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Phosphorylation of the lymphoid cell kinase p56^{lck} is stimulated by micromolar concentrations of Zn^{2+}

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In particulate fractions from LSTRA lymphoma cells, tyrosine phosphorylation of the lymphoid specific tyrosine kinase p56^{lck} is elicited by Zn^{2+} in the absence of other divalent cations. Zn^{2+} alone also induces autophosphorylation of immunoprecipitated p56lck. The effect of Zn^{2+} is dose dependent; it is detected at concentrations of Zn^{2+} as low as 5 μM and reaches a maximum at 100 μM Zn^{2+}. Among other divalent cations tested, Mn^{2+}, and Co^{2+} to a lesser extent, were also effective. Zn^{2+} also stimulated p56^{lck} phosphorylation in the presence of Mg^{2+} ions at physiological concentration, whereas orthovanadate had no effect. These results suggest that Zn^{2+} activates the autophosphorylation of p56^{lck}; this fact could be related with the stimulating effect of Zn^{2+} in the activation of T lymphocytes.

Zinc; Tyrosine kinase; Lymphoma cell

1. INTRODUCTION

Protein tyrosine kinases (PTK) constitute a class of enzymes thought to regulate growth and/or differentiation in a number of cell types [1]. In several well-documented cases, these activities are associated with transmembrane proteins transducing hormonal and/or growth signals; hence the binding of insulin or EGF to their specific membrane receptors stimulates tyrosine kinase activities in the cytosolic domain of the receptors [2]. On the contrary, the 'non-receptor' or 'src-like' tyrosine kinases are cytoplasmic proteins attached to the inner face of the plasma membrane through their N-terminal end. Their physiological roles as well as the pathways regulating their activity in the cell are poorly understood [3]. Activation of tyrosine kinase activity is correlated with autophosphorylation [2,3].

Recently, new insights in the regulation of 'src-like' protein kinases have come from studies of p56^{lck}, a lymphoid-specific kinase [4,5] involved in T cell function [6]; it was shown that the kinase activity is modulated by the interaction of p56^{lck} with surface proteins expressed in T lymphocytes (CD4 or CD8) [6,7]. The formation of these molecular complexes is critically dependent on the presence, in CD4/CD8 and p56^{lck}, of two pairs of cysteines coordinated by a divalent metal ion [8].

Like other protein kinases, PTKs require the presence of a divalent cation in order to function. The primary action of divalent cations is to form a complex with ATP, which constitutes the phosphate donor. Mg^{2+} or Mn^{2+} are usually efficient at concentrations of one to ten mM in the tyrosine kinase assays; Mg^{2+} is therefore assumed to be the physiological cation, as its intracellular concentration is in the same range (1–3 mM). Other metal ions are generally ineffective. Nonetheless Zn^{2+} was recently found to induce tyrosine kinase activity in platelet membranes, in the absence of other divalent cations [9].

We have investigated the effects of various metal ions on the tyrosine phosphorylation of p56^{lck}, the major non-receptor tyrosine kinase in lymphoid cells. We report here that, in particulate fractions from the murine lymphoma LSTRA (in which p56^{lck} is over expressed), in vitro phosphorylation of p56^{lck} is strongly stimulated by micromolar concentrations of Zn^{2+}, even in the absence of Mg^{2+}. This result is discussed in connection with the possible physiological role of Zn^{2+} in T cell response.

2. MATERIALS AND METHODS

2.1. *Cells*

The LSTRA tumor cell line was originally isolated as a transplantable tumor following infection of BALB/c newborn mice with Moloney murine leukemia virus (MoMulv) and maintained in vivo as an ascitic tumor by serial transplantation in syngeneic host. LSTRA cells express a high level of the lymphoid specific tryosine kinase p56^{lck} [10].

2.2. *Anti p56 antibodies*

Antisera were raised in rabbits against a peptide (residues 39–62) of the sequence of human p56^{lck}. These sera reacted in immunoprecipitation and immunoblotting experiments with a 56–58 kDa protein present in LSTRA cells and in T lymphoid cells of human and murine origin; the reaction was inhibited by preincubation of the serum with the 39–62 peptide used for immunization (D. Fradelizi and G. Gacon, unpublished results).

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2.3. *Materials*

Phenylmethylsulphonylfluoride (PMSF), aprotinine, and unlabelled ATP were obtained from Sigma laboratories. [γ-^{32}P]ATP (5000 Ci/mmol) was purchased from Amersham England. All reagents used for gel electrophoresis were from Bio Rad, X-ray films from Kodak and Fuji.

