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Acta Biochim Biophys Sin (2009): 846–851 | © The Author 2009. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmp071.
Advance Access Publication 30 August 2009

Regulation of membrane band 3 Tyr-phosphorylation by proteolysis of p72^{Syk} and possible involvement in senescence process

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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 (p72^{Syk}) to the 36 kDa (p36^{Syk}) 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 p72^{Syk} or p36^{Syk}. Effect of pre-phosphorylation of erythrocyte membrane band 3 protein by p36^{Syk} on p72^{Syk}-mediated phosphorylation and the effect of addition of a protease inhibitor (leupeptin) on p72^{Syk}-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 p36^{Syk} had a higher band 3 tyrosine phosphorylating activity compared with p72^{Syk}. Pre-phosphorylation with p36^{Syk} or p72^{Syk} increased band 3 phosphorylating activity. Protease inhibition treatment reduced p72^{Syk} but not p36^{Syk} 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 p72^{Syk} 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

Received: April 26, 2009 Accepted: June 6, 2009

Introduction

Apoptosis, senescence, and necrosis are distinct cell death mechanisms in normal physiology and during pathological distress. The nucleus itself is not required for apoptosis, since apoptotic stimuli induce apoptotic morphological features in anucleated cells [1]. While the apoptotic process has been studied in-depth in nucleated cells, the mechanism underlying apoptosis in anucleated cells is poorly understood.

Senescence is the apoptotic-like process involving circulating human erythrocytes in a sequence of alterations that provoke their removal through a process of antigen–antibody binding formation and macrophage-mediated removal. One of the proteins involved in antigen formation on the erythrocyte surface is protein band 3, which becomes a binding site for antibodies by means of various alterations, including oxidant-mediated clustering [2,3], caspase-induced proteolysis [4], or tyrosine phosphorylation (Tyr-P) as the system that can sterically alter the protein [5,6]. Band 3 Tyr-P is closely controlled by the antithetic activities of protein tyrosine kinases and phosphatases which maintain band 3 Tyr-P at very low levels in un-stimulated conditions [7,8].

Following stimulation in oxidative [7,9,10] or hyperosmotic [11,12] conditions, alteration of the balance between these two opposite activities results in triggering of Tyr-P of membrane proteins, mainly protein band 3. Diamide-induced oxidative stress increases the Tyr-P level of band 3 in a well-defined chain of events. First, tyrosine kinase Syk triggers band 3 Tyr-phosphorylation, catalyzing the so-called 'primary phosphorylation' of residues 8 and 21 which, once phosphorylated, recruit

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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 proteolysed from 72- to 36-kDa isoform, Syk is more active and it is able to prepare the membrane for the following p72^{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-^{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 consensus. 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 $MgCl_2$, 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^{syk}, p72^{syk}, and band 3 proteolytic fragments

p36^{Syk} was isolated from human erythrocyte cytosol as described previously [14]. p72^{Syk} was isolated from rat spleen as described previously [15]. The 40/45 kDa fragment of the cytoplasmic domain of band 3 (cdb3) 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^{syk} and p36^{syk}) to isolated human erythrocyte membranes (ghosts)

Human erythrocyte membranes (15 μg) pre-incubated with p72^{Syk} (20 ng) or p36^{Syk} (10 ng) at 0°C for 10 min in 50 mM Tris–HCl, pH 7.5, 10 mM $MnCl_2$, and 0.1 mM vanadate, were washed twice with 25 mM Tris, pH 8, 0.02% NaN_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 cdb3, at 30°C in 30-μl reaction mixtures containing 50 mM Tris–HCl, pH 7.5, 10 mM $MnCl_2$, 20 μM [γ-^{32}P]ATP (3×10^6 cpm/nmol) and 0.1 mM vanadate, in the presence of either p36^{Syk} (10 ng), or p72^{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 ^{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^{Syk} or p36^{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

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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 p36^{Syk} and its larger parent holoenzyme p72^{Syk} phosphorylate isolated cdb3 with the same apparent K_m but with different V_{max} and efficiency (**Table 1**), p72^{Syk} 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 [γ-^{32}P]ATP (**Fig. 2**). Comparing the extent of Tyr-P of membrane band 3 reached by p72^{Syk} (lane 2) with that obtained by p36^{Syk} (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 p72^{Syk} 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 p72^{Syk} in such a way as to prevent its optimal Tyr-P activity on band 3. Again, p36^{Syk}, lacking SH2 domains, can easily phosphorylate band 3, even when inserted in the membranes.

