Nerve growth factor stimulates tyrosine phosphorylation of paxillin in PC12h cells

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Abstract **Nerve growth factor (NGF) induces tyrosine phosphorylation of various cellular proteins to activate multiple signal transduction pathways. We show that one of these proteins is paxillin, a cytoskeletal component associated with adhesion plaques. Phospho-amino acid analysis showed that NGF stimulated phosphorylation of its serine in addition to tyrosine residues. Tyrosine phosphorylation of paxillin by NGF was blocked by the pretreatment of the cells with cytochalasin D, an inhibitor of actin polymerization. These results suggest that phosphorylation of paxillin is involved in the signaling pathway of NGF in PC12 cells.**

Key words: Nerve growth factor; PC12; Paxillin; Neuronal differentiation

1. Introduction

Nerve growth factor (NGF) is a neurotrophic factor required for the survival and differentiation of specific populations of neurons in the central and peripheral nervous systems. Its high affinity receptor has been identified as Trk A, a proto-oncogene product with intrinsic tyrosine kinase activity [1,2]. NGF stimulates its kinase activity and the phosphorylation level of its tyrosine residues, through which it triggers intracellular signal transduction pathways [3].

The PC12 cell line, which is derived from a pheochromocytoma, has been widely used as a model system to study the molecular basis of the action of NGF because it exhibits neuronal phenotypes in response to NGF [4]. Stimulation of the cells with NGF induces activation of the MAP kinase cascade [3], which was recently shown to be essential for NGF-induced differentiation of the cells [5]. In addition, a variety of signaling molecules, including *shc*, PLCγ and PI-3 kinase, are tyrosine-phosphorylated in response to NGF, resulting in the stimulation of other signal transduction pathways [3,6–8].

The Rous sarcoma virus carrying the *src* oncogene was shown to induce neuronal differentiation of PC12 cells [9], and that this effect was blocked by microinjection of neutralizing antibody against pp60$^{c\text{-}src}$ [10]. These findings suggest that pp60$^{c\text{-}src}$, a protein tyrosine kinase, is also involved in the signaling pathway of NGF. Its exact role in intracellular signaling is unknown, but there is increasing evidence that one of its targets may be actin binding sites in the cell membrane. A significant amount of pp60$^{v\text{-}src}$ can be recovered from *v-src*-transformed fibroblasts in a Triton X-100-insoluble fraction rich in cytoskeletal components [11,12]. Immunohistochemical studies have shown that pp60$^{c\text{-}src}$ is concentrated in intracellular membranes [13,14], cell-to-cell adherence junctions of hepatocytes [15], and the nerve growth cones of cultured retinal neurons [16]. Thus in cell-to-cell and cell-to-substrate adherence junctions, many proteins may be phosphorylated by pp60$^{c\text{-}src}$. In fact, in v-*src*-transformed cells and cells lacking CSK, which phosphorylate the negative regulatory sites of the src family of kinases, the level of tyrosine phoshorylation of proteins associated with adherence junctions such as cortactin and paxillin was found to be enhanced [17,18]. These findings indicate that the activated form of the src gene product is associated with adherence junctions and regulates their organization through phosphorylation of adherence junction-associated proteins.

The 68 kDa protein paxillin is a cytoskeletal component localized at points of focal contact of fibroblasts [18], and is thought to be involved in attachment of actin to them via an interaction with the carboxy-terminal rod of vinculin [19]. Tyrosine phosphorylation of paxillin was shown to be induced by expression of v-*src*, formation of focal contacts [20], and stimulation by growth factors and neuropeptides such as bombesin, vasopressin and platelet derived growth factor [21,22]. The tyrosine-phosphorylated form of paxillin could bind to the SH2-domains of v-*crk* and CSK [23,24]. Paxillin is also expressed at high levels in neuronal cells, but its function in the nervous system is still unknown. In this study, we examined whether paxillin is phosphorylated on stimulation by NGF.

