Characterization of the hypertonically induced tyrosine phosphorylation of erythrocyte band 3

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Human erythrocyte band 3 becomes rapidly phosphorylated on tyrosine residues after exposure of erythrocytes to hypertonic conditions. The driving force for this phosphorylation reaction seems to be a decrease in cell volume, because (1) changes in band 3 phosphotyrosine content accurately track repeated changes in erythrocyte volume through several cycles of swelling and shrinking; (2) the level of band 3 phosphorylation is independent of the osmolyte employed but strongly sensitive to the magnitude of cell shrinkage; and (3) exposure of erythrocytes to hypertonic buffers under conditions in which intracellular osmolarity increases but volume does not change (nystatin-treated cells) does not promote an increase in tyrosine phosphorylation. We hypothesize that shrinkage-induced tyrosine phosphorylation results either from an excluded-volume effect, stemming from an increase in intracellular crowding, or from changes in membrane curvature that accompany the decrease in cell volume. Although the net phosphorylation state of band 3 is shown to be due to a delicate balance between a constitutively active tyrosine phosphatase and constitutively active tyrosine kinase, the increase in phosphorylation during cell shrinkage was demonstrated to derive specifically from an activation of the latter. Further, a peculiar inhibition pattern of the volume-sensitive erythrocyte tyrosine kinase that matched that of $p72^{syk}$, a tyrosine kinase already known to associate with band 3 *in vivo*, suggested the involvement of this kinase in the volume-dependent response.

INTRODUCTION

The anion-exchange protein, band 3, is generally recognized as the major substrate *in situ* for human erythrocyte protein tyrosine kinases [1–4]. After exposure of erythrocytes to phosphatase inhibitors, tyrosine phosphorylation of band 3 has been shown to increase precipitously as phosphates are transferred to multiple tyrosine residues along the polypeptide's backbone. Although minor phosphorylation sites have been noted in the membrane-spanning and interdomain sequences of band 3, the preferred phosphorylation sites seem to be at the extreme N-terminus of the cytoplasmic domain, primarily on Tyr-8 [5]. At least two protein tyrosine kinases have been shown to phosphorylate band 3 *in vitro*, $p72^{syk}$ and $p53/56^{lyn}$ [4,6]; both are relatively active in mature erythrocytes [4]. In fact, not only does $p72^{syk}$ co-immunoprecipitate with band 3 from whole erythrocytes [4], but erythrocyte band 3 still serves as the most widely used substrate for the study of $p72^{syk}$ and related kinases from non-erythroid cells [7,8].

The physiological function of the tyrosine phosphorylation of erythrocyte band 3 is not fully understood and might, in fact, depend on the site on band 3 that is derivatized. Thus phosphorylation of Tyr-8, a residue within the glycolytic-enzyme-binding sequence on band 3 [9], has been shown to inhibit enzyme binding [3]. Consequently, the enhancement of tyrosine phosphorylation has frequently been proposed to modulate glycolysis in mature erythrocytes [3,10–12], although other interpretations of these data have also been offered [13]. Stimulants of band 3 tyrosine phosphorylation, such as pervanadate, have also been shown to promote erythrocyte shape changes (e.g. echinocytosis), leading to the hypothesis that one or more band 3 phosphorylation events could have an effect on erythrocyte morphology [14]. However, because the same stimulants that enhance band 3 phosphorylation can also inhibit the aminophospholipid translocase and several ion transporters [15–17], the above interpretation is by no means unique. In addition, tyrosine phosphorylation of band 3 could be involved in subtle regulatory processes not yet identified, or alternatively the phosphorylation events could simply represent vestigial reactions that were once important during erythroid development but no longer function in the mature erythrocyte. Although further research will obviously be required to resolve these several possibilities, the observations that tyrosine phosphorylation of band 3 is regulated by variables that change in mature erythrocytes (e.g. 2,3-diphosphoglycerate, H_2O_2, Mg^{2+}, Ins(4,5)P_2 and pH [2,18–21]) and that band 3 phosphorylation is a two-stage hierarchical process [6] that induces a structural change in the protein [22] argue that phosphorylation could perform a dynamic function in the circulating erythrocyte.

We observed recently that a decrease in erythrocyte volume was invariably accompanied by a concomitant increase in band 3 tyrosine phosphorylation [23]. In an effort to understand this possible functional linkage, in the present study we have examined the causal relationship between tyrosine phosphorylation, volume decrease and changes in erythrocyte morphology. We have also explored whether the increases in band 3 tyrosine phosphorylation result from the induction of a kinase or the suppression of a phosphatase, and are brought about by the modification of cell volume, or alternatively by the change in osmolarity of the cytosol.

EXPERIMENTAL

Materials

Nitrocellulose membranes were obtained from Sartorius; α-chymotrypsin (bovine pancreas), Nonidet P40 and anti-(band 3) monoclonal antibody (clone B-III139) were purchased from

Abbreviations used: cdb3, cytoplasmic domain of band 3; GST, glutathione S-transferase; Ht, haematocrit.

