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Syk kinase is phosphorylated in specific areas of the developing nervous system

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ABSTRACT

An increasing number of data involve immunoreceptors in brain development, synaptic plasticity and behavior. However it has yet to be determined whether these proteins in fact transmit an immunoreceptor-like signal in non-hematopoietic neuronal cells. The recruitment and activation of the Syk family tyrosine kinases, Syk and ZAP-70, being a critical step in this process, we conducted a thorough analysis of Syk/ZAP-70 expression pattern in nervous tissues. Syk/ZAP-70 is present in neurons of different structures including the cerebellum, the hippocampus, the visual system and the olfactory system. During the olfactory system ontogeny the protein is detected from the 16th embryonic day and persists in adulthood. Importantly, Syk was phosphorylated on tyrosine residues representative of an active form of the kinase in specialized neuronal subpopulations comprising rostral migratory stream neuronal progenitor cells, hippocampal pyramidal cells, retinal ganglion cells and cerebellar granular cells. Phospho-Syk staining was also observed in synapse-rich regions such as the olfactory bulb glomeruli and the retina inner plexiform layer. Furthermore, our work on cultured primary hippocampal neurons indicates that as for hematopoietic cells, Syk phosphorylation is readily induced upon pervanadate treatment. Therefore, Syk appears to be a serious candidate in connecting immunoreceptors to downstream adaptor/effector molecules in neurons.

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1. Introduction

The growing evidence that components originally considered characteristic of the immune system serve the nervous system confirms that they have much in common. Of interest is the immunoreceptor family of proteins that are used by hematopoietic cells to transduce extracellular signals, thereby controlling their homeostasis and functional outcome. Throughout these processes, immunoreceptors allow appropriate innate and adaptative immune responses. Strikingly, numerous components of the immunoreceptor family are expressed in brain tissues and moreover, most have already been shown to participate in brain development, synaptic plasticity and behavior (Chuang and Lagenaur, 1990; Huh et al., 2000; Yamada et al., 2001; Kitano et al., 2002; Kaifu et al., 2003; Syken and Shatz, 2003; Hamada et al., 2004; Roumier et al., 2004; Syken et al., 2006; Nakamura et al., 2007; Ogawa et al., 2007; Baudouin et al., 2008; Xu et al., 2010).

Classical and non-classical immunoreceptors are diverse but utilize a common signal transduction mechanism, which relies on conserved motifs mostly present in the signal-transducing subunits or part of the recognition-subunits themselves. Immunoreceptors can be either activator when present with immunoreceptor tyrosine-based activation motifs (ITAMs) or inhibitor when present with immunoreceptor tyrosine-based inhibition motifs (ITIMs). ITAM-based signaling depends on the coordinated interplays of protein tyrosine kinases (PTKs) of the Src and Syk families. Upon ligand recognition and receptor clustering, tyrosines within the ITAM are dually phosphorylated by Src family kinases, providing a docking site for the tandem SH2 domains of the Syk family kinases. These proximal events allow the recruitment and the activation of both kinase families and serve as a critical link to adaptor molecules capable of nucleating a multimolecular signaling complex. Downstream signaling that ensues comprises classical effector pathways including phospholipase (PL) C-gamma, protein

kinase C, phosphatidylinositol-3 kinase (PI3K), Ras, mitogen-activated protein kinase (MAPK), nuclear factor (NF) kappa-B and nuclear factor for activation of T cells (NFAT) (Turner et al., 2000; Humphrey et al., 2005). While most components of the ITAM-based signaling network depicted below are shared by neuronal cells, including Src PTKs, little is known regarding Syk PTKs.

