Spectrin Phosphorylation and Shape Change of Human Erythrocyte Ghosts

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Salt extraction of white ghosts reduced spectrin phosphorylation during shape change by 85-95%. Salt extraction did not alter crenation, rate of MgATP-dependent shape change, or the fraction (>80%) ultimately converted to disks and cup-forms after 1 h. Spectrin was partially dephosphorylated in intact cells by subjecting to metabolic depletion in vitro. Membranes from depleted cells exhibited normal shape-change behavior.

Shape-change behavior was influenced by the hemolysis buffer and temperature and by the time required for membrane preparation. Tris and phosphate ghosts lost the capacity to change shape after standing for 1-2 h at 0°C. Hemolysis in HEPES or N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid yielded ghosts that were converted rapidly to disks in the absence of ATP and did not undergo further conversion to cup-forms. These effects could not be attributed to differential dephosphorylation of spectrin, because dephosphorylation during ghost preparation and incubation was negligible.

These results suggest that spectrin phosphorylation is not required for MgATP-dependent shape change. It is proposed that other biochemical events induce membrane curvature changes and that the role of spectrin is passive.

The relationship of cell membrane properties to the biconcave shape of normal human erythrocytes has for many years engaged the attention of investigators from several disciplines. Recently, theoretical treatments (11, 16) and “macro” modeling (13) of the membrane have given satisfactory predictions of observed erythrocyte shapes and simple shape transformations. However, no acceptable explanation for these phenomena at the level of membrane molecular organization has yet been advanced. One of the central issues is the role of intracellular ATP. Nakao et al. (41) first described reversible shape transitions associated with changes in erythrocyte ATP content. Weed et al. (59) suggested that the physical basis for the metabolic dependence of both erythrocyte shape and deformability might be ATP-dependent sol-gel transformations at the inner surface of the membrane. The spectrin-actin meshwork beneath the membrane was found to have some properties of an actomyosin-like system (33, 34, 49, 53) and spectrin polypeptide 2 was shown to be phosphorylated (4, 29, 42, 45, 46). These findings raised the prospect that actomyosin-like ATPase activity or protein kinase-mediated phosphorylation would prove to be a link between energy metabolism and control of shape in normal erythrocytes (21, 30, 33, 37, 43, 44, 50, 53). Isolated erythrocyte membranes also undergo shape transformations induced by ATP (40).

The molecular basis of these phenomena was explored recently by Sheetz, Singer, and Birchmeier (9, 52). Sheetz and Singer (52) described conditions for assaying quantitatively the extent of conversion of crenated membranes (echinocytes) to biconcave disks (discocytes) and cup-forms (stomatocytes); they presented indirect evidence implicating altered spectrin organization in ATP-dependent shape change. Birchmeier and Singer (9) obtained correlative data suggesting that the ATP-dependence of the process reflects a requirement for spectrin phosphorylation. We have used the shape change assay system of Sheetz and Singer (52) to examine further the role of spectrin phosphorylation. Our approach has been to manipulate the level of phosphorylation by extracting the membrane-bound spectrin kinase and by incubating intact cells under conditions...
of metabolic depletion. We have found that the assay system is exquisitely sensitive to several variables, particularly to the conditions of hemolysis. Shape change is reproducible and invariably ATP dependent under the conditions described previously (52), but our results suggest that spectrin phosphorylation is not required.

MATERIALS AND METHODS

Reagents

\( ^{[32P]}\text{ATP} \) (NEN-002X) and carrier-free \( ^{[33P]}\text{P} \) orthophosphoric acid (NEN-054) were obtained from New England Nuclear Corp., Boston, Mass. Disodium ATP (no. A3127), HEPES (no. H3375), Tris (no. T1503), N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES, no. T1375), and dibutyryl-\( \beta \)-glycylglycine/25 mM NaHCO\(_3\)/1 MM MgSO\(_4\)/1 mM KH\(_2\)PO\(_4\)/4.8 mM KCl, \( 0.25 \text{M} \text{NaCl} \) in Tris (pH 7.4)/0.15 ml of 10 mM sodium phosphate/0.15 ml of 10 mM MgCl\(_2\)/2 mM EGTA in Tris (pH 7.4). Membrane incubation controls were prepared in the same way with omission of additional NaCl in sodium HEPES and sodium TES incubation buffer.

Membrane Preparation

Erythrocytes

Fresh heparinized blood was centrifuged at 3,000 g for 10 min at 4°C. The packed cells were washed with 10 ml of ice-cold NaF-buffer (140 mM NaCl/25 mM NaF/10 mM Tris/0.14 M KCl). The hemoglobin concentration of the cell suspension was 0.60 g/ml (hematocrit 57%). The packed cells were washed until the final hematocrit was 0.50 g/ml.

