Volume 332, number 1,2, 27–30 FEBS 13062 October 1993
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pp59^{fyn} and pp62$^{c\text{-}yes}$ are enriched in SH-SY5Y neuroblastoma growth cones but do not associate to the 38 kDa protein which complexes with pp60$^{c\ src}$ and pp60$^{c\text{-}srcN}$

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Received 4 August 1993

The tyrosine-specific kinases pp60$^{c\text{-}src}$ and pp60$^{c\text{-}srcN}$ (pp60^{src}) are slightly enriched and activated in growth cones isolated from neuronally differentiating SH-SY5Y neuroblastoma cells. In the growth cones the two *src* isoforms are associated with a 38 kDa protein. In this report, we have compared the subcellular distribution of pp59^{fyn} and pp62$^{c\text{-}yes}$ with that of pp60^{src} in differentiating SH-SY5Y cells. Like pp60^{src}, the other two tyrosine kinases were slightly enriched and activated in the growth cones as compared to the levels in the cell bodies. The kinase activites were 3- to 4-times higher in growth cones than in cell bodies. However, only pp60^{src} formed a complex with the 38 kDa protein while immunoprecipitation of pp59^{fyn} brought down an additional protein of 90 kDa. This may suggest that these related tyrosine kinases have different substrates and in part mediate different cellular responses in the growth cones of differentiating SH-SY5Y cells.

Tyrosine kinases; Growth cone; *src*; *fyn*; *yes*; Neuronal differentiation

1. INTRODUCTION

The *src*-family of non-receptor tyrosine kinases consist, at least, of nine structurally related members; c-*src*, c-*yes*, c-*fgr*, *fyn*, *lyn*, *lck*, *hck*, *blk* and *atk*. They all have a molecular weight of approximately 60 kDa, are myristylated in the amino-terminus, and are associated with the inside surface of the plasma membrane [1–3]. The precise functions of these kinases are not known, but pp56^{lck} and pp59^{fyn} are implicated in the intracellular signalling of antigen-activated T lymphocytes [4–6], while pp60$^{c\text{-}src}$, pp59^{fyn} and pp62$^{c\text{-}yes}$ in fibroblasts are involved in the signal transduction pathway via the platelet-derived growth factor receptor [7]. The most studied member, pp60$^{c\text{-}src}$, is expressed in all tissues tested, with the highest levels in the developing nervous system and in platelets [8–14]. In neuronal cells, alternative mRNA splicing generates three mRNA species and at least two protein products of the *src*-gene, pp60$^{c\text{-}src}$ and pp60$^{c\text{-}srcN}$ [15–17].

Human SH-SY5Y neuroblastoma cells differentiate neuronally in the presence of serum and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [18–21]. pp60$^{c\text{-}src}$ and pp60$^{c\text{-}srcN}$ (collectively called pp60^{src}) are activated early during differentiation, coinciding in time with the formation of neurites with growth cones [19], and pp60^{src} is slightly enriched and activated in these structures [22]. Furthermore, during differentiation pp60^{src} associates with a 38 kDa protein (pp38), and this complex is almost exclusively found in the growth cones [22]. A similar complex is also present in chromaffin granule membranes [23], and recently pp62$^{c\text{-}yes}$ was shown to associate with a 38 kDa phosphoprotein in adult rat cerebellum [24].

The expression profile and subcellular localization of the c-*src* isoforms suggest roles in the nerve cell function. However, mice homozygous for a null mutation in the c-*src* gene show no abnormalities in the nervous system [25], suggesting redundancy mechanisms presumably involving other members of the *src*-kinase family. We have therefore analysed the subcellular distribution of pp59^{fyn} and pp62$^{c\text{-}yes}$ in TPA-treated SH-SY5Y cells, and the potential association of these tyrosine kinases to pp38 previously shown to associate to pp60^{src}. We find that all kinases are slightly enriched and activated in the growth cones. However, neither pp59^{fyn} nor pp62$^{c\text{-}yes}$ appear to associate with a 38 kDa phosphoprotein.