ZAP-70 and Syk are the only two identified members of the Syk family sharing a common structure of two tandemly arranged Src homology 2 (SH2) domains and a carboxy-terminal kinase domain. Syk is widely expressed in all hematopoietic cells and several non-hematopoietic cells (Yanagi et al., 2001) whereas ZAP-70 expression is rather restricted, with the highest expression in T and natural killer cells (Chu et al., 1998). There are data in the literature suggesting a neuronal function of Syk. Syk was proposed as a candidate tyrosine kinase in alpha-synuclein and tau phosphorylation (Negro et al., 2002; Lebouvier et al., 2008). Furthermore, Syk was involved in RA-induced neurite formation, in P19 cells upon Syk overexpression (Tsujimura et al., 2001), and in CNTF-promoted nodose primary neuron neurite outgrowth (Gallagher et al., 2007). Interestingly, in the former model, tamalin, an ITAM-bearing scaffold protein that forms a multiple protein assembly including group 1 metabotropic glutamate receptors, was found endogenously phosphorylated and associated with Syk (Hirose et al., 2004). Although the physiological functional relevance of these findings still needs to be established, they are reminiscent of the described cross-regulation of signaling between ITAM-containing protein and heterologous receptors. These receptors are diverse and comprise cell adhesion molecules, cytokine and chemokine receptors, toll-like receptors, members of the TNF receptor family, plexins, lectin receptors and Ezrin/Radixin/Moesin proteins (Abram and Lowell, 2007; Ivashkiv, 2009). Syk kinases are central in this process of cross-regulation that extend ITAM-based signaling to non-immune receptors. In the present article, we describe the tissue distribution, the subcellular localization and more importantly the activation status of Syk family kinases in the nervous system.

2. Experimental procedures

2.1. Animals

Wistar-SPF rats (Charles River, St Germain sur l'Arbresle, France) were used. Animal care and procedures have been conducted according to the European Community Council Directive 86/609/EEC and the French Ethical Committee.

2.2. Preparation of tissue sections

Animals were deeply anesthetized with Equithesin 0.5 ml per 100 g body weight (i.p.). Embryos were taken out by Cesarean section at the indicated day of gestation, the day of conception corresponding to E1. Heads of embryos were fixed with periodate–lysine–paraformaldehyde (PLP) fixative, consisting of 2% paraformaldehyde, 75 mM lysine and 10 mM sodium m-periodate in 50 mM phosphate buffer (PB). Postnatal, day of birth corresponding to P1, and adult rats were anesthetized and perfused through the aorta with a Ringer's solution followed by PLP fixative. Nasal cavities, olfactory bulbs and brains were carefully dissected out and post-fixed overnight at 4 °C. Adult nasal cavities were decalcified by immersion in 50 mM PB containing 250 mM EDTA for 4 h at 4 °C. All tissues were cryoprotected in 100 mM PB 10% sucrose, frozen at −55 °C in nitrogen-cooled isopentane and cut in 12 μm thick sections with a cryostat.

2.3. Hippocampal primary neuron cultures

P2 rats were sacrificed under deep anesthesia induced by halothane inhalation and hippocampal cultures were prepared as previously described (Goslin et al., 1998). Briefly, hippocampi were dissected out and cells were dissociated by trypsin and trituration through Pasteur pipettes. Cells were maintained in serum-free Neurobasal medium supplemented with 1% N2, 2% B27, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (Invitrogen, Carlsbad, CA) and 1.6 mg/ml glucose.

For Syk subcellular distribution, cells were plated at 1×10^4 cells/cm^2 on poly-L-lysine and laminin (10 μg/ml each) coated glass coverslips. At the indicated time, cells were fixed in PBS 4% paraformaldehyde and processed for immunofluores-cence. For pervanadate activation, cells were plated on coated glass coverslips at 1×10^4 cells/cm^2 and after 3 days were either left unstimulated or stimulated with pervanadate (0.3 mM H_2O_2 and 0.1 mM Na_3VO_4) for 5 min. After two washes in cold PBS, cells were fixed in PBS 4% paraformaldehyde and stained with phospho-Syk Abs.

