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Metabolic depletion effect on serine/threonine- and tyrosine-phosphorylations of membrane proteins in human erythrocytes

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The response of serine/threonine-phosphorylation of the major transmembrane protein (band 3) in human erythrocytes to the metabolic state of the cells is different from that exhibited by the tyrosine-phosphorylation of the same protein. Precisely, both serine- and tyrosine-phosphorylation are decreased during metabolic depletion of the erythrocytes. However, the depletion-induced tyrosine-phosphorylation decrease of band 3 is not reversed by the subsequent metabolic repletion of the depleted cells, being accompanied by an irreversible inactivation of both membrane-bound and cytosolic tyrosine-protein kinase(s). By contrast, the depletion-induced phosphoserine-dephosphorylation is reversed by the following repletion, being accompanied by a reversible translocation of casein kinase(s) between cytosolic and membrane compartments. A possible functional correlation between the serine-phosphorylation state of band 3 protein and the band 3-mediated anion transport across the membrane is discussed.

Introduction

The band 3 protein, the major transmembrane protein (95 kDa) in human erythrocytes, is known to display several functions; i.e. the anion transport across the membrane, mediated by its membrane-spanning segment and the binding of underlying cytoskeletal and cytosolic proteins (hemoglobin and certain glycolytic enzymes), mediated by its extended cytoplasmic domain (43 kDa) which protrudes from the membrane bilayer into cytoplasm.

The cytoplasmic domain has been found to be phosphorylated not only on Ser/Thr residues [1] but also on Tyr residues [2–10].

Various specific Ser/Thr- and Tyr-protein kinases [11–23] as well as P-Ser- and P-Tyr-protein phosphatases [24–29] have been found to be differently distributed between cytoplasm and membrane structures of human erythrocytes and differently affected in vitro by ionic environment, by 2,3-bisphosphoglycerate (2,3-DPG) and other phosphorylated metabolites.

These findings prompted us to investigate whether the Ser/Thr- and Tyr-phosphorylation state of band 3 are controlled by the metabolic state of the cells.

The results reported here show that the response of Tyr-phosphorylation of band 3 to the metabolic depletion is different from that exhibited by Ser-phosphorylation of membrane proteins (band 3 and spectrin).

Precisely, both Tyr- and Ser-phosphorylations of band 3 in the human erythrocytes are decreased by prolonged starvation. However, the depletion effect on the Tyr-phosphorylation state of band 3, being accompanied by an inactivation of Tyr-protein kinase(s), is not reversed by the subsequent metabolic repletion of the depleted cells. By contrast, the depletion effect on Ser-phosphorylation state is reversed by subsequent repletion. This suggests that it is due to the large decrease of ATP [30,31] and 2,3-DPG (predominant metabolite) [31], which would alter the dynamic balance between proteinkinase and protein phosphatase activities in such a way as to cause P-Ser-dephosphorylation.

The reversible nature of Ser-phosphorylation of band 3 suggests that it might be functionally correlated with the reversible change of anion transport which has been shown to occur during the metabolic depletion followed by repletion [31].

Methods and Materials

Human erythrocytes were prepared by centrifugation (at 750 × *g* for 3 min) of fresh blood (20 ml) collected from healthy donors. To minimize contamination by

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leucocytes and platelets, the packed red cells were washed three times by centrifugation in buffer A (25 mM Hepes buffer (pH 6.7), 1.5 mM $MgSO_4$, 90 mM KCl, 48.5 mM Na_2SO_4, 0.1 mM EGTA, 25 μg/ml chloramphenicol and 0.1 mg/ml streptomycin) and discarding the buffy coat and the upper third of packed red cell layer.

Phosphorylation of membrane proteins in intact human erythrocytes in the presence of [^{32}P]P_i

For ^{32}P-labelling of membrane proteins with [^{32}P]P_i, the erythrocytes (1.5 ml packed cells) were incubated (at a 10% hematocrit) in the buffer A for 4 h at 37 °C to deplete endogenous ATP stores and then allowed to incubate with [^{32}P]P_i for 16 h at 37 °C in the same buffer A containing glucose (12 mM), 1 mM adenosine and 0.4–0.5 mCi [^{32}P]P_i, with continuous gentle stirring.

After 16 h of equilibration, the red cells were washed three times with buffer A to remove [^{32}P]P_i, glucose and then resuspended in the buffer A (zero time of depletion period).

