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Purification of a 72-kDa Protein-Tyrosine Kinase from Rat Liver and Its Identification as Syk: Involvement of Syk in Signaling Events of Hepatocytes[1]

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Syk protein-tyrosine kinase (PTK) has been implicated in a variety of hematopoietic cell responses including immunoreceptor signaling. However, so far, there has been no evidence of the expression of Syk or Syk-related PTK in non-hematopoietic tissues. In this study, we have purified from blood cell–depleted rat liver a 72-kDa cytoplasmic PTK which shows cross-reactivity with anti-Syk antibody. Partial amino acid sequence analysis revealed that this 72-kDa PTK is identical to Syk. Immunohistochemical and RT-PCR analyses demonstrated that Syk is expressed in human hepatocytes and two rat liver-derived cell lines, JTC-27 and RLC-16. Furthermore, Syk is significantly tyrosine-phosphorylated in response to angiotensin II in JTC-27 cells, and angiotensin II-induced MAP kinase activation is blocked by the treatment of cells with a Syk-selective inhibitor, piceatannol. These results suggest that Syk plays an important role in signaling events of hepatocytes, such as signaling steps leading to MAP kinase activation by G-protein–coupled receptors. This is the first report of the expression of Syk in non-hematopoietic tissue.

Key words: MAP kinase, angiotensin II, hepatocytes, protein-tyrosine kinase, Syk.

Protein-tyrosine kinases (PTKs) play an important role in signal transduction, leading various kinds of cells to activation, proliferation, and differentiation (*1*, *2*). In the hematopoietic cells, two structurally distinct families of PTKs, the Src and Syk families, are required for various types of signaling (*3–5*). In contrast to Src family PTKs, the Syk family PTKs (Syk and ZAP-70) are characterized by the presence of two tandemly arranged Src homology 2 (SH2) domains and have no membrane localization motifs (*6*, *7*). Tissue and subcellular distribution of Syk evoked the speculation that Syk may specifically participate in signal transduction initiated by cell surface receptors expressed on hematopoietic cells. In particular, recent efforts have in large part focused on the role of Syk in the antigen receptor-initiated signal transduction in B cells (*8*). A growing body of evidence demonstrates that Syk is essential for development and function of several hematopoietic cells, and it becomes activated through tandem SH2 interaction with immunoreceptor tyrosine-based activation motifs (ITAMs) in immune response receptors. The important role of Syk in immunoreceptor signaling has been well documented, and further established by the descriptions of Syk-deficient mice (*9*, *10*).

Evidence is accumulating that suggests the multiple function of Syk in a variety of signal transduction pathways. In hematopoietic cells, Syk has been reported to be activated through integrins, which do not contain ITAMs, suggesting a unique role for integrins in Syk function (*11*, *12*). Our previous studies also demonstrated that Syk is activated by stimulation with thrombin, thromboxane A2, or platelet-activating factor in platelets, suggesting that Syk may be one of the key molecules through which heterotrimeric G protein-coupled receptors act (*13–15*). Furthermore, Wan *et al.* recently showed that in Syk-deficient cells, both m1 and m2 muscarinic acetylcholine receptors failed to stimulate MAP kinase kinase and MAP kinase in DT40 chicken B cell lines, indicating that Syk is essential for the G(i)- and G(q)-coupled pathways (*16*). In addition, we have suggested that Syk is involved in oxidative and osmotic stress signaling in DT40 chicken B cell lines (*17*, *18*). Thus, the function of Syk is not limited to immunoreceptor signaling, and this evidence also raises the possibility that Syk or Syk-related PTK may be expressed in non-hematopoietic tissues.

In this paper, we report the expression of Syk in the non-hematopoietic hepatocytes and present evidence that Syk is involved in signaling events other than those mediated by immunoreceptors, such as G-protein–coupled receptor signaling.

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Abbreviations: PTK, protein-tyrosine kinase; SH2, Src homology 2; ITAM, immunoreceptor tyrosine-based activation motif; PBS, phosphate buffered saline; GAPDH, glyceraldehyde-phosphate dehydrogenase; TFA, trifluoroacetic acid; TGN, *trans*-Golgi network.