Salt Extraction of Membranes

Packed Tris-washed ghosts were diluted with four volumes of ice-cold 10 mM Tris/0.65 M KCl/0.1 mM EDTA/0.25 mM DTE (pH 7.4 at 0°C). After 15 min of incubation on ice, the membranes were pelleted, using the Sorvall SS-34 rotor (2 min, 27,000 g \( r_b \)). The supernate was drawn off, and the salt-stripped membranes were gently resuspended for washing in 10 mM Tris/0.14 M KCl/0.25 mM DTE (pH 7.4). The final pellet (as well as unretracted membranes prepared at the same time) was prepared for shape-change assay by addition of an equal volume of 10 mM Tris/0.14 M KCl (pH 7.4).

Protein Kinase Assay

The complete salt extraction mixtures, supernatants, and resuspended membranes were sampled by removal of 0.1-ml aliquots. These were diluted into 0.9 ml of 10 mM sodium phosphate/0.15 mM MgCl\(_2\)/0.3 M sucrose/1.5 mg/ml bovine serum albumin (pH 7.5) and stored at -60°C. The samples were assayed for CAMP-independent protein kinase activity by measuring phosphorylation of the exogenous substrate, casein. The procedure described previously (5) was followed, except that the reaction mixtures contained NaCl at 0.35 M instead of 0.5 M.

Analysis of Membrane Phosphorylation under Shape-change Assay Conditions

For observation of membrane phosphorylation during shape change, separate 0.6-ml reaction mixtures containing \( ^{[32P]}\text{ATP} \) (16 Ci/mol) were prepared as described above with appropriate volume reductions. These were incubated in parallel with shape-change assay mixtures. At intervals from 2 to 45 min, 0.1-ml samples were withdrawn and mixed rapidly with 0.3 ml of a termination and electrophoresis sample preparation solution containing (final concentration) 10 mM sodium borate (pH 9.3)/1 mM EDTA/5 mM N-ethylmaleimide/0.25 M sucrose/1.5% SDS. The solubilized membranes were incubated for 2 min at 96°-100°C. DTE (pH 8) was then added to 65 mM, and the high-temperature incubation was repeated. Samples containing 10 μg of membrane protein were fractionated electrophoretically on Tris-acetate-EDTA gels (23) polymerized with 4% acrylamide and containing 0.2% SDS. Staining with Coomassie Brilliant Blue R-250 and destaining were done as described previously (23), except that the acetic acid concentration was reduced to 5%, 1 mM H\(_2\)PO\(_4\) was present throughout, and no dye was added to destaining solutions.

In Vivo Incubation and Labeling of Intact Erythrocytes

Fresh heparinized blood was centrifuged as described above to sediment cells. The serum and buffy coat were removed and the erythrocytes were washed three times in a modified K medium (4). The cells were then incubated with a 3% solution of \( ^{[32P]}\text{Pi} \) in Tris/HCl, pH 7.4 (2%). The isotope was contained in 100 mM glycylglycine/25 mM NaHCO\(_3\)/1 mM MgSO\(_4\)/1 mM KH\(_2\)PO\(_4\)/4.7 mM KCl.
plus NaCl (-103 mM) to yield a 308 mosM solution. The pH was adjusted to 7.4 at 37°C under 5% CO₂ and gentamicin was added to 20 µg/ml. For ATP depletion, 10% cell suspensions in this medium were incubated without added energy source at 37°C under 5% CO₂ in nitrogen. For ATP maintenance, the medium was supplemented with 5 mM glucose/10 mM inosine/1 mM adenine.

In labeling experiments, [³²P]orthophosphate was present at 350 µCi/ml. The labeled membranes were prepared by hemolysis and washing in Tris as described above. In preparing samples for electrophoresis, 50 µl of packed ghosts resuspended in 120 µl of hemolysis buffer was mixed with 50 µl of 50 mM sodium borate (pH 8.3)/5 mM EDTA/1.25 mM sucrose/25 mM N-ethylmaleimide, 20 µl of 20% SDS was added and the samples were incubated for 2 min at 96°–100°C and then frozen. Before the gels were loaded, 10 µl of 1 M DTE was added, the samples were heated again for 2 min at 96°–100°C, and 5 µl of Pyronine Y tracking dye concentrate was added. Electrophoresis and measurement of ³²P in transverse gel slices were performed as described above. In determining spectrin specific radioactivity, net exchangeable spectrin-bound phosphate, and labeling increments in membranes from incubated cells, counts were normalized to the absorbance of Coomassie Blue eluted (24) from the spectrin zone of stained gels.

Analysis of Spectrin Dephosphorylation

Erythrocytes were labeled with [³²P]orthophosphate, using ATP maintenance medium as described above, but with 20 mM HEPES instead of glycylglycine as auxiliary buffer. After 17 h, the cells were sedimented and divided into three portions. These were washed in saline buffered with Tris, phosphate, or HEPES as in preparation for shape-change assays. Hemolysis was carried out at 0°C by rapid mixing of packed cells with 29 vol of hypotonic Tris, phosphate, or HEPES. The membranes were washed twice with the same buffers and diluted 1:20 in 0.14 M KCl/0.02 M NaCl (final concentrations) containing 10 mM Tris, 10 mM sodium phosphate, or 25 mM sodium HEPES (pH 7.4 at 25°C). The suspensions were then incubated as in shape-change assays at 0°C or 37°C and sampled at intervals from 0 to 30 min for membrane solubilization and electrophoresis. The initial 0°C hemolysates were sampled immediately after addition of hypotonic buffer. In all cases, the samples were prepared for electrophoresis as described for intact cell labeling experiments. The ³²P specific radioactivity in spectrin was determined as described for analysis of phosphorylations during shape change.