Metabolic depletion and subsequent repletion

The metabolic depletion was obtained by incubating the red cell suspension (at a 10% hematocrit) in buffer A (lacking glucose, adenosine) for 21 h at 37 °C with gentle stirring. After 21 h incubation (depletion period), 12 mM glucose and 1 mM adenosine were restored, thus allowing the metabolic repletion to occur for 7 h (repletion period).

Aliquots (5 ml) of red cell suspension (0.5 ml of packed cells) were withdrawn from incubation mixture at the indicated times (i.e., at zero time and after 21 h depletion and after 7 h repletion), washed five times in buffer A in the cold to remove excess of radioactivity and then hemolysed in 14 ml of hypotonic 5 mM phosphate buffer (pH 8) containing 0.02% NaN_3 and 0.03 mM phenylmethylsulphonyl fluoride (PMSF). The membranes were recovered by centrifugation (at 20000 × *g* for 20 min), washed twice in hypotonic lysis buffer and in 25 mM Tris buffer (pH 7.5) containing 0.03 mM PMSF and 0.02% NaN_3 and then solubilized by addition of 2% sodium dodecylsulfate (SDS) and 1% β-mercaptoethanol (final concentration), followed by a 5-min treatment at 100 °C, as described in Ref. 23.

40 μg of solubilized membranes were submitted to electrophoresis on 0.1% SDS/10% polyacrylamide slab gels, essentially according to Laemmli [32]. After electrophoresis, the slab gels were stained with Coomassie brilliant blue (CB), according to Laemmli [32], dried under vacuum, as previously described [23], and submitted to autoradiography at −80 °C with intensifying screens.

Endogenous phosphorylation of membrane proteins in the ghosts in the presence of [γ-^{32}P]ATP

The endogenous Ser- and Tyr-phosphorylation of membrane proteins as well as the casein kinase and Tyr-protein kinase activity assays reported below were performed in the presence of [γ-^{32}P]ATP in separate experiments using white ghosts and cytosol prepared from human erythrocytes that had been previously submitted to depletion and subsequent repletion under the same conditions as above described, except that radioactive [^{32}P]P_i was omitted.

At the times indicated in Tables I and II, aliquots of red cell suspension (0.5 ml of packed cells) were taken from incubation mixture and lysed under conditions above described and the membranes were recovered by centrifugation.

A part (3 ml) of red hemolysate supernatant was dialyzed overnight against 25 mM Tris buffer (pH 7.5) containing 0.03 mM PMSF and 0.02% NaN_3, prior to use for the casein kinase assay.

The remaining hemolysate supernatant (approx. 10 ml) was brought to 60% saturation with ammonium sulfate and the precipitate was dissolved in (0.7 ml) and dialyzed overnight against buffer A previously described [9], prior to use for the Tyr-protein kinase activity assay.

The endogenous Ser-phosphorylation of membrane proteins was tested by incubating the white ghosts (80 μg protein) at 30 °C for 3 and 6 min in 125 μl reaction mixture containing 100 mM Tris-HCl buffer (pH 7.5), 10 μM vanadate, 10 mM $MgCl_2$ and 5 μM [γ-^{32}P]ATP ($6 \cdot 10^6$ cpm/nmol).

Tyr-phosphorylation of membrane proteins was tested under the conditions followed for Ser-phosphorylation, except that 50 μM EDTA was added and $MgCl_2$ was replaced by 5 mM $MnCl_2$.

Incubation was stopped by addition of 2% SDS and 1% β-mercaptoethanol (final concentration) followed by a 5-min treatment at 100 °C, as described in Ref. 23.

40 μg of solubilized membranes were subjected to electrophoresis in 0.1% SDS/10% polyacrylamide slab gels, essentially according to Laemmli [32], as previously described [9]. After electrophoresis, the slab gels were stained with Coomassie brilliant blue according to Laemmli. Some gels were treated in a 2 M NaOH solution at 55 °C for 1 h and fixed again. Dried gels were autoradiographed at −80 °C with intensifying screens.

Casein kinase assay

Membrane-associated and cytosolic casein kinase were assayed by incubating 80 μg of white ghosts and, respectively, 50 μl of dialyzed hemolysate supernatant in the presence of 0.65 mg/ml whole casein as substrate, under the conditions above described for the endogenous Ser-phosphorylation of membrane proteins in the isolated ghosts.

