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THE LECTIN CONCANAVALIN A STIMULATES A PROTEIN-TYROSINE KINASE p72^{syk} IN PERIPHERAL BLOOD LYMPHOCYTES

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We report that the activation of porcine peripheral blood lymphocytes (PBL) by lectin concanavalin A (Con A) led to the increase in tyrosine phosphorylation on 84-, 72-, 55-, 40-, and 33-kDa proteins. A non-receptor protein-tyrosine kinase (PTK), p72^{syk} (Taniguchi *et al.* (1991) *J. Biol. Chem.* **266**, 15790-15796), was detected in PBL around 0.1 % of total protein and distributed in both particulate and cytosolic fractions. Furthermore, Con A induced a rapid activation of p72^{syk} within 1 min in a manner similar to the time course of Con A-induced protein-tyrosine phosphorylation. These results suggest that p72^{syk} plays a certain role in the activation of PBL and that p72^{syk} may be one of the major non-receptor PTKs in T cells as well as in B cells.

The peripheral blood lymphocytes (PBL) are the kind of circulating white blood cells to play important roles mainly in the immune system against invasion of microorganisms. When the system encounters some antigens, lymphocytes proliferate, maturate, and produce lymphokines. These phenomena are considered to be regulated through exquisite coordination of intracellular signaling pathways in which protein phosphorylation is considered to play important roles. In particular, protein-tyrosine phosphorylation is believed to be indispensable in lymphocytes activation, since many agonists of PBL such as interleukin-2 or antibodies against CD4 cause a rapid and significant increase in protein-tyrosine phosphorylation (1).

The mitogenic lectin concanavalin A (Con A), derived from the jack bean, mimics the action of antigens and induces the polyclonal activation of lymphocytes (2). In addition, Con A induces rapid phosphorylation of multiple cellular proteins including that on tyrosine residues in human T cell lines (3). Since Con A is a tetravalent lectin to bind specifically to D-glucopyranoside and D-mannopyranoside, it is believed to trigger the cellular responses by oligomerizing cell-surface glycoproteins.

Recently, the oligomerization of cell-surface molecules has been proposed as an activation mechanism of protein-tyrosine kinase (PTK). Especially in the case of the non-receptor PTK,

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Abbreviations: Con A, concanavalin A; PTK, protein-tyrosine kinase; PBL, peripheral blood lymphocytes; SH2 and SH3, *src* homology region 2 and 3, respectively.

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association with cell-surface proteins is also thought to transduce the signal from cell exterior leading to the activation of the enzyme; such as $p56^{lck}$ with CD4/CD8 (4) or interleukin-2 receptor (5), $p60^{fyn}$ with T cell receptor (6), $p56^{blk}$, $p56^{lyn}$, and $p60^{fyn}$ with B cell receptor (7, 8). We have also reported that $p72^{syk}$ is associated with membrane IgM in tonsillar cells and rapidly activated after engagement of IgM with anti-IgM antibodies (9).

A non-receptor 72-kDa PTK, $p72^{syk}$ is expected to have some different physiological functions or regulations from other members of the non-receptor family, since it has a unique structural character; the second *src* homology region 2 (SH2) instead of SH3 in its amino acid sequence (10). The expression of $p72^{syk}$ is limited in hematopoietic cells and the activation of $p72^{syk}$ is shown in platelets upon the stimulation with wheat germ agglutinin (11) or thrombin (12) and B-cell dominant cells such as tonsillar cells upon the stimulation with anti-IgM antibodies (9) and splenocytes upon the stimulation with wheat germ agglutinin (13). These results suggest that $p72^{syk}$ is involved in physiological responses of hematopoietic cells such as hemostasis and phylaxis.

In this study, we report that $p72^{syk}$ is expressed in porcine PBL and is activated upon Con A stimulation within minutes in a manner similar to the time course of Con A-induced protein-tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

Materials and chemicals --- Con A was purchased from Sigma. Methyl α-mannopyranoside was obtained from Wako Pure Chemicals. Percoll was a product of Pharmacia. Anti-phosphotyrosine antibodies (14) and anti-CPTK40 antibodies (10) that recognize both $p72^{syk}$ and its catalytic fragment, 40-kDa kinase (15), were prepared as described previously. Porcine blood was obtained at a local slaughterhouse.

Isolation of porcine PBL --- Porcine PBL were prepared from freshly obtained blood by centrifugation on a gradient of Percoll (16). The microscopic analysis showed that the purity was more than 98 % and the trypan blue extrusion test also indicated that the viability was more than 95 %. PBL were finally suspended in Hank's balanced salt solution (HBSS; 136.7 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM dextrose, 4.2 mM $NaHCO_3$, pH 7.4).

