Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 681-685

# RELATIONSHIP BETWEEN MEMBRANE PROTEIN PHOSPHORYLATION AND INTRACELLULAR TRANSLOCATION OF CASEIN KINASE IN HUMAN ERYTHROCYTES

L. Bordin,* G. Clari,* B. Baggio,** G. Gambaro,** and V. Moret*

*Dipartimento di Chimica Biologica, Universita' di Padova, 35121 Padova (Italy)

**Istituto di Medicina Interna, Universita' di Padova, 35121 Padova (Italy)

Received July 1, 1994

The present paper shows that an increased phosphorylation of the membrane proteins, promoted by the okadaic acid (strong inhibitor of P-Ser/Thr-protein phosphatase(s)), is accompanied by a release of casein kinase from the membrane into cytosol.
Such an intracellular translocation might provide a feedback mechanism for the regulation of the casein kinase catalyzed phosphorylation of membrane proteins in the human erythrocytes. 

Casein kinase activity, the major responsible for the membrane protein phosphorylation in human erythrocytes, is distributed between the cytosol and the membrane.
In the normal fresh human erythrocytes about 40% of total cellular casein kinase activity is bound to the membrane structures, and the intracellular partitioning is under metabolic control.
In fact, the metabolic depletion, decreasing the content of ATP, 2,3-DPG (predominant metabolite) (1) and the level of membrane protein phosphorylation (2,3), promotes an increase of membrane-bound casein kinase (2,3) at expense of cytosolic counterpart (3).
These depletion effects are reversed by the subsequent metabolic repletion of the depleted cells (3), which, besides increasing the content of ATP, 2,3-DPG enhances also the phosphorylation level of membrane proteins (3) and promotes a release of casein kinase from the membrane into the cytosol (3).
The present research was carried out to understand whether the phosphorylation level of erythrocyte membrane proteins *per se* is the regulatory mechanism of casein kinase translocation.

Abbreviations: 2,3-DPG, 2,3-diphosphoglycerate; DMF, dimethylformamide; PMSF, phenylmethylsulphonylfluoride; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis.

0006-291X/94 $5.00

At this aim we used okadaic acid as a tool modifying the erythrocyte membrane protein phosphorylation level. In fact, okadaic acid is a well known strong inhibitor of P-Ser/Thr-protein phosphatase(s) (4), which, entering the erythrocytes, markedly increases the membrane protein phosphorylation without affecting the level of ATP (4) and, quite likely, of phosphorylated glycolytic metabolites.

## Materials and Methods

Human erythrocytes were prepared, as described in (5), by centrifugation (at 750xg for 3 min) of fresh blood from laboratory personnel and 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, 140 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 24 mM Glucose, 1 mM Adenosine, 100 μg/ml Streptomycin, 25 μg/ml Cloramphenicol).

Samples of packed red cells (1 ml), resuspended in 9 ml of the same buffer A (10% hematocrit) were incubated at 35°C for 1, 3 and 6 h respectively, in the presence of okadaic acid (1 μM, final concentration) dissolved in dimethylformamide (DMF), whereas the control samples were resuspended and incubated under the same conditions except that okadaic acid solution was replaced by the same volume of solvent (DMF).

After incubation the samples were washed once in the same buffer A and then hemolysed in 28 ml hypotonic buffer B (5 mM phosphate, pH 8, 0.03 mM PMSF, 0.02% $NaN_3$ and 30 μM vanadate).

The membranes were recovered by centrifugation (at 20000xg for 20 min) and washed twice in hypotonic buffer B and twice in 25 mM Tris-HCl buffer (pH 8) containing 0.03 mM PMSF, 0.02% $NaN_3$ and 30 μM vanadate.

### Endogenous phosphorylation of membrane proteins in the isolated ghosts.

Endogenous phosphorylation of membrane proteins was tested by separately incubating the white ghosts (80 μg proteins) from okadaic acid-treated and -untreated erythrocytes at 30°C for 5 min in 125 μl reaction mixture containing 100 mM Hepes buffer (pH 7.5), 10 μM vanadate, 10 mM $MgCl_2$ and 20 μM [$\gamma$-$^{32}$P]ATP ($6x10^6$ cpm/nmole). Incubation was stopped by addition of 2% SDS and 1% β-mercaptoethanol (final concentration) followed by 5 min treatment at 100°C as described in (3).

