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ACTIVATION OF p72^{syk} BY THROMBIN IN A CELL-FREE SYSTEM

Kiyonao Sada°, Shigeru Yanagi° and Hirohei Yamamura°§[1]

°Department of Biochemistry, Fukui Medical School, Mastuoka, Fukui 910-11, Japan

§Biosignal Research Center, Kobe University, Kobe 657, Japan

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Thrombin dramatically activated p72^{syk} in a time- and dose- dependent fashion in extracts of resting porcine platelets in the presence of EDTA. Separation analysis using Sephacryl S-300 column chromatography has demonstrated that p72^{syk} may exist as large (complex) and small (monomer) forms in resting platelets, and activation of p72^{syk} was only observed in the fraction of large form. Pretreatment with ATP scavenger, GDPβS and protein phosphatase inhibitors had no effect on this activation. Furthermore, washed immuno-precipitates of large form p72^{syk} were also activated by thrombin or fibrinogen. These results suggest that p72^{syk} may associate with thrombin receptor or other agonist receptors and there may be a novel activation mechanism of non-receptor type protein-tyrosine kinase, which does not require the modification by other protein kinases, protein phosphatases and GTP binding proteins. © 1994 Academic Press, Inc.

Platelets provide a useful model for investigating the mechanisms involved in signaling pathway through protein-tyrosine kinases (PTKs). Several groups have reported that increases in the phosphorylation of proteins on tyrosine residues are early events in the stimulation of platelets by various agonists, especially thrombin (1-3). p72^{syk} (4), p125^{FAK} (5) and Src family kinases detected in platelets are candidate enzymes that may be involved in agonist-induced protein-tyrosine phosphorylation. The dramatic activation of p72^{syk} might greatly contribute to agonist-stimulated tyrosine phosphorylation in platelets (4).

We previously isolated a gene encoding a non-receptor type 72-kDa PTK (p72^{syk}) that has second src homology region 2 (SH2) instead of SH3 in its amino-acid sequence (6). The expression of p72^{syk} has been detected mainly in hematopoietic cells (7-11), and both particulate and cytosolic fractions in platelets or lymphocytes, although many protein-tyrosine kinases so far reported are associated with plasma membranes or particulate fractions (8). Consideration of its structure, tissue and subcellular localization, p72^{syk} is expected to have different physiological regulation system from other Src family PTKs.

[1]All correspondence should be addressed to Department of Biochemistry, Fukui Medical School, Matsuoka, Fukui 910-11, Japan.

Abbreviations: PTKs, protein-tyrosine kinases. SH2 and SH3, *src* homology regions 2 and 3, respectively.

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Since the phosphorylation of tyrosine 527 within the C-terminal regulatory domain suppresses the kinase activity of pp60$^{c\text{-}src}$, the activation mechanism may involve the dephosphorylation of tyrosine 527 (12-17). Therefore, the activities of Src family kinases seem to be regulated by a change in the balance of phosphorylation or dephosphorylation of tyrosine residues on C-terminus. In contrast, this C-terminal sequence (QYQ) of pp60^{src} is lack in the sequence of p72^{syk}. Recently, p72^{syk} (11, 18-21), ZAP 70 which is the second member of the Syk family kinases (22, 23) and PTK 72 (24, 25) also have been reported to associate with cell surface membrane receptors in immune cells. However the exact molecular mechanism of p72^{syk} activation is still unknown.

In the present study we suggest that p72^{syk} undergoes dramatic activation, which does not require the modification by other protein kinases or protein phosphatases following stimulation by thrombin, and p72^{syk} may associate with thrombin receptor or other agonist receptors. Implications of these phenomena in a mechanism for activation of p72^{syk} are briefly discussed.

EXPERIMENTAL PROCEDURES

Materials and chemicals—Bovine thrombin, human fibrinogen and apyrase were purchased from Sigma. Ammonium molybdate and sodium fluoride were from Nakarai Chemicals. Sodium orthovanadate and collagen type I solution were from Wako Pure Chemicals. Hirudin was from Biopharm Biochemicals. Leupeptin was from Peptide Institute Inc. (Osaka). Guanosine-5'-O-(3-thiotriphosphate) (GTPγS) and Guanosine-5'-O-(2-thiodiphosphate) (GDPβS) were from Boehringer Mannheim GmbH (Germany). Polyclonal anti-p72^{syk} antibodies were prepared as described previously (6). Porcine blood was obtained at a local slaughterhouse.

