteins might induce less favorable conformations for both band 2 and 3 substrates or alter accessibility of the kinase to them. However, a change in shape per se did not produce a deficiency in phosphorylation since, as noted, phosphorylation was not depressed in acquired spherocytosis or hereditary elliptocytosis.

The relative activity of protein phosphatase could also influence the level of phosphorylation. The erythrocyte membrane does contain protein phosphatase that is stimulated by magnesium and inhibited by EDTA (showing a divalent metal ion requirement), but appears to be insensitive to cAMP. However, examination of dephosphorylation in one preparation of hereditary spherocytosis membranes has shown dephosphorylation activity similar to controls ($111 \pm 17 \text{ pm/mg/hr}$ for controls versus $120 \pm 21 \text{ pm/mg/hr}$ for HS). Finally, alteration in kinetic parameters of the phosphorylation enzyme or the concentration of the enzyme could also affect the extent of reaction. We have not yet attempted a comparison of such parameters between normal and HS enzyme preparations in terms of kinetic analyses with variations in ATP concentration or exogenously supplied protein substrates.

The protein phosphorylation reaction has been described in a number of membranes,\textsuperscript{43} but the effects of phosphorylation on membrane proteins are not known. Modification by phosphorylation could alter enzymatic or structural functions of membrane proteins. For example, altered calcium stimulated adenosine triphosphatase (Ca$^{2+}$ ATPase) has been reported in HS membrane,\textsuperscript{44,45} and it has been suggested that spectrin contains Ca$^{2+}$ ATPase activity. It is interesting and perhaps relevant that phosphorylation of myosin in platelets is reported to regulate the actin-dependent Ca$^{2+}$ ATPase of myosin.\textsuperscript{46} A structural role for spectrin in red cell membranes is strongly suggested by the extreme spherocytosis found in hereditary spherocytes in the common house mouse, which is deficient in the membrane protein spectrin.\textsuperscript{47} Structural changes affecting spectrin are suggested by reduced extractability of this protein from red cell ghosts prepared from ATP-depleted cells.\textsuperscript{48}

Direct correlations between protein phosphorylation and the crucial physiologic function controlling cell deformability and shape control have not been made. However, several features of the phosphorylation reactions suggest a possible involvement in these functions. These include the energy dependence of the reactions, the presence of a reversible phosphorylation–dephosphorylation cycle of membrane proteins in intact cells,\textsuperscript{19} and the involvement of a structural protein, spectrin, as a major substrate of the reaction. Moreover, the finding of defective phosphorylation in hereditary spherocytosis membrane preparations supports a working hypothesis that this disorder of cell shape and deformability is mediated by a defect in the phosphorylation system.
ACKNOWLEDGMENT

We gratefully acknowledge the excellent technical assistance of Mary Rossi. We wish to thank Dr. William Mentzer and Dr. Bertram Lubin for supplying numerous clinical samples.

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