Other Methods

Membrane resealing was estimated by assaying the inaccessibility of inner surface markers. For Tris ghosts sedimented through a sucrose barrier and Tris-EDTA ghosts prepared after hemolysis at 37°C, glyceraldehyde-3-phosphate dehydrogenase was assayed in the presence and absence of Tris X-100 (54). In salt extraction experiments, NADH-cytochrome c oxidoreductase was assayed in the presence and absence of saponin (54). The concentration of ATP in neutralized perchloric acid extracts was measured by the method of Beutler (7). To increase the sensitivity, the cells were pelleted and resuspended in 0.9% NaCl to one-fourth the original volume before addition of perchloric acid. The specific radioactivity of the γ-phosphate of [³²P]ATP in the extracts was determined by use of the phosphorylase kinase reaction (19). Residual [³²P]ATP in the membranes from incubated cells was extracted by perchloric acid and isolated by thin-layer chromatography (14). The concentration of membrane-bound ATP was calculated from the ³²P counts in the ATP spot using the measured specific radioactivity, assuming equilibration of α-, β-, and γ-phosphates (3).

RESULTS

Spectrin Phosphorylation and Shape Change

Erythrocyte ghosts prepared by hemolysis in 10 mM Tris, pH 7.4, are mainly spherical and smooth contoured. When fresh ghosts are suspended in the isotonic shape-change assay medium at 0°C, they rapidly assume the highly crenated, echinocytic configuration. At 0°C, these populations of echinocytes are highly uniform and stable for several hours. But incubation of fresh crenated ghosts at 37°C in the presence of MgATP results in conversion to smooth biconcave disks. Both forms are readily visualized by dark-field light microscopy (Fig. 1 a and b). As described by Sheetz and Singer (52), the shape transformation does not occur in the absence of ATP and is not promoted by 1 mM GTP or by the ATP analogue AMP-PMP (data not shown). During the first 30 min of incubation, the degree of crenation appears to change progressively in the majority population. Thus, in contrast to Sheetz and Singer (52), we recognize intermediate stages of membrane expansion during the echinocyte to discocyte transformation. We have confirmed that the ATP-dependent shape-change
Spectrin phosphorylation and inhibition of shape change, suggesting that with exogenous phosphatases resulted in spectrin dephosphorylation and inhibition of shape change, probably because we scored the expanded, smooth-constricted intermediate forms as disklike.

In membranes undergoing shape change in the presence of \( [\gamma^{32}P]ATP \), polypeptide 2 of spectrin is rapidly labeled (Fig. 2 and 3a). Within 20–30 min, this reaction reaches a plateau that is equivalent to a net increment in phosphorylation of ~0.7 mol of phosphate/mol of spectrin polypeptide 2 (assuming that dephosphorylation is negligible; see below). Under shape-change assay conditions, labeling of band 3 is also detectable, but other polypeptide phosphorylations are suppressed (4).1

In parallel incubations we prepared unlabeled samples for glutaraldehyde fixation and dark-field light microscopy. Counting the fraction of disklike forms in the population yielded the kinetics of shape change shown in Fig. 3a. Typically, the process is ~50% complete at 30 min, and stomatocytes do not appear in significant numbers (>30%) until after 45 min. These observations replicate those of Sheetz, Singer, and Birchmeier (9, 52), except that, in our hands, shape change in Tris proceeds more or less linearly without a prominent lag phase (Figs. 2b and 3a); although labeling is detectable in salt-stripped ghosts, the apparent increment at 45 min is only 0.04–0.08 mol of phosphate/mol of spectrin 2. Surprisingly, this dramatic reduction in the rate of spectrin phosphorylation is not associated with any visible alterations in the shape-change process. The extracted ghosts are initially indistinguishable morphologically from control ghosts in the isotonic medium at 0°C (cf. Fig. 1a and c) and the rates and endpoints of shape change are identical (cf. Fig. 1b and d and Fig. 3d).

Membrane resealing might significantly affect the efficiency of spectrin kinase extraction and the rate of spectrin phosphorylation under shape-change conditions. However, the decreased spectrin phosphorylation rate after salt extraction cannot be attributed to resealing of the membranes to ATP, because the accessibility of an inner membrane marker, NADH-cytochrome c oxidoreductase (54), exceeds 85% for both extracted ghosts and controls (data not shown). The possible role of membrane resealing was further examined in two ways. In one experiment, control and salt-extracted Tris ghosts were sedimented through a 0.3 M sucrose layer (48). The leaked fraction penetrating the barrier was then used for analysis of shape change and phosphorylation. In a second experiment, ghosts were prepared by hemolysis at 37°C in 10 mM Tris/1 mM EDTA/0.25 mM DTE (pH 7.4).

