

# Interleukin 2 mediates p72<sup>syk</sup> activation in peripheral blood lymphocytes

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## Abstract

The ability of interleukin 2 (IL-2) to stimulate p72<sup>syk</sup> activity in intact porcine peripheral blood lymphocytes was examined. We demonstrated that IL-2 activated p72<sup>syk</sup> in a time- and dose-dependent manner, for which its peak time and maximum responsive dose were 5 min and 100 U/ml, respectively. This activation was observed only in cytosolic fractions and not in membrane ones. However, IL-2 failed to induce calcium mobilization. Moreover, IL-2-inducible p72<sup>syk</sup> activation was not affected when extra- and intracellular calcium was depleted. These data suggest that the IL-2 signaling pathway through p72<sup>syk</sup> in peripheral blood lymphocytes is different, at least in part, from other agonists, such as concanavalin A in polymorphonuclear neutrophils which can trigger both the activation of p72<sup>syk</sup> and intracellular calcium mobilization.

**Key words:** Interleukin 2; Protein-tyrosine kinase p72<sup>syk</sup>; Peripheral blood lymphocyte

## 1. Introduction

Interleukin-2 (IL-2) is a lymphokine produced by T cells that induces proliferation and differentiation of T, B, and NK cells. Unlike many other growth factor receptors, such as platelet-derived growth factor and epidermal growth factor receptors, the IL-2 receptor (IL-2R) consists of at least three distinct polypeptides, the IL-2R $\alpha$ (p55)-, IL-2R $\beta$ (p75)- and IL-2R $\gamma$ (p64)-chains [1–4]. Among them, none of the cytoplasmic regions of the IL-2R subunits contain any apparent catalytic motifs, such as kinase consensus sequence [3,5,6], however, IL-2 stimulation leads to the rapid phosphorylation of tyrosine residues on several cellular proteins, including the IL-2R $\beta$ -chain itself [7], and increases the activities of non-receptor protein-tyrosine kinase(s) (PTK). The well-characterised PTK which non-covalently associates with IL-2R $\beta$  is p56<sup>lck</sup> [8,9], a member of the *src* family in T cells. In addition to p56<sup>lck</sup>, p59<sup>lyn</sup> is also a candidate in T and B cells [10,11] and p53/56<sup>lyn</sup> in a p56<sup>lck</sup>-negative pro-B cell line [12]. These results implicate some flexibility or redundancy in the ability of various members of the *src* family to participate in IL-2 signal transduction in a cell lineage-specific manner. This urges us to search for other non-receptor PTK(s) that could be candidate(s) of IL-2 signaling events.

The Syk family of kinases, including Syk [13] and ZAP-70 [14], were recently identified as non-receptor PTKs which have the characteristics of bearing two SH<sub>2</sub> domains and no N-terminal myristylation site. We have reported that p72<sup>syk</sup> is associated with the B cell IgM receptor [15] and activated by thrombin in porcine platelets [16] or concanavalin A in porcine peripheral blood lymphocytes (PBLs) [17]. In this paper we explored whether p72<sup>syk</sup> has roles in IL-2-mediated signal transduction. Our data suggested that IL-2 stimulation could lead to activation of p72<sup>syk</sup> in a time- and dose-dependent manner but failed to induce calcium mobilization in porcine PBLs. Furthermore, p72<sup>syk</sup> existed in both cytosolic and particulate fractions, however, the activation of p72<sup>syk</sup> was observed only in cytosolic fractions.

## 2. Materials and methods

### 2.1. Materials and chemicals

Porcine blood and spleen were obtained from a local slaughterhouse. Anti-CPTK40 antibodies, raised against the synthetic partial polypeptides of p72<sup>syk</sup>, were prepared as previously described [13]. Ionomycin was purchased from Sigma. Pansorbin cell suspension was from Calbiochem Corp. Lymphocyte separation solution (d = 1.077) was from Nacalai Tesque Inc. Percoll solution was from Pharmacia. Recombinant human interleukin 2 was kindly provided by Takeda Chemical Industries. Acetoxymethyl esters of 5,5'-diethyl-bis-(*O*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA-AM) and Fura 2-AM were from Wako Pure Chemicals. Anti-phosphotyrosine antibody (4G10) was obtained from UBI.