EXPERIMENTAL PROCEDURES

Materials—All materials and chemicals for the purification procedure used in this study were prepared as described previously (*19*). Adult SD rats were purchased from SLC Inc. Polyclonal anti-Syk antibody (N-19) and immunogenic peptide were purchased from Santa Cruz. Aprotinin, piceatannol, phenylmethylsulfonyl fluoride, protein A–agarose beads, and angiotensin II were from Sigma. Anti-phosphotyrosine monoclonal antibody 4G10 and 4G10-agarose were purchased by Upstate Biotechnology Inc. Anti-phospho-MAP kinase antibody was from New England Biolabs Inc. Rat liver-derived cell lines, JTC-27 and RLC-16 were provided by RIKEN cell bank (Tsukuba). HuH7 and HLF cells were provided by the Health Science Research Resources Bank (Osaka).

Cell Culture and Harvest—JTC-27, RLC-16, HuH7, and HLF cells were maintained in DMEM medium supplemented with 10% (v/v) FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified 95% air, 5% CO_2 atmosphere. For experiments, cells were washed once in phosphate-buffered saline (PBS), then resuspended in FCS-free DMEM medium, pH 7.4.

Immunoprecipitation and Immunoblot Procedures—The lysates from cultured cells were clarified by centrifugation at 100,000 ×*g* for 10 min and immunoprecipitated with anti-Syk antibody or anti-phosphotyrosine antibody. Immunoprecipitates were washed three times with lysis buffer, once with 10 mM Hepes/NaOH pH 8.0 containing 0.5 M NaCl, and finally with 10 mM Hepes/NaOH pH 8.0. Samples were boiled with electrophoresis sample buffer for 3 min, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore), followed by detection with anti-phosphotyrosine antibody or anti-Syk antibody as described previously (*20*).

Peptide Phosphorylation Assay—Phosphorylation of [Val^5]angiotensin II was carried out as described previously (*19*).

Immunohistochemistry—A sample was collected from a patient with hepatocellular carcinoma after informed consent, and a non-tumorous lesion was used for immunohistochemical analysis. Human liver specimens were fixed in 10% neutral buffered formalin followed by Bouins solution. Serial sections 4 μm thick were cut and stained on glass slides. Sections were immunostained using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was removed by a 60-min incubation in methanol containing 0.3% H_2O_2. Nonspecific immunoglobulin binding was blocked by incubating sections for 15 min in PBS containing 5% normal goat serum at room temperature. Anti-Syk antibody was applied at 1:400 dilution and incubated overnight at 4°C. Following extensive washing, sections were incubated with biotinylated goat anti-rabbit IgG, then with avidin-enzyme complex. Antibody binding was visualized by incubating the slides in 3′,3-diaminobezidine (Sigma Chemical Co., St. Louis, MO) solution including hydrogen peroxide. Normal rabbit IgG (Lipshaw Immunon, Pittsburgh, PA) at the same concentration was used as a negative control. All tissue sections were immunostained simultaneously; incubation and development times were the same for all sections. Antibody specificity was confirmed by absorbing antiserum with a fivefold excess (by weight) of immunogenic peptide in PBS. The antiserum was incubated with the peptide (0.1 mg/ml) at room temperature for 2 h, centrifuged, and applied to the tissue samples.

RT-PCR—Total RNAs were isolated from JTC-27 and RLC-16 and reverse transcribed using oligo-dT primers. Primers used for amplification of rat Syk cDNA were synthesized as follows: primer (sense) was 5′-TCCATGGCAACATCTCCAG-3′; primer (antisense) was 5′-GACATGGTACCGTGAGGA-3′. Amplification was carried out at 94°C for 0.5 min, 50°C for 0.5 min, and 72°C for 1 min in 30 cycles. Primers used for amplification of rat glyceraldehyde-phosphate dehydrogenase (GAPDH) were synthesized as follows: primer (sense) was 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′; primer (antisense) was 5′-CATGTAGGCCATGAGGTCCACCAC-3′. Amplification was carried out at 94°C for 0.5 min, 60°C for 1 min, and 72°C for 1 min in 30 cycles. PCR products were electrophoresed on a 1.0% agarose gel containing ethidium bromide.