Spectrin phosphorylation is required for shape change. We had noted earlier that salt extraction of ghosts removes cAMP-independent protein kinase activity selectively and sharply reduces autophosphorylation of spectrin in isolated membranes (5, 21). This suggested that analysis of the shape-change behavior of salt-extracted ghosts could be useful in evaluating the functional significance of spectrin phosphorylation.

As shown in Table I, one washing with 0.5 M KCl removes ~90% of the cAMP-independent casein kinase of the membrane. In the autophosphorylation reaction under shape-change assay conditions, the initial rate of \( ^{32}P \) incorporation into spectrin polypeptide 2 is reduced to a comparable extent (Figs. 2b and 3a); although labeling is detectable in salt-stripped ghosts, the apparent increment at 45 min is only 0.04–0.08 mol of phosphate/mol of spectrin 2. Surprisingly, this dramatic reduction in the rate of spectrin phosphorylation is not associated with any visible alterations in the shape-change process. The extracted ghosts are initially indistinguishable morphologically from control ghosts in the isotonic medium at 0°C (cf. Fig. 1a and c) and the rates and endpoints of shape change are identical (cf. Fig. 1b and d and Fig. 3d).

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1 The amount of radioactivity below the tracking dye (Fig. 2b) is variable. This zone is believed to contain \([\gamma^{32}P]P\)polyporphinositol phosphates (58), but \([\gamma^{32}P]P\)orthophosphate and \([\gamma^{32}P]ATP\) have nearly the same mobility and may persist in trace amounts.
extracted, (b) the rate of residual spectrin phosphorylation, or (c) the kinetics of shape change.

**Shape Change of Membranes from Metabolically Depleted Cells**

The results of the salt extraction experiments indicated that most of the apparent increment in spectrin phosphorylation is dispensable in membrane shape change. They did not, however, exclude the possibility that preexisting spectrin phosphorylation is so near a critical threshold level that the very small increment in salt-stripped ghosts might be sufficient. We attempted to resolve this question by measuring phosphorylation and shape change in membranes from erythrocytes incubated in vitro to alter initial levels of spectrin phosphorylation.

Erythrocytes incubated with $^{32}$Porthophosphate in a modified Krebs-Ringer-bicarbonate medium incorporate $^{32}$P into spectrin and other phosphopeptides (5, 21). In the experiment presented in Table II, the cells were first labeled for 36 h under conditions that maintain ATP levels. They were then washed and divided into two portions for continued incubation with $^{32}$Porthophosphate in the presence and absence of energy sources. During the second incubation in maintenance medium, cellular ATP levels increased progressively. After 24 h in depletion medium, however, the ATP concentration had fallen to 2% of normal, and 89% of the cells were markedly crenated. At intervals during both incubations, samples were taken for measurement of spectrin labeling and ATP specific activity. At the lowest ATP levels, the precision of the latter measurement is low. However, because the organic phosphate pools of the cells had been equilibrated with $^{32}$Porthophosphate during the first incubation, the ATP specific activities did not differ in maintained and depleted cells and did not change significantly after 7 h in the second incubation. Accordingly, we used the specific activities and spectrin labeling data to estimate net exchangeable spectrin-bound phosphate. As shown in Table II, spectrin labeling in maintained cells was maximal at 16 h. In cells undergoing ATP depletion, it rose to the same level initially, but declined sharply between 16 and 24 h. The results imply that the membranes from ATP-depleted cells contained spectrin that was $\sim$50% dephosphorylated relative to spectrin in the membranes from cells incubated with maintenance of ATP.

**Measurements of shape-change capacity and spectrin label-**

### Table I
**Salt Extraction of Casein Kinase from Erythrocyte Ghosts**

| Hemolysis condition | Total activity (pmol $^{32}$P/h/mg ghosts) | Distribution | %
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris, pH 7.4, 0°C</td>
<td>453</td>
<td>85</td>
</tr>
<tr>
<td>Tris, pH 7.4, 0°C</td>
<td>419‡</td>
<td>90</td>
</tr>
<tr>
<td>Tris-EDTA, pH 7.4, 37°C</td>
<td>406</td>
<td>82</td>
</tr>
</tbody>
</table>

* Percent of total activity in supernatant and pellet fractions after salt extraction as described in Materials and Methods.

‡ Leaky membrane fractions from salt-extracted (pellet) and unextracted ghosts sedimented (15 min, 14,000 g) through a 0.3M sucrose cushion containing 10 mM Tris (pH 7.4)/0.14M KCl/0.25mM DTE (48).