Protein determination --- Protein concentration was determined by the method of Bradford (17) using bovine serum albumin as a standard.

Subcellular fractionation of PBL --- PBL were homogenized in cold homogenizing buffer (50 mM Tris/HCl pH 7.5, 0.25 M sucrose, 5 mM EDTA, 10 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride) with ultrasonic burst for 10 sec using a ultrasonic disrupter UD 200 (TOMY) at the output power of 3 and centrifuged at 5,000 x g for 10 min at 4 °C. The supernatant was then centrifuged at 100,000 x g for 60 min at 4 °C and the resultant supernatant was collected as a cytosolic fraction. The pellets were resuspended in the homogenizing buffer as a particulate fraction.

Stimulation, immunoprecipitation kinase assay and phosphoamino acid analysis --- After PBL (4 x 10^6 /ml) were stimulated with Con A at 37 °C for indicated time, cells were collected by a quick centrifugation and the resulting pellets were lysed with the lysis buffer (2 % Triton X-100, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 100 μM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride) and followed by ultrasonic burst for 5 sec. The lysates were immunoprecipitated with anti-CPTK40 antibodies and phosphorylated as described (10). The phosphorylation reaction was terminated by boiling for 3 min with electrophoresis sample buffer and separated using 12.5 % sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by autoradiography. One dimensional phosphoamino acid analysis was performed as described (10).

Immunoblot procedure --- Separated samples on a 12.5 % gel were transferred onto Immobilon P membrane (Millipore) that was probed with anti-phosphotyrosine antibodies (14) or with anti-CPTK40 antibodies as described previously (10).

RESULTS

The effects of Con A on protein-tyrosine phosphorylation --- Con A caused an increase of protein-tyrosine phosphorylation in porcine PBL within 1 min (Fig. 1), which was observed in the proteins with molecular masses of 84, 72, 55, 40, and 33 kDa. They reached maximum level at 5 min after stimulation and gradually decreased by 10 min. This phenomenon disappeared when methyl α-mannnopyranoside coexisted with Con A (Fig 1, lane 6).

The expression of $p72^{syk}$ *in porcine PBL* --- As shown in Fig. 2a, immunoreactive protein band around 72 kDa was recognized in the lysate of PBL as well as splenocytes and was observed in both particulate and cytosolic fractions of PBL. Moreover, a 72-kDa PTK activity was precipitated from lysate of porcine PBL (Figs. 2b and 2c). We, therefore, concluded that $p72^{syk}$ is expressed in PBL.

Estimation of $p72^{syk}$ *abundance in porcine PBL* --- We estimated the quantity of $p72^{syk}$ in PBL, based on the densitometry of immunoblot using the 40-kDa kinase as a standard. Densitometric analysis showed that densities of the standard bands were dose dependent (Fig. 3, lanes 3-5) in a linear fashion from 10 to 30 ng of the standard. Under this condition, the concentrations of $p72^{syk}$ in lysate, particulate, and cytosolic fractions were calculated to be 10, 15, and 8 pmol/mg protein, respectively. From the results of independent several experiments, we concluded that the amount of $p72^{syk}$ occupies around 0.1 % of total protein in porcine PBL.

Activation of $p72^{syk}$ *upon Con A stimulation* --- As shown in Fig. 4a, Con A induces an rapid increase in the activity of $p72^{syk}$. The activity began to rise within 1 min, reached maximal level at 5 min and decreased by 10 min. Densitometric analysis revealed about 3-fold increase in the activity of $p72^{syk}$. This activation disappeared when methyl α-mannopyranoside was included in the medium. The amount of immunoprecipitated $p72^{syk}$ was not affected by Con A stimulation (Fig. 4b). The effect of Con A on the activity of $p72^{syk}$ was dependent on the concentration (Fig. 4c). These results suggest that an increase of specific activity of $p72^{syk}$ was induced by the binding of Con A to surface glycoproteins of PBL.