2.4. *Particulate fractions of LSTRA cells*

Cells were washed twice in PBS, and lysed with a Dounce homogenizer in 10 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1% aprotinine, 1 mM PMSF, (1 ml for 2×10^8 cells) at 0°C. Nuclei were removed by low-speed centrifugation in 250 mM sucrose and the post-nuclear fraction was centrifuged at 400000 × g for 15 min. The pellet was resuspended in 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1% aprotinine and 1 mM PMSF. Aliquots were stored in liquid nitrogen.

2.5. *Phosphorylation and analysis of phosphoproteins*

Prior to phosphorylation, particulate fractions were washed twice with 2 mM EDTA and resuspended in the phosphorylation buffer (50 mM HEPES, pH 7.4). Particulate fractions (10 μg of protein) were preincubated for 3 min in the presence of the indicated metal ions. Phosphorylation reaction was started by adding 0.3 μCi of [γ-^{32}P]ATP, (0.5 μM ATP) allowed to proceed for 5 min at 0°C, and stopped by adding SDS sample buffer.

Phosphoproteins were resolved on 10% polyacrylamide SDS gels and revealed by autoradiography of the dried gels. In some cases, gels were alkali treated after glutaraldehyde fixation [11], i.e. soaked in 2 M NaOH at 55°C for one hour, prior to drying and film exposure.

Phosphorylated bands were quantitated by densitometric scanning of the X-Ray films using a Shimadzu densitometer.

2.6. *Immunoprecipitation*

Particulate fractions of LSTRA cells were solubilized in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP 40, 1% sodium deoxycholate (DOC), 1 mM dithiothreitol (DTT), 1% aprotinine); p56 antibodies were added and allowed to react for 4 h under continuous mixing. Immunocomplexes were collected on protein A Sepharose beads, washed 3 times in RIPA and once in 50 mM HEPES, pH 7.4.

2.7. *Protein content*

The protein concentration was determined according to Bradford [12].

3. RESULTS

Mg^{2+} and Zn^{2+} were compared for their ability to elicit protein phosphorylation in membranes from LSTRA cells. In particulate fractions previously washed in 2 mM EDTA (i.e. in the absence of divalent cation) no detectable phosphorylation occured upon in vitro incubation with [γ-^{32}P]ATP (see Fig. 3). As shown in Fig. 1(Ia) (lane 1), the addition of Mg^{2+} (10 mM) to LSTRA fractions induces protein phosphorylation, specifically in one major band of M_r 55000-60000