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Sigma Chemical Co.; anti-phosphotyrosine monoclonal antibody (clone 4G10) was from UBI; rabbit anti-(mouse IgG) (H+L), herbimycin A, tyrphostin A25, genistein and staurosporine were obtained from Calbiochem; nystatin was from Fluka; horseradish peroxidase-conjugated goat anti-(mouse IgG) (H+L) was from Bio-Rad Laboratories; and enhanced chemiluminescence Western blotting detection reagents were purchased from Amersham Life Science. GSH–Sepharose was from Pharmacia; Grace's insect medium was from Gibco-BRL. Other reagents were of analytical grade.

Erythrocyte treatments

Fresh blood from normal volunteers was collected and filtered on cellulose as previously described [23]. Cells were washed with 10 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl and 2 mM $MgCl_2$ (referred to below as Tris buffer). For some applications, portions of the washed, packed erythrocytes were resuspended at 10% haematocrit (Ht) in Tris buffer containing different concentrations of salts or other osmolytes to obtain solutions of different osmolarity, as described in the text. Before and after each incubation, a 50 μl aliquot of suspension was used to evaluate the Ht after centrifugation at 6800 ***g*** for 5 min at room temperature in an ALC4223 microhaematocrit centrifuge. When desired, staurosporine was also added to the 10% Ht erythrocyte suspensions from a 3 mM stock solution in DMSO to a final concentration of 3–6 μM. To increase the intracellular osmolarity of the erythrocytes under isovolumetric conditions, the following procedure was adopted [24,25]. Erythrocytes at 5% Ht in high-potassium buffer [10 mM potassium phosphate/12 mM NaCl/135 mM KCl/25 mM sucrose (pH 7.4), approx. 335 m-osM] were incubated in the presence of 30 μg/ml nystatin (added from a freshly prepared 25 mM stock solution in DMSO) for 10 min at 4 °C. The cells were then rapidly sedimented at 4 °C; 80% of the original volume of suspension was then discarded as cell-free supernatant. The cells were resuspended in the remaining supernatant (final Ht 25%). A final osmolarity of 475 m-osM was then attained by three separate additions of 600 m-osM high-potassium buffer (made hypertonic with KCl) containing 80 μg/ml nystatin, each followed by a 5 min incubation of the suspension at 4 °C. The final nystatin concentration was 60 μg/ml in a 10% Ht cell suspension. The cells were then washed four times with 475 m-osM nystatin-free high-potassium buffer containing 0.3% BSA at 37 °C, resuspended in the same buffer, then incubated for 15 min at 37 °C. The microhaematocrit of the suspensions was monitored at each step. Additional cell suspensions were treated either with hypertonic potassium buffer in the absence of nystatin or with isotonic potassium buffer, as positive and negative controls respectively. Cells were then processed for SDS/PAGE and immunodetection of phosphotyrosine.

Chymotryptic treatment of whole erythrocytes was performed as described in [23].

Electrophoresis and immunoblotting

The phosphotyrosine content of erythrocyte membrane proteins was assayed after SDS/PAGE by the method of Laemmli [26] and Western blotting [27] of erythrocyte samples, as previously described [23]. Films exposed to chemiluminescence were digitized and the bands integrated with NIH-Image software.

Expression and purification of recombinant proteins

Recombinant baculovirus expressing the catalytic domain of murine $p72^{syk}$ ($p35^{syk}$), corresponding to residues 326–629 of the tyrosine kinase, was a gift from Dr. Robert L. Geahlen (Purdue University, West Lafayette, IN, U.S.A.). The catalytic domain was expressed as a glutathione S-transferase (GST) fusion protein with a thrombin cleavage site between GST and the kinase domain [28]. The recombinant baculovirus was amplified, then titred by plaque-assay, in insect cells (line SF9) grown in stationary culture in Grace's insect medium containing 10% (v/v) fetal calf serum. The high-titre recombinant baculovirus suspension was then used to infect insect cells grown in suspension in spinning flasks at 28 °C in Grace's insect medium in accordance with standard protocols [29]. At approx. 60 h after infection, the cells were harvested, divided into aliquots (approx. 10^8 cells per aliquot) and frozen at −80 °C. Purification of GST–$p35^{syk}$ was performed by lysing approx. 10^8 cells with 1 ml of 50 mM Tris/HCl (pH 7.6)/150 mM NaCl/1 mM EDTA/1% (v/v) Triton X-100/20 μg/ml PMSF/2 μg/ml leupeptin/2 μg/ml pepstatin/1 mM dithiothreitol. The clarified supernatant was incubated with GSH–Sepharose for 1 h at room temperature, then washed extensively with the same buffer without Triton X-100. To cleave $p35^{syk}$ from the fusion protein, thrombin (5 NIH units/ml) was added and the mixture was incubated for up to 3 h at room temperature. The kinase was recovered from the resin by washing with the same buffer.

Expression and purification of the cytoplasmic domain of band 3 (cdb3) were conducted as described previously [30].

