Syk protein-tyrosine kinase is involved in neuron-like differentiation of embryonal carcinoma P19 cells

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Abstract Syk has been implicated in activated immunoreceptors to downstream signaling events in hematopoietic cells. Here we report that Syk is expressed in neuron-like cells and involved in neuron-like differentiation of embryonal carcinoma P19 cells. Immunoblot, RT-PCR, and Northern analysis indicated that Syk is expressed in mouse brain, PC12 and P19 cells. In addition, Syk was found to be tyrosine phosphorylated during neuron-like differentiation of P19 cells. Furthermore, adenovirus-mediated overexpression of Syk induced supernumerary neurite formation and extracellular signal-regulated kinase (ERK) activation in P19 cells. These results suggest that Syk plays an important role in signaling steps leading to ERK activation in P19 cells.

Key words: Protein-tyrosine kinase; Syk; Neuron-like differentiation; P19 embryonal carcinoma cell; ERK

1. Introduction

The Syk family protein-tyrosine kinases (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 [1,2]. 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 [3,4].

On the other hand, accumulating evidences suggest the multiple function of Syk in a variety of signal transduction pathways. In the 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 [5,6]. Our group have also suggested that Syk is involved in oxidative and osmotic stress signaling in DT40 chicken B cell lines [7,8]. Recent work has shown that Syk exhibits a more widespread expression pattern in non-hematopoietic cells including epithelial cells, hepatocytes, fibroblasts and breast tissues, suggesting a general physiological function for this kinase [9–11]. Most recently, we suggested that Syk plays an important role in signaling events of hepatocytes, such as signaling steps leading to extracellular signal-regulated kinase (ERK) activation by G-protein-coupled receptors [9]. Thus, the function of Syk is not limited in immunoreceptor signaling, and these evidences raise the possibility that Syk plays an important role in the non-hematopoietic tissues.

In this paper, we report the expression of Syk in neuron-like cells and present an evidence that Syk is involved in signaling steps leading to ERK activation during neuron-like differentiation of P19 cells. The implication of these phenomena in signal transduction is briefly discussed.

2. Materials and methods

2.1. Materials

Adult Wistar mice were purchased from SLC Inc (Shizuoka, Japan). Polyclonal anti-Syk antibody (Ab) was purchased from Santa Cruz Biotechnology (CA, USA). Aprotinin, phenylmethylsulfonyl fluoride (PMSF), retinoic acid (RA) and protein A-agarose beads were from Sigma (MO, USA). Anti-phosphotyrosine monoclonal Ab (4G10) and 4G10-agarose were purchased from Upstate Biotechnology Inc (NY, USA). Anti-phospho-ERK Ab was from New England Biolabs Inc (MA, USA). All cell lines are provided from the American Type Culture Collection.

2.2. Cell culture

P19 mouse embryonic carcinoma cells were maintained in α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum (FCS) at 37°C, in 5% CO_2, in a humidified chamber. To promote differentiation, cells were first allowed to aggregate in bacterial grade dishes in α-MEM containing 10% FCS and 1 μM RA. Two days later, floating aggregates were collected, washed with α-MEM, replated onto bacterial grade Petri dishes and incubated with RA for 2 more days. The aggregates were then dispersed into single cells by treatment with 0.05% trypsin/0.02% EDTA. The cell suspension was then plated onto a 100-mm tissue culture grade dish to induce neural differentiation. PC12 rat pheochromocytoma cells were maintained on collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum and 5% FCS. WEHI231 cells were maintained in RPMI 1640 medium supplemented with 10% FCS.