Preparation of extracts from resting porcine platelets —Porcine platelets were prepared by the methods described previously (4) and about 10^9 cells of purified packed platelets were quickly sedimented, and lysed with 1 ml of lysis buffer (2% Triton X-100, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 100 μM sodium vanadate, 1 mM phenylmethyl-sulfonyl fluoride (PMSF)) and kept at 4°C for 30 min. Then the lysates were centrifuged at 100,000 × g for 30 min. The supernatant was collected carefully and used as the extracts of resting porcine platelets. All subsequent procedures were carried out at 4°C.

Gel filtration — About 0.5 ml of purified packed platelets prepared from 300 ml of porcine blood was lysed with 5 volumes of lysis buffer, and kept at 4°C for 30 min. Then the lysates were centrifuged at 100,000 × g for 30 min and the supernatant was applied to a column of Sephacryl S-300 high resolution type equilibrated with buffer A (0.1 % Triton X-100, 150 mM NaCl, 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol, 10 μM sodium vanadate, 10% glycerol, 0.1% PMSF). Elution was performed downward with the buffer A.

Stimulation procedure in a cell-free system — Thrombin was added to resting platelets extracts or washed immunoprecipitates of complex formed-p72^{syk}. The incubation was carried out at 4°C for 60 min, and terminated by addition of 10 μg/ml of leupeptin (thrombin) or by centrifugation (other agonists) (10,000 × g for 2 min) of immunoprecipitates.

Immunoprecipitation kinase assay and immunoblotting — The lysates or samples were immunoprecipitated with anti-p72syk antibodies and we phosphorylated as described (8). After separation of samples on a polyacrylamide (12.5%) gel electrophoresis in the presence of 0.1 % sodium dodecyl sulfate, the activity of p72^{syk} was estimated by autoradiography. Immunoblotting using anti-p72^{syk} antibodies was carried out as described (6).

RESULTS AND DISCUSSION

Thrombin induced a time- and dose- dependent activation of p72^{syk} in the resting porcine platelet extracts in the presence of EDTA to chelate bivalent cations (Fig. 1a and b). The

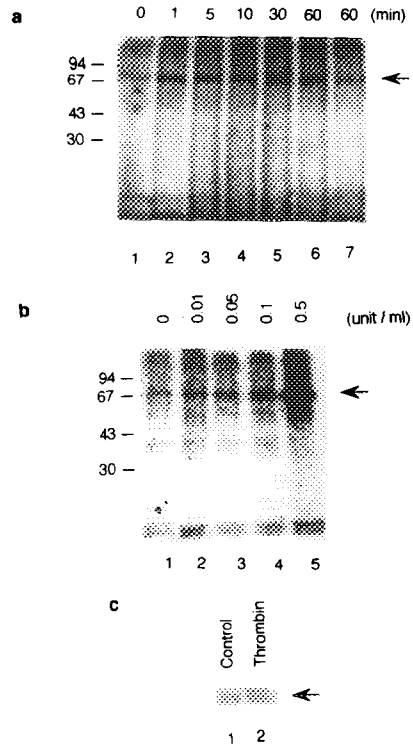

Fig. 1. Activation of p72^{syk} by thrombin in extracts of resting porcine platelets. The extracts of resting porcine platelets were incubated for the indicated times (min) with 0.5 unit/ml of thrombin (a), or with the indicated dose (unit/ml) of thrombin for 60 min (b). In lane 7 (Fig. 1a) extract was pretreated with 2 units/ml hirudin. The activity of p72^{syk} was assessed in an immunoprecipitation kinase assay as described under "EXPERIMENTAL PROCEDURES". (c) The extracts were incubated without (lane 1) or with (lane 2) 0.5 unit/ml of thrombin, and anti-p72^{syk} immunoprecipitates were analysed by immunoblotting with anti-p72^{syk} antibodies as described under "EXPERIMENTAL PROCEDURES". Molecular markers are shown in kilodaltons in the left. The arrows indicate the positions of p72^{syk}.

