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Zn^{2+} enhances protein tyrosine kinase activity of human platelet membranes

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In human platelet membranes enhanced tyrosine phosphorylation of certain proteins was observed when Zn^{2+} instead of Mg^{2+} or Mn^{2+} was used as a divalent cation for the kinase reaction. An enhanced level of phosphate incorporation into tyrosine residues occurred into a 68 kDa polypeptide besides the 45 kDa and 105 kDa proteins. Preincubation of platelet membranes with TBR-IgG showed a concentration-dependent inhibition of the phosphorylation of the 45, 68 and 105 kDa proteins. Moreover, $pp60^{c\text{-}src}$, representing the major protein tyrosine kinase activity in platelets, was found to be stimulated by Zn^{2+}. The data, thus, support the assumption that $pp60^{c\text{-}src}$ kinase is responsible for Zn^{2+} stimulated tyrosine phosphorylation.

Protein tyrosine kinase; Phosphotyrosyl-protein phosphatase; Zn^{2+}; Phosphoprotein $pp60^{c\text{-}src}$; (Human platelet membrane)

1. INTRODUCTION

Protein tyrosine kinase (PTK) activity is associated with cellular growth hormone receptors and many viral and cellular *onc* gene products, suggesting an important role in cell proliferation and differentiation [1–5]. High levels of PTK activity have also been found in nonproliferating terminally differentiated blood cells [6–9].

In human platelets the major PTK activity has been ascribed to $pp60^{c\text{-}src}$, the normal cellular homologue of the transforming protein of Rous sarcoma virus (RSV), $pp60^{v\text{-}src}$ [9,10]. However, other, as-yet unidentified PTKs may be responsible for the extensive tyrosine phosphorylation of platelet membrane proteins [6].

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Abbreviations: PTK, protein tyrosine kinase; $pp60^{c\text{-}src}$/$pp60^{v\text{-}src}$, the 60 kDa phosphoprotein encoded by the cellular (c-) or viral (v-) src gene; TBR serum, serum from tumor-bearing rabbits immunoprecipitating $pp60^{c\text{-}src}$; PTP, phosphotyrosyl-protein phosphatase

Here, we show that tyrosine phosphorylation of certain proteins in human platelet membranes is strongly enhanced when Zn^{2+} is used as the only divalent cation for the kinase reaction, indicating that Zn^{2+} acts not only as a phosphotyrosyl-protein phosphatase inhibitor but also as a stimulator of PTK activity.

2. MATERIALS AND METHODS

2.1. *Materials*

Mops, Na_3VO_4, *O*-phosphoserine and *O*-phosphothreonine were obtained from Sigma (München). *O*-Phosphotyrosine was prepared as in [11]. [γ-^{32}P]ATP (3000 Ci/mmol, 1 Ci = 3.7×10^{10} Bq) was purchased from Amersham Buchler (Braunschweig).

2.2. *Membrane isolation*

Platelet-rich plasma (PRP) from healthy volunteers was obtained 24 h after venipuncture from the local blood bank. PRP was centrifuged at $480 \times g$ for 20 min. The sedimented platelets were resuspended in a buffer containing 10 mM sodium phosphate, 125 mM NaCl, 5 mM KCl, 5 mM EDTA (pH 7.4) and washed twice. Membranes from human platelets were prepared by three different methods described by Golden et al. [9], Waldmann et al. [12] and Resh and Erikson [13]. All buffers used contained in addition 100 μM Na_3VO_4 and 5 mM EDTA. The membranes were finally resuspended in 25 mM Mops (pH 6.5) and kept frozen until use.

