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FUNCTIONAL LINK BETWEEN PHOSPHORYLATION STATE OF MEMBRANE PROTEINS AND MORPHOLOGICAL CHANGES OF HUMAN ERYTHROCYTES

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The Tyr-phosphorylation of the cytoplasmic domain of the major membrane-spanning band 3, rather than the Ser/Thr-phosphorylation of the membrane proteins (spectrin and band 3 itself),might be functionally related to certain morphological changes of human erythrocytes.
This view is supported by the following lines of evidence:
a) vanadate or its derivative pervanadate (vanadyl hydroperoxide), which markedly increase the Tyr-phosphorylation of band 3 (without practically affecting the Ser/Thr-phosphorylation of spectrin) promotes a crenation of human erythrocytes;
b) okadaic acid, which selectively increases the Ser/Thr-phosphorylation of spectrin and other membrane proteins, does not promote any shape change, at least at a level detectable with scanning electron microscopy.

The normal biconcave disk shape of human erythrocytes is thought to be essentially controlled by the membrane protein cytoskeleton acting as a scaffolding upon which the rest of membrane is built.

The cytoskeletal network underlying the lipid bilayer is composed of spectrin (band 1 and 2) tetramers cross-linked by junctions containing actin oligomers and other proteins (termed 4.1, 4.9, according to indexing system of Fairbanks et al. (1)).

The normal shape of the red cells may undergo a variety of changes induced by alterations of metabolic state or by several drugs.

For instance, since long time it is well known that some shape-change processes are dependent on ATP content. Up to now, however, the molecular mechanism of such ATP-dependence is still a matter of controversy, since conflicting results have been reported concerning this point.

A correlation between ATP-dependent shape-changes and phosphorylation state of cytoskeletal spectrin had been suggested by Birchmeier and Singer (2), using [γ-^{32}P]ATP-

Abbreviations: PMSF, phenylmethylsulphonyl fluoride; SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electophoresis.

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treated ghosts of human erythrocytes. Howewer, other subsequent studies did not support this idea (3,4).

This discrepancy prompted us to further investigate this problem, using the whole human erythrocytes, taking into account that both shape-changes and membrane protein phosphorylation might be controlled by factors lost during the preparation of their ghosts.

To this aim, the intact red cells have been incubated in the presence of okadaic acid, or of vanadate, or vanadyl hydroperoxide (pervanadate), very useful drugs for identifying whether a cellular process is likely correlated with Ser/Thr-phosphorylation or with Tyr-phosphorylation of membrane proteins, since these reagents, penetrating the cells, markedly and selectively increase Ser/Thr-phosphorylation or Tyr-phosphorylation of proteins, the okadaic acid being selective and strong inhibitor of some P-Ser/Thr-protein phosphatases (5-7), while the vanadate and pervanadate strongly and selectively inhibit P-Tyr-protein phosphatases (8-10).

The results reported here would suggest that quite likely Tyr-phosphorylation of band 3 cytoplasmic domain (rather than its Ser/Thr-phosphorylation) might be involved in the regulation of certain human erythrocyte shape-changes.

METHODS AND MATERIALS

Preparation of ^{32}P-labeled human erythrocytes.

Human erythrocytes were prepared by centrifugation (at 750xg for 3 min) of fresh blood collected from healthy adult volunteers. To minimize contamination by leucocytes and platelets, the packed red cells were washed three times by centrifugation in buffer A (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 0.1 mg/ml streptomycin, 25 μg/ml chloramphenicol) and discarding the buffy coat and the upper third of packed red cell layer.

Packed red cells (400 μl) were preincubated in 3.6 ml buffer A for 4 h at 35°C to deplete endogenous ATP stores, then centrifuged at 750xg for 3 min., and resuspended in 3.6 ml of the same buffer A (10% hematocrit) containing glucose (24 mM) and adenosine (1 mM) in the presence of [^{32}P]P_i (0.3 mCi) for 14 h at 35°C

Effect of pervanadate.