activity of p72^{syk} increased within 1 min, reached a maximum at 10 min and then plateau for up to 60 min after thrombin stimulation. Activation of p72^{syk} was observed even at the concentration of 0.01 unit/ml thrombin, then greatly enhanced in a dose-dependent manner. Pretreatment of the extracts with 2 units/ml hirudin, a specific antagonist of thrombin for 1 min completely canceled this activation (Fig. 1a, lane 7), and no apparent proteolytic breakdown was observed on p72^{syk}. Phosphorylated amino acid of p72^{syk} was exclusively tyrosine in all lanes (data not shown). Autoradiogram of the phosphorylation of H2B histone by immunoprecipitated p72^{syk} showed an increase in the activity for exogenous substrate phosphorylation (data not shown). The amount of precipitated p72^{syk} did not change judging from the immunoblot analysis (Fig. 1c). In addition, we could detect neither the activation of other known PTKs in a cell-free system nor their co-precipitations with p72^{syk} judged by immunoprecipitation kinase assay and immunoblot, respectively (data not shown). Thus, we concluded that the increase in the activity of p72^{syk} was evoked specifically through activated thrombin receptor in a cell-free system.

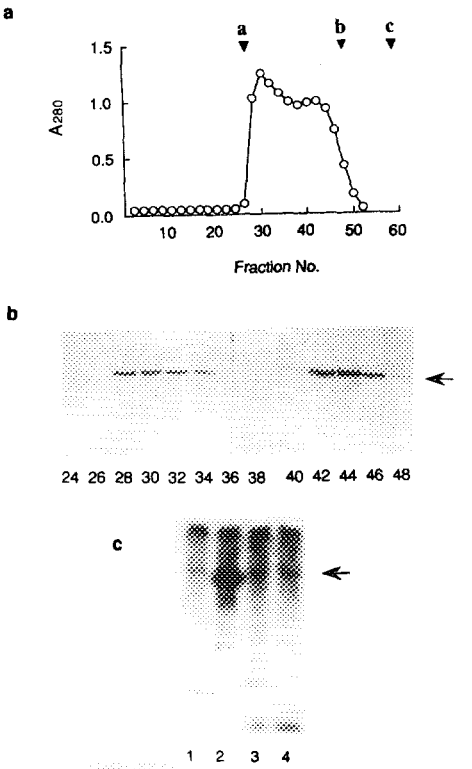

Fig. 2 . Sephacryl S-300 column chromatography of p72^{syk}. The extracts of resting porcine platelets were applied to a Sephacryl S-300 column and elution was performed as described under "EXPERIMENTAL PROCEDURES". a, Protein concentration was monitored by adsorption of UV detector (A_{280}). The molecular markers are (a) blue dextran, (b) hemoglobin, and (c) cytochrome C. b, Immunodetection of p72^{syk}. Aliquots from each fraction were analysed by immunoblotting with anti-p72^{syk} antibodies as described under "EXPERIMENTAL PROCEDURES". c, Effect of thrombin on the activity of p72^{syk} in the pooled fractions. The pooled fractions (1 ml) containing fractions 28-32 (lanes 1 and 2) or 42-46 (lanes 3 and 4) were incubated without (lanes 1 and 3) or with (lanes 2 and 4) 0.5 unit/ml of thrombin for 60 min. The activity of p72^{syk} was assessed in an immunoprecipitation kinase assay as described under "EXPERIMENTAL PROCEDURES". The arrows indicate the positions of p72^{syk}.

Next, we undertook a partial purification of p72^{syk} from the extracts of platelets by gel filtration methods. The extracts (3 ml) were applied to a column (1.5 × 93 cm) of Sephacryl S-300 high resolution type equilibrated with buffer A. Elution was performed downward with the same buffer, and fractions of 2.5 ml each were collected (Fig. 2a). Samples of eluted fractions were analysed by immunoblotting with anti-p72^{syk} antibodies (Fig. 2b). The position of p72^{syk} was separated into two peaks, one peak appeared high molecular weight near the void (fractions 28-32) and the other appeared around 72-kDa (fractions 42-46). Separation analysis of Sephacryl S-300 column chromatography suggested that p72^{syk} might exist as a large and a small forms in resting platelets. The large form might consist of various components, and the small form seemed to be p72^{syk} itself. Activation of p72^{syk} by thrombin was only observed in the pooled fractions (1 ml) containing the large form p72^{syk} but not in the small form (Fig. 2c). However, the large form p72^{syk} was eluted in the inclusion volume from another sizing column,

Table 1 Effects of pretreatment with inhibitors for protein kinases, protein phosphatases or GTP binding proteins. The pooled fractions (1 ml) containing fractions 28-32 were pretreated with various inhibitors for 10 min at 4 °C, and then stimulated without or with thrombin for 60 min at 4 °C. The activity of p72^{syk} was assessed in an immunoprecipitation kinase assay as described under "EXPERIMENTAL PROCEDURES" and analyzed by digital optical scanning of the autoradiograms developed from the gels on a BAS-2000. Results are representative of three independent experiments. – and + show the stimulation without and with thrombin (0.5 unit/ml), respectively.

