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Zinc-induced tyrosine phosphorylation of hippocampal p60$^{c\text{-}src}$ is catalyzed by another protein tyrosine kinase

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Tyrosine phosphorylation of p60$^{c\text{-}src}$ induced by Zn^{2+} in rat hippocampal membranes is shown to inhibit *Src* tyrosine kinase activity. Zn^{2+} catalyzes the phosphorylation of p60$^{c\text{-}src}$ in the membranes but does not activate autophosphorylation of p60$^{c\text{-}src}$ immunoprecipitated with anti-*Src* monoclonal antibody. Moreover, the immunoprecipitated *Src* kinase has no Zn^{2+}-induced activity in phosphorylation of exogenous substrate, enolase. Cyanogen bromide cleavage of p60$^{c\text{-}src}$ phosphorylated in the presence of Zn^{2+} yields a 4-kDa phosphopeptide corresponding to phosphorylation of a carboxy-terminal tyrosine residue of *Src* kinase. In conclusion, hippocampal membranes contain a Zn^{2+}-stimulated protein tyrosine kinase capable of regulating the p60$^{c\text{-}src}$ activity.

Zinc; Protein phosphorylation; Phosphotyrosine; p60$^{c\text{-}src}$; Rat hippocampus

1. INTRODUCTION

Brain contains an abundance of the transition metal zinc, which is primarily accumulated in hippocampus. The concentration of zinc in mossy fibers of human and rat hippocampus has been estimated at 0.22–0.30 mM [1]. Zn^{2+} is actively taken up and released from the mossy fiber terminals during stimulation of nerve fiber tracts [2–4], but functional significance of these events is still not clear. We have recently observed that Zn^{2+}, at concentrations 0.2 mM and greater, induces tyrosine phosphorylation of p60$^{c\text{-}src}$ and 49-kDa protein associated with p60$^{c\text{-}src}$ in rat hippocampal membranes [5]. A product of cellular *Src* proto-oncogene, p60$^{c\text{-}src}$, is highly expressed in neural tissues [6], particularly in hippocampal pyramidal cells [7]. It is a membrane-associated protein tyrosine kinase with two principal sites of tyrosine phosphorylation: Tyr^{416} and Tyr^{527} [8,9]. Tyr^{416} is a site of p60$^{c\text{-}src}$ autophosphorylation in vitro [8]. Phosphorylation of Tyr^{416} is a characteristic of activated forms of p60$^{c\text{-}src}$ [10]. Carboxy-terminal Tyr^{527} is a major site of the in vivo p60$^{c\text{-}src}$ phosphorylation leading to inhibition of the enzyme activity [9]. Phosphorylation of p60$^{c\text{-}src}$ on Tyr^{527} is catalyzed by another protein tyrosine kinase(s), distinct from p60$^{c\text{-}src}$ [11,12]. In the present study, we characterized the type of p60$^{c\text{-}src}$ tyrosine phosphorylation induced by Zn^{2+} in hippocampal membranes.

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2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP (>5000 Ci/mmol) was purchased from Amersham. The anti-*Src* monoclonal antibody LAO was obtained from NCI Repository Microbiological Associates, Inc (Bethesda). Membrane fraction from rat hippocampi was prepared as described before [5].