Morphological analysis of erythrocytes by scanning electron microscopy

Samples subjected to both isotonic and non-isotonic treatment were fixed for 2 h at room temperature by immersion in 1.4% (v/v) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, plus sufficient NaCl to match the osmolarity of the treatment. Erythrocytes were post-fixed for 1.5 h in 1% (w/v) osmium tetroxide in 0.1 M sodium cacodylate, pH 7.4. The samples were dehydrated by immersion into serial ethanol concentrations [50–100% (v/v)], air-dried, fixed with a conductive tape on aluminium stubs and covered with a gold layer with a Sputter Coater Edwards S 150 A. The electron microscope was a Cambridge Stereoscan 250 operating at 20 kV.

RESULTS

Time course and osmolarity dependence of cell volume and band 3 phosphorylation changes after hypertonic treatment of erythrocytes

In a previous study it was observed that erythrocyte shrinkage was accompanied by an increase in the tyrosine phosphorylation of band 3 [23]. In an attempt to understand the source and consequences of this phosphorylation, we have examined the kinetics and dependence of the process on the composition and concentration of osmolytes in the medium. Whereas the decrease in cell volume brought about by the increase in extracellular osmolarity with non-penetrating osmolytes is virtually instantaneous (as is generally accepted owing to the high permeability of erythrocyte membrane to water [31]), Figure 1(A) shows that the increase in band 3 tyrosine phosphorylation induced by hypertonic NaCl reached a plateau only after 15 min in 600 m-osM buffer [the scanning data, corresponding to the intensity of signal on band 3 in Figure 1(A) were as follows, in arbitrary units of band 3 tyrosine phosphorylation and from lane 1 to lane 7: 0, 306, 689, 947, 994, 874, 981]. These results suggest that tyrosine phosphorylation is not required for cells to shrink osmotically, but rather that phosphorylation might be promoted by the decrease in cell volume.

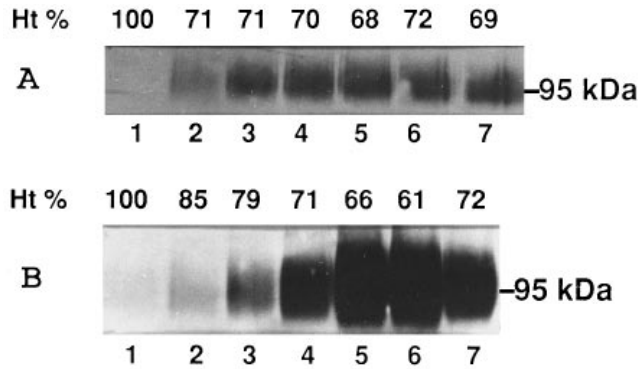

Figure 1 Tyrosine phosphorylation of band 3 in human erythrocytes treated with hypertonic buffer

(**A**) Erythrocytes (final Ht 10%) were incubated at 37 °C in Tris buffer containing sufficient NaCl to reach 600 m-osM for 1 min (lane 2), 5 min (lane 3), 10 min (lane 4), 15 min (lane 5), 30 min (lane 6) and 45 min (lane 7). Lane 1, control cells incubated for 45 min in isotonic buffer. (**B**) Erythrocytes (final Ht 10%) were incubated at 37 °C for 15 min in Tris buffer containing sufficient NaCl to reach 310 m-osM (lane 1), 400 m-osM (lane 2), 500 m-osM (lane 3), 600 m-osM (lane 4), 900 m-osM (lane 5), 1200 m-osM (lane 6) and 1500 m-osM (lane 7). Similar amounts of band 3, as tested by immunoblotting with anti-(band 3) monoclonal antibody, were present in all samples. Ht% is defined as the Ht of the erythrocyte suspension expressed as a percentage of the Ht of the control. After incubation, an aliquot of suspension was diluted with 9 vol. of SDS/PAGE sample buffer and treated at 60 °C for 15 min. In each well, 2 μl equivalent of packed cells was loaded. After SDS/PAGE and transfer to nitrocellulose, phosphotyrosine residues were revealed by immunodetection with anti-phosphotyrosine monoclonal antibody and enhanced chemiluminescence.

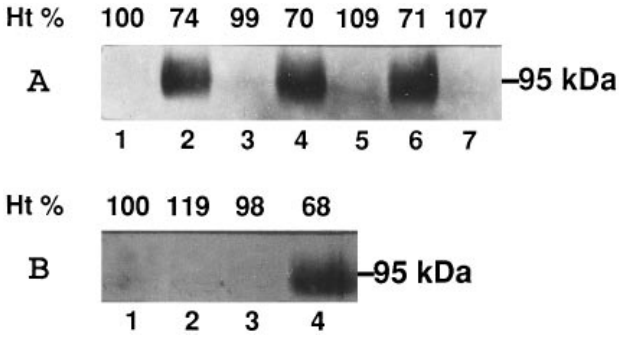

Figure 2 Reversibility of the hypertonicity-induced tyrosine phosphorylation of band 3 in human erythrocytes and the effect of hypotonic treatment

