Vol. 112, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1983 Pages 384-390

TWO STEPS IN ATP-DEPENDENT SHAPE CHANGE OF HUMAN ERYTHROCYTYE GHOSTS

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Received March 16, 1983

SUMMARY: Most human erythrocyte membranes (ghosts) prepared with 10 mM Tris-Cl buffer were spherocytic and changed shape through crenated to discoidal in the presence of 2 mM Mg-ATP at 37°C during 30 min under hypotonic conditions. These discocytic ghosts reverted to spherical form after being washed with 1 mM $MgCl_2$, although their membranes were phosphorylated and they were converted to discocytes again,immediately on addition of 2 mM Mg-ATP, even at 6°C. There seem to be 2 steps in the shape change of the ghosts; the first step proceeds gradually during incubation at 37°C for 30 min in the presence of a physiological concentration of Mg-ATP and the second step occurs rapidly after addition of Mg-ATP even at 6°C. This suggests that not only membrane phosphorylation but also specific ATP-binding (or hydrolysis) is necessary for erythrocyte shape change.

It has been proposed that the cytoskeletal network involving spectrin and actin may play an important role in determining erythrocyte shape and deformability (1-5). We obtained direct evidence that ankyrin is also necessary for erythrocyte shape change (6). However, the precise mechanism of ATP-dependent shape change described by Nakao et al.(7-9) is still unclear. Some authors suggested that Mg-ATP might be required for phosphorylation of spectrin (10,11) and others claimed that it might be required for phosphorylation of phosphatidyl inositol (12) or phosphatidic acid (13), because it takes a fairly long time for ghosts to change shape during incubation. The present paper deals with an immediate change of the shape induced by

0006-291X/83 $1.50

Mg-ATP under hypotonic as well as isotonic conditions after preincubation of ghosts with Mg-ATP followed by washing.

MATERIALS AND METHODS

Human erythrocytes obtained from ACD blood (4-8 weeks old) were washed three times with physiological saline and hemolyzed by 1:20 dilution with 10 mM Tris-Cl buffer (pH 7.4). The membranes were pelleted at 18000 rpm for 20 minutes and were washed twice with the hemolyzing buffer at 0°C. These ghosts were suspended in 10 volumes of various solutions and then incubated at 0°C or 37°C. For observation of shape change, 200 ghosts in each case were examined with a dark-field light microscope (Nikon Model S) at room temperature. All reagents added to the ghosts were first carefully adjusted to pH 7.4 with NaOH or HCl at room temperature.

RESULTS AND DISCUSSION

When ghosts were prepared with 10 mM Tris-Cl buffer (pH 7.4), they were mostly spherocytes (Fig. 1-a). Although these ghosts rapidly changed into echinocytes covered with large projections on addition of 2 mM Mg-ATP (pH 7.4) at 0°C (Fig. 1-b), they slowly changed to discocytes at 37°C over 20-30 min under hypotonic conditions (Fig. 1-c), as they did in isotonic NaCl. The value of K0.5 was very roughly estimated to be about 0.5 mM (Table I). At this incubation step, 2 mM Mg-ADP, Mg-AMP, Mg-GTP, Mg-UTP, Mg-CTP, Mg-cordycepin 5'-monophosphate, Mg-adenylylimidodiphosphate (AMP-PNP) or Mg-adenylyl-(ß-γ methylene) diphosphate (AMP-PCP) was ineffective, as shown in Table I. These discocytic ghosts reverted to spherocytes after being washed with 1 mM $MgCl_2$ (pH 7.4) (Fig. 1-d), and then changed to discocytes again immediately after addition of 2 mM Mg-ATP, even at 0°C (Fig. 1-e), although observation was performed at room temperature (about 25°C) or at 6°C in the cold room. The change in temperature of the slide glass was monitored with a thermocouple. The elevation was usually within one degree during observation for one minute. Under isotonic conditions, a slightly