40 μg of solubilized membranes were analized by 0.1% SDS-10% PAGE according to Laemmli (6) as previously described (3).

After electrophoresis the slab gels were stained with Coomassie Blue according to (6). Dried gels were autoradiographed at -80°C.

### Assay of casein kinase activity in the membranes and cytosol.

The membrane-associated and cytosolic casein kinase activities were assayed by incubating 80 μg of white ghosts, and, respectively, 50 μl of hemolysate supernatant prepared from the okadaic acid-treated and -untreated erythrocytes (see above) at 30°C for 5 min in the presence of 0.65 mg/ml whole casein as exogenous substrate under the conditions above

described for the endogenous phosphorylation of membrane proteins in the isolated ghosts. The reaction was stopped as above described for the $^{32}P$-labeled membranes.

Half of reaction mixture was analyzed by SDS-PAGE followed by autoradiography as described in (3) and $^{32}P$-labeled bands of casein, identified by autoradiography, were excised from the gels and counted for radioactivity in a liquid scintillation counter.

Other methods. Miscellaneous

Protein content was determined according to (7).

[$\gamma$-$^{32}P$] ATP was purchased from Amersham International (U.K.); casein was prepared from commercial powder (Merck) as described in (8).

## Results and Discussion

As previously reported (9), when the intact human erythrocytes are incubated with [$^{32}P$]$P_i$ in the presence of okadaic acid, the Ser/Thr-phosphorylation of membrane proteins (mainly cytoskeletal spectrin and transmembrane band 3 protein) is markedly higher than in the control, because the okadaic acid, while unaffecting the casein kinase (by far the major responsible for the membrane protein Ser/Thr-phosphorylation) (5), strongly inhibits the P-Ser/Thr-protein phosphatase(s) (5).

Such an enhanced phosphorylation, decreasing the number of the remaining phosphate-acceptor sites, may account for the decreased membrane protein phosphorylation occurring when the membranes ("ghosts"), isolated from okadaic acid-treated human erythrocytes are incubated in the presence of [$\gamma$-$^{32}P$]ATP (fig. 1)

Fig. 1 shows the time-course of the membrane protein phosphorylation decrease occurring in the presence of [$\gamma$-$^{32}P$]ATP in the ghosts from erythrocytes treated with okadaic acid for varying lenghts of time.

What is more interesting is that the higher phosphorylation level in the okadaic acid-treated erythrocytes is accompanied by a release of casein kinase from the membrane into cytosol (fig. 2), which also may account for the lower phosphorylation occurring in their isolated ghosts (fig. 1) when incubated with [$\gamma$-$^{32}P$]ATP.

Fig. 2 shows the time-course of casein kinase translocation from membrane into cytosol, i.e. the membrane-bound casein kinase (fig. 2A) decreases while the cytosolic counterpart (fig. 2B) increases to the same extent.

The above results, together with those reported in previous papers (2,3,9), show that in the human erythrocytes a decrease of membrane protein phosphorylation (promoted by metabolic depletion (2,3)) is accompanied by an increase of membrane-bound casein kinase at expense of cytosolic counterpart, whereas an increase of membrane protein phosphorylation (promoted both by metabolic repletion of depleted erythroytes (3) and by treatment of normal erythrocytes with okadaic acid (9)) is accompanied by a release of casein kinase from membrane into cytosol.

In conclusion, the above results would suggest that in the human erythrocytes an increase of casein kinase-catalyzed membrane protein phosphorylation causes a translocation of casein

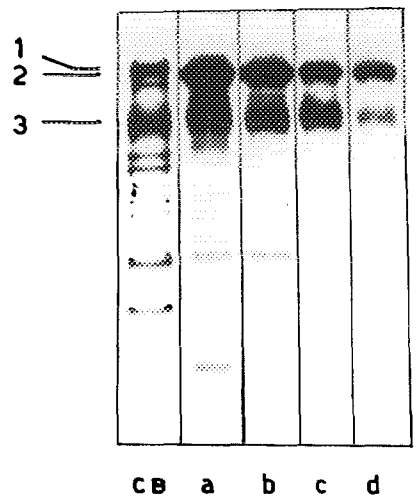


Fig. 1.