Determination of Internal Peptide Sequence of 72-kDa PTK—Purified 72-kDa PTK was acetone-precipitated, resuspended in 70% trifluoroacetic acid (TFA), and cleaved with cyanide bromide (10:1/by weight) at room temperature for 12 h under N_2. The cleaved protein was dried, resuspended in 25 μl of 8 M urea, 0.4 M NH_4HCO_3, reduced with 7 mM dithiothreitol, alkylated with 15 mM iodoacetamide, and diluted with H_2O to a final concentration of 2 M urea. Peptides were further digested with 1 μg of trypsin (Boehringer-Mannheim) for 12 h at 37°C. The same amount of trypsin was added after 10 h to ensure complete digestion. Peptide separation was performed with a 2.1 mm × 25 cm reverse-phase C18 column (Applied Biosystems) in a Hewlett Packard model 1090 high pressure liquid chromatographer. After a 15-min wash in 0.1% TFA, peptides were resolved using a gradient of 0 to 45% CH_3CN in 0.1% TFA over 60 min followed by a 45 to 90% CH_3CN gradient in 0.1% TFA over 15 min with a flow rate of 0.2 ml/min. The optical density profile at 215 nm was monitored, and fractions of 0.1 ml were collected. Amino acid microsequencing was performed with an Applied Biosystems Protein Sequencer (model 475A).

Purification Procedure of 72-kDa PTK—Rat liver (about 100 g wet mass) was depleted of blood cells by injection of PBS, then cut into small pieces in 5 volumes cold homogenizing buffer (50 mM Tris/HCl pH 7.5, 0.25 M sucrose, 5 mM EDTA, 10 μM vanadate, 1 mM phenylmethylsulfonyl fluoride) and homogenized in a mixer for 1 min. All subsequent procedures were carried out at 4°C. The homogenate was centrifuged at 8,000 ×*g* for 10 min to remove unbroken cells and fatty materials, and the supernatant was filtered through glass wool. The filtrate was then centrifuged at 100,000 ×*g* for 60 min. The supernatant was mixed with about 50 ml of gravity-packed phosphocellulose equilibrated with buffer A (50 mM Tris/HCl pH 7.5, 10 mM 2-mercaptoethanol, 1 mM EDTA, 10 μM vanadate, 0.1 mM phenylmethylsulfonyl fluoride) and stood on ice for 15 min. The mixture was washed with buffer A (500 ml) using a Buchner funnel and packed into a column. As shown in Fig. 1A, a 72-kDa protein that cross-reacted with anti-Syk antibody and the PTK activities were co-eluted from the phosphocellulose column (30 ml) with a linear gradient of 0 (200 ml) to 0.6 M NaCl (200 ml). Fractions (8 ml each) containing the 72-kDa PTK were pooled, and solid ammonium

sulfate was slowly added to the pooled fractions (60% saturation). After stirring for 30 min, the precipitates were collected by centrifugation at 10,000 ×*g* for 10 min, dissolved in a small volume of buffer A, and dialyzed against a large volume of the same buffer. The dialysates were clarified by centrifugation at 10,000 ×*g* for 10 min and applied to a column of Heparin-sepharose CL-6B (10 ml) equilibrated with buffer A. The column was thoroughly washed with 0.2 M NaCl in buffer A, and the protein was eluted with a linear gradient of 0.2–0.6 M NaCl (100 ml each) in Buffer A (Fig. 1B). Fractions (5 ml each) containing the 72-kDa PTK were pooled, and dialyzed against a large volume of buffer A. The dialysates were clarified by centrifugation at 10,000 ×*g* for 10 min and applied to a column of Casein-sepharose (2 ml) equilibrated with buffer A. The column was thoroughly washed with buffer A, then the protein was eluted with 0.4 M NaCl in buffer A. The eluted protein (10 ml) was incubated for 10 min at 4°C with 10 μM ATP and 10 μM $MnCl_2$. The phosphorylated protein was then applied to a column of 4G10 (anti-phosphotyrosine antibody)-agarose (1 ml) equilibrated with buffer A. The column was washed with buffer A, and the protein was eluted with 20 mM phenylphosphate in buffer A. The flow rate was about 1 ml/min, and 1-ml fractions were collected (Fig. 1C).

RESULTS

Purification of a Major Cytosolic PTK from Rat Liver—Previous Northern blot analysis demonstrated that the expression of Syk is detected in spleen and thymus but not in non-hematopoietic tissues including liver, kidney and brain (*6*). Subsequently, Syk has been demonstrated to be widely expressed in almost all hematopoietic cells, including B cells, T cells, platelets, mast cells, monocytes, polymorphonuclear cells, erythrocytes (*3*). In this study, we examined whether a novel Syk family PTK exists in the non-hematopoietic tissues like Src family PTKs that consist of many members ubiquitously expressed in almost all tissues.