In order to study the effect of pervanadate (vanadyl hydroperoxide), ^{32}P-labeled erythrocyte suspension, prepared as above described, was divided into 4 samples (1 ml) which were separately incubated for 20 min at 35°C in the presence of 1,5 mM hydrogen peroxide, or 1 mM sodium-orthovanadate, or pervanadate (vanadyl hydroperoxide)(a reagent prepared by mixing equal volumes of 3 mM hydrogen peroxide and 2 mM sodium orthovanadate).

After incubation, each sample of ^{32}P-labelled erythrocytes was solubilized by adding 4.5 ml buffer B (50 mM TRIS, pH 8.9, containing 5 mM EDTA, 380 mM Glycine and 176 mM SDS) and 1% β-mercaptoethanol (final concentration) followed by a 5 min treatment at 100°C.

Solubilized ^{32}P-labeled erythrocytes (180 μl) were submitted to electrophoresis on 0.1% SDS/10% polyacrylamide slab gels, essentially according to Laemmli (11).

After electrophoresis, the slab gels were stained with Coomassie brilliant Blue (CB) according to Laemmli (11). Some gels were treated in a 2 M NaOH solution at 55°C for 1 h and then fixed again. Dried gels were autoradiographed at -80°C with intensifying screens.

Effect of okadaic acid.

When the effect of okadaic acid was studied, 80 μl of 25 μM okadaic acid dissolved in ether, was added to a sample of ^{32}P-labeled erythrocyte suspension (2 ml), while the same amount of ether was added to another sample (control).

After 90 min incubation at 35°C, both samples were separately washed once in the same buffer A containing glucose and adenosine and then hemolysed in 6 ml of hypotonic 5 mM

phosphate buffer (pH 8), containing 0.02% NaN_3 and 0.03 mM phenylmethylsulphonyl fluoride (PMSF).

Membranes were recovered by centrifugation (at 20000 x g for 20 min), washed twice in hypotonic lysis buffer and twice in 25 mM Tris buffer (pH 8) containing 0.02% NaN_3 and 0.03 mM PMSF, and then solubilized by addition of 2% SDS and 1% β-mercaptoethanol (final concentration) followed by a 5 min treatment at 100°C.

Solubilized-^{32}P-labeled membranes (60 μg) were submitted to electrophoresis essentially according to Laemmli (11).

After electrophoresis, the slab gels were stained with Coomassie brilliant Blue (CB) according to Laemmli (11). Some gels were treated in a 2 M NaOH solution as above described and then fixed again.

Dried gels were autoradiographed at -80°C with intensifying screens.

Electron microscopy.

After incubation in the presence of pervanadate or okadaic acid, an aliquot of each sample was fixed overnight in 25% glutaraldehyde and postfixed in 1% osmiumtetroxide in 0.1 M sodium-cacodylate buffer for 2 h according to (12).

Postfixed samples were dehydrated in a graded ethanol series, dried at the critical point, coated with gold and examined with a scanning electron microscopy (SEM, Cambridge Stereoscan 260) operating at 12 KV.

Other methods. Miscellaneous.

Protein content was determined according to (13).

Okadaic acid was purchased from Calbiochem; [^{32}P]Pi from Amersham International.

RESULTS

Effect of vanadate and vanadyl hydroperoxide.

The normal smooth biconcave human erythrocytes (Fig. 1A), when treated with vanadate or with pervanadate (a membrane-permeant reagent prepared as described in the Methods), undergo the morphological change reported in Fig. 1

Fig.1 shows that several cells are converted by vanadate (fig. 1C) or by pervanadate (fig. 1D) from the normal smooth biconcave disk (Fig. 1A) (discocytes) to spiny form (echinocytes) (14).

Such a vanadate- or pervanadate-promoted crenation of intact erythrocytes is accompanied by the change of ^{32}P-labeling pattern of their membrane proteins reported in Fig. 2.

Fig. 2 shows the ^{32}P-labeling pattern of membrane proteins occurring when the whole human erythrocytes are incubated with [^{32}P]P_i in the presence or absence of vanadate or pervanadate (strong inhibitors of P-Tyr-protein phosphatases (8-10)), and then dissolved and analyzed by SDS-PAGE, as described in the Methods.