2. Materials and methods

2.1. Materials

PC12 h cells were kindly provided by Professor Hatanaka of our institute. NGF was obtained from Chemicon International (USA). Anti-phosphotyrosine antibody (4G10) was from Upstate Biotechnology (USA). Anti-paxillin monoclonal antibody and horseradish peroxidase-conjugated rabbit anti-mouse IgG were from Zymet (USA). Protein G-Sepharose FF was from Pharmacia (Sweden). Anti-crk antibody was from Transduction Laboratories (USA). A Renaissance chemiluminescence Western blotting detection kit, [^{32}P]orthophosphate and [^{32}P]ATP were from NEN Research Products (USA).

2.2. Cell culture

PC12h cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% horse serum and 5% calf serum for 4 days, and then serum-free DMEM for 1 day.

2.3. Immunoprecipitation

PC12h cells grown in 100 mm dishes were stimulated with 50 ng/ml of NGF for 5 min unless otherwise indicated and then washed three times with washing buffer consisting of 250 mM sucrose and 10 mM Tris-HCl (pH 7.4). The cells were lysed in 500 μl of lysis buffer consisting of 10 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl,

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1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate and 10 μM sodium molybdate. The cells were incubated on ice for 30 min and centrifuged at 15,000 rpm for 10 min. The supernatant was incubated with anti-paxillin monoclonal antibody and protein G-Sepharose FF for 2 h and then the immune complex was washed 3 times with lysis buffer.

2.4. Western blotting

Protein samples were separated by 8% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked overnight with Tween-TBS (10 mM Tris-HCl, pH 7.4, 135 mM NaCl, mM KCl and 0.1% Tween 20), containing 1% gelatin and 0.02% sodium azide, and incubated for 2 h with anti-phosphotyrosine monoclonal antibody 4G10 diluted 1:10,000 or anti-paxillin monoclonal antibody diluted 1:5000. Then the membrane was incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG and immunoreactive bands were detected with a Renaissance chemiluminescence detection kit. The molecular weight markers used were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa).

2.5. Cell labeling and phospho-amino acid analysis

PC12h cells were incubated in serum- and phosphate-free DMEM containing 0.1 mCi/ml [^{32}P]orthophosphate for 16 h. They were then stimulated with NGF for 5 min, washed 3 times with ice-cold phosphate buffered saline and lysed in 0.5 ml of lysis buffer. Paxillin was immunoprecipitated from the lysate, separated by SDS-PAGE, and detected by autoradiography.

For phospho-amino acid analysis, ^{32}P-labeled paxillin was excised from dried gels and hydrolyzed in 6 N HCl. Then phospho-amino acids were separated by electrophoresis on a cellulose plate as described previously [25].

3. Results

To examine the effect of NGF on tyrosine phosphorylation of cellular proteins, PC12h cells were stimulated with NGF and tyrosine-phosphorylated proteins were detected by Western blotting with anti-phosphotyrosine antibody (Fig. 1A). The major proteins phosphorylated in response to NGF were 42, 44, 54, 70, 120–130 and 140 kDa. From previous studies, we concluded that the 42 kDa and 44 kDa substrates correspond to two isoforms of MAP kinase [3,26] and that the 54 kDa substrate corresponds to shc [7]. The 140 kDa protein seemed to be Trk A, because its band was detected when the cells were stimulated with NGF but not with other growth factors such as EGF (data not shown).

Paxillin is a 68 kDa protein which is highly tyrosine-phosphorylated in v-*src*-transformed cells. To examine whether it is tyrosine-phosphorylated in response to NGF, we immunoprecipitated it from PC12h cells and determined its level of tyrosine phosphorylation by Western blotting with anti-phosphotyrosine antibody. As shown in Fig. 1A, paxillin was highly tyrosine-phosphorylated after 5 min stimulation of the cells with NGF. The tyrosine-phosphorylated form of paxillin immunoprecipitated from NGF-stimulated cells migrated slower than that from control cells. The same samples as for Fig. 1A were also analyzed by Western blotting with anti-paxillin antibody (Fig. 1B). Results showed that the band of paxillin from NGF-stimulated cells shifted upward and was more diffuse than that of control cells.