In preliminary experiments, we performed RT-PCR on the mRNA prepared from rat liver as a template using degenerated PCR primers designed for the conserved amino acid sequences of Syk, and screened two rat brain and liver cDNA libraries with a Syk probe under low-stringency conditions, but we could not find a novel Syk family PTK. On the other hand, we found that a 72-kDa PTK was specifically recognized by anti-Syk antibody from rat liver extract. To further examine whether this protein is related to Syk, we undertook a large-scale purification of this protein from rat liver using its immunological cross-reactivity with anti-Syk antibody and PTK activity assessed on [Val^5]angiotensin II as a substrate. The purification procedure involved sequential column chromatography on phosphocellulose, Heparin-sepharose, Casein-sepharose, and 4G10-agarose (Fig. 1). Surprisingly, the elution patterns of PTK activities were always correlated with the appearance in Western blot analysis of the 72-kDa PTK recognized by anti-Syk antibody, indicating that this 72-kDa PTK is a major cytosolic PTK in rat liver. As shown in Table I, the 72-kDa PTK was purified approximately 4,500-fold with an overall yield of about 0.7%. The results of SDS/PAGE obtained with the chromatographies described above are shown in Fig. 2. The final preparation contained a major protein with a molecular mass of 72 kDa (Fig. 2A).

Purified 72-kDa PTK Is Identical to Syk—As in the easlier purification steps described above, the purified 72-kDa PTK in the final preparation was specifically recognized by anti-Syk antibody (Fig. 2B), suggesting a close relationship between this protein and Syk. To further identify the protein, partial amino acid sequence analysis was performed. Peptides were generated by digestion with trypsin, separated by reversed-phase high pressure liquid chromatography (Fig. 3A), and subjected to amino acid microsequencing. Amino acid sequence analysis revealed the sequences of six peptide fragments, which completely corresponded to those of mouse Syk, indicating that this protein is identical to Syk (Fig. 3B).

Expression of Syk in Human Hepatocytes and Rat Liver-Derived Cell Lines—The fact that the purified protein is Syk itself was unexpected, because previous Northern blot analysis did not allow us to detect Syk mRNA in rat liver. This discrepancy may be due to low sensitivity of the previ-

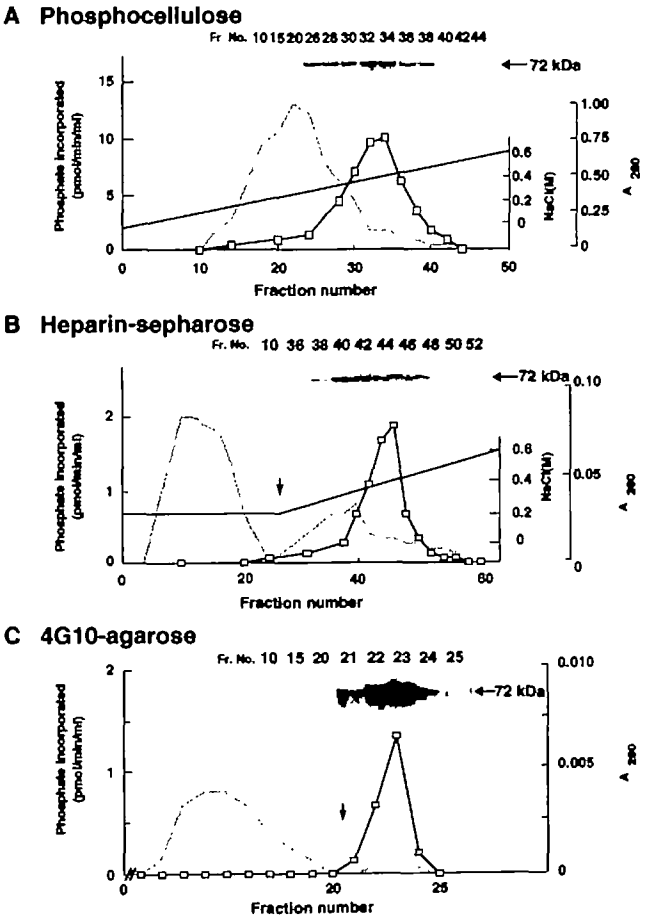


Fig. 1. **Purification of cytosolic 72-kDa PTK from rat liver.** (A) Phosphocellulose column chromatography. (B) Heparin-sepharose column chromatography. (C) 4G10-agarose column chromatography. The purification procedure of 72-kDa PTK was as described in "EXPERIMENTAL PROCEDURES." The fractions were assayed for protein kinase activity using [Val^5]angiotensin II as a substrate under the conditions described in "EXPERIMENTAL PROCEDURES." (□), PTK activity; (----), A_{280}; (—), NaCl concentration. Aliquots from indicated fractions of each column chromatography were electrophoresed and immunoblotted with anti-Syk antibody (shown above). The arrow indicates the start of the gradient (B) or batchwise elution (C).