### Table II
**Shape-change Behavior of Membranes from Incubated Erythrocytes**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>ATP concentration*</th>
<th>ATP specific activity‡</th>
<th>Spectrin labeling‡</th>
<th>Spectrin phosphorylation nmol $^{32}$P/stain</th>
<th>Echinocytic cells %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. First cell incubation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.890</td>
<td>0.116</td>
<td>7.26</td>
<td>0.063</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>1.33</td>
<td>0.152</td>
<td>10.2</td>
<td>0.067</td>
<td>0</td>
</tr>
<tr>
<td>36</td>
<td>1.45</td>
<td>0.130</td>
<td>10.2</td>
<td>0.079</td>
<td>0</td>
</tr>
<tr>
<td>B. Second cell incubation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maintained</td>
<td>7</td>
<td>1.20</td>
<td>0.177</td>
<td>14.1</td>
<td>0.080</td>
</tr>
<tr>
<td>Depleted</td>
<td>0.760</td>
<td>0.180</td>
<td>18.9</td>
<td>0.10</td>
<td>0</td>
</tr>
<tr>
<td>Maintained</td>
<td>16</td>
<td>1.40</td>
<td>0.163</td>
<td>18.9</td>
<td>0.12</td>
</tr>
<tr>
<td>Depleted</td>
<td>0.10</td>
<td>0.160</td>
<td>16.6</td>
<td>0.1</td>
<td>16</td>
</tr>
<tr>
<td>Maintained</td>
<td>24</td>
<td>1.81</td>
<td>0.188</td>
<td>17.1</td>
<td>0.091</td>
</tr>
<tr>
<td>Depleted</td>
<td>0.03</td>
<td>0.178</td>
<td>6.65</td>
<td>0.04</td>
<td>89</td>
</tr>
<tr>
<td>C. Membrane incubation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Salt-extracted</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24-h maintained</td>
<td>51</td>
<td>50</td>
<td>0.010</td>
<td>0.0021</td>
<td></td>
</tr>
<tr>
<td>24-h depleted</td>
<td>49</td>
<td>50</td>
<td>0.015</td>
<td>0.0026</td>
<td></td>
</tr>
</tbody>
</table>

The conditions for erythrocyte incubation are specified in Materials and Methods. After an initial 36-h incubation with $^{32}$Porthophosphate in ATP maintenance medium, the cells were divided into two equal portions. These were incubated for 24 h in the presence (maintained) and absence (depleted) of glucose-adenine-adenosine with $^{32}$Porthophosphate at the same specific activity. Samples were withdrawn at intervals for measurement of ATP concentration, ATP specific activity, and spectrin $^{32}$P-labeling as described in Materials and Methods. Unlabeled cells were incubated in parallel under identical conditions. Membranes prepared from these cells after the second incubation were used for analysis of shape-change capacity and for measurement of the spectrin phosphorylation increment under shape-change conditions.

* Single determination.

‡ Averages of duplicate measurements.
ing during shape change are also presented in Table II. Although the shapes of the ATP-depleted and maintained intact cells were markedly different at the end of the second incubation, the two populations of isolated ghosts were indistinguishable morphologically, both in hypotonic buffer and in 0.14 M KCl. They changed shape at the same rate under standard assay conditions, and neither the kinetics nor endpoint of shape change was altered by salt extraction. With continued incubation of these preparations, stomatocytes appeared and endocytosis was observed (data not shown), as described above for membranes from fresh cells. The rate of spectrin phosphorylation in the membranes from ATP-depleted cells was elevated in this experiment; typically, however, this reaction is unaffected or diminished slightly after depletion (21; also data not shown).

The data presented in Table I, together with similar experiments involving other conditions and time schedules of in vitro incubation, demonstrate that the level of spectrin-bound exchangeable phosphate can be manipulated physiologically over a wide range without altering the behavior of isolated membranes in the shape-change assay.

Effects of Variation in Buffer and Hemolysis

Temperature

In principle, the Tris buffer in the shape-change assay system could introduce artifacts resulting from its relatively poor buffering capacity at pH 7.4 and its high temperature coefficient of pH (the latter reflected in a pH drop of 1 when assay mixtures are transferred from ice to the 37°C bath). We therefore examined shape change in phosphate and HEPES buffers, which in this laboratory have been used more frequently than Tris in preparing erythrocyte membranes and in examining phosphorylation in cell-free systems. We also tested TES, which has chemical features of both Tris and HEPES—the tris(hydroxymethyl)methyl group and the ethanesulfonic acid group, respectively. All four buffers yield ghosts that initially appear as smooth, dimpled spheres under hypotonic conditions and that are converted to highly crenated spheres upon addition of KCI to isotonicity. However, HEPES ghosts resist crenation if allowed to stand in 10 mM HEPES, pH 7.4, for 3–4 h at 0°C or for 15 min at 37°C, a phenomenon alluded to also by Sheetz (47). Because of these effects, all shape-change assays were initiated within 1 h after hemolysis.