2. MATERIALS AND METHODS

2.1. *Cell cultures*

The SH-SY5Y cells, an adrenergic clone of the human neuroblastoma cell line SK-N-SH [26], was kindly provided by Dr. June Biedler, Sloan Kettering Institute, New York. The cells were routinely grown in Eagle's minimum essential medium supplemented with serum and antibiotics, and neuronal differentiation was induced by treatment with 16 nM TPA (Sigma) as described previously [27]. All cells were grown for 4 days in the absence or presence of TPA, before analysis.

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Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; pp60^{src}, pp60$^{c\text{-}src}$ + pp60$^{c\text{-}srcN}$.

2.2. *Subcellular fractionation*

The growth cone and the cell body fractions were prepared as described in [22]. Briefly, cells were suspended in ice-cold EDTA buffer (0.54 mM EDTA, 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 0.15 mM KH_2PO_4, pH 7.4) and homogenized in a Teflon glass homogenizer. The homogenate was layered onto a cushion of 20% sucrose and centrifugated at $500 \times g_{max}$ for 4 min. The growth cone fraction was collected from the material retained at the load/20% sucrose interface and the pellet in the bottom of the tube represented the cell body fraction. Each fraction was centrifugated at $20,000 \times g_{max}$ for 20 min before further analysis. The pelleted growth cones and cell bodies were solubilized in RIPA buffer (10 mM Tris-HCL, pH 7.2, 0.16 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EGTA, 1 mM EDTA and 100 μM pre-boiled sodium orthovanadate), and subjected to immunoprecipitation.

2.3. *Immunoprecipitation, kinase assay and immunoblotting*

$pp60^{src}$ was immunoprecipitated as in [19] using an excess of the monoclonal antibody 327 (MAb 327) [28] followed by incubation with rabbit anti-mouse Ig antiserum and formaldehyde-fixed *Staphylococcus aureus*. $pp59^{c\text{-}fyn}$ and $pp62^{c\text{-}yes}$ were immunoprecipitated with the polyclonal antisera, anti-*fyn*2 and anti-*yes*3 [7,29], respectively. The solubilized samples were incubated with an excess of the antibody for 90 min on ice, and then incubated with formaldehyde-fixed *S. aureus*, as described in [19].

For kinase assay the immunoprecipitates was resuspended in 20 μl kinase buffer (0.05 M PIPES, pH 6.8, 10 mM $MnCl_2$, 0.5 μM ATP, 5 mM $MgCl_2$) supplemented with 2.5 μCi [γ-^{32}P]ATP and incubated on ice for 20 min. The reactions were terminated with sample buffer for SDS-PAGE [30]. The proteins were separated on 10% polyacrylamide gels and the ^{32}P-labelled protein bands were identified by autoradiography and quantified by a phospho-imager (Molecular Dynamics).

For immunoblotting the immune-complexes were separated by SDS-PAGE, and electrophoretically transferred to nitrocellulose filters [31]. Then the filters were blocked in 1% non-fat dry-milk, dissolved in phosphate buffered saline (PBS) supplemented with 0.5% Tween-20, for 1 h at room temperature. The filters were incubated with either MAb 327, anti-*fyn*1 or anti-*yes*3 antibodies, diluted 1:100 in PBS, overnight at 4°C. The MAb 327 filters were incubated with rabbit anti-mouse Ig antiserum (1:200) for 2 h at room temperature. All filters were finally incubated with ^{125}I-labelled protein A (0.25 μCi/ml; Radiochemical Centre, Amersham, England), in PBS for 2 h at room temperature. The proteins were visualized by autoradiography. Three washings with PBS were performed between each antibody incubation. The ^{125}I-labeled protein bands were quantified by a phospho-imager.

2.4. *Specific kinase activity of $pp59^{fyn}$ and $pp62^{c\text{-}yes}$*

To examine the specific kinase activity of $pp59^{fyn}$ and $pp62^{c\text{-}yes}$, the immunoprecipitated proteins were divided into two parts and analysed for kinase activity and protein content as described above.

The radioactive protein bands, in the kinase and in the immunoblot analyses, were quantified using a phospho-imager analyser and the relative specific kinase activities were calculated as the ratio of relative kinase activity to relative protein content (see also [22]). The specific kinase activities of the cell body fraction were set to 1.0.