2.2. *Immunoprecipitation and assay of p60$^{c\text{-}src}$ kinase activity*

Proteins of hippocampal membranes (300 μg) were extracted from the membranes with extraction buffer containing 50 mM Tris-HCl, pH 8.1, 0.5 M KCl, 2% Nonidet P40, 10% glycerol, 0.2 mM Na_3VO_4, 1 mM EDTA and 2 mM dithiothreitol. After incubation for 2 h at 4°C with occasional mixing, the suspension was spun for 10 min at 11,000 × *g*. The supernatant was incubated with 2 μl of monoclonal antibody LAO to p60^{src} for 90 min on ice. Then 6 μl of Protein A-Sepharose suspended in the extraction buffer (1:1) was added and the mixture was incubated for 30 min on ice with occasional mixing. Sepharose beads were then washed three times with the extraction buffer and once with 50 mM Tris-HCl, pH 7.4. Twenty microliters of reaction buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 0.1 mM Na_3VO_4, 10 μM [γ-^{32}P]ATP (50 Ci/mmol) and (or not) 0.5 mg/ml of yeast enolase (denatured with 0.1 M acetic acid for 5 min at 30°C) were added to the washed Sepharose beads and the mixture was incubated for 10 min at 30°C. The reaction was terminated by adding SDS-sample buffer and boiling. The samples were subjected to electrophoresis on 10% SDS-polyacrylamide gels followed by the alkali treatment of the gel [13], staining with Coomassie blue, drying and exposure for autoradiography. The individual labeled bands of phosphoproteins were excised from the gel and counted.

2.3. *Protein phosphorylation assay*

The standard reaction mixture (20 μl) contained 50 mM HEPES-NaOH, pH 6.9, the membrane fraction proteins (1–2 mg/ml), 0.1 mM [γ-^{32}P]ATP (1 Ci/mmol) and 0.5 mM $ZnCl_2$ or 2.0 mM $MnCl_2$, or both 0.5 mM $ZnCl_2$ and 2.0 mM $MnCl_2$. After incubation for 10 min at 30°C, the reaction was terminated by adding SDS-sample buffer and boiling. The samples were subjected to SDS-PAGE on 10% gels followed by the alkali treatment of the gel [13], staining, drying and exposure for autoradiography.

2.4. *Cyanogen bromide cleavage analysis*

After phosphorylation and electrophoresis dried gel bands containing phosphorylated $p60^{c\text{-}src}$ were excised and subjected to cyanogen bromide treatment by the method of Takeya et al. [14], loaded onto 18.75% SDS-polyacrylamide gel containing 6 M urea prepared as described by Kadenbach et al. [15] and analyzed by electrophoresis.

3. RESULTS AND DISCUSSION

To characterize the Zn^{2+}-induced tyrosine phosphorylation of $p60^{c\text{-}src}$ we compared activities of *Src* kinase from rat hippocampal membranes phosphorylated by ATP in the presence of Zn^{2+} and from the control membranes. The kinase activity was measured after immunoprecipitation with $p60^{src}$-specific monoclonal antibody. Autophosphorylation of $p60^{c\text{-}src}$ (Fig. 1, lanes 1, 3, 5 and 7) as well as phosphorylation of enolase, the conventional exogenous substrate of $p60^{c\text{-}src}$ (Fig. 1, lanes 2, 4, 6 and 8) have been studied. The activity of $p60^{c\text{-}src}$ from the membranes preincubated with $ZnCl_2$ and nonradioactive ATP (Fig. 1, lanes 3 and 4) was reduced. With enolase as the phosphorylation substrate (Fig. 1, lane 4) the activity was as low as 31 ± 8% (n = 3) of the activity of $p60^{c\text{-}src}$ from untreated membranes (Fig. 1, lane 2) or from the membranes prephosphorylated by MgATP (Fig. 1, lane 8) or preincubated in the presence of $ZnCl_2$ or ATP alone (data not shown). Reduction of the kinase activity in the autophosphorylation after prephosphorylation of $p60^{c\text{-}src}$ in the presence of Zn^{2+} was not so prominent (Fig. 1, lane 3 in comparison with lanes 1 and 7). These results are in line with the known fact of the more drastic influence