As shown in Fig.2, the vanadate- or pervanadate-unaffected ^{32}P-labeling of spectrin (band 2) is due only to the phosphorylation of Ser-residues as indicated by the disappearance of the alkali-labile ^{32}P-labeling (15,16) in the alkali-treated gels (Fig. 2B, lanes a',b',c',d').

By contrast, band 3 is phosphorylated, predominantly, if not exclusively, on Tyr-residues as indicated by the alkali-stable ^{32}P-labeling (15, 16) in the alkali-treated gels (fig. 2 lanes c', d') of the erythrocytes incubated with vanadate or pervanadate.

This is confirmed by the phosphoaminoacid analysis of ^{32}P-labelled membrane proteins (15,16) and by immunoblotting analysis with anti-phosphotyrosine antibody of pervanadate-treated erythrocytes, as described in (17).

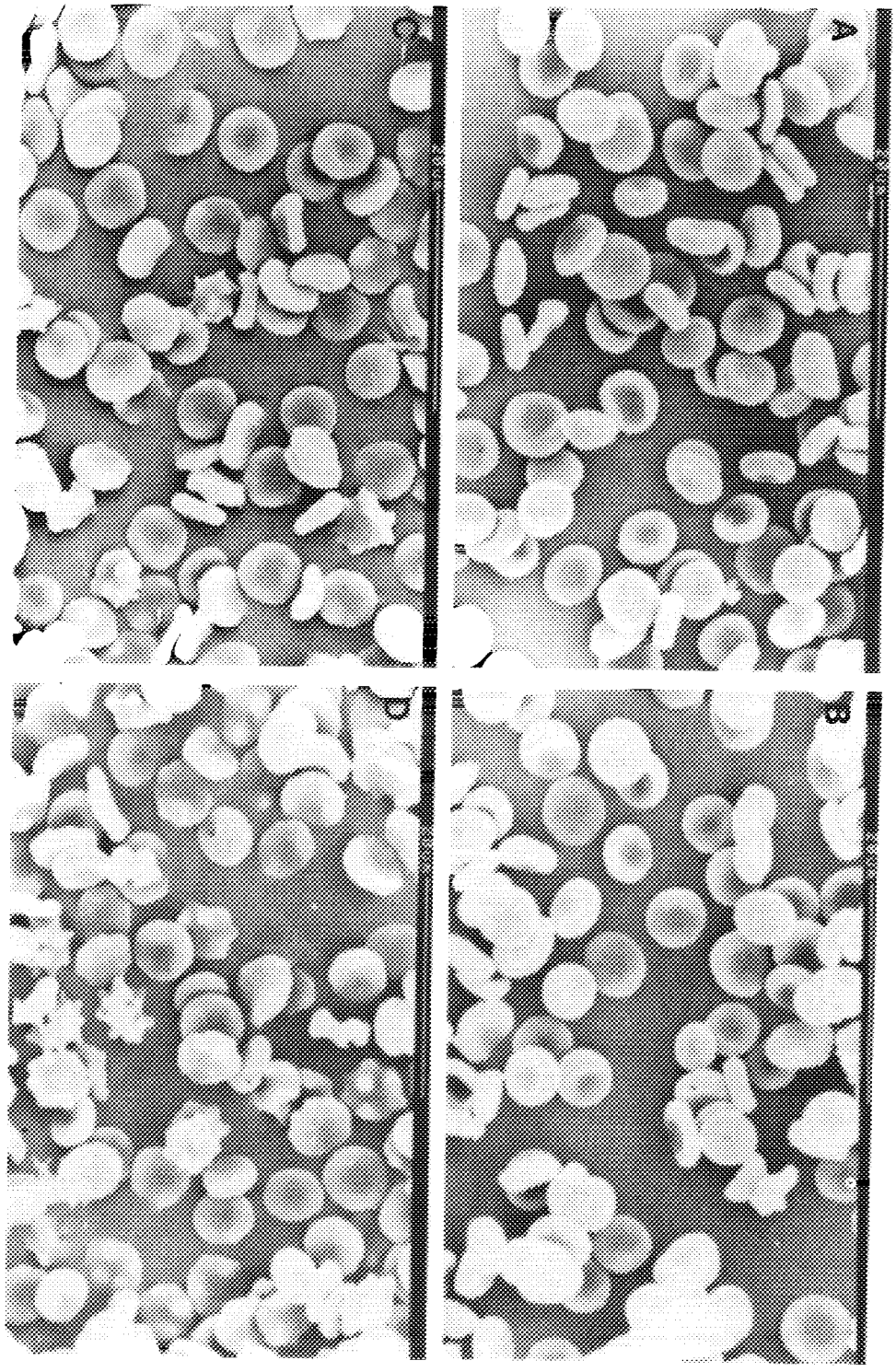

Fig. 1.
Scanning electron microscopy showing the shape of human erythrocytes treated with: no treatment (control) (A), H_2O_2 (B), vanadate (C), pervanadate (D), as described in the Methods.

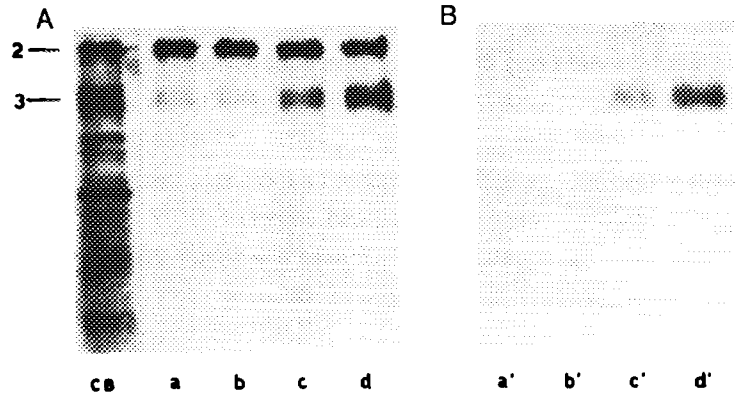

Fig. 2.
Autoradiograms showing the effect of H_2O_2, vanadate and pervanadate on erythrocyte membrane protein phosphorylation of intact human erythrocytes.
Intact human erythrocytes were incubated with [^{32}P]P_i alone (lanes a,a', controls), or in the presence of 1.5 mM H_2O_2 (lanes b,b'), or 1 mM sodium-orthovanadate (lanes c,c') or pervanadate (1.5 mM H_2O_2 and 1 mM vanadate)(lanes d,d'), under the conditions described in the Methods.
The whole ^{32}P-labelled erythrocytes were solubilized and analyzed by SDS-PAGE as described in the Methods.
Fig.2B lanes a'-d' show the alkali-treated gels corresponding to the alkali-untreated gels (2A, lanes a-d).