3. RESULTS

3.1. *$pp59^{fyn}$ and $pp62^{c\text{-}yes}$ kinase activity and protein levels in growth cone and cell body fractions from differentiating SH-SY5Y cells*

We have previously reported the enrichment and slight activation of $pp60^{src}$ in a growth cone preparation from differentiating SH-SY5Y cells [22]. To examine if other *src*-related tyrosine kinases were also enriched and/or activated in this cell compartment, a growth cone and a cell body preparation were analysed for $pp59^{fyn}$ and $pp62^{c\text{-}yes}$. The pelleted, fractionated material was solubilized, whereafter an equal amount of total protein was taken for immunoprecipitation followed by quantification of protein levels and kinase activities of the two tyrosine protein kinases. Western blotting data revealed that $pp62^{c\text{-}yes}$ and $pp59^{fyn}$ proteins were enriched 2.4- and 1.4-fold, respectively, in the growth cone relative to the level in the cell body fraction (Fig. 1 and Table I). In the growth cones, the specific activities of $pp62^{c\text{-}yes}$ and $pp59^{fyn}$ were 1.8- and 2.3-fold higher than in the cell bodies (Table I). Thus, like $pp60^{src}$ (Table I and [22]) the two examined *src*-related tyrosine kinase activities were enriched in SH-SY5Y growth cones.

3.2. *Unlike $pp60^{src}$, $pp59^{fyn}$ and $pp62^{c\text{-}yes}$ did not form complexes with a 38 kDa protein in the growth cones*

$pp60^{src}$ forms a complex with a 38 kDa protein (pp38) detected via its phosphorylation in a $pp60^{src}$-immunocomplex kinase assay. pp38, which presumably associated to $pp60^{src}$ via disulfide-bridges, is strongly phosphorylated in the growth cone fraction but just detectable in the cell bodies of SH-SY5Y cells (Fig. 2, lane 2, and [22]). To examine if the two other *src*-related kinases also were associated with pp38, kinase assays were performed using immunocomplexes obtained via anti-*fyn* and anti-*yes* immunoprecipitations of solubilized growth cone and cell body material. As seen in Fig. 2, pp38 was not detected in $pp59^{fyn}$ or $pp62^{c\text{-}yes}$ immunoprecipitates (lanes 3,4 and 5,6). Even after overexposure of the autoradiogram of the in vitro tyrosine kinase analysis the pp38 was undetectable (not shown). However, the $pp59^{fyn}$-immunocomplex kinase reaction

Table I

Specific kinase activity of $pp62^{c\text{-}yes}$, $pp59^{fyn}$ and $pp60^{src}$ in a growth cone and a cell body preparation

	Cell body	Growth cone*
$pp62^{c\text{-}yes}$		
Protein	1.0	2.4 ± 1.1
Autophosphorylation activity	1.0	4.3 ± 1.9
Specific autophosphorylation activity	1.0	1.8 ± 0.1
$pp59^{fyn}$		
Protein	1.0	1.4 ± 0.1
Autophosphorylation activity	1.0	3.1 ± 0.9
Specific autophosphorylation activity	1.0	2.3 ± 0.7
*$pp60^{src}$***		
Protein	1.0	1.5 ± 0.0
Autophosphorylation activity	1.0	3.2 ± 0.1
Specific autophosphorylation activity	1.0	2.1 ± 0.1

*Mean value (arbitrary units) of two separate experiments
**Data taken from [22].