(**A**) Lane 1, control cells; lane 2, erythrocytes (final Ht 10%) incubated at 37 °C for 15 min in Tris buffer containing sufficient NaCl to reach 600 m-osM; lane 3, the same cells resuspended in isotonic Tris buffer at 37 °C for 15 min. The hypertonic/isotonic cycle was repeated twice: erythrocytes after the second (lane 4) and third (lanes 6) incubations in the hypertonic buffer; erythrocytes after the second (lane 5) and third (lanes 7) incubations in the isotonic buffer. (**B**) Lane 2, erythrocytes (final Ht 10%) incubated at 37 °C for 15 min in Tris buffer containing sufficient NaCl to reach 220 m-osM; lane 3, the same cells resuspended at 37 °C for 15 min in isotonic Tris buffer; lane 4, erythrocytes (final Ht 10%) incubated at 37 °C for 15 min in Tris buffer containing sufficient NaCl to reach 600 m-osM. Lane 1, control cells. Ht% is defined as the Ht of the erythrocyte suspension expressed as a percentage of the Ht of the control. Samples were processed as described in the legend to Figure 1.

In an effort to explore further the possible induction of band 3 tyrosine phosphorylation by cell shrinkage, erythrocytes were subjected to a series of hypertonic buffers, assayed for cell volume in a microhaematocrit centrifuge and evaluated for band 3 phosphorylation by immunoblotting. As displayed in Figure 1(B), band 3 phosphorylation is relatively insensitive to small decreases in volume (i.e. at relative cell volumes above 79% of normal), but is markedly activated when cell volumes fall to less than 70% of normal. Maximum stimulation is seen when erythrocyte volume is only 61–66% of unmodified cells. In contrast, at very high osmolarity (1500 m-osM; Figure 1B, lane 7), at which the relative cell volume actually increases again (probably owing to mechanically induced ion leakage), band 3 tyrosine phosphorylation also decreases proportionally. Because the substitution of hypertonic buffers containing potassium rather than sodium salts, or the replacement of ionic solutes with sucrose, did not change any of the above responses (results not shown), we hypothesize that volume decrease itself promotes the observed increase in band 3 tyrosine phosphorylation.

A further test of the possible relationship between cell volume decrease and band 3 tyrosine phosphorylation was executed by cycling the same cell suspension several times between isotonic and hypertonic conditions, and then sampling cells for tyrosine phosphorylation after each transition (Figure 2A). As expected, initial exposure to hypertonic conditions promoted the phosphorylation of band 3 (compare lanes 1 and 2). More importantly, a return to isotonicity led to rapid dephosphorylation (Figure 2A, lane 3), which could subsequently be reversed by restoration of hypertonic conditions (lane 4). Further repetition of the same cycle yielded identical changes in band 3 phosphorylation, demonstrating that the sensory mechanism that controls the volume-regulated band 3 phosphorylation is repeatedly reversible. The results also demonstrate that both tyrosine kinases and tyrosine phosphatases are active in the mature erythrocyte and that the osmotically driven volume change somehow controls the balance between these two activities.

Hypotonicity does not directly induce tyrosine phosphorylation of band 3

One mechanism for communicating a change in cell volume to a change in membrane kinase or phosphatase activity might conceivably involve the modulation of membrane curvature. In this context, band 3 (and probably its kinase [4,21,22,32]) are membrane-bound proteins that could be sensitive to mechanical stimuli that might accompany a change in membrane topology. Although cell swelling and shrinking clearly involve different changes in membrane curvature, because the unmodified erythrocyte has regions of both concave and convex curvature, the conversion of a discocyte to a swollen spherocyte must require the stretching of the membrane skeleton. Therefore, if surface deformation constitutes a trigger for activating band 3 phosphorylation, one might expect to detect phosphorylation in swollen as well as in shrunken erythrocytes. However, as shown in Figure 2(B), neither cell swelling (lane 2) nor subsequent shrinking of the swollen cells to normal volume (lane 3) induced any measurable band 3 phosphorylation. It therefore seems that simple membrane deformations do not constitute the stimulus that triggers band 3 phosphorylation in hypertonically treated erythrocytes. However, we cannot rule out the possibility that an increase in band 3 tyrosine phosphorylation could occur in regions of the membrane where an extreme level of curvature is attained, such as at the rim of a flattened, shrunken erythrocyte, or in the protuberances of an echinocyte [23].