Lane CB shows the Coomassie Blue stained gel of solubilized whole erythrocyte proteins.
Autoradiograms were exposed for 15 h.

As shown in Fig. 2, the Tyr-phosphorylation of band 3 in the presence of vanadate (lane c) or pervanadate (lane d) is markedly higher than in the controls (lanes a and b), thus confirming the reports (17) appeared while this work was in progress.
It has to be underlined that, in agreement with (18), the ^{32}P-labeling of phospholipids (phosphoinositides)has been found to be unaffected by vanadate (not shown).
In conclusion, the above findings would indicate that vanadate-increased Tyr-phosphorylation of band 3 protein, rather than the vanadate-unaffected Ser-phosphorylation of spectrin and of other membrane proteins, may be functionally correlated with vanadate-promoted crenation of human erythrocytes.
This behavior is in agreement with the observation (18) that the vanadate blocks the ATP-promoted smoothing of isolated crenated ghosts in isotonic medium.
This idea is supported also by the following experiments with okadaic acid, a strong inhibitor of P-Ser-phosphatases (5-7).

Effect of okadaic acid.

When the intact human erythrocytes were incubated with [^{32}P] P_i in the presence or absence of 1 μM okadaic acid and, after incubation, their isolated ^{32}P-labelled membranes were dissolved and analyzed by SDS-PAGE, as described in the Methods, the P-labelling patterns reported in fig. 3 were obtained.

Fig. 3 A, lanes a,b show that under these conditions the membrane proteins, including band 3 protein, are phosphorylated only on Ser-residues (but not on Tyr-residues) as indicated by the total disappearance of alkali-labile ^{32}P-labelling in the alkali-treated gels (Fig. 3 B, lanes a',b').