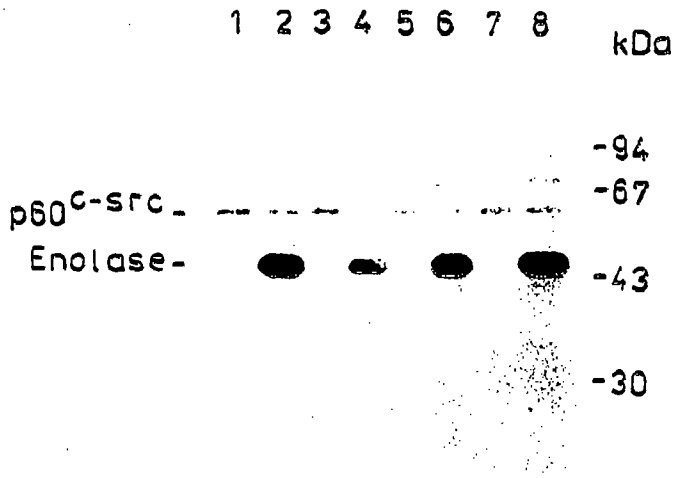

Fig. 1. Tyrosine kinase activity of the immunoprecipitated $p60^{c\text{-}src}$ in the autophosphorylation (lanes 1, 3, 5 and 7) and phosphorylated of enolase (lanes 2, 4, 6 and 8). Hippocampal membranes were preincubated in 50 mM HEPES-NaOH, pH 6.9, for 10 min at 30°C without ATP and bivalent cations (1, 2, 5 and 6) or with 0.1 mM ATP and 0.5 mM $ZnCl_2$ (3 and 4), or with 0.1 mM ATP and 1.0 mM $MgCl_2$ (7 and 8) before immunoprecipitation. Lanes 5 and 6 demonstrate the activity of $p60^{c\text{-}src}$ from hippocampal membranes isolated in the presence of 0.1 mM Na_3VO_4 (the inhibitor of protein tyrosine phosphatases).

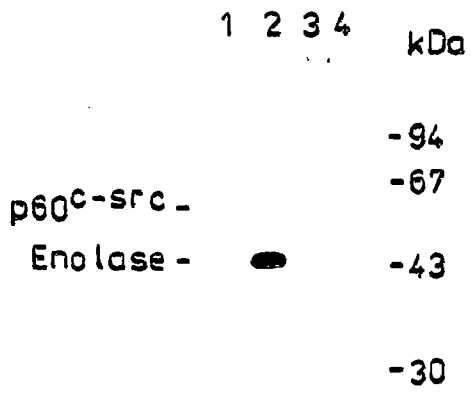

Fig. 2. Tyrosine kinase activity of the immunoprecipitated $p60^{c\text{-}src}$ in the autophosphorylation (lanes 1 and 3) and phosphorylation of enolase (lanes 2 and 4) in the presence of 10 mM $MgCl_2$, 3 mM $MnCl_2$ and 0.01 mM [γ-^{32}P]ATP (1 and 2) and in the presence of 0.5 mM $ZnCl_2$ and 0.1 mM [γ-^{32}P]ATP (3 and 4). Specific radioactivity of [γ-^{32}P]ATP in all experiments was equal to 50 Ci/mmol.

of inhibitory tyrosine phosphorylation of $p60^{c\text{-}src}$ on phosphorylation of exogenous substrates than on the autophosphorylation [16]. The activity of *Src* kinase of the membranes isolated from the hippocampi in the conditions preserving endogenous protein phosphotyrosyl (Fig. 1, lanes 5 and 6) was 62 ± 9% (n = 3) of that of the enzyme from the membranes isolated in the absence of a protein tyrosine phosphatase inhibitor (Fig. 1, lanes 1 and 2). These data demonstrate that Zn^{2+} induces the inhibitory phosphorylation of $p60^{c\text{-}src}$ and *Src* kinase is only partially inhibited in hippocampus.

Fig. 3. Cyanogen bromide cleavage analysis of $p60^{c\text{-}src}$ autophosphorylated after immunoprecipitation (lane 1) and phosphorylated in hippocampal membranes in the presence of Zn^{2+} (lane 2).