Identification of an inhibitor of hypertonically stimulated band 3 phosphorylation

To define further the nature of the stimuli leading to enhanced band 3 tyrosine phosphorylation, an inhibitor of the volume-

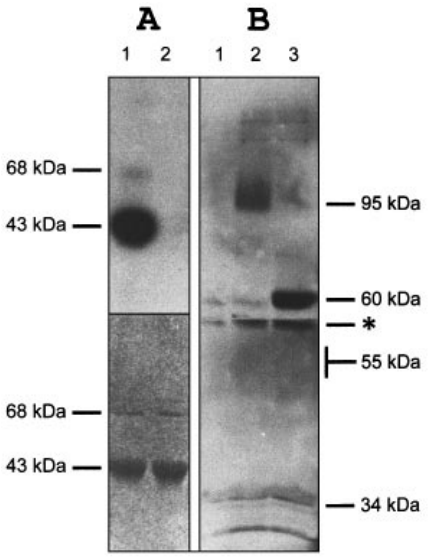

Figure 3 Effect of staurosporine on phosphorylation *in vitro* of recombinant cdb3 and localization of phosphorylation in band 3 after hypertonic treatment of erythrocytes

(**A**) The catalytic domain of $p72^{syk}$ ($p35^{syk}$), as GSH–Sepharose-bound GST–$p35^{syk}$, was used to phosphorylate the recombinant cdb3. The complete reaction mixture (50 μl) contained 25 mM Hepes, pH 7.4, 2 mM $MnCl_2$, approx. 5 μCi/nmol [γ-^{32}P]ATP (ATP concentration 100 μM), 500 μg/ml cdb3 and 10 μl of GSH–Sepharose slurry with bound GST–$p35^{syk}$. Lane 1, control; lane 2, containing 6 μM staurosporine. The reaction was started with the addition of ATP, continued for 30 min at 37 °C and was then stopped by addition of SDS/PAGE sample buffer. Samples were subjected to electrophoresis (each lane contained approx. 6 μg of cdb3). The gel was exposed to autoradiography film for 5 min (top panel). The bottom panel shows the same gel stained with Coomassie Blue before autoradiography. The 43 kDa arrow indicates recombinant cdb3; the 68 kDa arrow indicates GST–$p35^{syk}$. (**B**) Erythrocytes were treated for 15 min at 37 °C in the presence of isotonic NaCl (lane 1), 600 m-osM NaCl (lane 2) or 600 m-osM NaCl followed by treatment with chymotrypsin (lane 3). Cells were processed as described in the legend to Figure 1 for the electrophoresis and immunodetection of tyrosine-phosphorylated proteins. In lane 3 the proteolytic product of band 3 is visible: the approx. 60 kDa N-terminus is the sole peptide containing phosphotyrosine. The positive band (marked with an asterisk) just below the approx. 60 kDa band is due to a non-specific signal that was also present in control samples, probably owing to interference of the haemoglobin-rich samples with the chemiluminescence. Any phosphorylation in the membrane-spanning domain of band 3, if present, was expected to show up in the region marked as approx. 55 kDa.

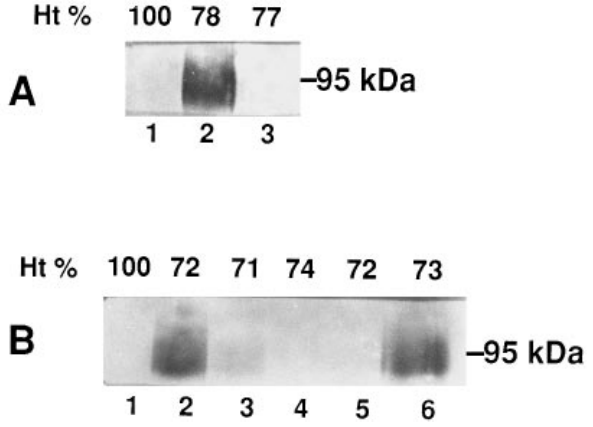

Figure 4 Tyrosine phosphorylation of band 3 in human erythrocytes: effect of staurosporine

(**A**) Erythrocytes (final Ht 10%) were incubated for 30 min at 37 °C in Tris buffer in the absence (lane 2) or the presence (lane 3) of 3 μM staurosporine and then incubated at 37 °C for 15 min in Tris buffer containing sufficient NaCl to reach 600 m-osM. Lane 1, control cells. (**B**) Erythrocytes (final Ht 10%) were incubated for 15 min at 37 °C in Tris buffer containing sufficient NaCl to reach a final osmolarity of 600 m-osM. An aliquot of suspension was taken before (lane 1) and after (lane 2) this step to monitor the phosphorylation of band 3 and cell volume. The suspension was divided into two parts and 3 μM staurosporine was added to one of the tubes. Aliquots of the staurosporine-treated suspension were taken 1 min (lane 3), 5 min (lane 4) and 15 min (lane 5) after the addition of the inhibitor. Lane 6 refers to cells left in the hypertonic buffer without the addition of staurosporine. Ht% is defined as the Ht of the erythrocyte suspension expressed as a percentage of the Ht of the control. Samples were processed as described in the legend to Figure 1.