The time-course of tyrosine phosphorylation of paxillin in PC12h cells on NGF stimulation for a period of 120 min is shown in Fig. 1C. Tyrosine phosphorylation increased for the first 15 min of NGF stimulation, and then decreased progressively. In the first 15 min, the electrophoretic mobility of paxillin decreased in parallel with its tyrosine phosphorylation.

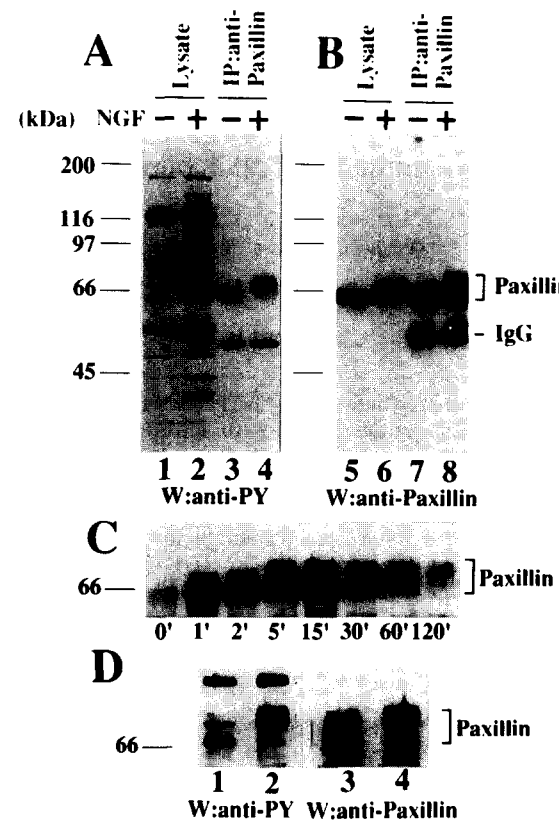

Fig. 1. Effect of NGF on tyrosine phosphorylation of paxillin. (A,B) PC12h cells were serum-starved for 16 h and stimulated with 50 ng/ml NGF for 5 min. Cell lysates were then prepared from control and stimulated cells and immunoprecipitated with anti-paxillin antibody. Lysates (lanes 1, 2, 5, 6) and immunoprecipitated materials (lanes 3, 4, 7, 8) were subjected to SDS-PAGE and phosphotyrosyl proteins were detected by Western blotting with anti-phosphotyrosine antibody (lanes 1–4) or anti-paxillin antibody (lanes 5–8). (C) PC12h cells were stimulated with NGF for the indicated periods. Paxillin was then immunoprecipitated and analyzed by Western blotting with anti-phosphotyrosine antibody. (D) PC12h cells were stimulated with NGF for 5 min. Lysates from control (lanes 1 and 3) and stimulated (lanes 2 and 4) cells were immunoprecipitated with anti-Crk antibody and analyzed by Western blotting with anti-phosphotyrosine (lanes 1 and 2) or anti-paxillin antibody (lanes 3 and 4).

After the first 30 min, the mobility stopped changing, but the level of tyrosine phosphorylation decreased, excluding the possibility that a shift in the paxillin band was due only to tyrosine phosphorylation.

Crk is a SH2-containing protein that can interact with several kinds of tyrosine-phosphorylated proteins. Immunoprecipitated materials with anti-Crk antibody contained some of these proteins (Fig. 1D, lanes 1 and 2). One of them was detected by Western blotting with anti-paxillin antibody, and the signal was enhanced and shifted upward by the stimulation with NGF (lanes 3 and 4).