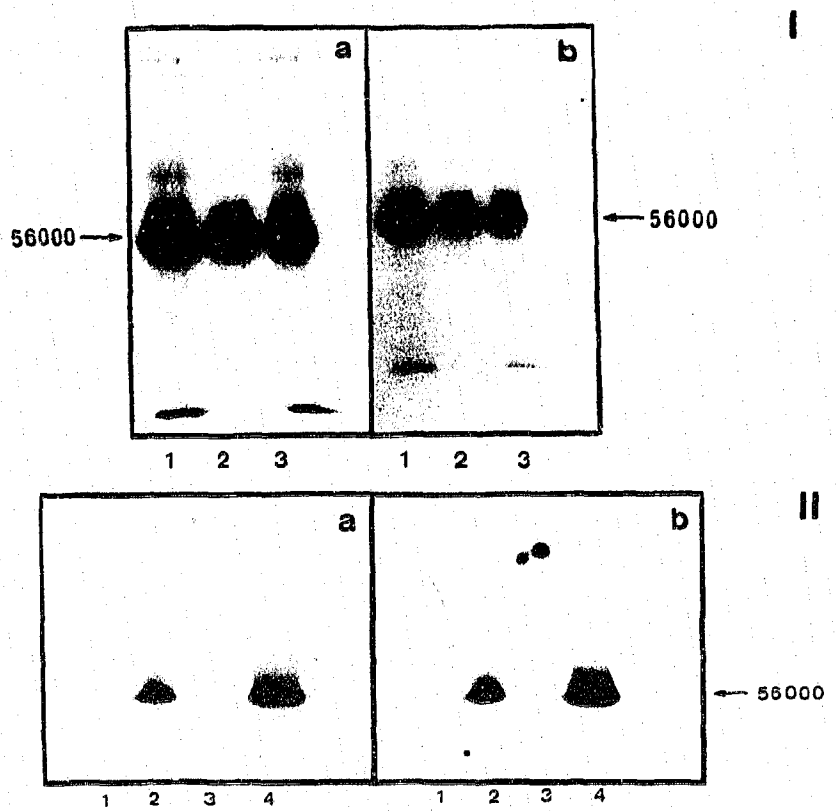

Fig. 1. Phosphorylation of particulate fractions from LSTRA cells; effects of Zn^{2+}. (I) Particulate fractions from LSTRA cells were washed with 2 mM EDTA and labelled with [γ-^{32}P]ATP for 5 min at 0°C, in the presence of either 10 mM Mg^{2+} (lane 1), 1 mM Zn^{2+} (lane 2) or both (lane 3). Phosphoproteins were analysed on SDS-PAGE as described in section 2. (a) Autoradiogram of the untreated gel; (b) autoradiogram of the alkali-treated gel. (II) Particulate fractions from LSTRA cells, phosphorylated in the presence of either 1 mM Zn^{2+} (lanes 1 and 2) or 10 mM Mg^{2+} (lanes 3 and 4) were immunoprecipitated with non-immune serum (lanes 1 and 3) and anti-p56^{lck} antiserum (lanes 2 and 4); immunoprecipitates were analyzed on SDS-PAGE. (a) Untreated gel; (b) alkali-treated gel.

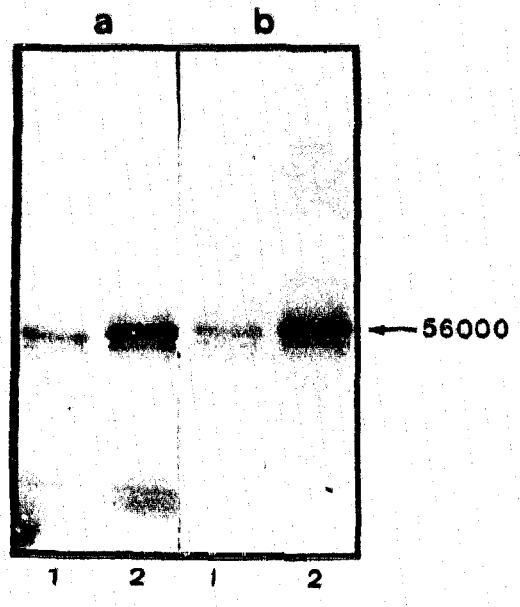

Fig. 2. Phosphorylation of immunoprecipitated $p56^{lck}$. $p56^{lck}$ was immunoprecipitated from LSTRA particulate fractions with anti-$p56^{lck}$ antiserum; immunoprecipitates were resuspended in 50 μl of 50 mM HEPES, pH 7.4, phosphorylated with 10 μCi of [γ-^{32}P]ATP (0.1 μM ATP) for 5 min at 0°C in the presence of 1 mM Zn^{2+} (lane 1) or 10 mM Mg^{2+} (lane 2) and analysed on SDS-PAGE. (a) Untreated gel; (b) alkali-treated gel.

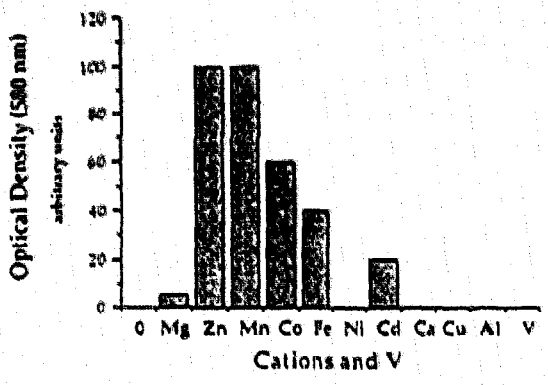

Fig. 4. Phosphorylation of $p56^{lck}$; effects of different metal ions and of vanadate. Particulate fractions from LSTRA cells were labelled in the presence of different cations and vanadate at the concentration of 100 μM. The intensity of the p56 band was recorded by scanning of the autoradiography. Ordinate values were expressed as percent of Zn^{2+}-induced labeling.

which is highly labelled. A similar phosphorylation is observed when Zn^{2+}(1 mM) is added instead of Mg^{2+} (Fig. 1(Ia), lane 2), except for minor bands which are labelled in the presence of Mg^{2+} but not in the presence of Zn^{2+} alone. The M_r 55 000-60 000 band labelled in the presence of Mg^{2+} or Zn^{2+} is resistant to the alkali treatment of the gel (Fig. 1(Ib), suggesting that it contains mainly phosphotyrosine as a phosphoamino acid; this has been confirmed by phosphoamino acid analysis (result not shown).