After 3 min incubation, the reaction was stopped as previously described [9]. Half of reaction mixture was

subjected to electrophoresis in 1% SDS/10% polyacrylamide slab gels to separate the ^{32}P-labelled casein from the other contaminating endogenous ^{32}P-labelled proteins. The corresponding ^{32}P-labelled band, identified by autoradiography, was excised from the gels and counted for radioactivity in a liquid scintillation counter.

Tyr-protein kinase assay

Membrane-bound and cytosolic Tyr-protein kinase were assayed by incubating at 30 °C for 5 min 80 μg of white ghosts and, respectively, 50 μl of dialyzed ammonium sulfate fraction (60% saturation) prepared from hemolysate-supernatant, in the presence of 8 μg poly(Glu,Tyr) 4:1 as substrate under the same conditions followed for the endogenous Tyr-phosphorylation of membrane proteins.

The incubation was stopped as previously described [9] and half of reaction mixture was subjected to electrophoresis in 1% SDS/10% polyacrylamide slab gels to separate the ^{32}P-labelled polymer poly(Glu,Tyr) 4:1 (molecular mass ranging from 20 to 50 kDa) from Tyr-phosphorylated band 3 protein (95 kDa). The corresponding diffuse ^{32}P-Tyr-labelled band, identified by autoradiography, was excised from the alkali-treated gels and counted for radioactivity in a liquid scintillation counter.

Other methods; miscellaneous

Protein content was determined according to Ref. 33.

Synthetic polymer poly(Glu,Tyr) 4:1 (molecular mass ranging from 20 kDa to 50 kDa) was purchased from Sigma; [γ-^{32}P]ATP and [^{32}P]P_i from Amersham International (U.K.); cellulose thin-layer plates (DC-Fertigplatten CE 20 × 20 cm) were from Riedel de Haen-Seelze (F.R.G.).

Whole casein was prepared from commercial powder (Merck) as previously described [14].

Results

Endogenous Ser- and Tyr-phosphorylation of membrane proteins in the isolated ghosts

When the ghosts prepared from fresh human erythrocytes after prolonged metabolic depletion and after the subsequent repletion were incubated with [γ-^{32}P]ATP in the presence of Mg^{2+} (as described in Methods and Materials) and then analyzed by SDS-PAGE, the protein phosphorylation patterns reported in Fig. 1 were obtained.

The ^{32}P-labelling in the presence of Mg^{2+} is due to the alkali-labile phosphorylation of serine residues, as indicated by its disappearance in the alkali-treated gels (lanes a′–f′). Relatively negligible phosphorylation of threonine residues was detectable under these conditions [9,10].

Fig. 1 clearly shows that the Ser-phosphorylation of spectrin (band 2) and of band 3 in the ghosts from the depleted erythrocytes (lanes b, e) is higher as compared with controls, i.e., ghosts prepared from fresh erythrocytes just prior to depletion (lanes a, d).

Such a depletion-induced change of Ser-phosphorylation is reversed by subsequent repletion in presence of glucose, the ^{32}P-labelling being decreased (lanes c, f) towards the values of the controls.

When the ghosts were incubated in the presence of 5 mM Mn^{2+}, (more effective than Mg^{2+} in activating the endogenous Tyr-protein kinase) the phosphorylation pattern of membrane proteins (Fig. 2) is different from that in the presence of Mg^{2+}.