ous traditional Northern blot method. Indeed, we could detect Syk mRNA by RT-PCR method on the mRNA prepared from rat liver as a template using specific PCR primers designed for rat Syk cDNA (data not shown). However, it is still unknown whether Syk is expressed in hepatocytes themselves or other cells such as Kuppfer cells or contaminating blood cells. To determine the distribution of Syk in liver, we performed immunohistochemical analysis of human liver with anti-Syk antibody. Since anti-Syk antibody for immunohistochemical analysis is only available for human tissues, we used a normal section of surgically resected human liver cancer. Antibody to human Syk was raised in rabbits against synthetic peptides corresponding to amino acid sequence mapping at the amino terminus of Syk of human origin. This antibody does not cross-react with ZAP-70 or other related PTKs. As shown in Fig. 4A, an immunohistochemical analysis of human liver showed the expression of Syk in the hepatocytic cytosol compartment. The specificity of the antibody was evidenced by the abolition of staining after antibody blockade with antigen peptide (Fig. 4B). To further confirm the expression of Syk in hepatocytes, we examined the expression of Syk in two rat liver–derived cell lines, JTC-27 and RLC-16, by immunoblot with anti-Syk antibody and RT-PCR method on the mRNA prepared from these cell lines as templates using specific PCR primers designed for rat Syk cDNA. As shown in Fig. 5, a low level of Syk expression was observed by both immunoblot and RT-PCR. Similar results were obtained with other human hepatoma cell lines, HuH7 and HLF (data not shown). These results provide the first evidence of Syk expression in human hepatocytes and liver-derived cell lines.

Involvement of Syk in Signaling Events of Hepatocytes—To understand the function of Syk in hepatocytes, we next examined whether Syk is activated by angiotensin II stimulation in JTC-27 cells. As shown in Fig. 6, Syk was significantly tyrosine-phosphorylated in response to angiotensin II stimulation in JTC-27 cells. Tyrosine-phosphorylation of Syk increased to a maximum at 3 min, then decreased. Equal amounts of Syk were present in each of the the immunoprecipitates, and the level of tyrosine-phosphorylation of Syk paralleled the activation state of Syk (data not shown). To further investigate the role of Syk, the effect of a Syk-selective inhibitor, piceatannol, on angiotensin II–induced MAP kinase activation was examined. As shown in Fig. 7, pretreatment of JTC-27 cells with piceatannol completely blocked angiotensin II–induced MAP kinase activation, as well as Syk immunoprecipitation by anti-phosphotyrosine antibodies. These results suggest that Syk may be

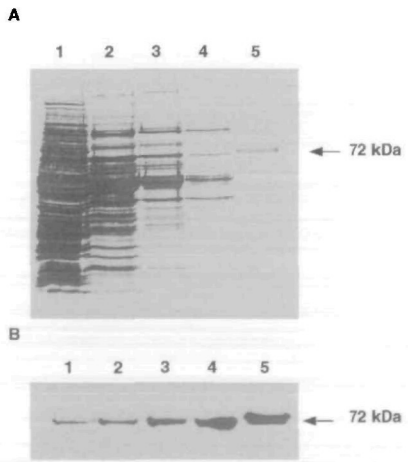
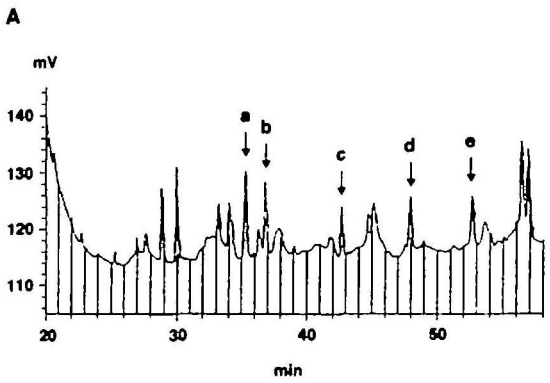


Fig. 2. **SDS/PAGE and immunoblot analysis of proteins from each stage of purification.** Aliquots from each step were subjected to 12.5% SDS/PAGE followed by silver-staining (A) or immunoblot probed with anti-Syk antibody (B) as described in "EXPERIMENTAL PROCEDURES." Lane 1, 1 μg 100,000 ×*g* supernatant; lane 2, 0.2 μg pooled fractions from phosphocellulose column; lane 3, 50 ng pooled fractions from Heparin-sepharose; lane 4, 10 ng pooled fractions from Casein-sepharose; lane 5, 2 ng pooled fractions from 4G10-agarose.



