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Antibodies Directed against ZAP-70 Cross-React with a 66 kDa Tyrosine Kinase in the Rat Brain[1]

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ZAP-70 is another member of Syk family tyrosine kinases which plays an essential role in growth, differentiation, and function of T lymphocytes. In this study, we report the specific expression of a 66 kDa tyrosine kinase that is specifically cross-reacted with anti-ZAP-70 antibodies in the developing neurons. By immunoblot and immunoprecipitation assay using various anti-ZAP-70 antibodies, a 66 kDa tyrosine kinase was detected in lysates from rat brain. During the development of rat brain, expression levels of this 66 kDa tyrosine kinase were highest around 3 weeks after birth and decreased thereafter in the adult. In addition, immunoblot analysis demonstrated that this 66 kDa tyrosine kinase was expressed almost solely in the nervous system. These results suggest that this ZAP-70-related tyrosine kinase may play an important role in growth and differentiation in the developing neurons. Our observations will provide the clue to approach the regulatory system common to neurogenesis and immune response.

Protein-tyrosine kinase (PTK) plays an important role in signal transduction, leading various kinds of cells to proliferation and differentiation (1, 2). In the immune system, two structurally distinct families of PTKs, the Src and Syk families, are required for antigen receptor 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). It has been demonstrated that ZAP-70 plays an essential role in growth, differentiation, and function of T cells (8). The important role of ZAP-70 in T cell receptor (TCR) function and T cell development is underscored by the descriptions of patients with an autosomal recessive form of severe combined immunodeficiency who lack ZAP-70 and mice lacking ZAP-70 (9).

In neurons, evidence is accumulating that cell adhesion molecules, extracellular matrix, and neurotrophic factors set in motion intracellular signaling cascades leading to neurite extension (10-12). Recently, PTKs have been implicated as both positive and negative signaling components of intracellular pathways in neurons. Neurons express several members of the Src family PTKs including Src, Fyn, Yes and Yrk (13). In these cells, they are expressed at high levels, and interestingly, a more active isoform of Src is found in neurons of the central nervous system. Conversely, a ubiquitous form of Fyn is found in neurons and a spliced form is expressed specifically in hematopoietic cells. On the other hand, the expression of ZAP-70 is highly expressed in tissues of T lymphoid origin and believed to be specific of immune cells (7, 14).

In this study, we report the specific expression of a ZAP-70-related tyrosine kinase in the developing neurons. The biological meaning of the expression of a ZAP-70-related PTK in the central nervous system is briefly discussed.

MATERIALS AND METHODS

Materials and chemicals. SD rats were employed in this study. Anti-ZAP-70 polyclonal antibodies (Abs) and their immunizing peptides were purchased from Santa Cruz Biotechnology. The anti-phosphotyrosine monoclonal Ab, 4G10 (Upstate Biotechnology) was used for the detection of phosphotyrosine-containing proteins. The anti-actin monoclonal Ab was from Calbiochem.

Northern blot analysis. RNAs were prepared from various rat tissues using thiocyanate method as described previously (15). Total RNA (20 μg) was separated in 1.2% formaldehyde gel, transferred

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Abbreviations: PTK, protein-tyrosine kinase; SH2, Src homology 2; TCR, T cell receptor.

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to Hybond-N membrane (Amersham) and probed with ^{32}P-labeled rat ZAP-70 cDNA and rat β-actin cDNA, as a control.

Immunoblot and immunoprecipitation analysis. Total lysates (10 μg) from rat brain and thymus were separated using 10 % SDS-PAGE, blotted onto Immobilon P (Millipore) and analysed with anti-ZAP-70 Abs and anti-phosphotyrosine Ab (4G10) as described previously (16).

Protein-tyrosine kinase assay. The lysates from rat brain were clarified by centrifugation at 100,000 × g for 10 min and immunoprecipitated with anti ZAP-70 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. The immunoprecipitates were incubated in a reaction mixture containing 100 mM Hepes/NaOH pH 8.0, 10 μM Na_3VO_4, 50 mM $MgCl_2$, 5 mM $MnCl_2$, and 50 μM ATP for 0 or 10 min at 30 °C. The reactions were terminated by boiling for 3 min with SDS-sample buffer and subjected to 10 % SDS-PAGE, blotted onto Immobilon P and analysed with anti-phosphotyrosine Ab (4G10) as described previously (16).