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2.3. *In vitro phosphorylation assay*

The phosphotransferase assay was performed for 20 min at 4 or 30°C in a total volume of 50 μl containing 25 mM Mops (pH 6.5), divalent cation and ATP concentrations as indicated and 5 μg membrane protein. The reaction was stopped with 20 μl SDS sample buffer and samples analysed by SDS-PAGE as described [11]. The dried gels were exposed to Kodak X-Omat films for autoradiography. Desired phosphoproteins were cut out from the dried gel and radioactivity determined by Cerenkov counting. Activity of $pp60^{c\text{-}src}$ PTK of human platelet membrane extracts was determined in solid phase according to Presek and Reuter [14], modified by incubating together protein A-Sepharose, TBR serum and antigenic material (60 μl) for 2 h. The phosphotransferase reaction was performed in the presence of 25 mM Mops (pH 6.5) instead of Tris buffer. ATP and ion concentrations were used as indicated. Membrane extracts were prepared by incubating the isolated membranes with 25 mM Mops (pH 6.5) containing 1% (v/v) Triton X-100 for 20 min on ice. The material was centrifuged for 15 min at 30000 × *g* and the supernatant used in the kinase assay. TBR sera (tumor-bearing rabbit sera) were prepared as in [15].

2.4. *Analysis of phosphoamino acids*

Separation and identification of [^{32}P]phosphoamino acids from ^{32}P-labeled proteins were performed according to Hunter and Sefton [16].

2.5. *Partial proteolytic peptide analysis*

One-dimensional peptide mapping of the 68 kDa phosphoprotein was performed according to Cleveland et al. [17].

2.6. *Dephosphorylation assay*

Membrane protein (140 μg) was phosphorylated for 30 min at 4°C in a total volume of 150 μl containing 25 mM Mops (pH 6.5), 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 3 μCi [γ-^{32}P]ATP. At the end of the incubation period, 200 μl of 1 mM ATP was added and the reaction mixture divided into aliquots containing 13 μg protein. Some aliquots were directly stopped with 20 μl of sample buffer as control values. The others were incubated for 20 min at 30°C in either the absence or presence of PTP inhibitors (200 μM Na_3VO_4, 0.02, 0.1 and 0.5 mM zinc acetate). The incubation was terminated with 20 μl sample buffer and samples analysed on SDS-PAGE.

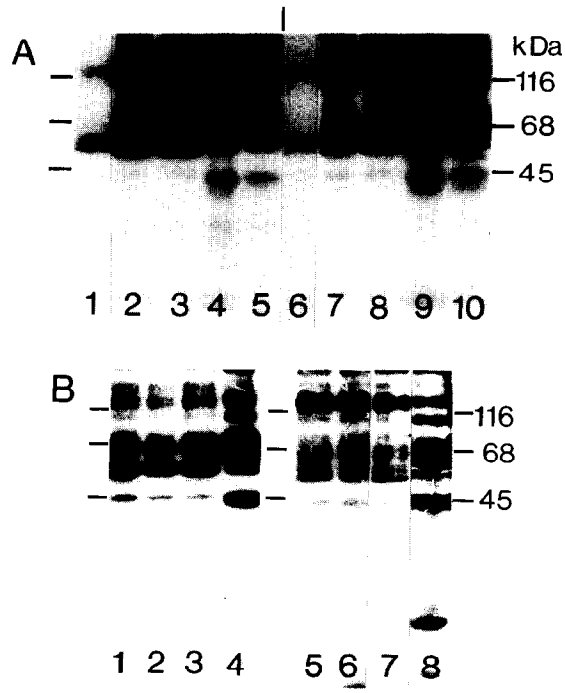

Fig.1. (A) Divalent cation requirement and temperature dependence of tyrosine phosphorylation of membrane proteins. Phosphotransferase reactions were performed in the presence of 6 nM ATP (3000 Ci/mmol) and various cations for 20 min at 4°C (lanes 1–5) and 30°C (lanes 6–10). Autoradiograms of the ^{32}P-labeled phosphoproteins analysed by SDS-PAGE are shown. Lanes: 1,6, 1 mM $MgCl_2$; 2,7, 1 mM $MnCl_2$; 3,8, 1 mM $MgCl_2$/1 mM $MnCl_2$; 4,9, 1 mM $MgCl_2$/1 mM $MnCl_2$/1 mM zinc acetate; 5,10, 1 mM zinc acetate (final concentrations). (B) Effect of phosphotyrosyl-protein phosphatase inhibitors. Platelet membranes were phosphorylated with 1 mM $MgCl_2$/$MnCl_2$ and 6 nM ATP in the presence of different phosphotyrosyl-protein phosphatase inhibitors for 20 min at 4°C (lanes 1–4) and 30°C (lanes 5–8). Autoradiogram of the phosphoprotein patterns is demonstrated. Lanes: 1,5, control; 2,6, 10 mM NaF; 3,7, 200 μM Na_3VO_4; 4,8, 1 mM zinc acetate.