As clearly shown in the Fig. 3, okadaic acid markedly increases the Ser-phosphorylation of membrane proteins (spectrin (band 2), band 3 and others).

However, this drug does not significantly promote any shape change, at least at level detectable with scanning electron microscopy (Fig. 4).

DISCUSSION

As known, when the whole human erythrocytes are incubated with [^{32}P]P_i, several membrane proteins are ^{32}P-labelled (Fig. 2 and 3), primarily the major cytoskeletal spectrin, β-subunit (band 2) and the major membrane-spanning band 3 protein (95-98 KDa), whose N-terminal cytoplasmic domain (ca.45-50 KDa), protruding into the cytosol as a flexible finger, provides binding sites for hemoglobin, some cytosolic glycolytic enzymes and, through ankyrin, for the underlying cytoskeletal spectrin-actin network.

Such a band 3 cytoplasmic domain is phosphorylated not only on Ser/Thr-residues (19), like the other membrane proteins (20), but also on Tyr-residues (8,21,22) unlike the other membrane proteins, by specific Tyr-protein kinase(s) (15,16,23-26).

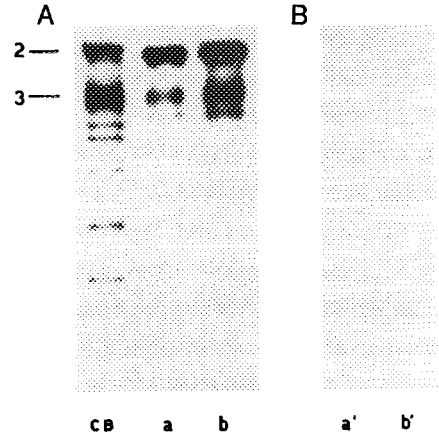

Fig. 3.
Autoradiograms showing the effect of okadaic acid on erythrocyte membrane protein phosphorylation.
Intact human erythrocytes were incubated with [^{32}P]P_i in the presence (lanes b,b') of 1 μM okadaic acid or of its solvent ether (lanes a,a') in the absence of okadaic acid, under the conditions described in the Methods.
^{32}P-labelled membraneproteins were recovered from hemolised erythrocytes and analyzed by SDS-PAGE as described in the Methods.
Fig.3B, lanes a',b' show the alkali-treated gels corresponding to the alkali-untreated gels (fig. 3A, lanes a,b).
Lane CB shows the Coomassie Blue stained gel of isolated membrane proteins.
Autoradiograms were exposed for 16 h.