RESULTS

Dominant expression of ZAP-70 mRNA in immune system. To examine the expression of rat ZAP-70 in the nervous system, we harvested total cellular RNA from various organs, and compared it by using Northern blot analysis with a rat ZAP-70 cDNA probe. Although a strong signal of ZAP-70 mRNA was detected in thymus, a significant signal was not observed in brain (Fig. 1). The low level of expression was presented in spleen (data not shown). This result is consistent with a previous report that human ZAP-70 is present exclusively in T and NK cell lines (14). Thus, it is unlikely that ZAP-70 is expressed in the nervous system.

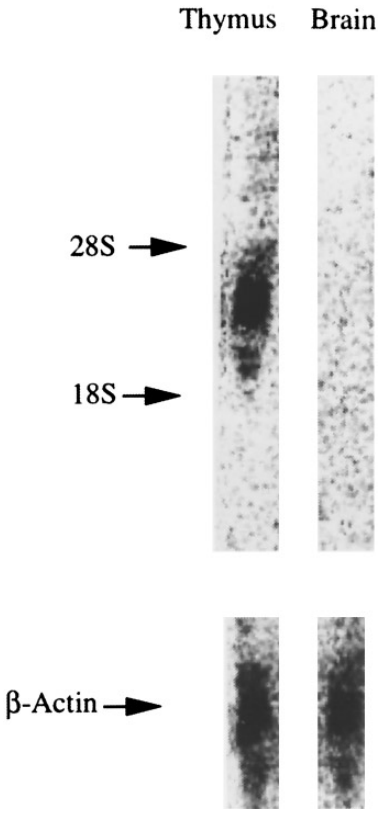

FIG. 1. Comparison of expression of ZAP-70 in thymus and brain. Total cellular RNA was harvested from thymus and brain in 3 week old SD rat by using thiocyanate method. Cytoplasmic RNA (20 μg) was hybridized with either rat ZAP-70 (top) or β-actin (bottom) cDNA probes.

Expression of ZAP-70 related protein in the brain. To examine the expression of ZAP-70-related tyrosine kinase in the central nervous system, cell lysates from rat brain were analysed by immunoblotting using various anti-ZAP-70 Abs (Fig. 2). As shown in Fig. 2B, several anti-ZAP-70 Abs specifically cross-react with a 66 kDa protein in lysates from rat brain. Particularly, Abs raised against linker lesion (326-343) and kinase domain (485-499) of ZAP-70 recognized a 66 kDa protein. However, an Ab against C terminus of ZAP-70 (600-618) did not recognize this 66 kDa protein. In order to provide the validity of these Abs for detecting this protein, the experiment was performed to show the effect of antigen peptides for their Abs on immunoblots. As shown in Fig. 2C, low concentration of specific peptides (1 μM) completely blocked the Abs to detect a 66 kDa protein, demonstrating the specific recognition of a 66 kDa protein by these Abs. Furthermore, anti-Syk Abs raised against two SH2 lesion cross react with partial purified 66 kDa protein (data not shown). These results suggested that this 66 kDa protein is closely related with ZAP-70.

Association of tyrosine kinase activity with immunoprecipitated 66 kDa protein. To examine whether this 66 kDa protein is associated with tyrosine kinase activity, immunoprecipitation kinase assay was performed. As shown in Fig. 3, immunoprecipitated 66 kDa protein with anti-Zap-70 Ab (485-499) exhibits autophosphorylation tyrosine kinase activity. Immunoprecipitated 66 kDa protein phosphorylates poly Glu-Tyr (4:1) peptides and tubulin on their tyrosine residues *in vitro*, however, the level of tyrosine kinase activity is very low in comparison with that of Syk or ZAP-70, suggesting that this 66 kDa protein may exist as an inactive state (data not shown). These results suggest that this 66 kDa protein may be a ZAP-70-related tyrosine kinase expressed in the central nervous system.