2.3. Immunoprecipitation and immunoblot procedures

Mouse tissues and cell cultures were homogenized by a high-speed blender or pipeting in an ice-cold homogenization buffer (20 mM Tris–HCl pH 7.5, 1% NP-40, 1 mM EDTA, 10 μM sodium orthovanadate, 10 μg/ml aprotinin, 1 mM PMSF). The homogenates were centrifuged at 8000×*g* for 10 min to remove unbroken cells and the

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Abbreviations: PTK, protein-tyrosine kinase; RA, retinoic acid; FCS, fetal calf serum; SH2, Src homology 2; ITAM, immunoreceptor tyrosine-based activation motif; N-CAM, neural cell adhesion molecule; Ab, antibody; ERK, extracellular signal-regulated kinase

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supernatants were used as cell lysates. For immunoprecipitation, the lysates were further centrifuged at 100 000×*g* for 60 min and the supernatant was immunoprecipitated with anti-Syk Ab or anti-phosphotyrosine Ab. 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. For in vitro tyrosine kinase assay, the immunoprecipitates were incubated in a reaction mixture containing 50 mM HEPES/NaOH pH 8.0, 10 μM Na_3VO_4, 50 mM $MgCl_2$, 5 mM $MnCl_2$, and 10 μM ATP for 10 min at 30°C. Samples were boiled with electrophoresis sample buffer for 3 min, separated in SDS–PAGE and transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore) followed by detection with anti-phosphotyrosine Ab (4G10) or anti-Syk Ab as described previously [12].

2.4. RT-PCR

Total RNA from WEHI231 cells, P19 cells and PC12 cells were reverse transcribed and amplified by PCR method using Syk cDNA-specific primers as described previously [9]. Briefly, primers used for amplification of mouse Syk cDNA were synthesized as follows: primer (sense) was 5′-TCCATGGCAACATCTCCAG-3′; primer (antisense) was 5′-GACATGGTACCGTGAGGA-3′. Amplification was carried out with 94°C for 0.5 min, 50°C for 0.5 min, and 72°C for 1 min at 30 cycles. Primers used for amplification of mouse glyceraldehyde-phosphate dehydrogenase (GAPDH) were synthesized as follows: primer (sense) was 5′-TGAAGGTCGGTGTCAACG-GATTTGGC-3′; primer (antisense) was 5′-CATGTAGGCCAT-GAGGTCCACCAC-3′. Amplification was carried out with 94°C for 0.5 min, 60°C for 1 min, and 72°C for 1 min at 30 cycles. PCR products were electrophoresed on a 1.0% agarose gel containing ethidium bromide.

2.5. Preparation of recombinant adenoviruses

An adenovirus vector encoding porcine Syk was constructed with the use of an adenovirus expression kit (Takara, Tokyo, Japan). A 2.7-kb cDNA fragment containing the entire coding sequence of porcine Syk was ligated into the pAxCAwt cosmid vector, which contains the modified chicken α-actin promoter with cytomegalovirus-IE enhancer (CAG promoter). Then the recombinant adenovirus pAxCAwt-Syk was prepared by homologous recombination of the expression cosmid cassette and parental viral genome, and amplified to achieve a stock with a titer of approximately 10^9 plaque-forming units/ml [13].

3. Results and discussion

3.1. Expression of Syk in mouse brain and neuron-derived cell lines

To examine whether Syk is expressed in neuron-like cells, we performed immunoblot analysis using anti-Syk Ab. As shown in Fig. 1A, a lower level of Syk expression was observed in mouse brain, rat pheochromocytoma PC12 cells and neuronally differentiated P19 embryonic carcinoma cells as compared with those of mouse spleen and WEHI231 cells, respectively. To further confirm Syk expression in neuron-like cells, we performed reverse transcription polymerase chain reaction (RT-PCR) analysis using mRNA prepared from PC12 and P19 cells. This analysis demonstrates that Syk mRNA is expressed in these cells (Fig. 1B). Quantitative immunoblot analysis suggested that Syk expression of P19 cells is approximately 20-fold lower than that of WEHI231 cells (Fig. 1A). Immunoprecipitation assay revealed that Syk possesses enzymatically autophosphorylation tyrosine kinase activity in P19 cells (Fig. 1C). These results provide the first evidence of expression of Syk in neuron-like cells. Since recent work has shown that Syk exhibits a more widespread expression pattern in non-hematopoietic cells including epithelial cells, hepatocytes, fibroblasts and breast tissues [9–11], Syk appears to play a general physiological function in various