3. RESULTS

Human platelet membranes prepared according to three different methods [9,12,13] showed no remarkable variations in phosphorylation pattern in the presence of Mg^{2+} and Mn^{2+}. In all further experiments, therefore, membranes prepared according to Golden et al. [9] were used. In the presence of Mn^{2+}, a drastic increase in phosphate incorporation into many proteins was observed as compared to Mg^{2+} (fig.1A). Among these, a 68 kDa protein was phosphorylated at tyrosine residues at 4°C but not at 30°C (fig.1). As a phosphotyrosyl-protein phosphatase (PTP) might be responsible for this phenomenon [18], we tested the effect of the phosphatase inhibitors NaF, Na_3VO_4 and Zn^{2+} upon 68 kDa phosphorylation. Only in the presence of Zn^{2+} ($ZnCl_2$ or zinc acetate) did tyrosine phosphorylation of that protein occur at 30°C and was enhanced about 3-fold at 4°C (fig.1B). Apart from the 68 kDa protein, enhancement of tyrosine phosphorylation was observed in the 45 kDa and 105 kDa proteins at both temperatures. In the case of NaF and Na_3VO_4, no change in phosphoprotein patterns

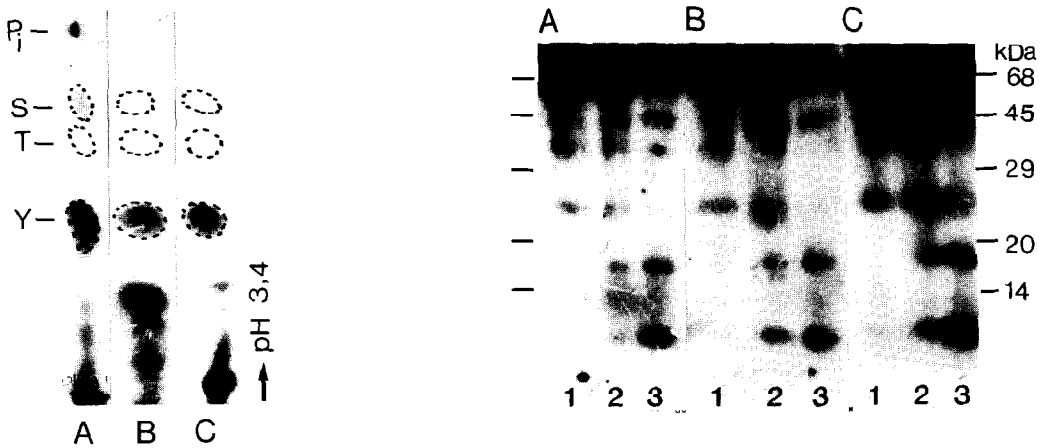

Fig.2. Analysis of the phosphorylated 68 kDa membrane protein. Platelet membranes were phosphorylated with 1 mM $MgCl_2$/1 mM $MnCl_2$ at 4°C (A), 1 mM zinc acetate at 4°C (B) and 1 mM zinc acetate at 30°C (C). Left: one-dimensional phosphoamino acid analysis [16]. The dashed circles show the positions of the standard phosphoamino acids identified by ninhydrin. Y, phosphotyrosine; T, phosphothreonine; S, phosphoserine; P_i, orthophosphate. Right: partial proteolytic peptide mapping by V_8 protease. Digestion was performed with increasing amounts of V_8 protease (Miles Labs). Lanes: 1, 5 ng; 2, 50 ng; 3, 500 ng.

was observed (fig.1B). When prelabeled membranes were incubated at 30°C in the absence of Zn^{2+}, drastic dephosphorylation of the ^{32}P-labeled proteins was observed, thus confirming the role of Zn^{2+} in PTP inactivation. Dephosphorylation was prevented by 0.5 mM Zn^{2+}, while 20 and 100 μM Zn^{2+} were without any effect (not shown).

