Erythrocyte senescence is characterized by exposure of cell surface epitopes on cell membrane proteins leading to immune mediated removal of red blood cells. One mechanism for antigen formation is tyrosine phosphorylation (Tyr-P) of the transmembrane protein band 3 by Syk kinase. Our aim was to test the hypothesis that proteolytic activation of Syk kinase by conversion from 72 kDa (p72Syk) to the 36 kDa (p36Syk) isoform enhances its phosphorylating activity independently of the association of Syk kinase with the cytoskeleton. Tyr-P assay was conducted using quantification of $^{32}$P uptake into the cytoplasmic domain of band 3 after addition of p72Syk or p36Syk. Effect of pre-phosphorylation of erythrocyte membrane band 3 protein by p36Syk on p72Syk-mediated phosphorylation and the effect of addition of a protease inhibitor (leupeptin) on p72Syk-mediated phosphorylation were studied by autoradiographic visualization of $^{32}$P uptake. Tyr-P by Syk isoforms of membrane skeletal and soluble fractions of band 3 was visualized by immunoblotting. It was found that p36Syk had a higher band 3 tyrosine phosphorylating activity compared with p72Syk. Pre-phosphorylation with p36 Syk or p72 Syk increased band 3 phosphorylating activity. Protease inhibition treatment reduced p72Syk but not p36Syk band 3 tyrosine phosphorylating activity significantly. Both soluble and membrane skeletal fractions of band 3 protein were equally tyrosine phosphorylated by each Syk isoform. In conclusion, we confirmed the hypothesis that proteolytic cleavage of p72Syk is an important regulatory step for band 3 Tyr-P and its independence of the association of band 3 with the cytoskeleton.

Keywords: band 3 Tyr-phosphorylation; Syk; proteolysis; human erythrocyte
and activate a second tyrosine kinase, Lyn, which phosphorylates band 3 residues 359 and 904 in the so-called 'secondary phosphorylation' [13]. If the mechanism that regulates Lyn-mediated activity has been well defined, what can trigger Syk activation must still be defined. In this study, we propose that protease-mediated Syk activation represents the missing link in defining the band 3 Tyr-P event. We demonstrated that, when proteolyzed from 72- to 36-kDa isoform, Syk is more active and it is able to prepare the membrane for the following p72\textsuperscript{Syk}-catalyzed phosphorylation of band 3. More interestingly, this process has been seen in isolated membranes in the absence of leupeptin, where a higher level of band 3 Tyr-P is found.

**Materials and Methods**

**Materials**

Anti-Syk (C-20) antibody raised against residues 616–635 of the Syk kinase family was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-Tyr-P antibody was from Upstate (Waltham, USA). [γ-\textsuperscript{32}P]ATP was from Amersham (Buckinghamshire, UK). Complete tablets containing several protease inhibitors with broad inhibitory specificity were obtained from Boehringer Mannheim. Other proteins and reagents were from Sigma (St. Louis, USA).

**Membrane preparation**

Human erythrocytes were prepared by centrifugation (750 g for 3 min) of fresh blood collected from healthy donors following their informed consent. To minimize contamination by leukocytes and platelets, packed red cells were washed three times by centrifugation in buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM KCl, 1 mM MgCl\textsubscript{2}, 5 mM glucose, 25 μg/ml chloramphenicol, and 0.1 mg/ml streptomycin) discarding the buffy coat and the upper third of the cell layer. The packed red cells were haemolysed and the membranes were prepared as previously described [8].

**Purification of p36\textsuperscript{Syk}, p72\textsuperscript{Syk}, and band 3 proteolytic fragments**

p36\textsuperscript{Syk} was isolated from human erythrocyte cytosol as described previously [14]. p72\textsuperscript{Syk} was isolated from rat spleen as described previously [15]. The 40/45 kDa fragment of the cytoplasmic domain of band 3 (cd3) was obtained by α-chymotrypsin-promoted proteolytic breakdown of inverted membrane vesicles derived from ghosts and isolated by DE52 chromatography [16].