### 2.2. Cell isolation and stimulation

Isolation protocols were as described previously [17]. Briefly PBLs were harvested from fresh blood by lymphocyte separation solution and Percoll gradient centrifugation followed by erythrocyte sedimentation with physiological saline solution containing 3% dextran. Residual erythrocytes were lysed in hypotonic condition. Cells were washed twice and finally suspended ( $5\text{--}10 \times 10^7$  cells/ml) in HBSS (136.7 mM NaCl, 5.4 mM KCl, 0.81 mM MgSO<sub>4</sub>, 1.3 mM CaCl<sub>2</sub>, 0.33 mM Na<sub>2</sub>HPO<sub>4</sub>,

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**Abbreviations:** PTK, protein-tyrosine kinase; HBSS, Hanks' balanced salt solution; IL-2, interleukin 2; IL-2R, interleukin 2 receptor; PBLs, peripheral blood lymphocytes; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular calcium concentration; BAPTA-AM, acetoxymethyl esters of 5,5'-diethyl-bis-(*O*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid.

0.44 mM  $\text{KH}_2\text{PO}_4$ , 5.6 mM dextrose, 4.2 mM  $\text{NaHCO}_3$ , pH 7.4). In the case of  $\text{Ca}^{2+}$  depletion, cells were suspended in  $\text{Ca}^{2+}$ -free HBSS. Porcine splenocytes were prepared from freshly obtained spleen by teasing against a wire screen followed by hemolysis and also suspended in HBSS. PBLs or splenocytes were stimulated by IL-2 at 37°C with gently stirring.

### 2.3. Measurement of intracellular calcium concentration ( $[\text{Ca}^{2+}]_i$ )

The cells were pretreated with Fura 2-AM at a final concentration of 5  $\mu\text{M}$  for 30 min at 37°C. The washed cell suspension ( $2 \times 10^6$  cells/ml) was incubated in a cuvette for 5 min at 37°C and stimulated by ionomycin or IL-2 under gently stirring. Fluorescence was monitored using a Shimadzu fluorescence spectrometer RF-5000 with standard monitor settings of 340 nm and 380 nm excitation and 490 nm emission.

### 2.4. Immunoblot procedures

Cell stimulation and immunoblot analysis were carried out as described previously [13].

### 2.5. Immunoprecipitation and kinase assays

Stimulated cells ( $1 \times 10^8$  cells/ml) were lysed in 500  $\mu\text{l}$  of RIPA buffer (5 mM EDTA, 150 mM NaCl, 2% Triton X-100, 100  $\mu\text{M}$   $\text{Na}_3\text{VO}_4$ , 2 mM phenylmethylsulfonyl fluoride, 10  $\mu\text{g}/\text{ml}$  leupeptin, 50 mM Tris, pH 7.4) followed by quick centrifugation. The lysates were precleared by incubation for 30 min with pansorbin cells. Nuclei and cellular debris were removed by centrifugation at  $16,000 \times g$  for 15 min at 4°C. The supernatant was immunoprecipitated with 0.4  $\mu\text{g}$  anti-CPTK40 antibody and 40  $\mu\text{l}$  of pansorbin for 1 h at 4°C. Immunoprecipitates were washed three times with RIPA buffer, once with 10 mM HEPES (pH 8.0) buffer containing 500 mM NaCl and once with the same HEPES buffer without NaCl, then immunoprecipitates were phosphorylated with or without 0.2 mg/ml H2B histone as previously described [13]. The gels were treated with 1 M KOH at 56°C for 1 h to remove phosphoserine and phosphothreonine [12] before exposure. Autoradiography was carried out with phosphorImager.

### 2.6. Preparation of subcellular fractions

Stimulated PBLs ( $1 \times 10^8$  cells/ml) were lysed in RIPA buffer without Triton X-100. After centrifugation at  $800 \times g$  for 5 min at 4°C, the post-nuclear fraction obtained was further centrifuged at  $100,000 \times g$  for 60 min at 4°C. The supernatant and pellet resuspended in RIPA buffer were the cytosolic and particulate fraction, respectively.

## 3. Results

### 3.1. IL-2 induces transient alteration in the specific activity of $p72^{\text{syk}}$ but not calcium mobilization in porcine PBLs

Immunoprecipitation kinase assays revealed that  $p72^{\text{syk}}$  activity was transiently elevated upon addition of IL-2. The  $p72^{\text{syk}}$  activity was detected within 15 s, peaked at 5 min and approached baseline levels within 20 min (Fig. 1A and B). Densitometric analysis revealed a 5- and 7-fold increase in the activity for auto- and exogenous substrate phosphorylation, respectively. Since the abundance of  $p72^{\text{syk}}$  by immunoblot did not change during the tested time-course (data not shown), the IL-2-induced  $p72^{\text{syk}}$  activity reflected a rise in the specific activity. The IL-2-triggered  $p72^{\text{syk}}$  activation was observed not only in porcine PBLs, but also in porcine splenocytes (data not shown).