sensitive erythrocyte tyrosine kinase was needed. Initially, several classical tyrosine kinase inhibitors were tested in whole cells, but none was found to inhibit the kinases measurably (10 μg/ml herbimycin A, 200 μM tyrphostin A25 or 500 μM genistein). Piceatannol, the most commonly employed inhibitor of $p72^{syk}$ kinase, a tyrosine kinase abundantly present in mature erythrocytes [4], was unfortunately incompatible with erythrocyte applications, because it immediately oxidized the haemoglobin and turned the erythrocytes brown. Therefore, in an effort to simplify the search for an effective inhibitor, an examination of the inhibitor sensitivity of the recombinant catalytic domain of $p72^{syk}$ ($p35^{syk}$) was conducted, on the basis of the conviction that $p72^{syk}$ might be the kinase responsible for band 3 phosphorylation *in vivo*. Consistently with the results in whole cells, classical tyrosine kinase inhibitors were found to be generally ineffective in blocking $p35^{syk}$ phosphorylation of the purified cdb3 (results not shown). In contrast, the common Ser/Thr kinase inhibitor, staurosporine, was observed to inhibit $p35^{syk}$ activity quantitatively at low micromolar concentrations (Figure 3A, lane 2). This unusual sensitivity to a Ser/Thr kinase inhibitor, but insensitivity to most tyrosine kinase inhibitors, constitutes a peculiarity of $p72^{syk}$ that could prove valuable in identifying the volume-sensitive tyrosine kinase *in vivo*. Indeed, as shown in Figure 4(A), staurosporine inhibits the hypertonicity-induced tyrosine phosphorylation of band 3 in whole erythrocytes without detectable effects on cell volume.

Is $p72^{syk}$ the volume-sensitive tyrosine kinase in erythrocytes?

The following lines of evidence led us to suppose, although a direct proof is not yet available, that the kinase responsible for the hypertonicity-induced tyrosine phosphorylation of band 3 is Syk: (1) previous experiments [22] showed that the endogenous membrane-bound kinase activity of purified erythrocyte ghosts incorporates phosphate almost exclusively in the cytoplasmic domain of band 3 with at least 80% incorporation within the first 56 residues from the N-terminal end; (2) the tyrosine kinases described so far in human erythrocytes co-immunoprecipitate with band 3 and have been identified as $p72^{syk}$ and $p53/56^{lyn}$ [4]; (3) Lyn kinase phosphorylates band 3 in isolated membranes to a very small extent compared with the purified catalytic domain of $p72^{syk}$ and moreover the K_m of the purified catalytic domain of $p72^{syk}$ for purified cdb3 is one-third that of Lyn for the same substrate [6]; (4) the results in Figure 3(B) show that virtually all the band 3 tyrosine phosphorylation induced by hypertonic treatment is localized in the approx. 60 kDa N-terminus of band 3, as has already been shown for Ca^{2+}/A23187-induced band 3 tyrosine phosphorylation [23]. From these considerations we therefore conclude that Syk is a good candidate for the kinase responsible for the phosphorylation of band 3 during the hypertonic treatment of human erythrocytes.

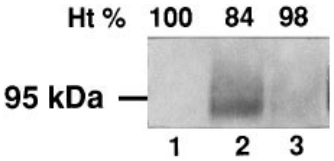

Figure 5 Effect of the osmolarity increase in the cytosol, under isovolumetric conditions, on the phosphorylation state of band 3

A 5% Ht erythrocyte suspension was treated at 4 °C in isotonic potassium buffer (see the Experimental section) in the presence of 30 μg/ml nystatin to permit the equilibration of univalent ions across the membrane. A final 475 m-osM osmolarity was gradually reached at 4 °C by the addition of hypertonic (600 m-osM) potassium buffer, and the cells were processed as described in the Experimental section. Lane 1, control cells; lane 2, erythrocytes treated with hypertonic (475 m-osM) potassium buffer in the absence of nystatin for 15 min at 37 °C; lane 3, attainment of the same hyperosmolarity under isovolumetric conditions, in the presence of nystatin. Ht% is defined as the Ht of the erythrocyte suspension expressed as a percentage of the Ht of the control.

Hypertonicity-induced band 3 tyrosine phosphorylation occurs through the stimulation of a kinase rather than inhibition of a phosphatase

With an inhibitor of the volume-sensitive tyrosine kinase identified, it was also possible to evaluate whether cell shrinkage was promoting the activation of a kinase or the inhibition of a phosphatase. To resolve this issue, erythrocytes were treated with hypertonic (600 m-osM) NaCl for 15 min at 37 °C to boost the tyrosine phosphorylation of band 3 (Figure 4B, lane 2). At this point an aliquot of the erythrocyte suspension was treated with staurosporine to inhibit the kinase activity while maintaining the decreased cell volume. If the phosphatase activity were inhibited by cell shrinkage, the phosphorylation level of band 3 would be expected to remain high for some time after inhibition of the kinase with staurosporine. In contrast, if the tyrosine kinase were activated by cell shrinkage, its inhibition by staurosporine should allow rapid dephosphorylation to occur, just as though the erythrocytes were cycled back to isotonic conditions, as seen in Figure 2(A). In fact, as shown in Figure 4(B) (lanes 3–5), within 1 min of treatment, most of the phosphotyrosine disappeared, and by 5 min after addition, band 3 was completely dephosphorylated. This rapid loss of phosphotyrosine after the addition of staurosporine demonstrates that the tyrosine phosphatase remains active in hypertonically shrunken erythrocytes, and that the observed increase in tyrosine phosphorylation must therefore derive from the activation of a kinase, presumably p72^{syk}.