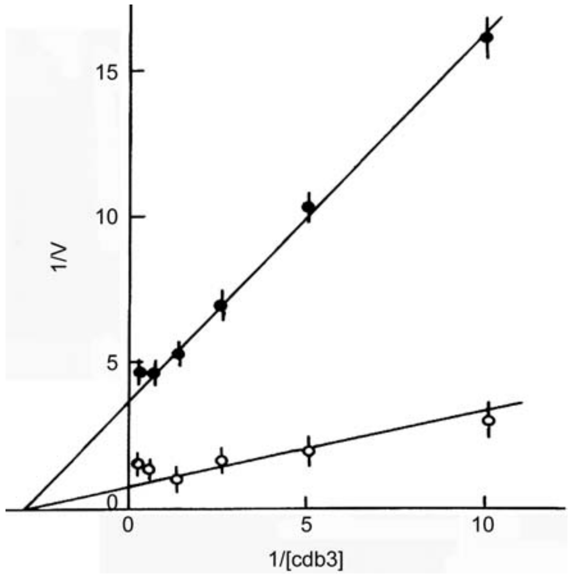

Figure 1 Lineweaver–Burk plot showing rate of cdb3 Tyr-phosphorylation by p72^{Syk} (filled circles) and p36^{Syk} (empty circles) For the determination of the kinetic constants, purified enzyme isoforms (10 ng of p36^{Syk} or 20 ng of p72^{Syk}) were incubated for 3 min in the presence of 20 μM [γ-^{32}P]ATP as indicated in Materials and Methods. ^{32}P-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 as 1/cpm × 10^{-3}. Values are the mean ± SD for four experiments performed in triplicate.

Table 1 Kinetic analysis of cdb3 as substrate of p72^{Syk} or p36^{Syk}

Syk isoform	V_{max} (pmol/min)	K_m (μM)	Efficiency (V_{max}/K_m)
p72^{Syk}	0.083	0.3	0.277
p36^{Syk}	0.444	0.3	1.48

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

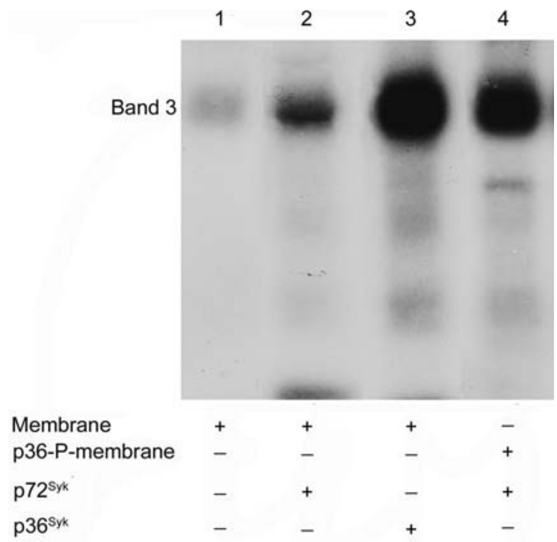

Figure 2 p72^{Syk}- and p36^{Syk}-catalyzed phosphorylation of erythrocyte membranes Isolated membranes (10 μg) were incubated alone (lane 1, control) or in presence of p72^{Syk} (20 ng) (lane 2) or p36^{Syk} (10 ng) (lane 3) in the reaction mixture as described in 'Materials and Methods'. To study p36^{Syk}-pre-phosphorylation, membranes (10 μg) were pre-incubated at 30°C for 10 min as described above, and p36^{Syk} (10 ng). After washing to remove both p36^{Syk} and ATP, pre-treated membranes were then re-incubated in the reaction mixture containing [γ-^{32}P]ATP and p72^{Syk} (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.

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In addition, when the membranes were pre-incubated with p36Syk and ATP before incubation with p72Syk and [γ-32P]ATP (lane 4), 32P-labeled band 3 is far higher than that obtained with membranes not pre-incubated with p36Syk (lane 2). This confirms the hypothesis that the lower activity of p72Syk 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, p72Syk results as efficiently as p36Syk 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 p72Syk (**Fig. 3**, lanes 3 and 4) with that obtained by p36Syk (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 p72Syk 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 p72Syk 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 p36Syk was tested, leupeptin had no effect in modulating p36Syk-mediated phosphorylation of membrane band 3 (lanes 5 and 6).

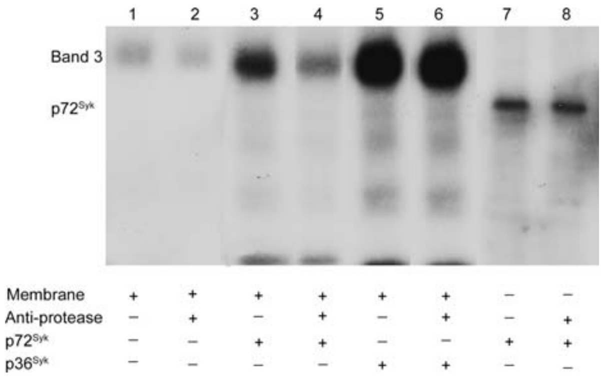

Figure 3 Anti-protease effect of leupeptin on p72Syk- and p36Syk-mediated membrane band 3 Tyr-phosphorylation Erythrocyte membranes, prepared in presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of leupeptin, were incubated in the reaction mixture (see 'Materials and Methods') alone (lanes 1 and 2), with p72Syk (20 ng) (lanes 3 and 4), or p36Syk (10 ng) (lanes 5 and 6) and [γ-32P]ATP at 30°C for 10 min. Samples were analyzed by SDS–PAGE, and gels were submitted to NaOH treatment (see Materials and Methods). Samples of p72syk (40 ng) (lanes 7 and 8) were incubated alone (lane 7) or in the presence of leupeptin (lane 8), to exclude protease contamination leading to p36Syk formation. Autoradiograms were exposed for 6 h. Figure is representative of four separate experiments.