B

Peptide	Amino acid sequence	Mouse Syk
a	ALRADENYYK	512-521
b-1	LSIPEGK	225-231
b-2	VLHYRIDRDK	212-221
c	ISDFGLSK	504-511
d	ENLIREYVK	124-132
e	MPWFHGNISRDESEQTVLIG	165-184

Fig. 3. **Peptide sequence of 72-kDa PTK.** (A) High pressure liquid chromatogram of 72-kDa PTK-derived peptide. Peptides a-e are denoted by arrows. (B) Amino acid sequence of 72-kDa PTK peptides separated in (A) and comparison of these peptides with mouse Syk sequence.

TABLE I. **Purification of 72-kDa PTK from rat liver.** PTK activity was measured as described under "EXPERIMENTAL PROCEDURES."

Purification step	Protein (mg)	Total activity (pmol/min)	Recovery (%)	Specific activity (pmol/min/mg)	Purification (fold)
Supernatant	8,156	632	100	0.08	1
Phosphocellulose	834	926	147	1.1	14
Ammonium sulfate	425	580	92	1.4	18
Heparin-sepharose	12.2	65.2	10.3	5.3	69
Casein-sepharose	5.2	42.1	6.7	8.1	104
4G10-agarose	0.012	4.2	0.7	350	4,517

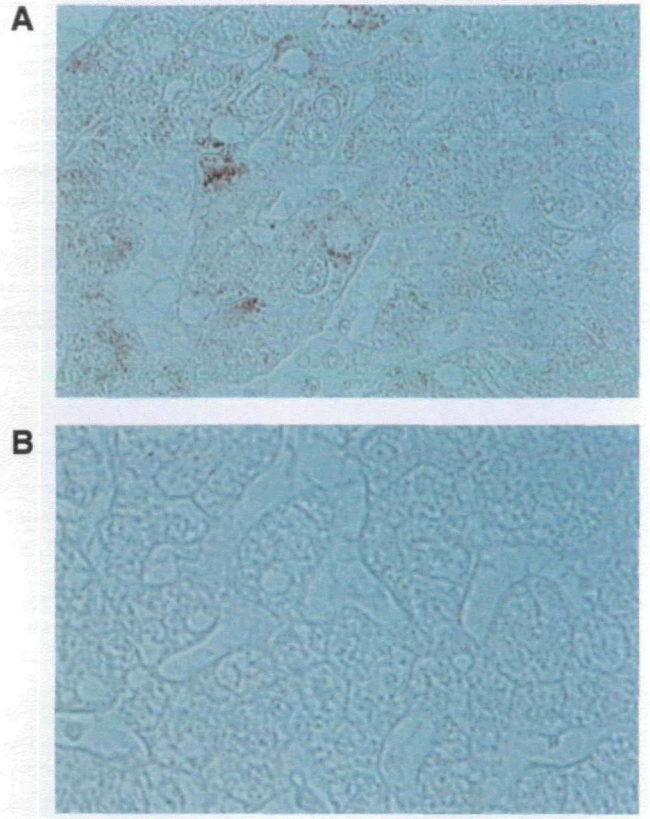


Fig. 4. **Immunohistochemical staining of Syk in human liver.** Serial sections were immunostained with specific antibodies and pre-absorbed antibodies to Syk, then photographed at high magnification (400×). Intense immunostaining with Syk antibodies was seen only in hepatocytes (A). Specimens treated with pre-absorbed antibodies did not show any immunoreactivity (B).