To confirm that IL-2 could mediate the activation of  $p72^{\text{syk}}$  in porcine PBLs, the dose-dependence of the

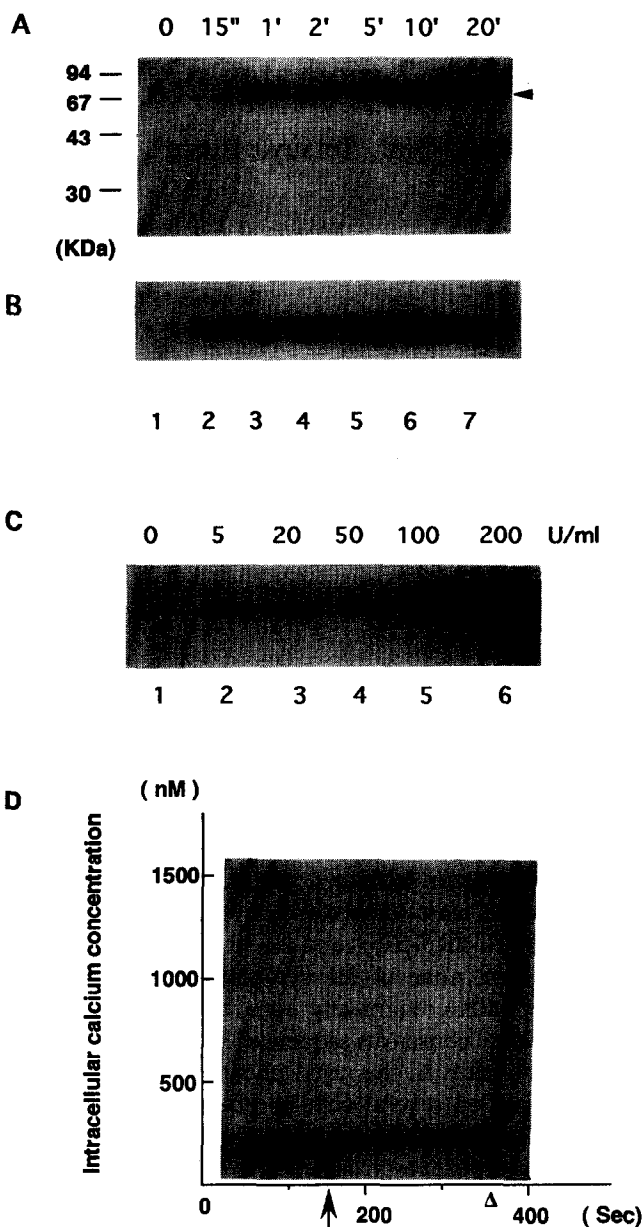


Fig. 1. IL-2 induces a transient alteration of  $p72^{\text{syk}}$  activity but not calcium mobilization in porcine PBLs. (A) Time-course. After  $1 \times 10^8$  cells/sample were stimulated with 100 U/ml IL-2 for the indicated time, they were processed for immunoprecipitation kinase assay as described in section 2. The samples were subjected to 12.5% SDS-PAGE and were visualized by autoradiography. Lane 1, control; lane 2, incubation of 15 s; lane 3, incubation of 1 min; lane 4, incubation of 2 min; lane 5, incubation of 5 min; lane 6, incubation of 10 min; lane 7, incubation of 20 min. The arrowheads and asterisk indicate the positions of  $p72^{\text{syk}}$  and H2B histone, respectively. (B) Autoradiograph of the phosphorylation of H2B histone by immunoprecipitated  $p72^{\text{syk}}$ . (C) Dose-dependence. Cells were stimulated for 5 min with various concentrations of IL-2 and in vitro kinase assays were performed as described in section 2. Lane 1, control; lane 2, 5 U/ml; lane 3, 20 U/ml; lane 4, 50 U/ml; lane 5, 100 U/ml; lane 6, 200 U/ml. (D) Cytosolic calcium response. Cells were loaded with 5  $\mu\text{M}$  of Fura 2-AM. Intracellular calcium concentration was quantified as described in section 2. 1  $\mu\text{M}$  ionomycin stimulation was used as a positive control. An arrow and an open triangle indicate the beginnings of IL-2 and ionomycin stimulation, respectively.