**Immunoblotting assay of binding of the two Syk kinases (p72\textsuperscript{Syk} and p36\textsuperscript{Syk}) to isolated human erythrocyte membranes (ghosts)**

Human erythrocyte membranes (15 μg) pre-incubated with p72\textsuperscript{Syk} (20 ng) or p36\textsuperscript{Syk} (10 ng) at 0°C for 10 min in 50 mM Tris–HCl, pH 7.5, 10 mM MnCl\textsubscript{2}, and 0.1 mM vanadate, were washed twice with 25 mM Tris, pH 8, 0.02% NaN\textsubscript{3}, and 0.03 mM phenylmethylsulphonyl fluoride [17], and then solubilized and analyzed by 8% sodium dodecyl sulfate (SDS)–PAGE, according to Laemmli [18]. The electrophoresed proteins were blotted and immuno-detected with appropriate antibody.

**Tyr-phosphorylation assays**

Tyr-phosphorylation assays of membrane proteins were performed by incubating white ghosts (10 μg), or increasing concentrations of cd3, at 30°C in 30-μl reaction mixtures containing 50 mM Tris–HCl, pH 7.5, 10 mM MnCl\textsubscript{2}, 20 μM [γ-\textsuperscript{32}P]ATP (3 × 10\textsuperscript{6} cpm/nmol) and 0.1 mM vanadate, in the presence of either p36\textsuperscript{Syk} (10 ng), or p72\textsuperscript{Syk} (20 ng) [17]. When required, 100 μg/ml leupeptin was added to the membranes before incubation [19]. Reactions were stopped by the addition of 2% SDS and 1% β-mercaptoethanol (final concentrations) incubated for 5 min at 100°C, and analyzed by 0.1% SDS/8% PAGE.

The resulting gels were stained with Coomassie brilliant Blue according to Laemmli [18], treated with 2 M NaOH at 55°C for 1 h, as described, and fixed again. Dried gels were autoradiographed at −80°C with intensifying screens.

Incorporation of \textsuperscript{32}P into the proteins was measured with a Packard Instant Imager. \(K_m\) and \(V_{max}\) values were determined by double-reciprocal plots, constructed from initial-rate measurements fitted to the Michaelis–Menten equation. The values reported are the means of at least three separate experiments and SE values were <10%.

**Preparation of membrane skeletal and soluble fractions**

Membranes, pre-phosphorylated by p72\textsuperscript{Syk} or p36\textsuperscript{Syk} and recovered as described above, were extracted with two volumes of buffer A containing 50 mM Tris, pH 7.5, 1% Triton X-100, 1 mM vanadate, and protease inhibitor cocktail for 1 h at 4°C. After the removal of an aliquot for western blot analysis, the remainder was centrifuged at 80,000 g for 40 min. Both supernatant, corresponding to the Triton-soluble fraction, and pellet, corresponding...
to the Triton-insoluble fraction (cytoskeleton), were then collected, and the pellet was re-suspended to the same soluble-fraction volume with buffer A. Total membrane (10 µg) and the corresponding soluble and cytoskeleton fractions were then subjected to either western blot analysis and immuno-revealed with anti-Tyr-P antibody, or to 10% SDS–PAGE followed by Coomassie coloration.

Results

Phosphorylation of isolated cdb3 and band 3 in ghosts

Figure 1 shows that the Tyr-protein kinase p36Syk and its larger parent holoenzyme p72Syk phosphorylate isolated cdb3 with the same apparent \( K_m \) but with different \( V_{\text{max}} \) and efficiency (Table 1), p72Syk showing substantially reduced efficiency toward this substrate.

When assayed for their ability to phosphorylate band 3 in red blood cell membranes, the same concentration of each kinase was added to isolated membranes with \([\gamma-\text{32P}]\text{ATP (Fig. 2). Comparing the extent of Tyr-P of membrane band 3 reached by p72Syk (lane 2) with that obtained by p36Syk (lane 3), the estimated about 5-fold lower phosphorylation efficiency of the former on cdb3 (Fig. 1) does not account for the dramatically reduced band 3 Tyr-P level induced by p72Syk on membranes. The SH2 domains in the holoenzyme may also have an indirect effect on phosphorylating activity: by mediating binding to the membrane, this SH2 tandem region may dislocate p72Syk in such a way as to prevent its optimal Tyr-P activity on band 3. Again, p36Syk, lacking SH2 domains, can easily phosphorylate band 3, even when inserted in the membranes.