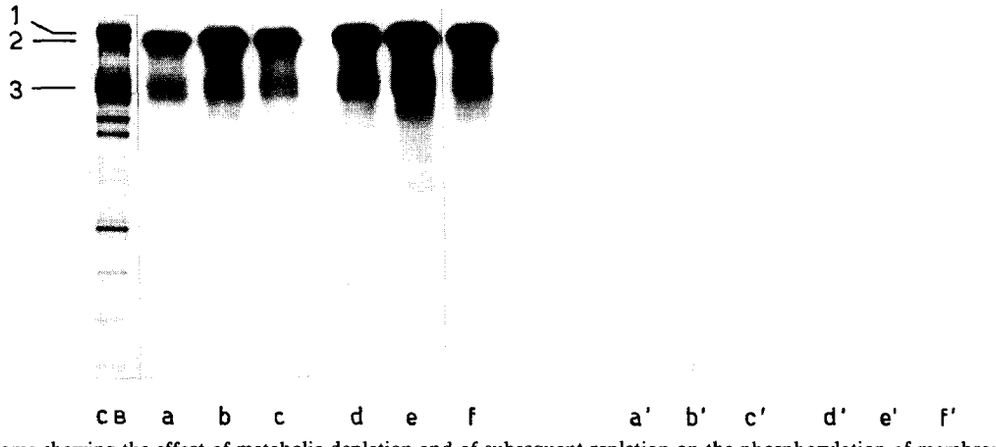

Fig. 1. Autoradiograms showing the effect of metabolic depletion and of subsequent repletion on the phosphorylation of membrane proteins in the ghosts incubated for 3 min (lanes a–c) and for 6 min (lanes d–f) with [γ-^{32}P]ATP in the presence of Mg^{2+} (10 mM). The ghosts were prepared from fresh human erythrocytes: just prior to depletion (lanes, a, d), after 21 h depletion (lanes b, e) and after 7 h repletion of the depleted cells (lanes c, f). Lanes a′–f′ show the alkali-treated gels corresponding to the alkali-untreated gels a–f. Lane CB shows the Coomassie blue-stained gel. Autoradiograms were exposed for 24 h.

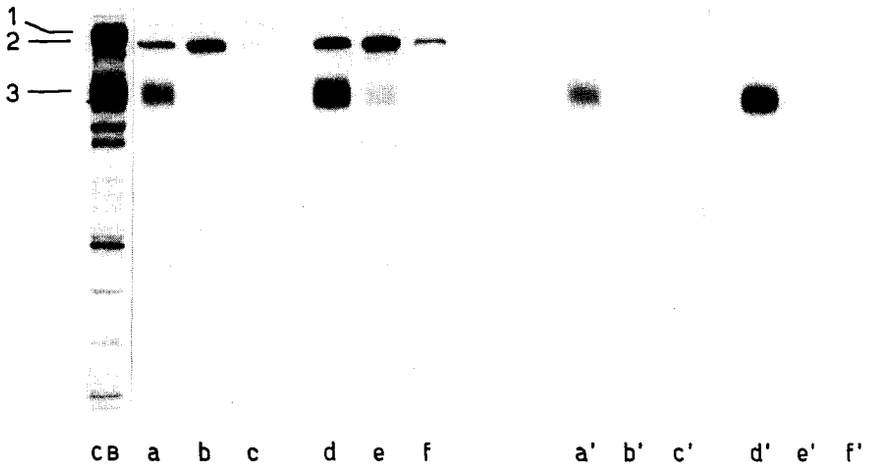

Fig. 2. Autoradiograms showing the effect of metabolic depletion and of subsequent repletion on the phosphorylation of membrane proteins in the ghosts incubated for 3 min (lanes a–c) and for 6 min (lanes d–f) with [γ-^{32}P]ATP in the presence of 5 mM Mn^{2+}. The ghosts were prepared from human erythrocytes: just prior to depletion (lanes a, d), after 21 h depletion (lanes b, e) and after 7 h repletion of the depleted cells (lanes c, f). Lanes a′–f′ show the alkali-treated gels corresponding to the alkali-untreated gels a–f. Autoradiograms were exposed for 44 h. Lane CB shows the Coomassie blue-stained gel.

Under these conditions, the ^{32}P-labelling of spectrin (band 2) is again due to the alkali-labile phosphorylation of Serine residues, as indicated by its disappearance in the alkali-treated gels (lanes a′–f′), whereas the ^{32}P-labelling of band 3 involves also the phosphorylation of tyrosine residues, as indicated by its alkali-stability in the alkali-treated gels (lanes a′–f′) and confirmed by the [^{32}P]phosphoamino acid analysis of the alkali-treated ^{32}P-labelled membranes [9,10].

Fig. 2 shows that also in the presence of Mn^{2+}, as in the presence of Mg^{2+} (Fig. 1), the Ser-phosphorylation of spectrin (lanes a–f) increases in the ghosts from depleted erythrocytes (lanes b, e) and the change is reversed in the ghosts from repleted erythrocytes (lanes c, f).