2.4. Antibodies

Syk, ZAP-70 or phospho-Syk Abs used included anti-Syk polyclonal Ab – N19 – (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Syk monoclonal Ab – clone 01 – (BioLegend, San Diego, CA), anti-ZAP-70 monoclonal Ab – clone 29 – (BD Biosciences, San Jose, CA), anti-phospho-Syk Y525/Y526 polyclonal Ab (Cell Signaling Technology, Danvers, MA) and anti-phospho-Syk Y348 monoclonal Ab (BD Biosciences). Mouse monoclonal Abs directed against the following markers was used: βIII tubulin (Sigma–Aldrich, Saint Louis, MO), GFAP (BD Biosciences), MAP-2 (Sigma–Aldrich), GAP-43 (Sigma–Aldrich), calbindin (Sigma–Aldrich), GAPDH (Millipore, Billerica, MA), phalloidin (Invitrogen) and synaptophysin (Sigma–Aldrich). Anti-MAP-2 rabbit polyclonal Ab was purchased from Chemicon (Millipore). Alexa Fluor 488- or 555-conjugated goat anti-mouse or anti-rabbit secondary Abs was purchased from Molecular Probes (Invitrogen). The experimental use of each antibody is summarized in Table 1.

2.5. Immunostaining

Samples were first permeabilized for 30 min at room temperature in PBS 0.3% triton X100 and incubated for an additional 60 min in blocking buffer (1–5% bovine serum albumin and 1–5% normal goat serum in PBS) containing 0.1% triton X100. Primary Abs were then added and left overnight at 4 °C. After 3 washes in PBS, appropriate Alexa Fluor-conjugated secondary Abs were diluted in blocking solution and applied for 90 min at room temperature. Samples were washed 3 times and 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI) staining of nucleus was performed before samples were mounted in Fluoroprep (BioMérieux, Lyon, France). For control experiments, samples were treated following the same procedure but primary Abs were omitted. Sections were examined with a Zeiss fluorescence microscope (Axio Imager.Z1) and images were recorded and analyzed using the Axiovision microscope software 4.7. Digitized images were processed for adjustment of brightness and contrast with image-editing softwares (ImageJ and Adobe Photoshop CS).

2.6. Western blot

P7 rat olfactory epithelium and bulb tissues and splenocytes isolated from adult animals were lysed in buffer containing 50 mM Tris–HCl pH 7.9, 120 mM NaCl, 0.5% NP-40, 1 mM EDTA, 0.1 mM NaF, 0.1 mM Na_3VO_4, 1 mM PMSF, 1 mM benzamidine and a protease inhibitor cocktail (Roche Applied Science). Lysates (30 μg of protein) were resolved on 9% SDS–PAGE and transferred to nitrocellulose membranes (PerkinElmer Life Sciences, Boston, MA). Membranes were blocked for 1 h in TBS-T (150 mM NaCl, 20 mM Tris and 0.1% Tween 20, pH 7.5) containing 5% milk and blotted against the indicated specific Abs (overnight at 4 °C), followed by incubation (1 h at room temperature) with horseradish peroxidase-conjugated goat anti-mouse secondary Abs (Amersham, Arlington Heights, IL). Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (ThermoFisher Scientific, Rockford, IL). Densitometric quantification was performed using Quantity One 4.1 software (Bio-Rad, Marnes-la-Coquette, France) and results were shown as mean OD values per mm^2 ± SD.

3. Results

3.1. Syk/ZAP-70 is expressed in differentiating and mature neurons

Syk and ZAP-70 expression was first examined by Western blot on protein extracts prepared from the olfactory bulb (OB) and olfactory epithelium (OE) of postnatal day 7 (P7) rats. As shown in Fig. 1A, Syk (72 kDa) was detected in these structures, in support to previous data reporting the presence of Syk in brain tissue (Tsujimura et al., 2001; Siegmund et al., 2007) and cultured primary nodose neurons (Gallagher et al., 2007). ZAP-70 expression was assessed on the same protein extracts, a faint band of the expected molecular weight (70 kDa) was revealed. Syk and ZAP-70 levels of expression were estimated by semi quantitative immunoblot analysis at respectively 10- and 20-fold lower than that of splenocytes, known to express a fair amount of the two kinases (Fig. 1A).

To characterize their spatial pattern of expression in the developing nervous system, we tested different commercially available