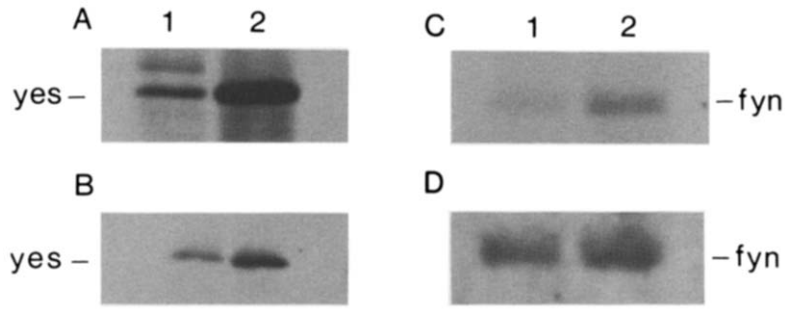

Fig. 1. Analysis of the specific pp62^{yes} and pp59^{fyn} kinase activities in a cell body and a growth cone fraction from TPA-treated SH-SY5Y cells. Growth cones and cell bodies prepared from differentiating SH-SY5Y cells were solubilized in RIPA buffer and normalized for total protein content. pp62$^{c\text{-}yes}$ and pp59^{fyn} were immunoprecipitated with anti-*yes*3 and anti-*fyn*1 antibodies, respectively, and each immunoprecipitate was divided into two parts; 0.1 mg of total protein was used for kinase assay, and 0.5 mg for immunoblotting. [^{125}I]Protein A was used for the detection of the immunoreactive proteins in the immunoblot analysis. (A) Kinase assay of pp62$^{c\text{-}yes}$, (B) immunoblot of pp62$^{c\text{-}yes}$, (C) kinase assay of pp59$^{c\text{-}fyn}$, and (D) immunoblot of pp59$^{c\text{-}fyn}$. (A–D) Lane 1, cell body; lane 2, growth cone fraction. The radioactivity was quantified using a phospho-imager analyzer and the values are presented in Table I.

identified a major additional protein with an approximate molecular weight of 90 kDa. This in vitro phosphorylated protein was particularily prominent in the growth cone fraction, but was also readily detected in the cell body preparation (Fig. 2, lanes 3 and 4). Electrophoretic analysis of the pp59^{fyn} immunoprecipitate under non-reducing conditions gave no indication that the 90 kDa protein was linked to pp59^{fyn} via disulfide-bridges (data not shown). In contrast, no major phosphorylated proteins appeared to associate to pp62$^{c\text{-}yes}$, as judged by the immunocomplex protein kinase analysis (Fig. 2, lanes 5 and 6). Some minor bands were detected, but phosphorylated proteins of similar molecular weights were also detected in the pp60^{src} and pp59^{fyn} immunocomplexes. Whether these phosphorylated proteins reflect true association to members of the *src*-family or not is an open question.

4. DISCUSSION

The enrichment of pp60^{src} [22,32] and the complex formation with a 38 kDa protein in neuronal growth cones [22], suggest specific roles for these proteins in the growth cone. However, mice homozygous for a null mutation in the c-*src* gene show no overt abnormalities in the nervous system [25]. It is reasonable to assume, though, that the function(s) of the c-*src* isoforms in the nervous system in these animals are compensated by protein activities that are interchangeable with the pp60^{src} kinase activity. Candidate proteins are pp59^{fyn} and pp62$^{c\text{-}yes}$ since they are biochemically closely related to pp60^{src}, and have to a certain extent similar tissue distributions as pp60^{src} [10,13,33–36]. The *fyn* and c-*yes* genes, respectively, have recently been knocked-out resulting in no (c-*yes*) or limited effects (*fyn*) on the nervous system. In the *fyn* knock-outs, long-term potentiation and the development of hippocampal neurons were affected [37,38]. However, ablation of two or more genes of the *src*-family might turn out to have profound effects on the development and function of the nervous system.