Differential expression and localization of 66 kDa tyrosine kinase. To examine the differential expression of 66 kDa tyrosine kinase during the development of rat brain, immunoblot analysis was performed using anti-Zap-70 Ab (485-499). As shown in Fig 4, expression levels of this 66 kDa protein were highest around 3 weeks after birth and decreased thereafter in the adult. Furthermore, a strong expression of 66 kDa protein was detected in the brain, but was not detected in other tissues. These results suggest that this 66 kDa

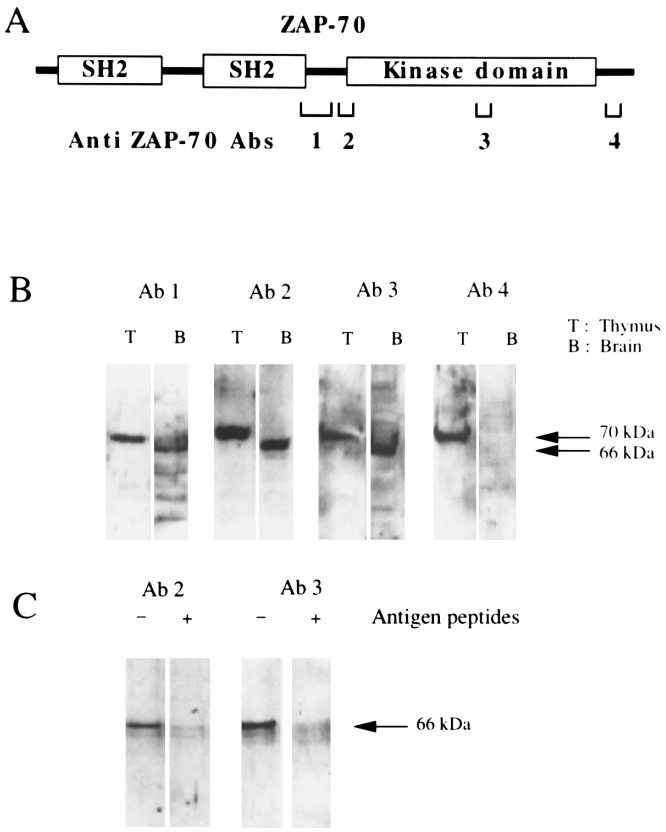

FIG. 2. Anti-ZAP-70 Abs recognize a 66 kDa protein from rat brain. (A) Recognition locuses of various anti-ZAP-70 Abs (1; 253-304, 2; 326-341 and 3; 485-499 of human ZAP-70 and 4; 600-618 of mouse ZAP-70). (B) Immunoblot analysis by anti-ZAP-70 Abs. Total lysates (10 μg) from rat brain and thymus were separated using 10 % SDS-PAGE, blotted onto Immobilon P and analysed with anti-ZAP-70 Abs as described "Materials and Methods". (C) Effect of the presence of antigen peptides on immunoblot probed with anti-ZAP-70 Abs. After blotting as described above, the individual lanes were separated and the sheets were incubated with 1 μM antigen peptide corresponding to 326-341 or 485-499 of human ZAP-70. Upon completion of incubations the sheets were analysed with anti-ZAP-70 Abs as described "Materials and Methods".

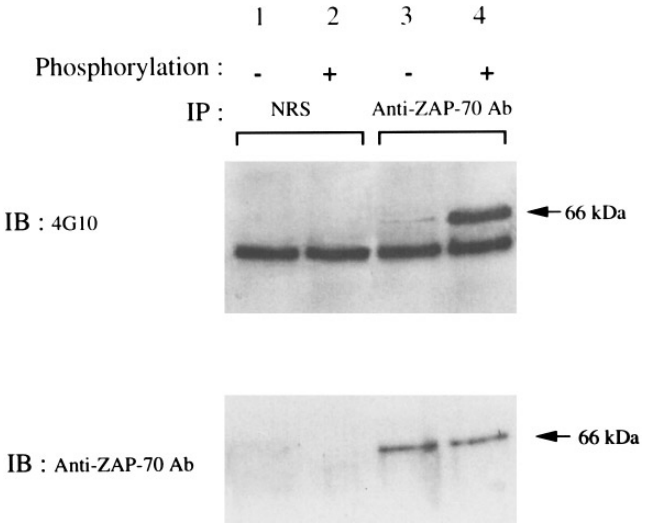

FIG. 3. Tyrosine kinase activity associated with immunoprecipitated 66 kDa protein by anti-ZAP-70 Ab. Lysates from rat brain were immunoprecipitated with normal rabbit serum (lane 1 and 2) or anti-ZAP-70 Ab 3 (lane 3 and 4). The immunoprecipitates were incubated with reaction mixture for 0 min (lane 1 and 3) or 10 min (lane 2 and 4) at 30 °C, and then subjected to 10 % SDS-PAGE, blotted onto Immobilon P and analysed with anti-phosphotyrosine Ab (4G10) or anti-ZAP-70 Ab as described "Materials and Methods".

tyrosine kinase is expressed mainly in the central nervous system.