The enhanced level of phosphate incorporation into the 45, 68 and 105 kDa proteins in the presence of Zn^{2+} was specific as none of the other divalent cations tested (Sr^{2+}, Pb^{2+}, Ni^{2+}, Fe^{2+}, Cs^{2+}, Co^{2+}, Ba^{2+}) induced phosphorylation of these proteins. The 68 kDa proteins phosphorylated in the presence of Mg^{2+}/Mn^{2+} or Zn^{2+} at 4 and at 30°C showed identical phosphopeptide patterns after partial proteolysis, indicating that the same protein substrate was phosphorylated by the kinase independently of the divalent cation used. Phosphorylation occurred exclusively at tyrosine residues (fig.2). Since Zn^{2+} also acts as an inhibitor of PTP activity, we could study the effect of Zn^{2+} upon phosphorylation of the 68 kDa pro-

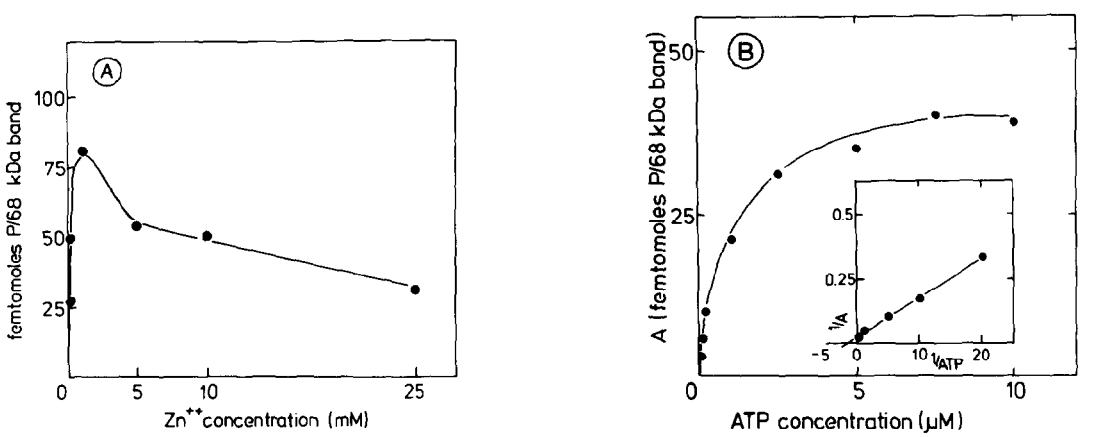

Fig.3. Zn^{2+} concentration (A) and ATP concentration (B) dependence of 68 kDa protein tyrosine phosphorylation. Experiments were performed at 30°C. ATP concentration used in (A) was 2 μM (10 Ci/mmol) and 1 mM zinc acetate was the final divalent cation concentration in (B). Enzyme activity is expressed as fmol phosphate incorporated into the 68 kDa protein.

tein at 30°C. This was a time-dependent process completed within 20 min of incubation time. The most effective ion concentration was 1 mM Zn^{2+}. Higher concentrations of Zn^{2+} did not stimulate PTK activity any further and were even inhibitory (fig.3A). The ATP concentration dependence of the phosphotransferase reaction gave a saturation curve as shown in fig.3B. The K_m for ATP was 0.7 μM.