Autoradiograms showing the time-course of membrane protein phosphorylation occurring in the ghosts from okadaic acid-treated human erythrocytes.

Membranes (80 μg) from erythrocytes treated for 1 h (lane b), 3 h (lane c) and 6 h (lane d) with okadaic acid (1 μM) dissolved in DMF or with its solvent (DMF) (control) (lane a) were incubated at 30°C for 5 min in the presence of $MgCl_2$ and [$\gamma$-$^{32}$P]ATP and finally (40 μg protein) analyzed by SDS-PAGE as described in (3).

Lane CB shows the Coomassie Blue stained gel.

Autoradiograms were exposed for 22 h.

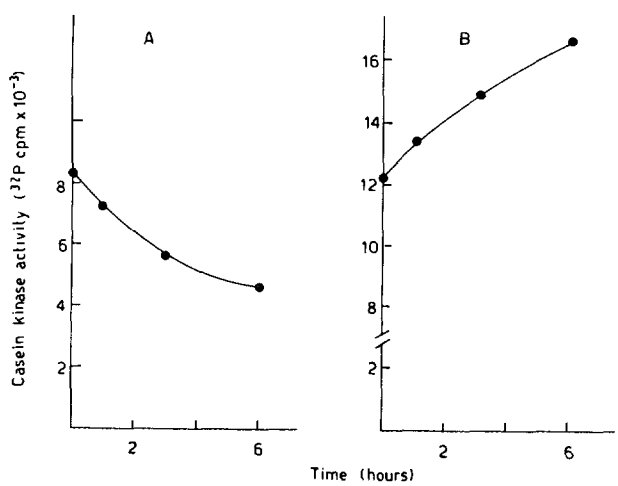


Fig. 2.

Casein phosphorylation by membranes (A) and by cytosol (B) from erythrocytes incubated 1, 3 and 6 h in the presence of okadaic acid (1 μM final concentration) dissolved in DMF.

The control was incubated in the presence of the solvent DMF.

kinase from membrane to cytosol, whereas a reverse translocation of casein kinase from cytosol to membrane is promoted by a dephosphorylation of membrane proteins.

In other words, a reversible translocation of casein kinase between membrane and cytosol may be regulated, through a feedback mechanism, by the phosphorylation state of membrane proteins.

It may be that the phosphorylation, introducing negatively charged phosphate groups in particular specific sites of the membrane proteins can remove, by electrostatic repulsion, the casein kinase from the membrane.

Such a repulsion mechanism might be involved also in the removal of casein kinase from isolated eryhtrocyte membranes (10) as well as from the "spectrin-casein kinase complex" (10) in solution, promoted by the binding of polyanionic 2,3-DPG to spectrin (10,11).

## Acknowledgments

This work has been supported by grants from the CNR Progetto Finalizzato (Invecchiamento).

Miss Carla Munari for the technical assistance, Mr. Giorgio Parajola for preparing the figures and Mr. Dario Spinello for supplying fresh blood from volunteers are gratefully acknowledged.

## References

1) Bursaux E., Hilly M., Bluze A. and Poyart C. (1984) Biochim. Biophys. Acta 777, 253-260.
2) Fairbanks G., Avruch J., Dino J.E. and Patel V.P. (1978) J. Supramol. Struct. 9, 97-112.
3) Clari G., Marzaro G. and Moret V. (1990) Biochim. Biophys. Acta 1023, 319-324.
4) Cohen P., Holmes C.F.B. and Tsukitani Y. (1990) Trends Biochem. Sci. 15, 98-102.
5) Bordin L., Clari G., Bellato M., Tessarin C. and Moret V. (1993) Biochem. Biophys. Res. Commun. 195, 723-729.
6) Laemmli U.K. (1970) Nature (London) 227, 680-685.
7) Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951) J. Biol. Chem. 193, 265-275.
8) Clari G., Michielin E. and Moret V. (1987) Biochem. Biophys. Res. Commun. 142, 587-594.
9) Baggio B., Bordin L., Clari G., Gambaro G. and Moret V. (1993) Biochim. Biophys. Acta 1148, 157-160.
10) Conway R.G. and Tao M. (1981) J. Biol. Chem. 256, 11932-11938.
11) Shaklai N., Benitez L. and Ranney H.M. (1978) Am. J. Physiol. 234, C36-43.