IL-2-induced elevation of the  $p72^{syk}$  activity was examined. As shown in Fig. 1C,  $p72^{syk}$  activation by IL-2 occurred in a dose-dependent manner. Within the range 0–100 U/ml of IL-2, the intensity of autophosphorylation activity was paralleled with an increase in IL-2 amount. In contrast,  $p72^{syk}$  activity declined when the dose of IL-2 was up to 200 U/ml (Fig. 1C, lane 6). Based on these observations, IL-2 was an activator of  $p72^{syk}$  in porcine PBLs.

Intracellular calcium mobilization was shown to be a PTK-dependent event and an initial signal in activated T or B cells [15,18]. It was of interest to know whether a similar phenomenon happens in the case of IL-2R-mediated activation. A representative profile is shown in Fig. 1D. Unexpectedly, we did not observe calcium mobilization induced by IL-2, however, a rapid increase in  $[Ca^{2+}]_i$  was observed after 1  $\mu$ M of ionomycin stimulation in the same sample (Fig. 1D). The failure of IL-2 to trigger cytosolic calcium elevation was further confirmed by using a high dose (1,000 U/ml) and longer incubation time (15 min), which also did not induce an increase in intracellular calcium concentration (data not shown). Therefore, it is reasonable to postulate that calcium is not involved in  $p72^{syk}$  activation by IL-2. Next, experiments were performed to confirm this possibility. Before stimulation, extra- and intracellular calcium was chelated with 2 mM of EGTA and 15  $\mu$ M of BAPTA-AM, respectively; the observed kinetic pattern of  $p72^{syk}$  activation (data not shown) was similar to that when calcium was present suggesting that IL-2-mediated  $p72^{syk}$  activation was independent of intracellular calcium elevation.

### 3.2. IL-2 enhances $p72^{syk}$ activity only in cytosolic fractions

$p72^{syk}$  exists in both particulate and cytosolic fractions in porcine PBLs, as confirmed by immunoblot anal-

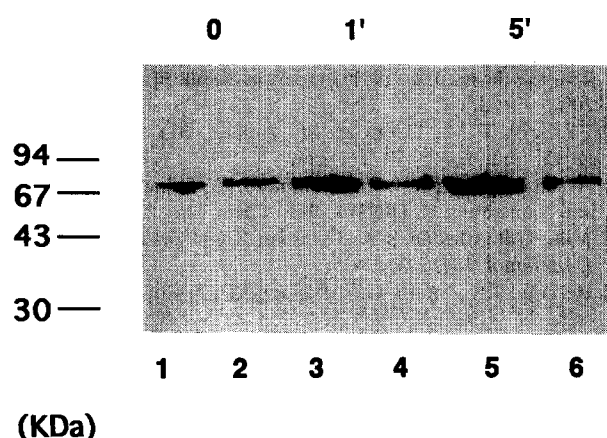


Fig. 2. IL-2 enhances  $p72^{syk}$  activity only in cytosolic fractions. Cells were stimulated with 100 U/ml IL-2 at the indicated times. Subcellular fractions were prepared and immunoprecipitation kinase was processed as described in section 2. Unstimulated cells (lanes 1, 2), 1 min (lanes 3, 4) and 5 min (lanes 5, 6) stimulation. Lanes 1, 3, 5 and lanes 2, 4, 6 were cytosolic and particulate fractions, respectively.

ysis [17]. The distribution ratio of cytosol-to-particulate matter was about 2 and remained unchanged after IL-2 stimulation (data not shown). This result might suggest that translocation did not occur after IL-2 stimulation. In addition, this observation raised the argument of which part(s) of  $p72^{syk}$  in terms of subcellular localization was responsible for the increase in kinase activity. Subcellular fractions were prepared from these cells treated with IL-2 and assayed for  $p72^{syk}$  activity. We observed that  $p72^{syk}$  activity was not significantly modified by IL-2 in particulate fractions compared with untreated cells (Fig. 2). In contrast, the increase in  $p72^{syk}$  activity was clearly observed in cytosolic fractions. As shown in Fig. 2, a ~5-fold increase in PTK activity was reached after 5 min stimulation, indicating that IL-2 perturbation only augmented  $p72^{syk}$  activity localized in the cytosol.

## 4. Discussion

We have previously reported the molecular cloning of a gene, *syk*, encoding the 72 kDa non-receptor PTK [13]. In this paper, we demonstrated that  $p72^{syk}$  was one of the PTKs participating in the IL-2-mediated signaling events. IL-2 induced a rapid increase in the activity of  $p72^{syk}$  in porcine PBLs; this activation was observed only in cytosolic fractions.