Table 1 Kinetic analysis of cdb3 as substrate of p72Syk or p36Syk

<table>
<thead>
<tr>
<th>Syk isoform</th>
<th>( V_{\text{max}} ) (pmol/min)</th>
<th>( K_m ) (µM)</th>
<th>Efficiency ( (V_{\text{max}}/K_m) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>p72Syk</td>
<td>0.083</td>
<td>0.3</td>
<td>0.277</td>
</tr>
<tr>
<td>p36Syk</td>
<td>0.444</td>
<td>0.3</td>
<td>1.48</td>
</tr>
</tbody>
</table>

The data represent the mean of three independent experiments with SD never exceeding 10%. Kinetic parameters were determined as indicated in ‘Materials and Methods’.

Figure 2 p72Syk- and p36Syk-catalyzed phosphorylation of erythrocyte membranes

Isolated membranes (10 µg) were incubated alone (lane 1, control) or in presence of p72Syk (20 ng) (lane 2) or p36Syk (10 ng) (lane 3) in the reaction mixture as described in ‘Materials and Methods’. To study p36Syk-pre-phosphorylation, membranes (10 µg) were pre-incubated at 30°C for 10 min as described above, and p36Syk (10 ng). After washing to remove both p36Syk and ATP, pre-treated membranes were then re-incubated in the reaction mixture containing \([\gamma-\text{32P}]\text{ATP and p72Syk (20 ng) at 30°C for 10 min (lane 4). Samples were analyzed by SDS–PAGE, and gels were submitted to NaOH treatment (see ‘Materials and Methods’). Autoradiograms were exposed for 6 h. Figure is representative of four separate experiments.

Figure 1 Lineweaver–Burk plot showing rate of cdb3 Tyr-phosphorylation by p72Syk (filled circles) and p36Syk (empty circles) For the determination of the kinetic constants, purified enzyme isoforms (10 ng of p36Syk or 20 ng of p72Syk) were incubated for 3 min in the presence of 20 µM \([\gamma-\text{32P}]\text{ATP as indicated in Materials and Methods.}^{32P}\text{-labeled cdb3 was separated by SDS–PAGE, excised from alkali-treated gels and counted for radioactivity in a liquid scintillation counter. [cdb3] was evaluated as µM, and } 1/V \text{ as } 1/\text{cpm} \times 10^{-3}. Values are the mean ± SD for four experiments performed in triplicate. |
In addition, when the membranes were pre-incubated with p36<sup>Syk</sup> and ATP before incubation with p72<sup>Syk</sup> and [\(\gamma-P\)]ATP (lane 4), 32P-labeled band 3 is far higher than that obtained with membranes not pre-incubated with p36<sup>Syk</sup> (lane 2). This confirms the hypothesis that the lower activity of p72<sup>Syk</sup> on membrane band 3 is prevented by a sort of steric hindrance. When electrostatic alteration of band 3 structure is induced, for example, by introducing negatively charged phosphate groups by pre-phosphorylating the protein, p72<sup>Syk</sup> results as efficiently as p36<sup>Syk</sup> in reaching high level of band 3 Tyr-P.

**Anti-protease effect on the Tyr-P of membrane proteins**

In order to verify if proteolysis is really a physiological process able to activate band 3 Tyr-P, we compared the extent of Tyr-P of membrane band 3 induced by p72<sup>Syk</sup> (Fig. 3, lanes 3 and 4) with that obtained by p36<sup>Syk</sup> (lanes 5 and 6) in membranes prepared with (lanes 4 and 6) or without (lanes 3 and 5) leupeptin, a well-known inhibitor of protease activities, and, in particular, of proteases involved in p72<sup>Syk</sup> degradation [19]. Also in this case, membrane band 3 Tyr-P turned out to be closely controlled by the proteolytic process. In fact, in the presence of leupeptin, the effect of the addition of p72<sup>Syk</sup> on band 3 Tyr-P (lane 4) was drastically lower when compared with the same effect without leupeptin (lane 3).

Parallel leupeptin-induced decrease in band 3 Tyr-P level was also observed in membranes incubated without the addition of the enzyme (lanes 1 and 2), where the Tyr-P level is mediated by endogenous enzyme contents. When p36<sup>Syk</sup> was tested, leupeptin had no effect in modulating p36<sup>Syk</sup>-mediated phosphorylation of membrane band 3 (lanes 5 and 6).