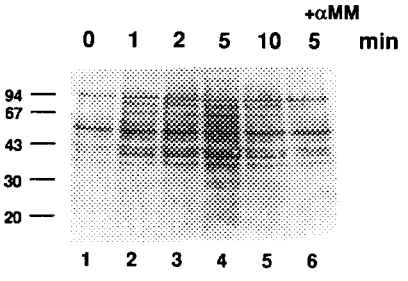

Fig. 1. Effects of Con A on tyrosine phosphorylation of PBL. The porcine PBL were incubated with 100μg/ml Con A at indicated times in the absence (lanes 1-5) or presence (lane 6) of 100 mM methyl α-mannnopyranoside for 0 (lane 1), 1 (lane 2), 2 (lane 3), 5 (lanes 4 and 6), and 10 min (lane 5). Protein-tyrosine phosphorylation was assessed on immunoblot with anti-phosphotyrosine antibodies as described in EXPERIMENTAL PROCEDURES. Positions of molecular mass markers are shown to the left in kilodaltons. αMM, methyl α-mannnopyranoside.

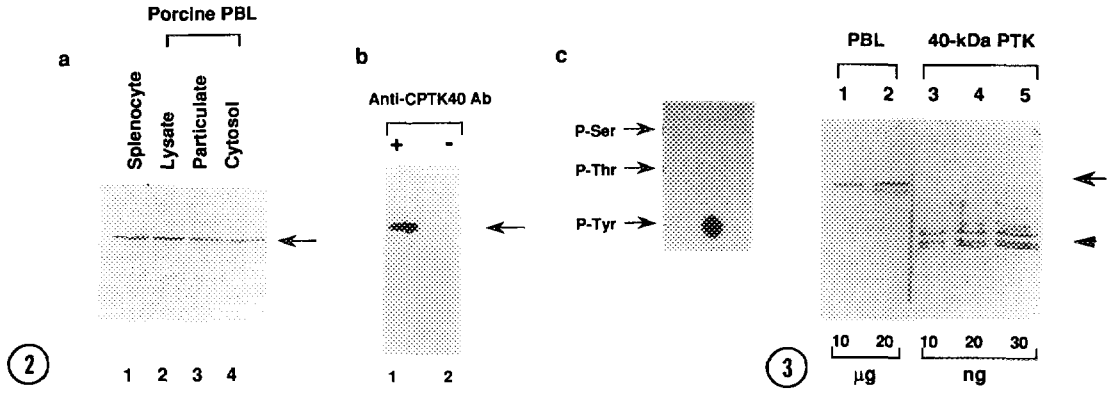

Fig. 2. (a) Immunoblot analysis of p72^{syk} in porcine PBL. Samples prepared from the lysates of splenocytes (lane 1) or PBL (lane 2) and particulate (lane 3) or cytosolic (lane 4) fractions of PBL were separated, blotted and probed with anti-CPTK40 antibodies as described in EXPERIMENTAL PROCEDURES. Each lane contained 10 μg of protein. An arrow indicates the position of p72^{syk}. **(b) Immunoprecipitation kinase assay of p72^{syk} in porcine PBL.** p72^{syk} was immunoprecipitated from PBL lysate with (lane 1) or without (lane 2) anti-CPTK40 antibodies and phosphorylated as described in EXPERIMENTAL PROCEDURES. **(c) Phosphoamino acid analysis.** Phosphorylated 72-kDa band of lane 1 in panel (b) was excised and phosphorylated amino acid was analyzed as described in EXPERIMENTAL PROCEDURES. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

Fig. 3. Estimation of p72^{syk} abundance in porcine PBL. The indicated amount of porcine PBL lysate (lanes 1 and 2) or a 40-kDa PTK purified from porcine spleen (lanes 3-5) was analyzed on immunoblot with anti-CPTK40 antibodies as described in EXPERIMENTAL PROCEDURES. An arrow and an arrowhead indicate the position of p72^{syk} and the 40-kDa kinase, respectively.