Pretreatment		Thrombin stimulation	Relative p72^{syk} activity
none		–	1.00 ± 0.00
none		+	3.59 ± 1.22
apyrase	(10 units / ml)	+	4.21 ± 0.35
ammonium molybdate sodium vanadate sodium fluoride	(50 μM) (100 μM) (10 mM)	+	4.04 ± 0.48
GDPβS	(50 μM)	+	3.97 ± 0.04
GTPγS	(50 μM)	–	1.15 ± 0.27

Sepharose CL-4B. Thus, molecular size of the large form p72^{syk} seemed to be over 200 kDa but not over 2,000 kDa (data not shown). Further experiments using specific antibodies will be needed to identify and characterize the components of the large form p72^{syk} and their association mechanisms.

Since it is reported that phosphorylation and dephosphorylation have an important role for the regulation of the activity of Src family kinases, we next examined the effects of various inhibitors of protein kinase and protein phosphatase (Table 1). Pretreatment with inhibitors for protein kinases (apyrase as ATP scavenger), protein phosphatases (sodium vanadate, ammonium molybdate and sodium fluoride) (26, 27) or GTP binding proteins (GDPβS) of the fraction containing the large form p72^{syk} had no effect on the activation of p72^{syk} induced by thrombin. GTPγS itself had no effect on the activity of p72^{syk}. EDTA to chelate intrinsic bivalent cations was originally contained in elution buffer.

In order to investigate these phenomena further, we examined the effect of various agonist stimmulation to washed immunoprecipitates from the fraction containing the large form p72^{syk} (Fig. 3). In the suspension of washed immunoprecipitates, thrombin and fibrinogen also caused the increase of the activity of p72^{syk}.

The results of this study strongly support the possibility that p72^{syk}, which has neither SH3 nor N-terminal myristration site, may associate with thrombin receptor or other agonist receptors especially integrin in resting platelets (5), and stimulated-receptors may directly or indirectly activate p72^{syk}. And the activation of p72^{syk} is not mediated by the modification of other protein kinases, protein phosphatases or by interaction with GTP binding proteins. There may be a novel regulation system of non-receptor type PTKs, such as comformational change of p72^{syk} through an interaction with receptor(s) or regulation factor(s), through a specific motif or

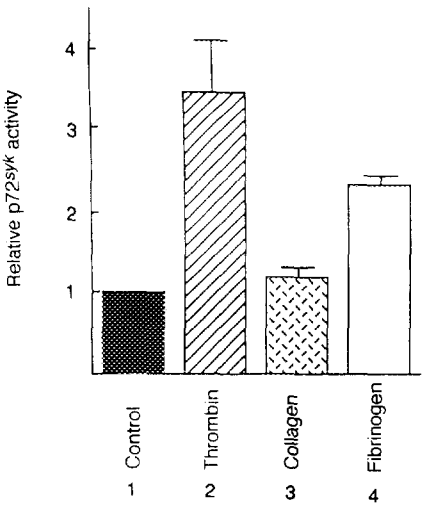

Fig. 3 . Effects of various agonists to washed immunoprecipitates of complex formed-p72^{syk} . The immunoprecipitates of p72^{syk} from pooled fractions (1 ml) containing fractions 28-32 were washed 3 times with lysis buffer and resuspended in 1 ml of the same buffer, then incubated without (lane 1) or with 0.5 unit/ml of thrombin (lane 2), 150 μg/ml of collagen with 2 mM Mg^{2+} (lane 3), 100 μg/ml of fibrinogen with 5 mM Ca^{2+} (lane 4) for 60 min at 4 °C. The activity of p72^{syk} was assessed in an immunoprecipitation kinase assay as described under "EXPERIMENTAL PROCEDURES" and analyzed by digital optical scanning of the autoradiograms developed from the gels on a BAS-2000. Results are representative of three independent experiments.

unknown mechanism. Before we can evaluate the molecular mechanisms of activating p72^{syk}, it is necessary to investigate further details involving association of these agonists receptors, cell surface proteins and p72^{syk} complex (the large form p72^{syk}) in human platelets. Our attempts in this direction are in progress.

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