Activation of the erythrocyte tyrosine kinase is mediated by a decrease in cell volume rather than an increase in intracellular salt concentration

When the erythrocyte shrinks in response to hypertonic NaCl, the decrease in cell volume is accompanied by an increase in the osmolarity of the cytosol. The question therefore arises: which of these two events, i.e. volume decrease or osmolarity increase, is primarily responsible for stimulation of the kinase? To discriminate between these possibilities we performed the following experiment. Erythrocytes were treated with isotonic KCl at 4 °C in the presence of nystatin to allow the equilibration of the salt between the extracellular and intracellular compartments [24,25]. By this method the KCl content of the erythrocytes was gradually elevated to 475 m-osM without promoting a loss of cell water or a change in cell volume. As shown in Figure 5, no phosphorylation of band 3 was detected during this isovolumetric increase in osmolarity, implying that the kinase is insensitive to intracellular osmolarity. We suggest instead that the kinase responds directly to a decrease in cell volume and the biochemical changes that accompany this transition.

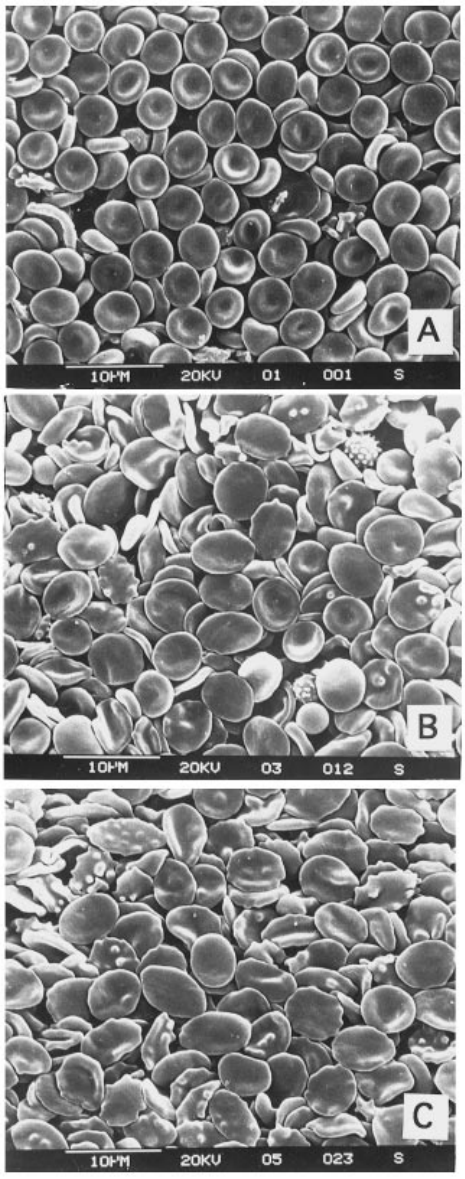

Figure 6 Scanning electron microscopy of human erythrocytes treated with hypertonic buffer

Human erythrocytes were treated with hypertonic buffer (600 m-osM) in the absence (**B**) and in the presence (**C**) of 3 μM staurosporine. (**A**) Control cells.

Analysis of the shape of erythrocytes treated with hypertonic buffer

An examination of erythrocyte shape by electron microscopy was performed to determine whether modification of the phosphorylation state of band 3 might be associated with any morphological changes in the shrunken cell. Figure 6 shows that erythrocytes incubated at 37 °C in hypertonic buffer partly lose their biconcavity and assume a flattened, slightly crenated, shape. Importantly, no significant difference in cell shape was detectable when erythrocytes were similarly shrunk in the presence of

staurosporine to suppress band 3 tyrosine phosphorylation. Therefore it can be concluded that the levels of tyrosine phosphorylation attained under hypertonic conditions do not directly induce changes in erythrocyte shape and that the observed modifications in cell shape are due to the decrease in cell volume consequent on the hypertonic treatment.

DISCUSSION

The objective of this study was to examine the mechanism of hypertonicity-induced tyrosine phosphorylation of band 3 in intact human erythrocytes. Several lines of evidence were presented to demonstrate that a change in cell volume functioned as the primary driving force for the stimulated phosphorylation. First, cell shrinkage was found to precede the induction of band 3 phosphorylation by several minutes. Secondly, isovolumetric increases in intracellular salt concentration promoted no change in phosphorylation, even though the same increases in solute concentration induced elevated kinase activity in cells that had not been treated with nystatin. Thirdly, phosphorylation of band 3 was equally up-regulated by several distinct solutes, including KCl, NaCl and the non-ionic osmolyte sucrose, indicating that the nature of the hypertonic solute was inconsequential. Lastly, phosphorylation changes were found to accurately chart changes in cell volume through three consecutive cycles of shrinking and swelling.