To examine the phosphorylation state of paxillin in vivo, we immunoprecipitated it from PC12h cells metabolically labeled with [^{32}P]orthophosphate (Fig. 2A). The radioactivity of paxillin in NGF-stimulated cells was 4 times that in control cells, as judged by scanning densitometry (data not shown), confirming that NGF induced phosphorylation of paxillin. To determine which amino acids were phosphorylated, we subjected the

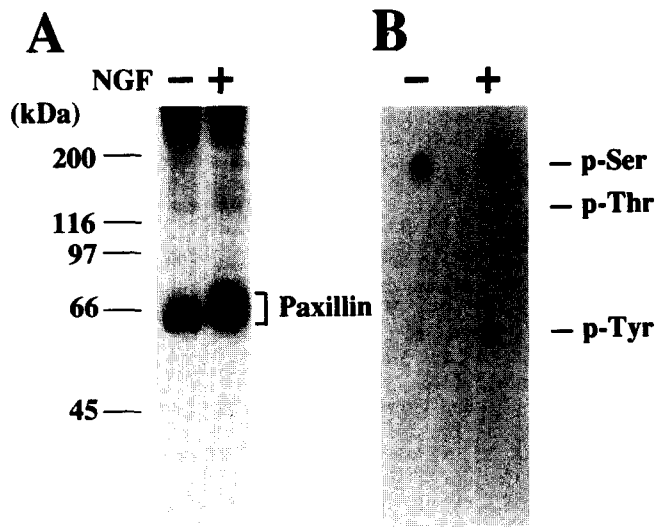

Fig. 2. NGF-stimulated ^{32}P incorporation into paxillin. PC12h cells were cultured in serum-free DMEM containing [^{32}P]orthophosphate for 16 h, then stimulated with 50 ng/ml NGF for 5 min. (A) Paxillin was immunoprecipitated with anti-paxillin antibody and detected by SDS-PAGE, followed by autoradiography. (B) ^{32}P-Labeled paxillin was recovered from the dried gel, hydrolyzed in 6 N HCl at 110°C for 1.5 h and separated by electrophoresis on a cellulose plate. Phospho-amino acids were detected by autoradiography.

labeled paxillin to phospho-amino acid analysis (Fig. 2B). Results showed that phosphorylation occurred mostly at the serine residues in resting cells, but at both serine and tyrosine residues in stimulated cells.

Cytochalasin D is known to disrupt actin filaments by binding to their barbed ends and to inhibit the polymerization of actin monomers to these ends [27]. Accordingly, we examined the effect of cytochalasin D on NGF-induced tyrosine phosphorylation to determine whether there is any relationship between cytoskeletal organization and tyrosine phosphorylation (Fig. 3). Cytochalasin D did not affect the NGF-induced tyrosine phosphorylations of MAP kinases and *shc*, but blocked the NGF-induced tyrosine phosphorylation of paxillin. Cytochalasin D stimulated the basal levels of tyrosine phosphorylation of several proteins such as the 66 kDa and 140 kDa proteins, but these proteins have not yet been identified.

4. Discussion

Tyrosine phosphorylation of cellular proteins is thought to be essential for neuronal differentiation induced by NGF. In the present study, we found that NGF stimulated the tyrosine phosphorylation of proteins of 42, 44, 54, 70, 120–130 and 140 kDa proteins. The 68 kDa component was shown to be paxillin by immunoprecipitation with anti-paxillin antibody. Paxillin is a component of adherence junctions by which actin filaments are anchored to plasma membranes. Thus the present results indicate that tyrosine phosphorylation of paxillin may be involved in the effect of NGF on cytoskeletal organization.