**Analysis of membrane band 3 subpopulations as target of both kinase isofoms**

When membranes previously phosphorylated by p72<sup>Syk</sup> (Fig. 4, lanes 1 and 2) or p36<sup>Syk</sup> (lanes 3 and 4) were extracted with Triton X-100 and analyzed for their phosphorylated band 3 subdivision, phosphorylation patterns clearly showed that both Syk isofoms were equally able to phosphorylate soluble band 3 (lanes 1 and 3) as well.
as cytoskeleton-bound band 3 (lanes 2 and 4), although to different extent, as expected.

Discussion

The process related to red cell senescence is triggered by the appearance of senescent erythrocyte-specific antigens generated during cell circulation by incoming alterations in membrane proteins. One of the most reliable antigen precursors is membrane protein band 3, which accumulates changes during the erythrocyte life-span, resulting in senescent cell antigen formation [3]. The first mechanism involves oxidation-mediated denaturation of hemoglobin in hemichromis which induce band 3 clustering by selective binding to the cytoplasm portion of the protein [20]. In the second mechanism, breakdown of band 3, induced by increased sensitivity of the protein to proteolysis, also leads to conformational changes, responsible for IgG binding [3]. A third hypothesis involves Tyr-P as a post-translation mechanism able to regulate multiple cell functions, not excluding erythrocyte life span [5,6].

The results reported in this work highlight the fact that p36Syk, proteolytic product of p72Syk, is the activated form of the protein kinase Syk, clearly represented by its higher efficiency demonstrated on cdb3 phosphorylation. Syk is maintained in an autoinhibited conformation by the interactions between the two SH2 regulatory regions with the inter-SH2-kinase linker and the catalytic domain that reduce the conformational flexibility required by the kinase domain for catalysis [21–23]. This explains the reduced phosphorylation efficiency of p72Syk in phosphorylating cdb3. In addition, the presence of the two tandem SH2 domains exerts further inhibitory effect in the phosphorylation of membrane band 3 by delocalizing the enzyme in unfavorable position. Lacking both SH2 domains, the proteolysed enzyme, p36Syk, can catalyze the reaction more efficaciously. In fact, when membranes were analyzed for their ability in retaining Syk isoforms, western blotting of membranes incubated with p72Syk or p36Syk and washed as described in Methods, showed that p72Syk (20 ng) were totally retained, while p36Syk (10 ng) was recovered in the supernatant (data not shown).

That proteolytic cleavage does not alter substrate preference is also evidenced by the fact that both p36Syk and p72Syk can phosphorylate both band 3 populations, distributed between the soluble fraction of the membrane and the cytoskeleton (Fig. 4). We hypothesize that the physiological function of p72Syk proteolytic cleavage is the triggering of band 3 Tyr-P. p72Syk, when added to membranes, binds through its SH2 domains [9] which, displacing the enzyme, represent a handicap for band 3 Tyr-P. The difficulty in catalyzing band 3 Tyr-P may be bypassed by proteolysis of the enzyme which, once reduced to the proteolytic isoform p36Syk, may act on its own and trigger band 3 Tyr-P. In the in vitro study, we tried to determine the increasing presence of p36Syk in cytosol from variously treated erythrocytes (data not shown), but unsuccessfully. This may be due to the fact that, during aging, erythrocytes lose cholesterol and phospholipids through the formation of microvesicles [24], embedding lipid membranes, band 3 and both protein tyrosine phosphatases and kinases, including 47 ± 13% of Syk [25]. Otherwise, proteolysis could only occur on a few molecules of the enzyme, just sufficient to prepare the membrane for the following event catalyzed by the holoenzyme, p72Syk, which would be far greater (Fig. 3). In fact, we show that the presence of leupeptin, which has been demonstrated to inhibit the proteolytic fragmentation of p72Syk [19], also prevents band 3 Tyr-P (Fig. 3), both in control membranes and in those incubated with p72Syk. This is evidence that isolated erythrocyte membranes contain proteolytic, leupeptin-sensitive enzymes, which can digest both endogenous and exogenously added p72Syk, but which do not affect p36Syk (Fig. 3). In addition, the extent of band 3 Tyr-P is closely controlled by this proteolytic activity, as highlighted by the net decrease of band 3 Tyr-P when leupeptin is added to the incubation.

In conclusion, we confirmed the hypothesis that proteolytic cleavage of p72Syk is an important regulatory step for band 3 Tyr-P and its independence of the association of band 3 with the cytoskeleton.

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