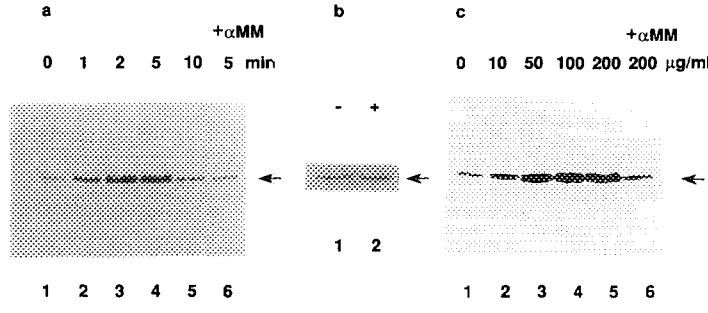

Fig. 4. Activation of p72^{syk} in response to Con A stimulation. (a) Time course. After PBL were incubated with 100 μg/ml Con A in the absence (lanes 1-5) or presence (lane 6) of 100 mM methyl α-mannopyranoside for 0 (lane 1), 1 (lane 2), 2 (lane 3), 5 (lanes 4 and 6), and 10 min (lane 5), the samples were processed for immunoprecipitation kinase assay as described in EXPERIMENTAL PROCEDURES. **(b) Immunoblot analysis of the immunoprecipitated samples.** After porcine PBL were incubated for 5 min in the absence (lane 1) or presence (lane 2) of 200 μg/ml Con A, the samples were immunoprecipitated as above. The precipitated samples were subjected to immunoblot with anti-CPTK40 antibodies as described in EXPERIMENTAL PROCEDURES. **(c) Dose-responsive activation of p72^{syk}.** Porcine PBL were incubated for 5 min in the presence of Con A at the concentration of 0 (lane 1), 10 (lane 2), 50 (lane 3), 100 (lane 4), and 200 μg/ml (lanes 5 and 6) without (lanes 1-5) or with 200 mM methyl α-mannopyranoside (lane 6). The samples were then processed as in (a). αMM, methyl α-mannnopyranoside. An arrow indicates the position of p72^{syk}.

DISCUSSION

When lymphocytes are exposed to Con A, the cross-linking of glycoproteins may occur on the cell surface resulting in induction of cellular responses (2). In this study, we observed the Con A-induced increases in tyrosine phosphorylation on the proteins with molecular masses of 84, 72, 55, 40, and 33 kDa in porcine PBL. This pattern is almost compatible with the previous report using murine T cell lines (3). Since T cells are the major population in PBL (70-80 %) and Con A can not cause the proliferation of B cells but that of T cells (2, 18), it is likely that this increase in protein-tyrosine phosphorylation mainly reflects that in T cells.

A member of non-receptor PTK, $p72^{syk}$, is also expressed and distributed in both particulate and cytosolic fractions (Fig. 2) and the abundance is around 0.1 % of the total protein in porcine PBL. Several other non-receptor PTKs including protein products of *src*, *fyn*, *lyn*, *lck* and *hck* have been detected in PBL (1, 19). However, since the amounts of those gene products have not been demonstrated, we can not compare those with that of $p72^{syk}$. In platelets, it is expressed at 0.1-0.2 % of total proteins and observed in both particulate and cytosolic fractions (11). This value corresponds to half of that of *src* gene product and is 2.5-5 times higher than other non-receptor PTKs in platelets (11). Taking this and the fact that $p72^{syk}$ is expressed mainly in hematopoietic cells (10) into consideration, it may be one of the major non-receptor PTKs in PBL. In this study, we have shown that $p72^{syk}$ is also expressed and activated by proper agonists in PBL which are T-cell dominant cells as well as in splenocytes (13) and tonsillar cells (9) which are B-cell dominant cells. It is, therefore, likely that $p72^{syk}$ is expressed and plays some important roles in T cells as well as in B cells.

Although the regulatory mechanism of $p72^{syk}$ is currently uncertain, $p72^{syk}$ is physically associated with B cell antigen receptor and it was rapidly activated following the engagement of membrane IgM (9). Similar mechanisms were reported between non-receptor PTKs and other cell-surface molecules such as high-affinity IgE receptor and T cell receptor (20, 21). It is, therefore, possible to speculate that $p72^{syk}$ is also associated with some PBL surface receptors and their cross linking by Con A results in the activation of this kinase.

Recently, Chan *et al.* have reported a 70-kDa PTK that associates with ζ chain of T cell receptor complex (ZAP-70) (22). The deduced amino acid sequence of ZAP-70 has two SH2 and shows the highest homology to that of $p72^{syk}$. However, the amino acid sequence of ZAP-70 dose not include the homologous part with that of the immunogen peptide against which anti-CPTK40 antibody was raised (10, 22). It is unlikely, therefore, the immunoreactive 72-kDa protein to anti-CPTK40 antibody is ZAP-70. Hutchcroft *et al.* have also reported a 72-kDa PTK (PTK72) associated with membrane IgM (23). PTK72 shares several common features with $p72^{syk}$ and might be a product of murine or human homologue of *syk*. However, the identity of PTK72 is depending solely on the immuno-cross reactivity with their antibody, that was raised against p40 kinase purified from bovine thymus (24). In addition, the homology between $p72^{syk}$ and ZAP-70 implies a certain subfamily including two or more members distinct from *src* family. Thus, we should wait to draw a conclusion until further information about the structure or enzymatic character of PTK72 is provided.

We report here that $p72^{syk}$ can be a responsible candidate of the PTKs in signal transduction of PBL. Further investigation is necessary for understanding the physiological function and regulation of $p72^{syk}$.

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