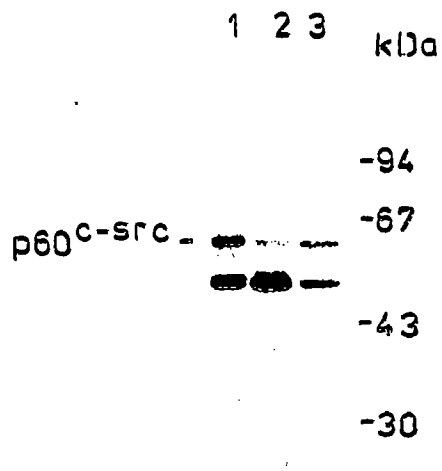

Fig. 4. Phosphorylation of $p60^{c\text{-}src}$ in hippocampal membranes in the presence of 0.5 mM $ZnCl_2$ (1), 2.0 mM $MnCl_2$ (2), or 0.5 mM $ZnCl_2$ and 2.0 mM $MnCl_2$ (3). The gel was treated with hot alkali [13] after electrophoresis to reduce the content of phosphoseryl and phosphothreonyl in the proteins.

Inhibitory tyrosine phosphorylation of $p60^{c\text{-}src}$ on carboxy-terminal tyrosyl is known to be catalyzed not by *Src* kinase itself but by another cellular protein tyrosine kinase(s) [11,12]. Immunoprecipitated $p60^{c\text{-}src}$ had no Zn^{2+}-induced kinase activity in the autophosphorylation (Fig. 2, lane 3) as well as in phosphorylation of enolase (Fig. 2, lane 4). Besides, there was no phosphorylation of 49-kDa protein (Fig. 2, lane 3), which was shown to be tyrosyl-phosphorylated in hippocampal membranes in the presence of Zn^{2+} and co-immunoprecipitated with $p60^{c\text{-}src}$ [5]. These results favored the suggestion that $p60^{c\text{-}src}$ and 49-kDa protein are phosphorylated in the hippocampal membranes not by $p60^{c\text{-}src}$, but by another protein tyrosine kinase, stimulated by Zn^{2+} and not co-immunoprecipitated with $p60^{c\text{-}src}$. Cyanogen bromide cleavage of the phosphorylated *Src* kinase provided an additional confirmation. The carboxy-terminal Tyr^{527} is contained within the 4-kDa peptide, but Tyr^{416} within the 10-kDa fragment after this treatment [11]. We observed that the cleavage of $p60^{c\text{-}src}$ phosphorylated by ZnATP in hippocampal membranes did produce 4-kDa phosphopeptide (Fig. 3, lane 2). Control cleavage of the autophosphorylated $p60^{c\text{-}src}$ produced a 10-kDa rather than 4-kDa phosphopeptide (Fig. 3, lane 1). Based on these observations, we concluded that Zn^{2+} in hippocampal membranes activates a protein tyrosine kinase which catalyzes inhibitory phosphorylation of $p60^{c\text{-}src}$ on the carboxy-terminal tyrosine residue.

Recently, a protein tyrosine kinase, named *CSK*, that specifically phosphorylates the negative regulatory site of $p60^{c\text{-}src}$ has been found and characterized in neonatal rat brain [17,18]. However the activity of *CSK* is maximal in the presence of Mn^{2+} and is inhibited by Zn^{2+} [17]. Phosphorylation of $p60^{c\text{-}src}$ in hippocampal membranes was lower in the presence of Mn^{2+} than in the presence of Mn^{2+} and Zn^{2+} and much lower than in the presence of Zn^{2+} alone (Fig. 4). Besides, *CSK* was reported to be a cytosolic protein [18]. So, the nature of the Zn^{2+}-activated protein tyrosine kinase catalyzing negative regulatory phosphorylation of *SRC* kinase in hippocampal membranes remains unclear. The existence of the Zn^{2+}-stimulated protein tyrosine kinase capable of regulating $p60^{c\text{-}src}$ kinase activity in the membranes of hippocampal neurons makes it likely that Zn^{2+} may participate in hippocampal neurotransmission causing changes in the protein tyrosine kinase activities.

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