As shown in Figs. 4 and 5, hemolysis in these four buffers and subsequent analysis of shape change in the same buffers revealed dramatic differences in both shape-change kinetics and ATP dependence. Shape change in phosphate buffers is clearly dependent on ATP, but the initial rate of conversion is consistently higher than that observed with Tris. In comparable experiments using HEPES buffer, shape change is extremely fast (complete in 15 min) and requires no added ATP. The results in TES buffer are similar, in that the initial rate of conversion is independent of ATP; but, in the absence of ATP, shape change in TES is only ~75% complete at 60 min.

Observation of the conversion of discocytes to stomatocytes also revealed major differences in the effects of these buffers. In phosphate, nearly 40% of the membranes are stomatocytes after 30 min, while, in Tris, a comparable degree of conversion is observed only after 45 min. Conversion to stomatocytes is not seen after hemolysis in HEPES or TES, in either the presence or absence of ATP (Table III).

In several experiments, we attempted to identify the stage at which the buffers exert differential effects. The results presented in Figs. 4 and 6 and in Table IV demonstrate the importance of the conditions of hemolysis. When ghosts are prepared by lysis at 0°C in Tris or phosphate and are assayed promptly in HEPES buffer, shape change requires ATP (Fig. 6a and b), and the kinetics closely resemble those obtained when both lysis and shape change are carried out in Tris or phosphate (Fig. 5a and c). On the other hand, even though HEPES cannot support ATP-independent shape change when it is present only during the assay, membranes prepared by lysis in HEPES exhibit ATP-independent shape change when assayed in Tris (Table IV). This suggests that the dominant factor determining both the degree of ATP dependence and the initial rate of the shape-change process is the buffer used in the hemolysis and initial washing.

The effect of hemolysis temperature is somewhat less clear cut. Membranes prepared by hemolysis at 37°C in Tris or phosphate are initially slightly larger and smoother in shape-change medium (cf. Fig. 4g vs. 4e and 1a). Their subsequent shape-change behavior in Tris or phosphate (Figs. 4h and 6e and f) is identical to that observed after 0°C hemolysis (Fig. 5a and c). However, 37°C hemolysis yields membranes that undergo significant ATP-independent shape change when assayed in HEPES; under these conditions, the extent of conversion to discocytes is >80% (Figs. 4h and 6e and d). This effect of HEPES is associated with an acceleration in the rate of shape change in the presence of ATP. Thus, it appears that lysis at 37°C does not in itself facilitate ATP-independent shape change, but renders the membranes more susceptible to the shape-change-promoting effect of HEPES.

Conversion of discocytes to stomatocytes is preconditioned by the hemolysis buffer rather than by temperature. Thus, shape change of membranes assayed in Tris after hemolysis in HEPES (Table IV) is arrested at the discocyte stage, whereas hemolysis in Tris or phosphate at 0° or 37°C (Fig. 6) yields membranes that undergo ATP-dependent shape change to stomatocytes in the presence of Tris, phosphate, or HEPES. However, ATP-independent shape change in HEPES after 37°C hemolysis (Fig. 6c and d) yields only discocytes (Table III).

The HEPES ghosts used in most of these experiments were pink, suggesting that increased membrane retention of endogenous ATP or other cytoplasmic components might be responsible for the ATP-independence of shape change after HEPES hemolysis. Certain observations seem to rule out this possibility. Thus, rapid, ATP-independent shape change is exhibited by several types of ghost preparation that should be relatively free of cytoplasmic contamination: (a) white HEPES ghosts produced by increasing the buffer:membrane ratio during washing; (b) salt-extracted HEPES ghosts; and (c) HEPES ghosts from ATP-depleted cells (data not shown). Furthermore, the measured concentration of endogenous ATP in Tris and HEPES ghosts is not well correlated with ability to change shape in the absence of added ATP (Table V).

Spectrin Dephosphorylation under Shape-Change Assay Conditions

We have reported (21, 27) that spontaneous spectrin dephosphorylation in isolated membranes is relatively sluggish. The initial rate is <1%/min, both for membranes labeled with [γ-32P]ATP after isolation and for membranes isolated from intact cells labeled with [32P]orthophosphate. Fig. 7 displays
results of an experiment designed to measure the extent of dephosphorylation during membrane preparation and during incubation in isotonic shape change media containing Tris, phosphate, and HEPES. It can be seen that no significant loss of radioactivity occurs after hemolysis and that the decline in specific activity during 37°C incubation in all three buffers is
barely perceptible. Accordingly, the buffer effects described above cannot be attributed to differential effects on spectrin dephosphorylation.

**DISCUSSION**

**Shape-change Assay**

The shape-change assay of Sheetz and Singer (52) was attractive because it promised to facilitate studies on the regulation of the spectrin phosphorylation-dephosphorylation system in the context of spectrin function. However, our experiments revealed several unforeseen complications that probably reflect the interplay of multiple variables in shape determination. The effects of HEPES and TES buffers are particularly striking. Membranes incubated with HEPES resist salt-induced crenation, and both HEPES and TES ghosts undergo accelerated echinocyte-discocyte transformations independent of...
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One of the most troublesome observations is that the capacity of isolated membranes to change shape is not stable but undergoes a spontaneous decline over a period of 1–2 h, even when the membranes are held at 0°C. Because spectrin dephosphorylation is negligible under these conditions, the altered membrane behavior must be attributed to other physical or biochemical changes.