Since $pp60^{c\text{-}src}$ is highly active in platelet membranes [9], we suggest that the kinase responsible for tyrosine phosphorylation of the various proteins in the presence of Mg^{2+}/Mn^{2+} and Zn^{2+} as well is $pp60^{c\text{-}src}$. In the common kinase assay with immobilized $pp60^{c\text{-}src}$ [4,9,11,14,16], we observed cation concentration-dependent TBR-IgG phosphorylation exclusively at tyrosine residues with Zn^{2+}, Mg^{2+} and Mn^{2+} (fig.4A). In the presence of Zn^{2+} the kinase was most active using 10 nM ATP. When higher concentrations of ATP were used, Mg^{2+} was the most stimulating cation for $pp60^{c\text{-}src}$ PTK. Surprisingly, Mn^{2+} and Zn^{2+} had a lower but similar efficiency (fig.4B). Furthermore, we studied the $pp60^{c\text{-}src}$ dependence of protein phosphorylation in platelet membranes. As shown in fig.5, strong inhibition of protein phosphorylation was observed in a concentration-dependent manner by TBR-IgG but not by control IgG. A weak unspecific effect was also evident with greater amounts of control IgG only. The phosphorylation of the 45, 68 and 105 kDa proteins is totally blocked by the highest TBR-IgG concentration used. In contrast, phosphorylation of the 120 kDa protein was not completely inhibited. This may be due to the activation by Zn^{2+} of another PTK besides $pp60^{c\text{-}src}$.

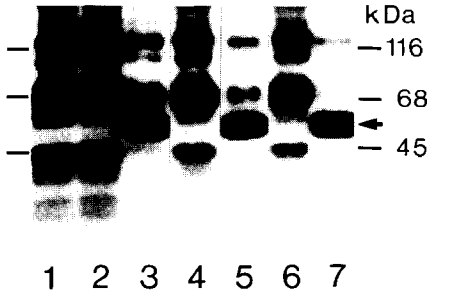

Fig.5. TBR-IgG-dependent inhibition of protein tyrosine phosphorylation in platelet membranes. Membranes were incubated for 2 h with different concentrations of TBR-IgG or IgG of control serum at 4°C and phosphorylated for 20 min at 30°C in the presence of 1 mM zinc acetate. Autoradiogram of the SDS-polyacrylamide gel is shown: Lanes: 1, control (25 mM Mops, pH 6.5); 2, control (C)-IgG (0.1 mg/ml); 3, TBR-IgG (0.1 mg/ml); 4, C-IgG (0.2 mg/ml); 5, TBR-IgG (0.2 mg/ml); 6, C-IgG (0.4 mg/ml); 7, TBR-IgG (0.4 mg/ml). Arrow indicates the phosphorylated heavy chains of TBR-IgG.

4. DISCUSSION

High PTK activity has been reported in terminally differentiated platelets [6,7,9,10] which resulted in tyrosine phosphorylation of various proteins [6,9]. In the presence of Zn^{2+} as the only divalent cation enhanced tyrosine phosphorylation