DISCUSSION

In this report, we demonstrated the specific expression of a ZAP-70-related tyrosine kinase in the developing neurons. It seems important to understand the biological meaning of the expression of a ZAP-70-related PTK in the central nervous system since the critical role of ZAP-70 is well established in TCR function and T cell development.

Interestingly, there are some evidences that indicate the similar biological regulation between central nervous system and immune system. For instance, Src family PTKs, which are highly expressed in the neurons and hematopoietic cells, have been shown to regulate functions of both neuronal and immune cells. Moreover, it has been shown recently that neural cells produce various immunoregulatory cytokines, including in-

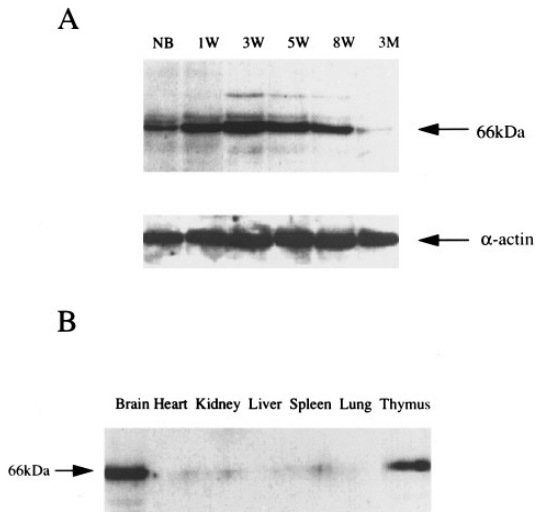

FIG. 4. Differential expression and tissue localization of 66 kDa tyrosine kinase. (A) Change in the level of expression of 66 kDa tyrosine kinase in the rat brain obtained at various age. NB, newborn rat. (B) Expression of 66 kDa tyrosine kinase in various tissues of rat (3 weeks). Total lysates (10 μg) from rat brain and various tissues were separated using 10 % SDS-PAGE, blotted onto Immobilon P and analysed with anti-ZAP-70 Ab (485-499) or anti-α-actin Ab as described "Materials and Methods".

terleukin-1, 2, 3, 4, 6, tumor necrosis factors, interferons, colony stimulating factors and transforming growth factor β (17). These cytokines regulate functions of both neuronal and glial cells as autocrine or paracrine mediators, and form a unique cytokine network in the central nervous system. Thus, these evidences suggest the regulatory system common to neurogenesis and immune response, and raise a possibility that novel Syk family PTK(s) may be required in the central nervous system and play an essential role in function of central nervous system as well as immune system.

Recent studies in the immune system have demonstrated that the oligomerization of TAMs (tyrosine-based activation motifs) allows the phosphorylation of two tyrosine residues found in this motif and these phosphotyrosine residues act as a bidentate docking site for the paired SH2 domains present in the Syk family PTKs (Syk and ZAP-70). From these investigations, we predicted the expression of brain-specific molecules that have two cytoplasmic TAMs. Indeed, it has been reported that a novel brain molecule, named BIT, has two cytoplasmic TAMs (18). Since BIT is believed to be a specific receptor molecules located just upstream of SHP-2, it does not seem to be a physiological target of Syk family PTK(s). However, further studies may contribute to identify novel brain molecules containing two cytoplasmic TAMs which are responsible for upstream of Syk family PTK(s) expressed in the central nervous system.

What is a physiological function of this 66 kDa tyrosine kinase in the neurons? During the development of rat brain, expression levels of this kinase were highest around 3 weeks after birth and decreased thereafter in the adult. Furthermore, in the preliminary result, this kinase was mainly localized in the growth cone fraction (data not shown). Therefore, one possibility is that this kinase may be involved in the nerve growth cone guidance. Recent findings have revealed that L1 and NCAM induce neurite outgrowth by activating intracellular signaling pathways in the growth cone mediated by Src family PTKs, $pp60^{c\text{-}src}$ and $p59^{fyn}$ (19, 20). Thus, as well as immune system, two structurally distinct families of PTKs, the Src and Syk families, may be required for neural cell adhesion molecule signaling and dictate their distinctive molecular interactions with cell adhesion molecules and signal components. Further studies on the purification and molecular cloning of 66 kDa tyrosine kinase may contribute to elucidate a physiological role of this kinase in the central nervous system.

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