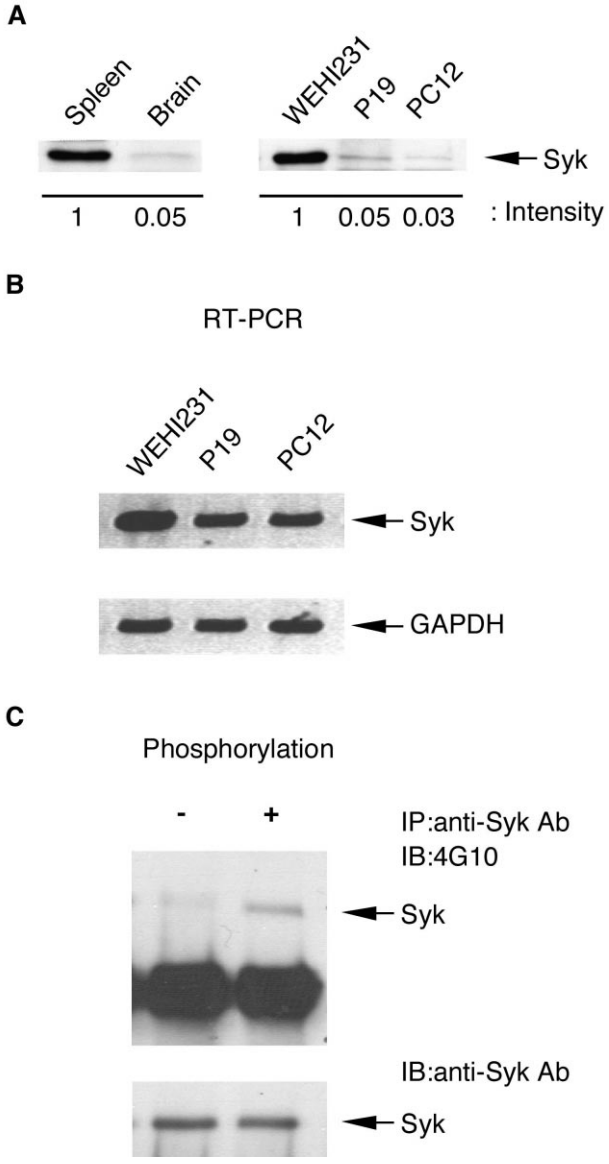

Fig. 1. Expression of Syk in mouse brain and neuron-like cell lines. A: Immunoblot analysis of Syk in mouse spleen, brain, WEHI231 cells, P19 and PC12 cells. Tissues and cell extracts (10 μg of protein) were analyzed by immunoblotting with anti-Syk Ab. B: RT-PCR, Northern blot analysis of Syk mRNA in WEHI231 cells, P19 and PC12 cells. The positions of Syk and glyceraldehyde-phosphate dehydrogenase (GAPDH) as control are indicated. C: In vitro tyrosine kinase assay of immunoprecipitated Syk from P19 cells. Immunoprecipitated Syk was phosphorylated and analyzed by immunoblotting with anti-phosphotyrosine Ab (4G10) or anti-Syk Ab. These experiments were repeated three times with similar results.

tissues. Indeed, Syk-deficient mice died shortly after birth and the mechanism of this lethal phenotype is unknown. Therefore, Syk may also play some roles in the developmental process of central nervous system. To further understand the role of Syk in neuronal cells, we are currently investigating the nerve cells of Syk-deficient mice.