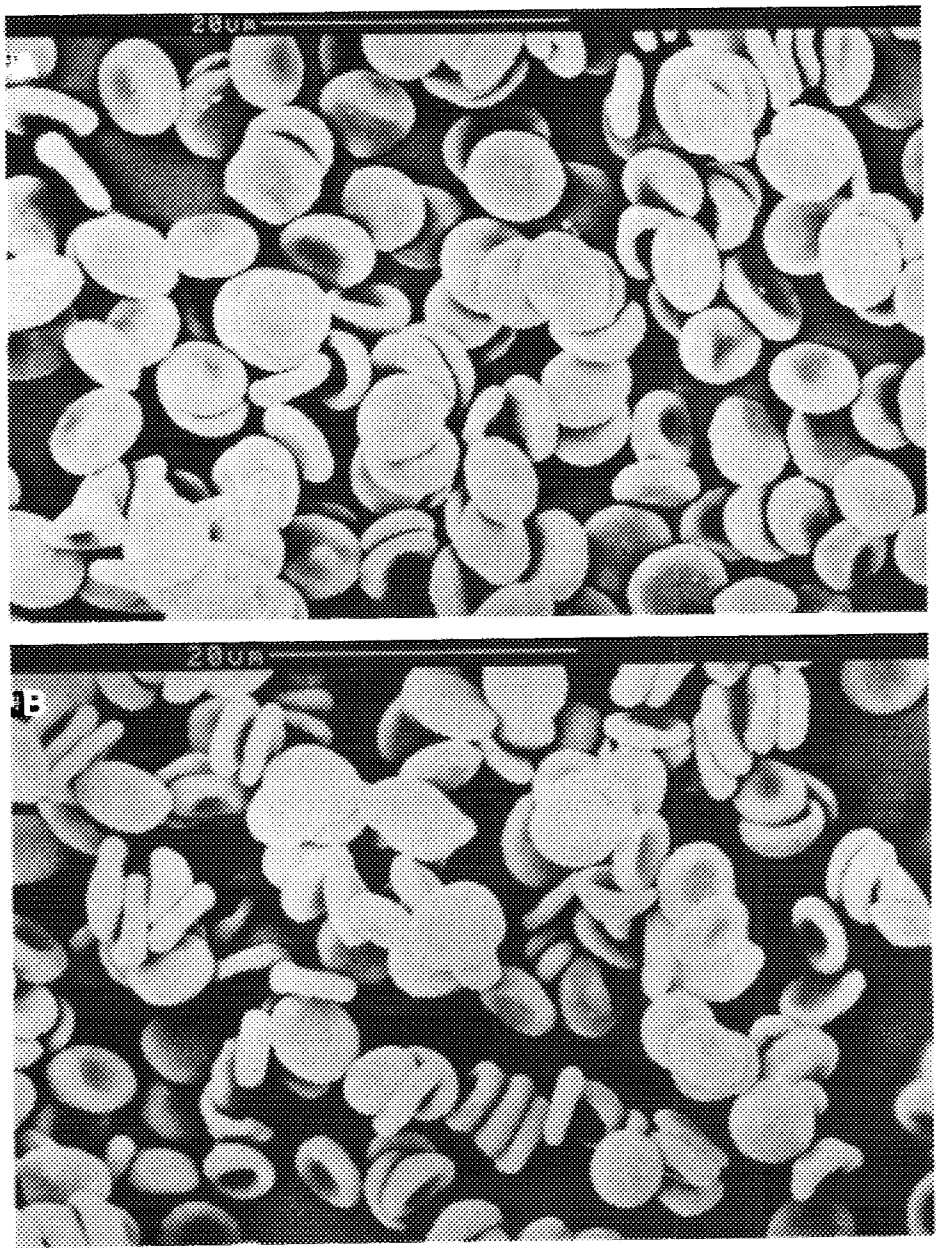

Fig. 4.
Scanning electron microscopy showing the shape of human erythrocytes incubated in the presence (fig. 4B) or in the absence (fig. 4A) of 1 μM okadaic acid.

However, in the whole human erythrocytes, this latter Tyr-phosphorylation, unlike the Ser/phosphorylation, appears to be negligible at all (Fig. 2B lanes a',b' and 3B, lane a', controls), because the Tyr-kinase activity (although being considerable when assayed in vitro) is kept in check in vivo by the counteracting P-Tyr-phosphatases (10,27-30).

Only in the presence of a strong P-Tyr-phosphatase inhibitor (such as vanadate Na_3VO_4 (8-10) or its derivative pervanadate (17)) the Tyr-phosphorylation is promoted, becoming well detectable also in vivo.

Moreover, the functional role of band 3 Tyr-phosphorylation is still unknown.

It has been reported (31) that the in vitro Tyr-phosphorylation of cytoplasmic domain prevents the binding of various glycolytic enzymes to the band 3 amino-terminal region, thus suggesting a possible involvement in the regulation of glycolysis (31-33).

Taking into account that in our experiments vanadate- and pervanadate-promoted Tyr-phosphorylation increase is accompanied by a crenation of the erytrhrocytes, it is tempting to suggest that the Tyr-phosphorylation of cytoplasmic domain of band 3 involved in the binding of ankyrin (only about 15% of total band 3 monomers) (34), may modulate the ankyrin-mediated binding of cytoskeletal network, thus altering the structural organization of the membrane in such a way as to produce the above reported spiny shape of echinocytes.

Of course, it can't be ruled out that other shape-changes of human erythrocytes may involve the phosphorylation state of membrane phospholipids (35).

None of the manipulations (± adenosine) affecting lipid phosphorylating activity in the ghosts produced a significant reduction of ATP-dependent shape-change activity in the ghosts (18).

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