Overall, these observations serve to emphasize that the shape-change mechanism is labile. At this stage, it is not clear that all significant variables have been recognized and subject to experimental control in the assay system. Nonetheless, we have confirmed that, in the Tris system, shape change is invariably ATP-dependent and reproducible in its kinetics and endpoint. In other respects, our results differ from those of Sheetz and Singer (52). In particular, we observe ATP-dependent shape change of phosphate ghosts, as well as Tris ghosts, and we note the occurrence of intermediate forms, suggesting that the shape transformation of individual membranes is progressive rather than all-or-none.

Role of Spectrin Phosphorylation

Many independent observations have contributed to general acceptance of the view that the spectrin-actin complex has an important function in maintaining membrane integrity (33, 37, 44, 53). Lux et al. (38) reported that the abnormal shape of irreversibly sickled erythrocytes is retained in spectrin, actin-enriched Triton skeletons of the isolated membranes. This implicated spectrin in the control of cell shape and membrane deformability in a pathological state, and it was plausible to assume that the condition observed represented an extreme along a continuum of regulation in normal cells. Considering the metabolic dependence of cell shape and deformability (59), it was also appropriate to explore the possibility that spectrin phosphorylation and dephosphorylation reflect enzymatic regulation of normal states of the spectrin-actin complex. As the system has been examined with increasingly sophisticated techniques, however, several predictions based on these ideas have not been substantiated. Thus, it has been reported recently that changes in spectrin phosphorylation state have no effect on spectrin self-associations (3, 15, 17, 57), spectrin-actin interaction (12, 15, 17), and spectrin binding to the cytoplasmic face of the membrane (3, 6).

Our principal finding was that membranes depleted in spectrin kinase activity are not altered in their capacity to undergo ATP-dependent shape change. In the experiments described, salt extraction reduced the initial rate of spectrin phosphorylation by 90% and reduced plateau labeling by 80–95%. The subpopulation of leaky ghosts exhibited the same behavior, indicating that membrane resealing is not required for shape change and is not responsible for the retention of residual spectrin kinase activity.

The absence of any effect of salt extraction on shape change is striking but not totally inconsistent with some of the postulated functions of spectrin phosphorylation in the shape-change process. It is conceivable that the residual phosphorylation observed is necessary and sufficient for the nucleation of a structure transition in the spectrin-actin meshwork. Such a reorganization could be a prerequisite for subsequent force generation in the lattice (50). This model has some plausibility, but it is clearly difficult to reconcile it with the observation of unaltered shape-change kinetics in the face of a sharp decline in the initial phosphorylation rate. It may be relevant to compare the energy derived from phosphorylation in extracted membranes with Evans’ (20) estimate of the change in interfacial free energy required for membrane bending. The residual protein kinase activity in salt-stripped ghosts phosphorylates up to 5% of the 215,000 chains of spectrin polypeptide 2 on each 140–150 μm² membrane. Even if all the free energy of ATP hydrolysis (about −32 kJ/mol for MgATP) could be recovered at the membrane, the change in interfacial free energy resulting from residual spectrin phosphorylation would be only 0.4 × 10⁻² erg/cm². This is near the calculated requirement (10⁻² erg/cm²) for initiating minimal membrane bending, but it is two orders of magnitude below the theoretical requirement for further membrane bending to the curvatures observed in creation and endocytosis (20, 30). Hence, the prospect that shape change in salt-stripped membranes could be driven energetically by spectrin phosphorylation is quite remote.

One potential problem with the approach we have taken is that exposure to 0.5 M salt may significantly perturb membrane organization. It could be argued that the salt treatment, while removing spectrin kinase, glyceraldehyde-3-phosphate dehydrogenase, and traces of residual cytoplasmic proteins (23, 53), might alter the spectrin-actin meshwork in a way that would partially compensate for the reduced phosphorylation in the later assay of shape change. This cannot be excluded, but it
appears unlikely in view of the unaltered crenation response and shape-change kinetics of extracted membranes.

We also examined the relationship between phosphorylation and shape change in experiments on membranes from erythrocytes in which spectrin was partially dephosphorylated by in vitro ATP depletion. Although ATP depletion resulted in removal of >50% of the exchangeable phosphate groups on spectrin and was associated with erythrocyte crenation, the membranes isolated from depleted cells exhibited the same shape-change behavior (initial morphology, salt-induced crenation, and kinetics of MgATP-dependent conversion to discocytes and stomatocytes) as membranes from fresh cells or cells incubated in vitro with maintenance of ATP. Because the initial phosphorylation level is clearly not critical and the phosphorylation increment is dispensable, we conclude that spectrin phosphorylation is incidental to MgATP-dependent shape change in isolated membranes.3