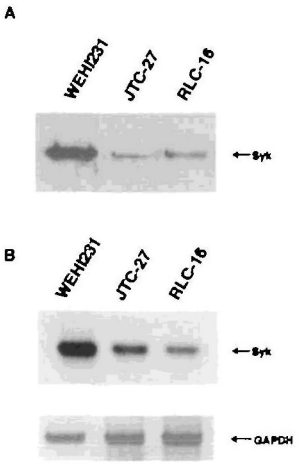


Fig. 5. **Expression of Syk in the rat liver–derived cell lines.** (A) Immunoblot analysis of Syk in mouse B cell line WEHI 231 and two rat liver–derived cell lines, JTC-27 and RLC-16. Cell extracts (10 μg of protein) were subjected to 12.5% SDS/PAGE followed by immunoblot probed with anti-Syk antibody as described in "EXPERIMENTAL PROCEDURES." (B) Total RNA (5 μg) from three cell lines was reverse transcribed and amplified during 30 cycles of PCR using Syk cDNA–specific primers. The PCR products of the expected size (263 bp) could be detected in all cell lines. The positions of Syk and GAPDH are indicated by arrows.

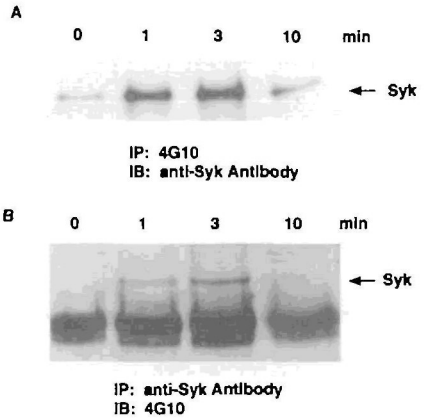


Fig. 6. **Involvement of Syk in angiotensin II signaling.** Tyrosine-phosphorylation of Syk in response to angiotensin II. JTC-27 cells (1×10^6 cells) were stimulated by angiotensin II (10 nM) for the indicated time, then lysates were immunoprecipitated with antibody to phosphotyrosine (A) or Syk (B), fractionated by PAGE, transferred to nitrocellulose filter, and analyzed with anti-Syk antibody (A) or anti-phosphotyrosine antibody (B) as described under "EXPERIMENTAL PROCEDURES." An arrow indicates the position of Syk.

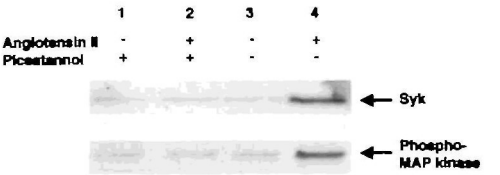


Fig. 7. **Effect of selective Syk inhibitor on angiotensin II–induced MAP kinase activation.** JTC-27 cells (1×10^6 cells) were incubated for 10 min in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of 100 μg/ml of piceatannol, and stimulated without (lanes 1 and 3) or with (lanes 2 and 4) angiotensin II (10 nM) for 3 min. (Upper panel) Cell lysates were immunoprecipitated with antibody to phosphotyrosine (4G10), fractionated by polyacrylamide gel electrophoresis, transferred to nitrocellulose filter, and analyzed with anti-Syk antibody. (Lower panel) Cell lysates were subjected to 12.5% SDS/PAGE followed by immunoblot probed with anti–phospho MAP kinase antibody as described under "EXPERIMENTAL PROCEDURES."

involved in signaling steps leading to MAP kinase activation by angiotensin II.

DISCUSSION

In this report, we demonstrate the expression of Syk in mammalian hepatocytes. This result was unexpected, because previous Northern blot analysis indicated the specific expression of Syk in hematopoietic cells (*6*). The relatively low sensitivity of Northern blots, compared to RT-PCR, could explain why Syk could not previously be detected in non-hematopoietic tissues such as hepatocytes. Although previous work suggested that Syk is present in low levels in lung and other non-hematopoietic tissues, this finding was thought to result from contamination by blood cells (*21*). However, our immunohistochemical data and the use of several liver-derived cell lines make contamination by blood cells very unlikely. In a previous study, Wong and Goldberg also reported the purification of p72/75 PTK from rat liver cytosol as a major species of PTK in liver (*22*). It is

highly possible that this p72/75 PTK is identical to Syk, since enzymatic properties of p72/75 kinase, including substrate specificity and the apparent molecular mass, closely resemble those of Syk. Furthermore, in spite of its low expression, Syk represents 30–40% of total [Val^5]angiotensin-II–phosphorylating activity in blood cell–depleted rat liver cytosolic fraction (data not shown). Therefore, Syk may be a major cytosolic PTK and play an important role in signaling in hepatocytes.