According to previous studies [4,5], the major alkali-resistant phosphoprotein of M_r 55 000-60 000 in the LSTRA particulate fraction, corresponds to the autophosphorylation on tyrosine of the lymphoid specific tyrosine kinase $p56^{lck}$, which is overexpressed in LSTRA cells. Therefore, we attempted to immunoprecipitate $p56^{lck}$ from LSTRA fractions previously phosphorylated in the presence of Zn^{2+} or Mg^{2+}. The result, shown in Fig. 1(IIa), confirms that the protein phosphorylated in both conditions is $p56^{lck}$ and thus $p56^{lck}$ is indeed phosphorylated in the presence of Zn^{2+} as sole divalent cation. The immunoprecipitated phosphoprotein is alkali resistant to the same extent in Mg^{2+}- and Zn^{2+}-dependent phosphorylations (Fig. 1(IIb)). Moreover, as shown in Fig. 2, Zn^{2+} can elicit alkali-

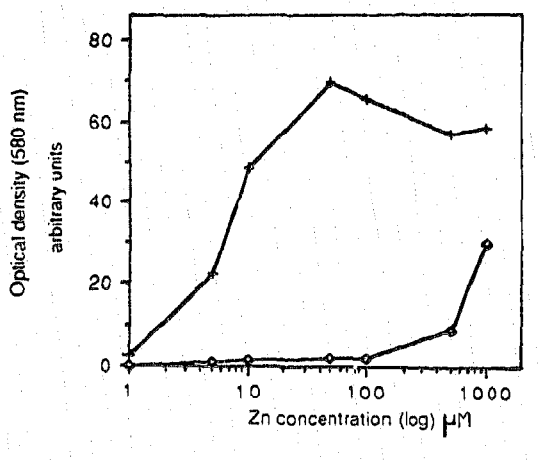

Fig. 3. Phosphorylation of $p56^{lck}$; effects of Mg^{2+} and Zn^{2+}. Particulate fractions from LSTRA cells were phosphorylated in the presence of Mg^{2+} (○) or Zn^{2+} (+) ions, at various concentrations. The intensity of the p56 band was recorded by scanning of the autoradiography.

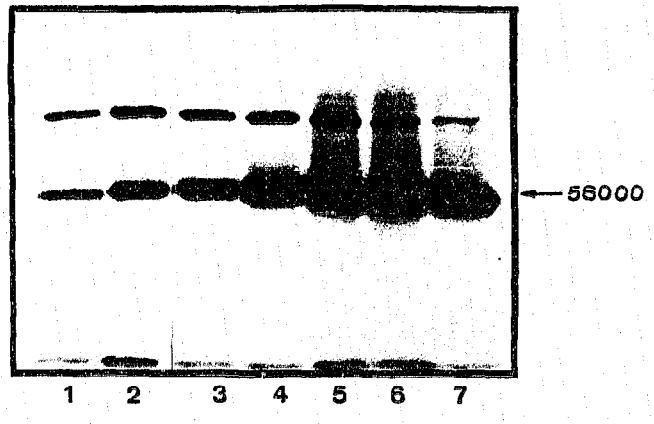

Fig. 5. Phosphorylation of particulate fractions from LSTRA cells; effects of Zn^{2+} in the presence of 1 mM Mg^{2+}. Particulate fractions from LSTRA cells were phosphorylated in the presence of 1 mM Mg^{2+} and increasing concentrations of Zn^{2+}. (1) no Zn^{2+}; (2) 1 μM Zn^{2+}; (3) 10 μM Zn^{2+}; (4) 50 μM Zn^{2+}; (5) 100 μM Zn^{2+}; (6) 500 μM Zn^{2+}; (7) 1 mM Zn^{2+}.

stable phosphorylation in $p56^{lck}$ previously purified by immunoprecipitation; the pattern appears very similar to that obtained by immunoprecipitation of previously phosphorylated membranes (Fig. 1(II)). These results indicate that Zn^{2+} alone is capable of inducing autophosphorylation of $p56^{lck}$.

The effect of Zn^{2+} in eliciting $p56^{lck}$ phosphorylation is concentration dependent; Zn^{2+} concentrations as low as 1–5 μM induce a clear phosphorylation of $p56^{lck}$ (Fig. 3) and the protein is maximally labeled at Zn^{2+} concentrations of 100–500 μM. By contrast, with Mg^{2+}, no phosphorylation is detectable in the μM range, and the maximum labelling is reached at concentrations higher than 1 mM.