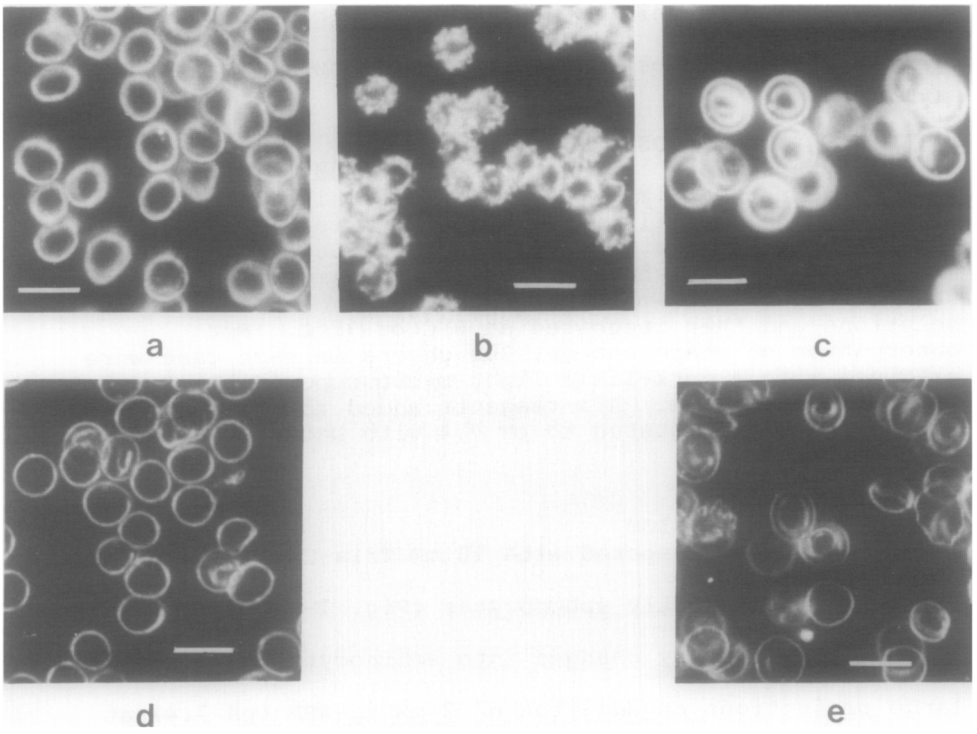

Fig.1

Shape changes of human erythrocyte ghosts

Dark-field light micrographs illustrate ghosts which were prepared with 10 mM Tris-Cl buffer (a) and suspended immediately in 2 mM Mg-ATP at 0°C (b), then incubated in 2 mM Mg-ATP at 37°C for 30 min (c). The ghosts were then washed with 1 mM $MgCl_2$ after the incubation with Mg-ATP (d) and resuspended immediately in 2 mM Mg-ATP at 0°C (e). (bar = 10 μm)

different phenomenon was observed at the second step. When the spherocytic ghosts after being washed with 1 mM $MgCl_2$ (pH 7.4) were incubated with 2 mM Mg-ATP at 0°C for 10 min and then 1/10 volumne of 1.54 M KCl was added, the shape became discocytic immediately at 0°C. Without incubation for 10 min at 0°C, spherocytic ghosts were converted only to echinocytes. This difference may be due to some difficulty in passage of ATP across the cell membrane. As

Table I

Effects of various reagents on ghost shape change

Addition	Step I* crenate→disc	Step II** sphere→(crenate)→disc
2mM $MgCl_2$	-	-
2mM $MgCl_2$+2mMATP	+++	+++
2mM $MgCl_2$+1mMATP	+++	+++
2mM $MgCl_2$+0.5mMATP	++	++
2mM $MgCl_2$+0.1mMATP	-	-
2mM $MgCl_2$+2mM ADP	+	-
2mM $MgCl_2$+2mM AMP	-	-
2mM $MgCl_2$+2mM Pi	-	-
2mM $MgCl_2$+2mM PPi	-	-
2mM $MgCl_2$+2mM Na_2SO_4	-	-
2mM $HgCl_2$+2mM2'-DeoxyATP	+++	+++
2mM $MgCl_2$+2mM Cordycepin 5' monophosphate	-	+++
2mM $MgCl_2$+2mM CTP	-	+
2mM $MgCl_2$+2mM GTP	-	+
2mM $MgCl_2$+2mM UTP	-	+
2mM $MgCl_2$+2mM AMP-PNP	-	-
2mM $MgCl_2$+2mM AMP-PCP	-	-

-= Disc 0%
+= Disc 0-20%
++= Disc 20-80%
+++= Disc >80%

*) The ghosts, prepared with 10 mM Tris-Cl buffer, were suspended in the indicated solutions and their shapes were observed after incubation for 30 min at 37°C.

**) The ghosts, preincubated in 2 mM Mg-ATP for 30 min at 37°C, were washed once with 1 mM $MgCl_2$ and then suspended in the indicated solutions at 0°C and their shapes were immediately observed.

shown in Table 1, the physiological concentration of Mg-ATP is also necessary for shape change at the second step (very roughly, K0.5=0.5 mM) and the same concentration of other nucleotides, such as AMP, ADP, AMP-PNP or AMP-PCP, was also ineffective at the second step. In contrast to AMP, cordycepin 5'-monophosphate (deoxy AMP) was effective, and UTP, GTP or CTP was slightly effective, as listed in Table I. The ability of these ghosts to change into discocytes was lost within one hour if they were stored in 1 mM $MgCl_2$ solution at 0°C. The shape-change process, composed of 2 steps, is schematically presented in Fig. 2. The first step requires incubation at 37°C for 20 or 30 min, and the second one is a rapid change following the first step. The