By contrast, the Tyr-phosphorylation of band 3 (lanes a′–f′) decreases in the ghosts of depleted cells (lanes b′, e′) and is not restored by repletion (lanes c′, f′), thus suggesting that Tyr-protein kinase and/or its activator(s) are irreversibly inactivated during the depletion. This is confirmed by the observation that in the intact erythrocytes both the membrane-associated and the cytosolic Tyr-protein kinases activities (assayed on poly(Glu,Tyr) 4:1) are decreased by depletion in an irreversible fashion, being not restored by repletion (Table I).

On the contrary, the membrane-associated casein kinase activity (the major responsible for spectrin phosphorylation) (assayed on whole casein) in the ghosts from depleted cells is enhanced at expense of the cytosolic counterpart (Table II).

Moreover, such a depletion effect on the partitioning of casein kinase between membrane and cytosolic compartments is reversed by repletion. It is noteworthy that the total cellular casein kinase activity does not change markedly during depletion followed by repletion.

TABLE I

Effect of metabolic depletion and of the following repletion on the cytosolic and membrane-bound Tyr-protein kinase activity

The Tyr-protein kinase activity was assayed (as described in Methods and Materials) in the cytosol and in the ghosts prepared from 10 μl of packed human erythrocytes: just prior to depletion (control), after 21 h depletion and after 7 h repletion of the depleted cells. The gels prior to autoradiography were alkali-treated, as described in Methods and Materials. The Tyr-protein kinase activity is expressed as counts/min incorporated into poly(Glu,Tyr) 4:1, under conditions described in Methods and Materials.

	Tyr-protein kinase activity (expressed as cpm incorporated into poly(Glu,Tyr) 4:1)	
	membranes	cytosol
Control (zero time)	3100	7800
Depleted (21 h)	570	1810
Repleted (7 h)	510	1310

TABLE II

Effect of the metabolic depletion and of the following repletion on the intracellular distribution of casein kinase activity

The casein kinase activity was assayed (as described in Methods and Materials) in the cytosol, and, respectively, in the ghosts, prepared from 10 μl of packed erythrocytes: just prior to depletion (control), after 21 h depletion and after 7 h repletion of the depleted cells. The casein kinase activity is expressed as counts/min incorporated into casein under conditions described in Methods and Materials.

	Casein kinase activity (expressed as cpm incorporated into whole casein)	
	membranes	cytosol
Control (zero time)	7050	28050
Depleted (21 h)	13200	17100
Repleted (7 h)	9000	22300

This suggests that the higher membrane protein Ser-phosphorylation displayed by the isolated ghosts from depleted cells when incubated in the presence of added [γ-^{32}P]ATP (Fig. 1), might be accounted for by the parallel reversible increase of membrane-associated casein kinase activity.

However, it may reflect also an increased phosphate-acceptor capacity of membrane protein substrates due to their progressive P-Ser-dephosphorylation occurring during the depletion in their parent erythrocytes prior to the ghost-isolation procedure.

This view is supported by the experiments with intact erythrocytes where the phosphorylation state of membrane proteins is depending also on cytosolic protein kinases and protein phosphatases, taking into account that by far most of the P-Ser-protein phosphatase activity is located in the cytosol [24–26].

Phosphorylation of membrane proteins in intact erythrocytes

Fig. 3 shows the SDS-PAGE patterns of ^{32}P-labelled membrane proteins obtained from fresh human erythrocytes after 16 h equilibration with [^{32}P]P_i in the presence of glucose (lane a) and after the following prolonged metabolic depletion by removal of glucose (Fig. 3, lane b), as described in Methods and Materials.

The ^{32}P-labelling of membrane proteins in the depleted cells (lane b) was much lower than that in the controls, i.e., ^{32}P-labelled membrane proteins prepared from erythrocytes just prior to depletion (lane a), although the specific radioactivity of endogenous [^{32}P]ATP has been found to be markedly enhanced by depletion [34].