In this report we show that pp59^{fyn} and pp62$^{c\text{-}yes}$ were

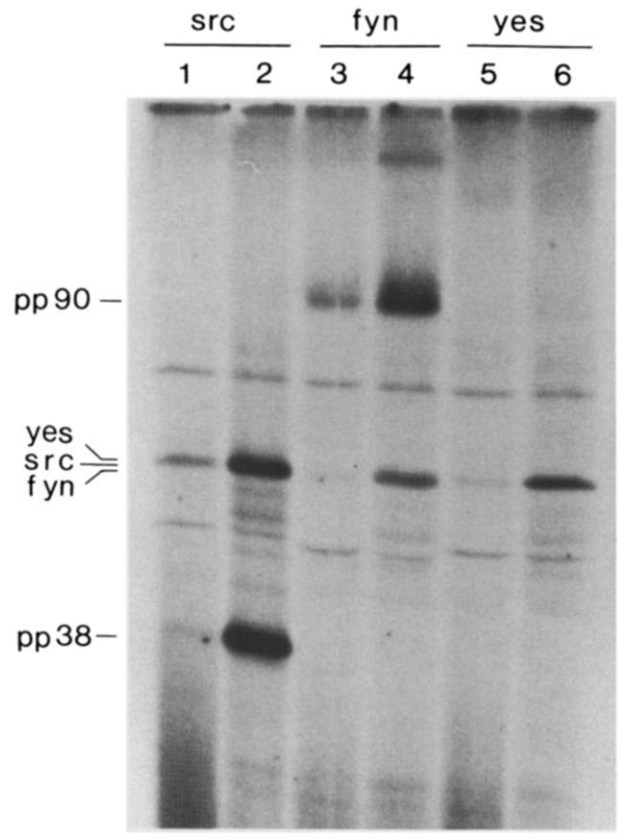

Fig. 2. pp60^{src}, but not pp59$^{c\text{-}fyn}$ and pp62$^{c\text{-}yes}$, forms a complex with pp38 in the growth cone fraction. The cell body and the growth cone fractions were solubilized, divided into three parts, and immunoprecipitated using the following antibodies; MAb 327 against pp60^{src} (lanes 1,2), anti-*fyn*1 against pp59^{fyn} (lanes 3,4), and anti-*yes*3 against pp62$^{c\text{-}yes}$ (lanes 5,6). The immunocomplexes were analysed for kinase activity and the phosphorylated proteins were analysed by SDS-PAGE and autoradiography. Lanes 1, 3 and 5, represent cell body; lanes 2, 4 and 6, represent the growth cone material.

slightly enriched and activated in growth cones from differentiating SH-SY5Y cells. Qualitatively, the same distribution and activation patterns were observed for these two kinases as for pp60^{src}, as determined previously [22]. Thus, our data could support the idea that redundancy mechanisms explain why animals lacking the *src* gene still have the capacity to form neuronal growth cones. However, neither pp59^{fyn} nor pp62$^{c\text{-}yes}$ was associated with the 38 kDa protein in growth cones or in cell bodies, which distinguishes pp60^{src} from the two other kinases. The result was somewhat unexpected since pp62$^{c\text{-}yes}$ complexes with a 38 kDa protein in adult rat cerebellum [24]. A number of reasonable explanations can be suggested. One is that the cerebellar pp38 is not identical to pp38 in SH-SY5Y cells, and that the former pp38 is not expressed in these cells. Another explanation would be that the pp62$^{c\text{-}yes}$–pp38 complex is formed at later developmental stages than the corresponding complex with pp60^{src}, and that SH-SY5Y cells represent a differentiation stage at which pp38 does not complex with pp62$^{c\text{-}yes}$. A third possibility is that pp62$^{c\text{-}yes}$ and pp60^{src} have different localizations in the SH-SY5Y growth cones, and that pp60^{src} co-localizes with pp38.

Immunoprecipitates of pp59^{fyn} contained a 90 kDa protein that was heavily phosphorylated in the SH-SY5Y growth cones as detected by the in vitro kinase assay. In contrast, no major additional phosphorylated proteins were found in the pp62$^{c\text{-}yes}$ immunocomplex. Thus, the *src*-related tyrosine kinases in SH-SY5Y growth cones differed in their associations to other proteins, and presumably this reflects differences in their specific growth cone functions. The isolation and structural characterization of pp38 or corresponding cDNA might therefore disclose the role(s) of pp60^{src} in growth cones, and shed some light over its function in neurons.

Acknowledgements: We thank Irja Johansson for excellent technical assistance; Dr. June Biedler, Sloan Kettering Institute, New York, for kindly providing the SH-SY5Y cells; Dr. Joan Brugge, ARIAD Pharmaceuticals Inc., Cambridge, MA, for the Mab 327; and Dr. Sara Courtneidge, European Molecular Biology Laboratory, Heidelberg, for the anti-*fyn*2 and anti-*yes*3 antibodies. This work was supported by grants from Swedish Cancer Society, Children Cancer Foundation of Sweden, HKH Kronprinsessan Lovisas förening för barnasjukvård, Hans von Kantzows, and Ollie och Elof Ericssons Stiftelser.

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