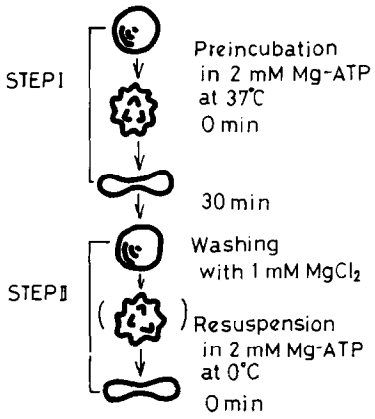

Fig. 2

Shape changes of human erythrocyte ghosts.

This is a schematic representation of the shape changes based on the dark-field light microscopic observations.

ghosts prepared from fresh erythrocytes in the presence of Mg-ATP at hemolysis immediately changed to discocytes after addition of Mg-ATP even at 6°C, coldroom temperature, but those prepared in the absence of Mg-ATP did not. Preincubation with Mg-ATP was necessary in the latter case. This phenomenon could not be explained by Sheetz and Singer (10), but can now be understood in terms of two steps, presumably one involving phosphorylation and the other involving ATP binding. When the ghosts were incubated with Mg-(γ-^{32}P)-ATP at 37°C for 30 min, the amount of membrane phosphorylation was 0.38 μmol per mg protein, whereas no significant phosphorylation was detected at 0°C. This result is consistent with the idea that some component in ghosts are phosphorylated during incubation at 37°C, before the shape change can occur. Spectrin (10,11), phosphoinositol (12) and diglyceride (13) have already been proposed as candidates for phosphorylation. Besides preincubation, the presence of Mg-ATP is necessary for shape

change at the second step, although the nucleotide specificity was not as strong as in the first step (Table I). As significant membrane phosphorylation was not detected at 0°C for 30 min, as described above, ATP binding to the membrane is presumably necessary at the second step, although a very small amount of hydrolysis of ATP cannot be excluded completely. Haley et al. showed that ATP bound to spectrin and band 5 by using a photo-affinity ATP analog (14). Therefore, ATP binding to some components in ghosts may occur and induce the shape change only when the ghosts are phosphorylated. The effect of Mg-ATP cannot be attributed to elimination of Ca^{2+} from the system, because the same phenomenon was observed even in the presence of calcium-EGTA buffer containing 10^{-5}-10^{-7}M free calcium ion [20 mM Tris-maleate buffer (pH 7.4) containing 1 x 10^{-3}M EGTA and 1 x 10^{-3} M $CaCl_2$ at various ratios, according to Ogawa (15)].

It remains to be determined which component is phosphorylated, to which component ATP is bound, and whether or not a very tiny amount of ATP hydrolysis is necessary for the shape change.

ACKNOWLEDGMENT
This study was supported by a grant in aid for Scientific Research (57612205) from the ministry of Education, Science and Culture of Japan.

REFERENCES

1. Sheetz, M.P. (1979) Biochim.Biophys.Acta 557, 122-134.
2. Tsukita, S., Tsukita, S. and Ishikawa, H. (1980) J.Cell.Biol. 85, 567-576.
3. Tsukita, S., Tsukita, S., Ishikawa, H., Sato, S. and Nakao, M. (1981) J.Cell Biol. 90, 70-77.
4. Cohen, C.M., Tyler, M.M. and Branton, D. (1980) Cell 21, 875-8u83.
5. Branton, D. (1981) Cell 24, 24-32.
6. Jinbu, Y., Sato, S., Nakao, T. and Nakao, M. (1982) Biochem.Biophy.Res.Commun. 104, 1087-1092.
7. Nakao, M., Nakao, T. and Yamazoe, S.(1969) Nature 187, 945-946.

8. Nakao, M., Nakao, T., Yamazoe, S. and Yoshikawa, H. (1961) J.Biochem. 49, 487-492.
9. Nakao, M., Nakao, T., Tatibana, M. and Yoshikawa, H. (1960) J.Biochem. 47, 694.
10. Sheetz, M.P. and Singer, S.J. (1977) J.Cell Biol. 73, 638-646.
11. Birchmeier, W. and Singer, S.J., (1977) J.Cell Biol. 73, 647-659.
12. Quist, E.E. and Reece, K.L.(1980)Biochem.Biophys.Res. Commun. 95, 1023-1030.
13. Fairbanks, G., Petel, V. and Dino, J. (1981) Scand.J.Clin.Lab.Invest. 41, 139-144.
14. Haley, B.E. and Hoffman, J.F. (1974) Proc.Nat.Acad.Sci.USA 71, 3367-3371.
15. Ogawa, Y. (1968) J.Biochem. 64, 255.