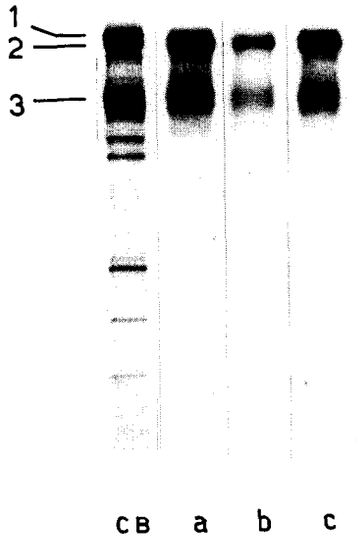

Fig. 3. Autoradiograms showing the effect of metabolic depletion and of the subsequent repletion on the membrane protein phosphorylation in intact human erythrocytes incubated with [^{32}P]P_i in the presence of Mg^{2+}. The erythrocytes, after metabolic equilibration with [^{32}P]P_i, were depleted and subsequently repleted under the conditions described in Methods. The ^{32}P-labelled membranes were prepared from the erythrocytes: just prior to depletion (lane a), after 21 h depletion (lane b) and after 7 h repletion of the depleted cells (lane c). Autoradiograms of the alkali-untreated gels were exposed for 44 h. Lane CB shows the Coomassie blue-stained gel.

This depletion effect is reversed by the subsequent repletion (lane c), thus suggesting that it is likely a consequence of the depletion-induced large decrease of ATP and 2,3-DPG content [30,31,34] which, besides reducing the supply of phosphoryl donor for the kinase reaction, may activate the cytosolic P-Ser-protein phosphatase by removing 2,3-DPG, ATP and other phosphorylated metabolites which, at physiological concentrations, have been shown to strongly inhibit this phosphatase activity in vitro [13,24,25].

Discussion

In conclusion, the above results indicate that in the human erythrocytes the response of the Tyr-phosphorylation of band 3 protein to the metabolic depletion is different from that exhibited by Ser-phosphorylation of membrane proteins.

Both Ser-phosphorylation and Tyr-phosphorylation of band 3 are progressively decreased by prolonged metabolic depletion. However, the depletion-induced P-Tyr-dephosphorylation of band 3, being accompanied by inactivation of both membrane-associated and cytosolic Tyr-protein kinase(s) or by destroying of some unidentified Tyr-protein kinase activator(s), is not reversed by the subsequent metabolic repletion. By contrast, the P-Ser-dephosphorylation of band 3 is accompanied by an increase of membrane-bound casein kinase activity (the major responsible for the Ser-phosphorylation). In addition, it is reversed, like the membrane binding of the casein kinase, by the subsequent repletion, as already shown for the dephosphorylation of spectrin [30].

This indicates that the reversible Ser-phosphorylation decrease is due to the large depletion of ATP and 2,3-DPG [30,31,34] which causes an imbalance between the Ser-phosphorylation (reduced by the poor ATP supply) and the prevailing P-Ser-dephosphorylation presumably activated by the removal of 2,3-DPG and other phosphorylated metabolites which, at physiological concentration, strongly inhibit the P-Ser-protein phosphatase in vitro [13,24,25]. The resulting P-Ser-dephosphorylation, besides increasing the number of phosphate-acceptor sites, might promote also the membrane binding of casein kinase, thus accounting for the higher Ser-phosphorylation occurring in the isolated ghosts from depleted cells when incubated in the presence of added 5 μM [γ-^{32}P]ATP.

The depletion effect on both the Ser-phosphorylation and the binding of casein kinase to the membranes is reversed by the subsequent metabolic repletion which, partially restoring the ATP, 2,3-DPG pools, restores

also the phosphorylation of membrane proteins and concomitantly the partial release of membrane-bound casein kinase into the cytosol. However, the reversible translocation of casein kinase between membrane and cytosol might be modulated by intracellular 2,3-DPG level also through a direct mechanism, 2,3-DPG being able to dissociate the casein kinase from spectrin-kinase complex in vitro [19]. The above findings raise the question of their biological significance.

In this context, it seems to us of interest to point out that:

(1) The prolonged metabolic depletion, which causes P-Ser-dephosphorylation of band 3, has been found [31] to decrease also the band 3-mediated anion transport across the erythrocyte membrane;

(2) the subsequent metabolic repletion of the depleted erythrocytes, which restores the Ser-phosphorylation of band 3, has been found [31] to restore also the anion transport.

Such a parallelism between the Ser-phosphorylation state of band 3 and the band 3-mediated anion transport across the membrane, although not necessarily implying a cause-effect relationship, would support the idea [31,35,36] that band 3-mediated anion transport across the membrane and the Ser-phosphorylation-dephosphorylation of band 3 may be functionally correlated.

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