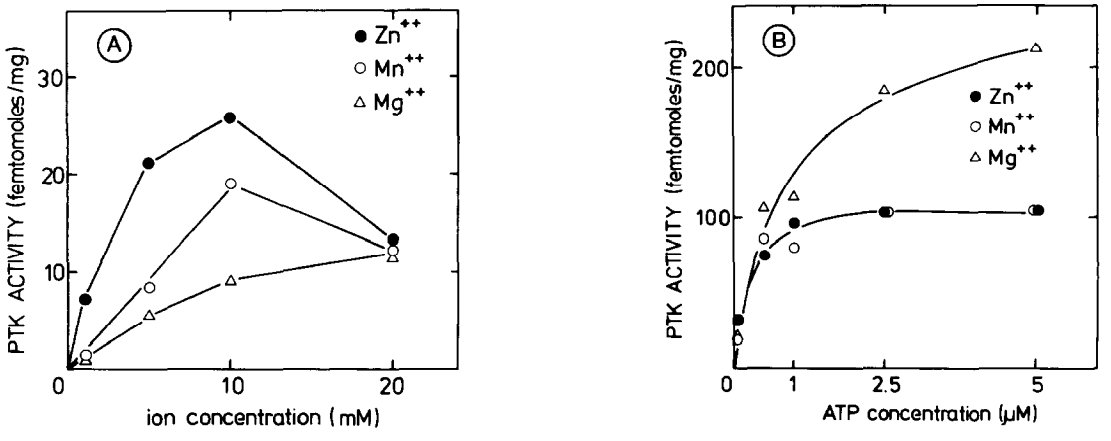

Fig.4. Cation and ATP dependence of $pp60^{c\text{-}src}$ PTK activity of platelet membranes. 40 μg platelet membrane protein were used in the solid-phase assay. (A) Ion concentration dependence was assayed with 10 nM ATP (3000 Ci/mmol). (B) ATP concentration dependence determined in the presence of 10 mM final concentration of ion.

occurred at certain human platelet membrane proteins (fig.1). Zn^{2+} has been found to be a potent phosphotyrosyl-protein phosphatase (PTP) inhibitor at micromolar concentrations [18–23]. As Zn^{2+} was able to inhibit dephosphorylation of the 45, 68 and 105 kDa proteins, we conclude that the observed increase in tyrosine phosphorylation by Zn^{2+} is the result of simultaneous PTK activation and PTP inhibition. Mn^{2+}, which stimulates PTK activity of human platelets [6,7], may have similar effects, since it also acts as a PTP inhibitor [24]. We found that Zn^{2+} alone was as effective as Mn^{2+} on immunoprecipitated $pp60^{c\text{-}src}$ activity in the common solid-phase assay (fig.4). Mg^{2+} was more effective when higher concentrations of ATP were used. This may be the reason why Golden et al. [9] performed phosphorylation of their platelet membranes in the presence of 5 mM Mg^{2+} and 5 μM ATP to analyse $pp60^{c\text{-}src}$ phosphorylation. The discrepancy in cation optimum between $pp60^{c\text{-}src}$ TBR-IgG phosphorylation measured in the solid-phase assay and 68 kDa protein phosphorylation in situ may be caused by an alteration in sensitivity of the immobilized enzyme under the solid-phase assay conditions. The observed TBR-IgG-dependent inhibition of Zn^{2+}-induced membrane protein phosphorylation (fig.5) further indicated that $pp60^{c\text{-}src}$ is involved in the Zn^{2+}-dependent protein phosphorylation. Richard et al. [25] mentioned that the purified degradation product of $pp60^{v\text{-}src}$, p54, can be activated by Zn^{2+}, but the efficiency was only 5% of that obtained with Mg^{2+}. The feline sarcoma virus (TP1-FeSV) *gag-onc* fusion product also exhibited autophosphorylation at tyrosine residues in the presence of Zn^{2+} [26]. Zn^{2+}, on the other hand, had no effect on the activity of an unidentified PTK of HL 60 cells [27]. Inhibition of PTK activity by Zn^{2+} was reported for tyrosine phosphorylation of the epidermal growth factor receptor [28] and autophosphorylation of the insulin receptor which was completely blocked by 10 μM Zn^{2+} [29]. In contrast, Avruch et al. [30] could not demonstrate an inhibitory effect of Zn^{2+} upon insulin receptor tyrosine phosphorylation.

A sequence within the catalytic domain of $pp60^{c\text{-}src}$ [1], Cys(487)-X_4-His-X_3-Cys-X-Cys [31], is similar to one of those reported in nucleic acid binding proteins which potentially could form metal-binding domains [32]. Lys 295 which forms part of the ATP-binding site of $pp60^{c\text{-}src}$ [1] is not present within this sequence, but the binding of Zn^{2+} to $pp60^{c\text{-}src}$ may lead to a conformational change in the molecule resulting in enhanced PTK activity. A similar mechanism has been discussed for PKC activation by Zn^{2+} [33]. Our results indicate that Zn^{2+} can activate $pp60^{c\text{-}src}$ and may be an efficient tool for detecting unknown protein substrates of PTKs in vitro. The physiological significance of Zn^{2+}-dependent PTK activation in intact platelets, however, remains unknown and may be an interesting subject for further investigation.

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