Paxillin was first identified as a major tyrosine-phosphorylated protein in RSV-transformed cells in which v-*src* is highly expressed [18,19]. Later paxillin was shown to bind not only to the SH3 domain of $pp60^{c\text{-}src}$ [28], but also to the SH2 domains of Crk and Csk [23,24]. The present results indicate that association of paxillin and Crk might occur on NGF stimulation. It was shown by Hempstead et al. [28] that paxillin was tyrosine-phosphorylated when PC12 cells were stimulated with NGF for 2 weeks, but they did not detect a change in its tyrosine phosphorylation after short-term stimulation. On the other hand, the present results showed that paxillin was tyrosine-phosphorylated within 2 min of NGF stimulation. The discrepancy may have arisen because they did not use the immunoprecipitation technique to examine the short-term effect of NGF on paxillin

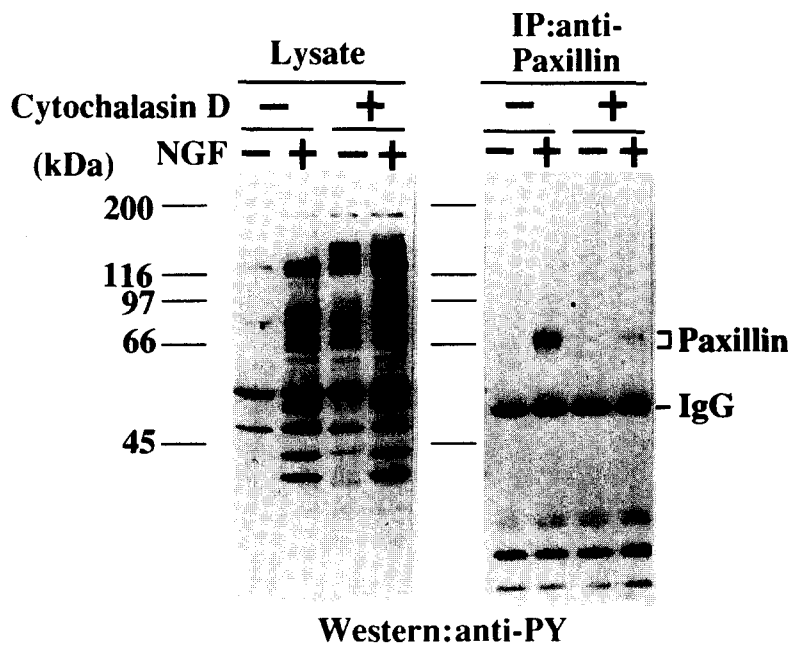

Fig. 3. Effect of cytochalasin D on tyrosine phosphorylation of paxillin. PC12h cells were serum-starved for 16 h, then further incubated in the presence or absence of 1 μM cytochalasin D for 15 min. The cells were stimulated with 50 ng/ml NGF for 5 min. Lysates (left panel) or anti-paxillin antibody-precipitated materials (right panel) were analyzed by Western blotting with anti-phosphotyrosine antibody .

Tyrosine phosphorylation of paxillin is also enhanced in cells lacking Csk, a negative regulator of the src family of tyrosine kinases [17]. These findings suggest that the Src family of proteins are candidates as kinase of paxillin. PC12h cells contain at least three of the src family of kinases, $pp60^{c\text{-}src}$ $pp59^{fyn}$ and $pp62^{c\text{-}yes}$ [25]. However, we could not detect any change in the activities of these kinases in response to NGF (data not shown). Thus further studies are needed to determine which tyrosine kinases are responsible for the NGF-induced tyrosine phosphorylation of paxillin. In this connection, it is noteworthy that NGF stimulated phosphorylation of serine residues. Thus some serine kinase, in addition to tyrosine kinases, must be involved in NGF signaling to paxillin.

Tyrosine phosphorylation of paxillin was also stimulated by EGF, which did not cause differentiation, but induced proliferation of the cells (data not shown). Thus the actions of NGF and EGF are different, although both cause membrane ruffling (data not shown). Since paxillin is localized in regions of focal contact of fibroblasts, its tyrosine phosphorylation may be correlated with the reorganization of actin filaments in response to the two factors. This idea is supported by the finding that its tyrosine phosphorylation is inhibited by cytochalasin D, which binds to the plus ends of actin filaments, blocking polymerization of actin monomers to these ends [27]. It will be interesting to determine whether the distribution of paxillin changes in response to NGF in PC12h cells.

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