3.2. Tyrosine phosphorylation of Syk in neuron-like differentiation of P19 cells

To understand the function of Syk in neuron-like differentiation, we next examined whether Syk is tyrosine phosphor-

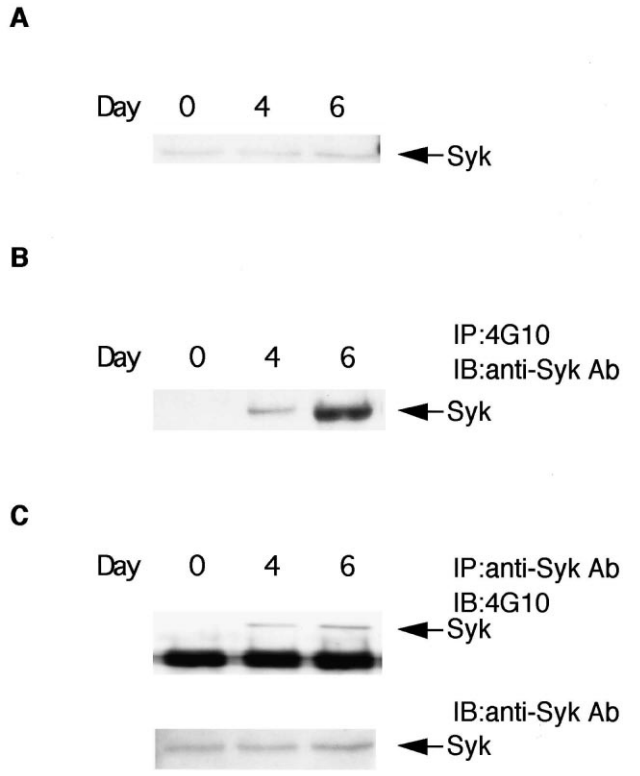

Fig. 2. Tyrosine phosphorylation of Syk in neuronally differentiated P19 cells. A: P19 cells (1×10^6 cells) were stimulated by RA (1 μM) for indicated time, and then cell lysates (10 μg of protein) were immunoblotted with anti-Syk Ab. B: Cell lysates were immunoprecipitated with anti-phosphotyrosine Ab (4G10), and then immunoprecipitates were immunoblotted with anti-Syk Ab. C: Cell lysates were immunoprecipitated with anti-Syk Ab, and then immunoprecipitates were immunoblotted with anti-phosphotyrosine Ab or anti-Syk Ab. The experiment was repeated three times with similar results.

ylated in P19 cells that can be induced to assume a neuron-like phenotype. P19 cells are developmentally pluripotent and after exposure to RA develop neuron-like properties. Immunoblot analysis indicated the almost equal amounts of Syk expression in each of stages during neuron-like differentiation of P19 cells (Fig. 2A). As shown in Fig. 2B and C, prior to the induction of neuron-like differentiation, Syk tyrosine phosphorylation was undetectable, but after 4 and 6 days of exposure to RA, Syk was significantly tyrosine phosphorylated. For the induction of neuroectoderm cells, P19 cells were allowed to form aggregates by culturing on bacterial grade Petri dishes in the presence of RA for 4 days, and then the aggregates were replated on tissue culture dishes to induce neural differentiation. Importantly, Syk tyrosine phosphorylation was detectable after replating the cells. Therefore, it is considered that Syk activation might occur after the adhesion of P19 cells to the extracellular matrix. In the hematopoietic cells, Syk has been reported to be activated through integrins, sug-