The time-course of  $p72^{syk}$  activation in porcine PBLs is similar to that of  $p56^{lck}$  in an IL-2-dependent human T cell clone after IL-2 engagement [19]. The increase in  $p72^{syk}$  activity was proportional to the augmentation of IL-2 dose over the range 0–100 U/ml. When 200 U/ml IL-2 was applied, in contrast,  $p72^{syk}$  activity decreased. At present, it is difficult to explain the low activity induced by high doses of IL-2. However, the same phenomenon was also observed in IL-2-stimulated activation of mitogen-activated protein kinase-2 in human PBLs [20]. However, we observed subtle changes of tyrosine-residue phosphorylation in 1–2 protein(s) (data not shown), reflecting one possibility that the total level of tyrosine phosphorylation might be modified by other IL-2-triggered signals in porcine PBLs. Further investigation showed that IL-2 stimulation increased the  $p72^{syk}$  activity only in cytosolic fractions. This observation led us to consider that the activation pathway of  $p72^{syk}$  was mediated by other transducers, such as the *src* family non-receptor PTKs, although we could not exclude the possibility of direct interaction between  $p72^{syk}$  and IL-2R $\beta$ . Since the redundancy and cell-lineage specificity exist in IL-2 signaling through non-receptor PTKs, it is unclear how IL-2R $\beta$  commits the IL-2 binding signal to each individual non-receptor PTK. For example, F7 and 14B, two transfectants with IL-2R $\beta$  cDNA derived from an IL-3-dependent pro-B cell line, BAF-B03, have some contrasting features in  $p53/56^{lyn}$  and  $p59^{fyn}$  activation by

IL-2. In F7 cells, only p53/56<sup>lyn</sup> was activated and physically associated with IL-2R $\beta$  [12]. However, in 14B cells, both p53/56<sup>lyn</sup> and p59<sup>lyn</sup> were activated but only p59<sup>lyn</sup> physically associated with IL-2R $\beta$  [11].

The elevation of intracellular calcium has been established to be one of the initial signals through the T cell receptor [18] or oligomerization of neutrophil cell surface glycoproteins by concanavalin A [21]. Our data demonstrated that IL-2 was capable of inducing an early, transient change in specific activity of p72<sup>syk</sup> in a dose-dependent manner within the range 0–100 U/ml. Nevertheless, when extra- and intracellular Ca<sup>2+</sup> was chelated by the presence of 2 mM EGTA and 15  $\mu$ M BAPTA-AM, respectively, IL-2-triggered p72<sup>syk</sup> activation was similar to that when calcium was present, suggesting that p72<sup>syk</sup> activation was not affected by the intracellular calcium level. Moreover, IL-2 itself also failed to induce Ca<sup>2+</sup> mobilization, further confirming that IL-2-dependent activation of p72<sup>syk</sup> neither resulted in nor required an increase in [Ca<sup>2+</sup>]<sub>i</sub>. Our result was consistent with the report by Mills et al. [22] that in fresh or established IL-2-dependent human PBL cell lines, incubation with IL-2 did not lead to an increase in [Ca<sup>2+</sup>]<sub>i</sub> at any time during the 24 h. During this time period, however, IL-2-induced proliferation progressed normally even under the condition that calcium was clamped [22]. These data suggested that the IL-2 signaling pathway was different from that of mitogenic agents such as phytohemagglutinin and concanavalin A for which initial calcium movement was required for proliferation [23]. In addition, the deactivation pattern of p72<sup>syk</sup> induced by IL-2 in PBLs was different from that induced by thrombin and TXA<sub>2</sub> in platelets [16,24] and concanavalin A in neutrophils [21] when extra- and intracellular calcium was depleted. In the former, deactivation was the same as when calcium was present, but in the latter, no deactivation after maximal p72<sup>syk</sup> activity was observed. This discordance might result from the difference in cells, ligands, signaling pathways and so on. Although the exact deactivation mechanism was unclear, it seemed that the ligand and signaling pathway might play a more important role in regulating p72<sup>syk</sup> deactivation since calcium was also not involved in IL-2 signaling in human PBLs and IL-2-sensitive murine cell lines [22].

In summary, we have demonstrated that IL-2 can activate p72<sup>syk</sup> in addition to p56<sup>lck</sup>, p59<sup>lyn</sup> and p53/56<sup>lyn</sup>. This activation does not require the involvement of an intracellular calcium increase. The mechanism by which IL-2 regulates p72<sup>syk</sup> activity through its receptor is still obscure. Further investigation will be necessary to elucidate this.

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