Evidence was also presented that the enhanced phosphorylation of band 3 in shrunken cells was due to the activation of tyrosine kinase rather than the inhibition of tyrosine phosphatase. Thus fully phosphorylated, hypertonically shrunk erythrocytes were found to dephosphorylate rapidly when the endogenous tyrosine kinase activity was inhibited. These results also argue that the endogenous tyrosine phosphatase must be constitutively active and fully capable of reversing any tyrosine phosphorylation of band 3 as soon as the responsible kinase is down-regulated. Importantly, previous results demonstrate that the band 3 tyrosine kinase is also constitutively active *in situ*, because phosphorylation can be rapidly elevated by inhibition of the phosphatase (e.g. by treatment with certain oxidizing agents or vanadate [2,10,14,33,34]). Consequently the tyrosine phosphorylation status of band 3 *in situ* must reflect a delicate balance between a constitutively active tyrosine kinase and constitutively active tyrosine phosphatase. On the basis of anti-phosphotyrosine immunoblots of unstimulated cells, the phosphatase must predominate under normal circumstances. However, as shown here, a decrease in erythrocyte volume can activate the kinase, allowing it to increase measurably the steady-state level of phosphorylated band 3 in the cell.

Although molecular details are still lacking, there is now strong evidence that band 3 physically associates with both a tyrosine kinase and a tyrosine phosphatase in the mature erythrocyte. Thus several authors have observed that a tyrosine kinase activity co-purifies with erythrocyte band 3 [1,2,32], and immunoprecipitation experiments have shown that the protein tyrosine kinase, $p72^{syk}$, consistently co-pellets with the erythrocyte anion exchanger [4]. Conversely, a protein tyrosine phosphatase 1B has also been observed to co-isolate with erythrocyte band 3 [35]. Given their likely co-localization on the anion transporter, it will be of interest to determine how their relative spatial arrangements participate in controlling the level of tyrosine-phosphorylated band 3.

One of the most unusual characteristics of the tyrosine phosphorylation of band 3 *in situ* is its potent inhibition by a classical serine/threonine kinase inhibitor (staurosporine) and its apparent indifference to many commonly used tyrosine kinase inhibitors. Although initial interpretation of these results might imply the existence of a tyrosine kinase under the control of a staurosporine-sensitive serine/threonine kinase, our studies *in vitro* with the catalytic domain of $p72^{syk}$ argue against this supposition. Thus the ability of purified $p35^{syk}$ to phosphorylate the isolated cdb3 was blocked by staurosporine but was not measurably affected by genistein. This peculiar inhibition pattern was also observed for the hypertonically induced phosphorylation of band 3 in intact erythrocytes, suggesting the participation of $p72^{syk}$ in the volume-regulated phosphorylation.

Although a decrease in cell volume, not an increase in ionic strength, was shown to cause the increase in erythrocyte tyrosine phosphorylation, few additional clues were obtained to clarify the molecular mechanism of the activation process. If the driving force were simply an increase in intracellular ATP concentration, multiple phosphorylation reactions should have been enhanced. However, not even the tyrosine phosphorylation of protein 4.1, a known tyrosine kinase substrate [36], was measurably affected.

It was also conceivable that a change in membrane curvature or conformation could have stimulated the kinase. In fact, some regions of the membrane undergo extreme deformation when the cell loses its biconcavity and becomes flat in hypertonic buffer. Another explanation could be that the marked increase in thermodynamic activity coefficients that occurs as a consequence of the exponential rise in molecular crowding in the cell [37,38] might enhance the interaction of $p72^{syk}$ with band 3. If the interaction between band 3 and $p72^{syk}$ were submaximal in the unmodified erythrocyte (where the phosphatase activity prevails), any decrease in cell volume might promote an increase in their association. Therefore a cell-shrinkage-induced enhancement of band 3 tyrosine phosphorylation, independent of intracellular osmolarity, might ensue, as a consequence of molecular crowding or changes in membrane curvature, or both.

Finally the question naturally arises regarding the possible function of enhanced band 3 tyrosine phosphorylation during cell shrinkage. The observation (previously reported) that tyrosine phosphorylation of band 3 leads to the displacement of bound glycolytic enzymes and their consequent activation [10] provides little resolution to the question, because a need to increase erythrocyte glycolysis whenever the erythrocyte shrinks is not immediately obvious. Protection against shrinkage-promoted membrane deformations might also not constitute the intended activity, at least over short periods, because, as noted above, major differences in morphology between erythrocytes exposed to hypertonic saline in the presence and absence of staurosporine were not detected. One possible function worthy of further consideration might involve a phosphorylation-dependent increase in membrane flexibility that would enable a shrunken erythrocyte to traverse the hypertonic passages deep within the kidney more readily.

Clearly, more information is needed on the structural consequences of band 3 phosphorylation before an answer to this question can be expected.

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