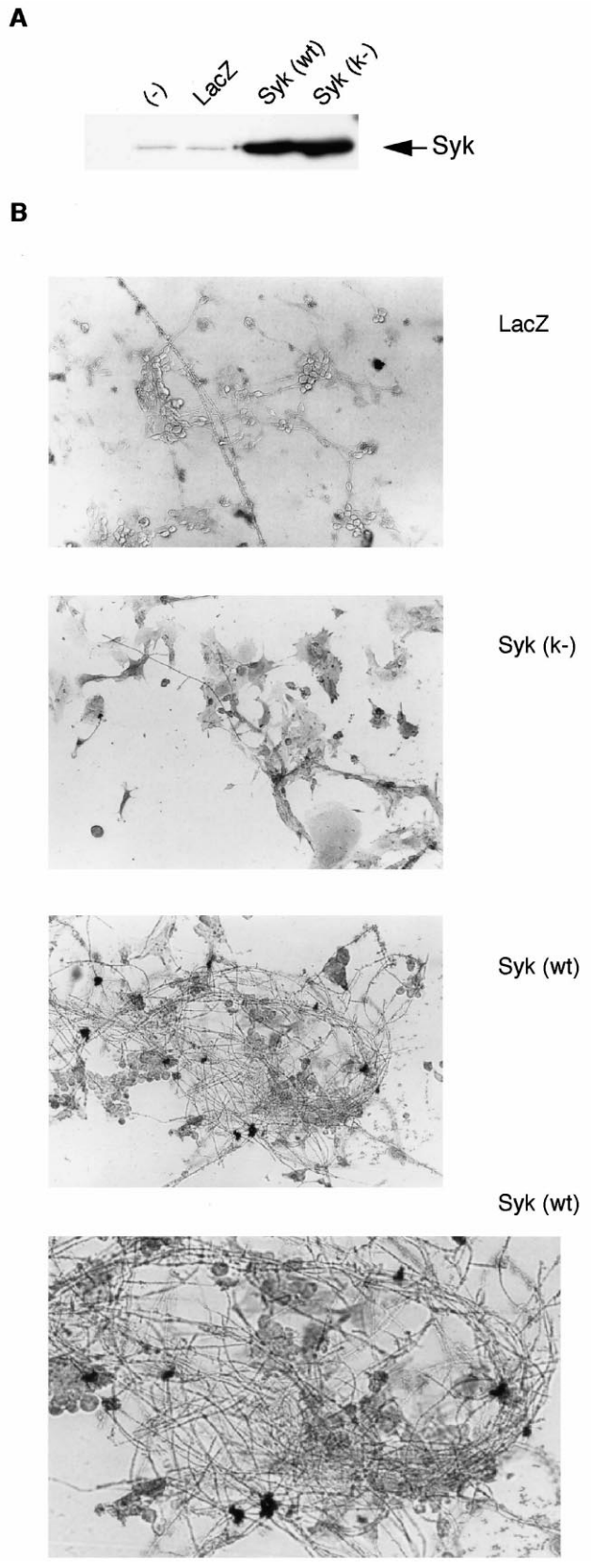

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Fig. 3. Effect of adenovirus-mediated overexpression of Syk on the differentiation of P19 cells. A: RA (1 μM)-induced P19 cells (at day 4) were equally infected with 20 multiplicity of infection (MOI) of LacZ, Syk wild-type (wt) and Syk kinase negative mutants (k−), and 2 days later, expression of Syk wild-type and Syk kinase negative were confirmed by immunoblot analysis probed with anti-Syk Ab. B: Each transfectant was photographed at 2 days after transfection. Photograph of cells overexpressing Syk (wt) at high magnification is shown. Note that Syk (wt), but not Syk (k−), induces supernumerary neurites.

gesting a unique role for integrins in Syk function [5,6]. Thus, Syk may be involved in signaling event of the cell adhesion. On the other hand, Src family PTKs have been implicated in differentiation and function of neural cells [14]. It was observed that neurons derived from Src-deficient mice exhibited a defect in neurite extension on a cell adhesion molecule, L1 [15], and that neurons from Fyn-deficient mice showed impaired neurite outgrowth on neural cell adhesion molecule (N-CAM) [16]. These results suggest that Src family PTKs play certain roles in cell-to-cell interaction mediated by neural adhesion molecules. Together with these observations, it is possible that Syk may be involved in signaling event of the cell-to-matrix or cell-to-cell interaction.