Other metal ions were examined for their capacity to induce phosphorylation of $p56^{lck}$. All cations were tested at a concentration of 100 μM; this is shown in Fig. 4: Mn^{2+} was as effective as Zn^{2+}, Co^{2+} was also effective but to a lesser extent, Fe^{2+} and Cd^{2+} showed a moderate activity, whereas Ni^{2+}, Ca^{2+}, Cu^{2+} and Al^{3+} were ineffective. Otherwise orthovanadate did not show any activity.

The effect of Zn^{2+} ions was also analysed in the presence of 1 mM Mg^{2+}. As illustrated in Fig. 5, a stimulation of $p56^{lck}$ phosphorylation by low concentrations (1 and 10 μM) of Zn^{2+} ions is clearly visible with maximal labelling of $p56^{lck}$ also obtained at concentrations of 100–500 μM Zn^{2+} ions.

4. DISCUSSION

In the data reported here, Zn^{2+} at micromolar concentrations is shown to stimulate tyrosine phosphorylation of the $p56^{lck}$ protein in LSTRA particulate fractions, in the absence of other divalent cations. Similar results have been obtained in particulate fractions from peripheral blood lymphocytes (result not shown). Clearly, Zn^{2+} ions do not have the same effect on all tyrosine kinases. Phosphorylation of EGF receptor in hepatocyte membranes or of insulin receptor β subunit are not stimulated by Zn^{2+} [15,16]. By contrast, it was recently reported that a tyrosine kinase activity, present in platelate membranes, most probably $p60^{src}$, was stimulated by Zn^{2+} ions [9]. To our knowledge, serine/threonine protein kinases cannot be activated by Zn^{2+} alone. Since Zn^{2+} is capable of eliciting phosphorylation of $p56^{lck}$ in the absence of other divalent cations, it substitutes for Mg^{2+} in the formation of a phosphate donor complex with ATP. This may implicate a particular conformation of the ATP binding site of $p56^{lck}$.

However, the stimulating effect of Zn^{2+} ions in the presence of a large excess of Mg^{2+} ions may involve a different mechanism. For instance, as described in the case of protein kinase C [17,18], binding of Zn^{2+} ions to $p56^{lck}$, could result in a simulation of the kinase activity. Recent studies have shown that, in the plasma membrane of T cels, $p56^{lck}$ is linked to proteins CD4 or CD8; these interactions involve two pairs of cysteine residues present on partner proteins, which are thought to form a metal ion binding site [6–8]. It is thus tempting to assume that such protein/protein interactions mediated by a Zn^{2+} ion, might stimulate the autophosphorylation of the lck kinase. Thus, Zn^{2+} would act at least by two distinct mechanisms: (i) the formation of a Zn-ATP complex; and (ii) activation of the kinase through another binding site. Such a dual effect has been previously described for Mg^{2+} with casein kinases I and II [19], and for Mn^{2+} with the insulin receptor tyrosine kinase [20].

Zn^{2+} is known to inhibit some phosphotyrosine phosphatase activities [13] (although it activates the CD45 tyrosine phosphatase (leucocyte common antigen) [14]). Hence, the stimulation of phosphorylation of $p56^{lck}$ by Zn^{2+} might be due to this inhibitory effect. However, Co^{2+} which does not inhibit phosphatase activities [13] is also able to promote phosphorylation of $p56^{lck}$. Moreover, sodium orthovanadate, a phosphotyrosine phosphatase inhibitor, unlike Zn^{2+}, does not stimulate the phosphorylation of $p56^{lck}$ (Fig. 4). Finally, we have observed that Zn^{2+} can elicit tyrosine phosphorylation in $p56^{lck}$ purified by immunoprecipitation (Fig. 2). Thus, the effect of Zn^{2+} must be, at least partly, related to an increased tyrosine phosphorylation of $p56^{lck}$. Since in vitro phosphorylation of $p56^{lck}$ is known to occur through autophosphorylation [13], it is likely that Zn^{2+} stimulates $p56^{lck}$ autokinase activity.

Our experiments clearly show that Zn^{2+} at very low concentrations can act as a modulatory agent even in the presence of Mg^{2+} at physiological concentration. The requirement of Zn^{2+} for the development of the immune response and more specifically for activation of T lymphocytes has been documented. Several studies have shown that low concentrations of Zn^{2+} (ca. 0.1 mM) are capable of restoring T cell mitogen responsiveness or even of inducing mitogenesis [21–23]. It is therefore conceiveable that the activation of $p56^{lck}$ plays a role as a target of Zn^{2+} action. Further work is needed to analyze this hypothesis.

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