Analysis of membrane band 3 subpopulations as target of both kinase isoforms

When membranes previously phosphorylated by p72Syk (**Fig. 4**, lanes 1 and 2) or p36Syk (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 isoforms were equally able to phosphorylate soluble band 3 (lanes 1 and 3) as well

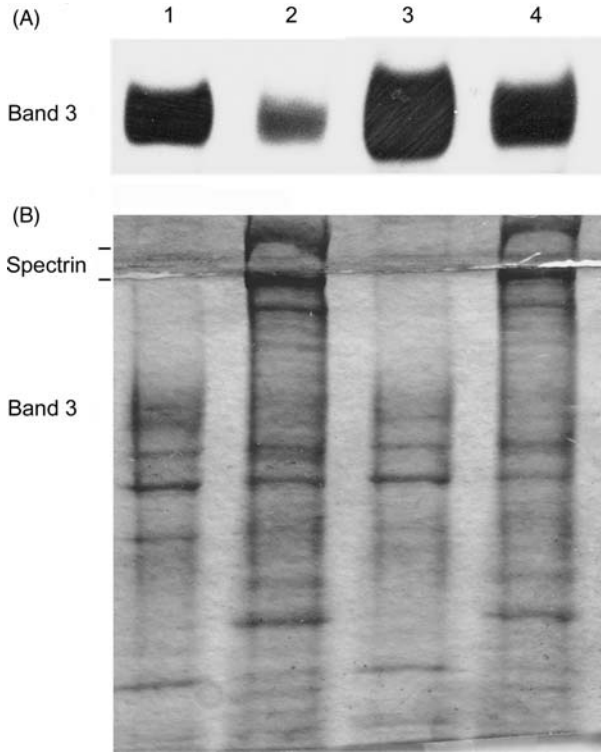

Figure 4 Phosphorylation pattern of Triton-soluble and cytoskeletal band 3 Tyr-phosphorylation by p72Syk and p36Syk Isolated membranes (15 μg) were incubated with p72Syk (20 ng) (lanes 1 and 2) or p36Syk (10 ng) (lanes 3 and 4) in the reaction mixture (see 'Materials and Methods') for 10 min at 0°C. After washing to remove unincorporated radionucleotide, both samples were then treated with Triton X-100 (1% final concentration) for 5 min at 0°C and centrifuged at 80,000 *g* for 40 min. Pelleted insoluble residues were resuspended in Tris buffer to same starting volume. Fractions of both Triton extract (lanes 1 and 3) and cytoskeleton (lanes 2 and 4) were analyzed by SDS–PAGE and either western blotting analysis and immunorevelation with anti P-Tyr antibody (A) or Coomassie Blue staining (B).

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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 p36^{Syk}, proteolytic product of p72^{Syk}, 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 p72^{Syk} 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, p36^{Syk}, 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 p72^{Syk} or p36^{Syk} and washed as described in Methods, showed that p72^{Syk} (20 ng) were totally retained, while p36^{Syk} (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 p36^{Syk} and p72^{Syk} 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 p72^{Syk} proteolytic cleavage is the triggering of band 3 Tyr-P. p72^{Syk}, 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 p36^{Syk}, may act on its own and trigger band 3 Tyr-P. In the *in vivo* study, we tried to determine the increasing presence of p36^{Syk} 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, p72^{Syk}, 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 p72^{Syk} [19], also prevents band 3 Tyr-P (**Fig. 3**), both in control membranes and in those incubated with p72^{Syk}. This is evidence that isolated erythrocyte membranes contain proteolytic, leupeptin-sensitive enzymes, which can digest both endogenous and exogenously added p72^{Syk}, but which do not affect p36^{Syk} (**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.

Ca^{2+}/ionophore-induced *in vitro* erythrocyte senescence has also been demonstrated to be mediated by activation of proteolysis [26]. Consequently, rearrangements of membrane structure with aging, together with the probable activation of proteolytic enzymes, may induce p72^{Syk} recruitment to membranes [9], as well as proteolysis-mediated p36^{Syk} formation, culminating in band 3 Tyr-P. This, in turn, causes further band 3-mediated membrane modifications, and may represent a mechanism for their selective recruitment in large membrane clusters [6], acting as a prelude to rapid markers of senescent erythrocyte removal.

In conclusion, we confirmed the hypothesis that proteolytic cleavage of p72^{Syk} is an important regulatory step for band 3 Tyr-P and its independence of the association of band 3 with the cytoskeleton.

Acknowledgements

The authors thank Claudio Bettella and Giancarlo Ruffato for supplying fresh blood from volunteers.

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Funding

This work was supported by a grant from the Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST).

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