3.3. *Adenovirus-mediated overexpression of Syk induced supernumerary neurite formation and ERK activation in P19 cells*

To address the role of Syk in neuron-like differentiation, the effect of overexpression of Syk wild-type and Syk kinase negative on the neuron-like differentiation of P19 cells was examined using adenovirus-mediated expression system. Syk kinase negative construct contained the ATP binding site mutant of Syk (K395R). LacZ-expressing adenoviral vectors were used as controls. After 4 days of exposure to RA, P19 cells were equally infected with 20 multiplicity of infection (MOI) of these vectors. After 2 days infection, quantitative expression of Syk wild-type and Syk kinase negative mutants in P19 cells were confirmed by both immunoblot and immunohistochemical methods using anti-Syk Ab (Fig. 3A). Interestingly, overexpression of Syk wild-type, but not Syk dominant negative mutants, led to the formation of supernumerary neurites (Fig. 3B). Thus, these results suggest that Syk plays an important role in signaling events of neurite induction and outgrowth of P19 cells.

To further investigate the Syk-mediated signaling mechanism, the effect of overexpression of Syk wild-type and Syk dominant negative mutants on ERK activation was examined. As shown in Fig. 4, adenovirus-mediated expression of Syk wild-type (wt) induced ERK activation in a dose-dependent manner. Oppositely, adenovirus-mediated expression of Syk dominant negative mutants (k−) suppressed ERK activation in a dose-dependent manner. These results suggest that Syk plays an important role in signaling steps leading to ERK activation during neuron-like differentiation of P19 cells. Since it has been well known that treatment of PC12 cells with nerve growth factor stimulates ERK activation and induces neuron-like differentiation [17], Syk-ERK pathway may contribute the enhancement of RA-induced neural differentiation of P19 cells. On the other hand, it has been recently reported that differentiation of P19 cells in response to RA is regulated by Gα 12/13 and is associated with activation of c-Jun N-terminal kinase [18]. Therefore, it is also possible that neuron-like differentiation by Syk overexpression is associated with not only ERK activation but also other molecules including Gα 12/13 and c-Jun N-terminal kinase activation. Alternatively, Syk may be closely involved in the regulatory mechanism of cytoskeletal system. Neuronal differentiation is accompanied by extensive reorganization of the cytoskeleton to initiate the extension of neuritic processes. Indeed, Syk has been reported to be translocated to cytoskeleton and phosphorylate cortactin, an F-actin binding protein which is involved in signaling events of reorganization of the actin cytoskeleton [19]. Furthermore, it has been reported that Syk has the capacity to interact with microtubule networks within the B-lymphocyte and catalyzes the phosphorylation of the α-tubulin subunit [20]. Thus, in neuronal cells, Syk-dependent phosphorylation of microtubules may affect the ability of the microtubule cytoskeleton to induce the formation of supernumerary neurites. Further investigation is needed in order to clarify the exact role of Syk in the signaling pathway of neuronal differentiation.

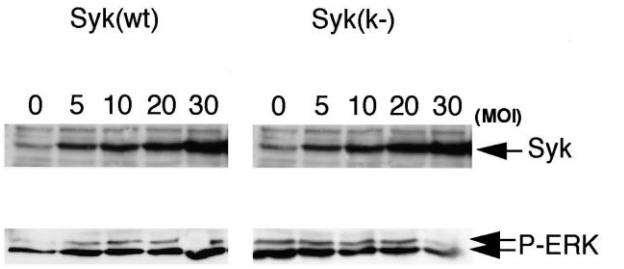

Fig. 4. Effect of adenovirus-mediated overexpression of Syk on the ERK activation. RA (1 μM)-induced P19 cells (at day 4) were infected various multiplicity of infection (MOI) of Syk wild-type (wt) and Syk kinase negative mutants (k−), and 2 days later, expression of Syk and ERK activation was examined by immunoblot analysis probed with anti-Syk Ab and anti-phospho-ERK Ab, respectively. To make clear the inhibitory effect of Syk (k−) expression on ERK activities, the exposure time of chemiluminescent detection by anti-phospho-ERK Ab is longer than that of Syk (wt) expression. The experiment was repeated three times with similar results.

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