The search for Syk or novel Syk family PTKs in non-hematopoietic cells was prompted by the recent findings of the multiple function of Syk in a variety of signal transduction pathways, including G-protein coupled receptor signaling. For instance, in platelets, Syk is activated by stimulation with thrombin, thromboxane A2, or platelet-activating factor, suggesting the involvement of Syk in G-protein coupled receptor signaling (*13–15*). Moreover, Wan *et al.* recently reported that both m1 and m2 muscarinic acetylcholine receptors failed to stimulate MAP kinase kinase and MAP kinase in Syk-deficient DT40 chicken B cells, indicating that Syk is essential for both the Gi- and Gq-coupled pathways in this cell system (*16*). In this study, we demonstrated that Syk is tyrosine-phosphorylated in response to angiotensin II in rat liver–derived cell line, which is usually correlated to Syk activation, and that angiotensin II–induced MAP kinase activation is blocked by treatment with a Syk-selective inhibitor, piceatannol. Our results suggest that Syk is involved in signaling steps leading to MAP kinase activation by G-protein–coupled receptors in hepatocytes. However, since the expression of Syk in hepatocytes is very low compared to that of hematopoietic cells, other PTK(s) may display some functional redundancy for G-protein–coupled receptor signaling. Indeed, Li *et al.* reported that EGF receptor–specific tyrosine kinase inhibitors blocked angiotensin II–dependent EGF receptor tyrosine phosphorylation and MAP kinase activation in GN4 rat liver epithelial cells (*23*). Thus, G-protein–coupled receptor to EGF receptor pathway may play a central role in signaling steps leading to MAP kinase activation in hepatocytes.

Syk is well known as a critical effector of immunoreceptor-mediated cell signaling through ITAMs. Recently, Bonnerot *et al.* reported that blocking of Fc receptor gamma-chain–mediated Syk activation impaired FcR transport from endosomes to lysosomes, indicating that Syk regulates FcR-associated gamma-chain lysosomal targeting (*24*). Thus, ITAMs are involved in both protein targeting and, via SH2 domain binding, intracellular signaling. On the other hand, TGN38 is a type 1 integral membrane protein that cycles between the *trans*-Golgi network (TGN) and plasma membrane. The sequence -SDYQRL- (amino acids 331–336) within the cytosolic domain of TGN38 has previously been shown to be required for efficient internalization from the cell surface and targeting back to the TGN (*25*). Interestingly, the sequence of TGN38 (-pYQRL) has significant similarity to the sequence of the predicted phosphopeptide motif for interaction with the C-terminal SH2 domain of Syk. Indeed, Stephens and Banting reported that the cytosolic domain of TGN38 can be phosphorylated by the insulin receptor *in vitro*, and tyrosine-phosphorylated TGN38 can specifically bind to the SH2 domains of Syk (*26*). Since the insulin receptor and TGN38 are commonly expressed in hepatocytes, these observations together with our results further support a potentially important role of Syk in liver cell insulin signaling pathway and underline the physiological relevance of the TGN38–Syk interaction. It is difficult to speculate on the role of TGN38–Syk interaction since the function of TGN38 remains unknown. However, it is possible that tyrosine phosphorylation of TGN38 and subsequent TGN38–Syk interaction might be an important mechanism for modulating intracellular trafficking of the protein.

Syk may also be expressed in various cells besides hematopoietic cells and hepatocytes, since a Syk band was also detected by immunoblot with anti-Syk antibody in several cells including human umbilical vein endothelial cells, differentiated embryonal carcinoma P19 cells, rat vascular smooth muscle cells (A10), and COS-7 fibroblasts (data not shown). This preliminary result suggests the ubiquitous function of Syk in a variety of cell types. In particular, the possible expression of Syk in vascular tissue suggests a critical role of Syk in maintaining vascular integrity, since Syk-deficient mice showed severe petechiae *in utero* and died shortly after birth (*9*, *10*). The bleeding seen in Syk-deficient mice is unlikely to be due to a platelet deficiency or dysfunction, because mice lacking platelets do not show *in utero* bleeding. Rather, it is more likely to be caused by a defect in vascular tissue.

In conclusion, we demonstrate the expression of Syk in the non-hematopoietic tissue hepatocytes and raise the possibility of an important role of Syk in signaling events of hepatocytes. Further studies are currently underway to elucidate the physiological role of Syk in hepatocytes and other non-hematopoietic cells.

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