The relationship between the shape-change process we have studied and the shape transformations of intact erythrocytes has not been established. However, in a recent study, Anderson and Tyler (3) demonstrated that, during ATP depletion, most incubated erythrocytes have lost biconcave shape long before spectrin dephosphorylation is detectable. Independence of shape from spectrin phosphorylation state is thus seen in both systems. Our findings do not exclude a possible role of spectrin phosphorylation in the maintenance of membrane integrity and prevention of membrane rigidity. However, it is difficult to conceive of arrangement in which membrane material properties and shape are not interdependent at the molecular level. Furthermore, it has been demonstrated recently that ATP depletion does not alter certain membrane microeheologic parameters measurable by refined pipette aspiration and membrane tethering techniques (31, 39). Overall, it appears that recent work in this field has not yet identified and examined the physiological context in which spectrin phosphorylation is relevant. This suggests, in turn, that spectrin might have major functions other than the structural role and mechanochemical activity already postulated for it.

Spectrin Dephosphorylation

We believe that dephosphorylation of spectrin in isolated membranes is not a significant factor in any of the shape-change phenomena we have described. We have not reproduced the rapid initial dephosphorylation reported by Birchmeier and Singer (>7% per min; Fig. 7 and reference 9), and Sheetz has also observed relatively slow dephosphorylation under shape-change assay conditions.5 The discrepancies in the data from various laboratories probably reflect variation in the physical state of spectrin and/or in the level of cytoplasmic phosphospectrin phosphatase activity retained in the membranes (21, 27). Under the conditions we describe, most of the exchangeable phosphate groups on spectrin do not turn over. Accordingly, we suspect that the plateaus in spectrin labeling reached in shape-change incubations represent the saturation of previously unfilled sites or, possibly, a dynamic equilibrium involving a small subclass of sites accessible to both spectrin kinase and phosphospectrin phosphatase.

ATP Dependence of Endocytosis

The appearance of stomatocytes followed by endocytosis under shape-change conditions has been regarded as an extension of the echinocyte-discocyt e transformation in which the net curvature of the entire system (including endocytic vesicles) is continuously decreasing (8, 43). In our experiments, stomatocytes undergoing endocytosis were frequently seen and estimates of their numbers were recorded. It was noted that endocytosis did not invariably follow the formation of discocytes. Thus, stomatocytes never appeared in membrane populations prepared by lysis in HEPES or TES; and membranes prepared at 37°C could not undergo conversion from discocytes to stomatocytes in the absence of ATP. Under conditions we have tested, the dependence of endocytosis on ATP appears absolute (Table III). However, the results on salt-stripped membranes and membranes from ATP-depleted cells clearly imply that spectrin phosphorylation is not required.

Mechanism of Shape Change

In considering the possible contributions of spectrin to the ATP-dependent shape change of ghosts, it is useful to distinguish between active and passive roles of the protein complex. We have inferred from our results that protein kinase–mediated phosphorylation does not initiate or drive the shape-change process. We cannot exclude the possibility that shape change and endocytosis involve an actomyosin-like mechanoschématic system, as postulated by others (29, 32, 48, 49). However, the physical and antigenic relationships between spectrin and the myosins are remote (28, 34, 49), and there is little evidence that either the intact membrane or isolated spectrin bears actomyosin-like ATPase activity (30, 33, 34). In any case, it is unnecessary to invoke an active role of spectrin in shape change. There is considerable evidence that the submembrane skeleton restricts lateral migration of transmembrane proteins (18, 37, 55). If shape change involves reactions requiring such migration, then the overall shape-change process may be passively controlled by the organization of the spectrin–actin complex and by its binding to the membrane. In this model, spectrin phosphorylation is incidental, but a variety of experimental manipulations—including exposure to antispectrin antibodies (52) and exogenous actin (8)—may have complex indirect effects.

The elucidation of the mechanism of ATP-dependent shape change will involve the identification of reaction sites in the membrane where utilization of ATP can induce the required bending moments. It has been observed that interconversions of polyphosphoinositides, phosphatidylinositol, 1,2-diacylglycerol, and phosphatidic acid in the erythrocyte membrane are associated with vesiculation and shape change under various conditions (1, 2). The rapid transbilayer diffusion of 1,2-diacylglycerol provides a potential mechanism for changing the relative areas of the two membrane leaflets (1), and biosynthesis of phosphatidate and polyphosphoinositides requires ATP (2, 30). It will be of interest to determine the relationship of these reactions to the phenomena we have described.

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3 We have recently reexamined spectrin phosphorylation during shape change, using very low membrane concentrations to minimize depletion of free ATP resulting from hydrolysis and binding to the membrane. Under these conditions, the $K_d$(ATP) for phosphorylation ($\sim 60 \mu M$) is lower than the $K_d$(ATP) for shape change ($\sim 250 \mu M$) measured at the same time. These and other demonstrations of a dissociation of the two processes will be reported separately (reference 22 and V. P. Patel and G. Fairbanks, manuscript in preparation).
REFERENCES

1. Allan, D., P. Thomas, and R. H. Michell. 1976. Rapid transblot diffusion of 1,2-


