

Phosphorylation of paxillin by p38MAPK is involved in the neurite extension of PC-12 cells

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Cell adhesions play an important role in neurite extension. Paxillin, a focal adhesion adaptor protein involved in focal adhesion dynamics, has been demonstrated to be required for neurite outgrowth. However, the molecular mechanism by which paxillin regulates neurite outgrowth is unknown. Here, we show that paxillin is phosphorylated by p38MAPK in vitro and in nerve growth factor (NGF)-induced PC-12 cells. Ser 85 (Ser 83 for endogenous paxillin) is identified as one of major phosphorylation sites by phosphopeptide mapping and mass spectrometry. Moreover, expression of the Ser 85 → Ala

mutant of paxillin (pax_{S85A}) significantly inhibits NGF-induced neurite extension of PC-12 cells, whereas expression of wild-type (wt) paxillin does not influence neurite outgrowth. Further experiments indicate that cells expressing pax_{S85A} exhibit small, clustered focal adhesions which are not normally seen in cells expressing wt paxillin. Although wt paxillin and pax_{S85A} have the same ability to bind vinculin and focal adhesion kinase, wt paxillin more efficiently associates with Pyk2 than pax_{S85A}. Thus, phosphorylation of paxillin is involved in NGF-induced neurite extension of PC-12 cells, probably through regulating focal adhesion organization.

Introduction

Paxillin is a focal adhesion-associated adaptor protein involved in adhesion organization and cell migration (for review see Schaller, 2001). Its structure features five copies of a 13-amino acid sequence, called the LD motif in the NH₂-terminal half and four LIM domains in the COOH-terminal half (Turner and Miller, 1994; Salgia et al., 1995). A number of adhesion or signaling molecules, such as vinculin, integrin α 4 and 9, FAK, cell adhesion kinase β , integrin-linked kinase, PTP-PEST, paxillin-kinase linker, and its homologous Git1 and Git2 proteins, bind to paxillin largely through LD motif or LIM domain interactions (for review see Schaller, 2001).

The NH₂-terminal half of paxillin also contains a large number of Ser-Pro epitopes, which are potential substrates for proline-directed protein kinases (Pearson and Kemp, 1991; Seger and Krebs, 1995). Indeed, it has been demonstrated that c-jun NH₂ terminus kinase (JNK) phosphorylates paxillin at Ser178 in vitro and in vivo (Huang et al., 2003). Several protein kinases including Erk and p38MAPK, have been proposed to phosphorylate paxillin based on observations using chemical inhibitors (Vadlamudi et al., 1999; Ku and

Meier, 2000; Liu et al., 2002), but due to the potential lack of specificity of these inhibitors, the results remain to be confirmed more directly. Paxillin is also a potential substrate for cdk5, a proline-directed protein kinase that is enriched in neuronal tissues and regulates neurite outgrowth (Nikolic et al., 1996, 1998; Zukerberg et al., 2000), because paxillin contains a consensus sequence (S/T)PX(K/H/R) for cdk5.

Integrin-mediated adhesions are essential for the neurite outgrowth (Ivankovic-Dikic et al., 2000; Rhee et al., 2000; Vogelesang et al., 2001). Thus, it is important to understand how signaling pathways regulate cell adhesion dynamics during the process of neurite outgrowth. Paxillin is a focal adhesion-associated adaptor protein involved in the regulation of focal adhesion dynamics (Liu et al., 1999; Schaller 2001; Hagel et al., 2002; Huang et al., 2003). It has been demonstrated that paxillin plays a key role in neurite outgrowth (Ivankovic-Dikic et al., 2000). Moreover, expression of the v-crk oncogene protein, the binding partner for tyrosine phosphorylated paxillin, in PC12 cells promotes neurite outgrowth by both NGF and EGF-dependent pathways (Hempstead et al., 1994), but tyrosine phosphorylation of paxillin does not seem to be essential for the neurite outgrowth of PC-12 cells (Ivankovic-Dikic et al., 2000). Phosphorylation of Ser 178 on paxillin by JNK has been shown to play a key

The online version of this article contains supplemental material.

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Key words: neurons; nerve growth factor; focal adhesions; neurite outgrowth; mass spectrometry

Abbreviations used in this paper: 2-D, 2-dimensional; JNK, c-jun NH₂ terminus kinase; wt, wild-type.

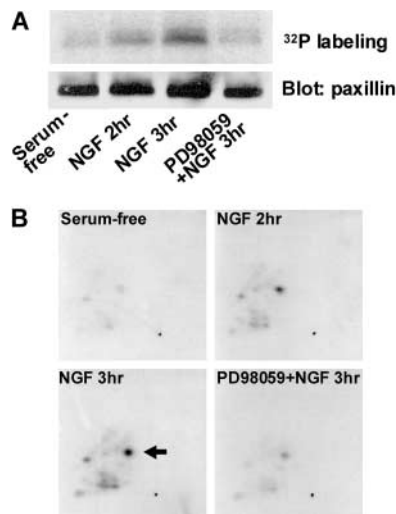


Figure 1. NGF-stimulated paxillin phosphorylation in PC-12 cells and PD98059 partially inhibited the phosphorylation. Cells were cultured in tissue culture dishes, labeled with $25 \mu\text{M}$ PD98059 as indicated for 10 min, and stimulated with 100 ng/ml NGF for times as indicated. Cells were then lysed and paxillin was immunoprecipitated from cell lysates with antipaxillin mAb. Paxillin phosphorylation was analyzed as described in Materials and methods. Total phosphorylation of paxillin (A) and the corresponding phosphopeptide mapping analysis (B) were shown. The origins are marked by dots. One major spot is pointed to by an arrow.

role in cell migration (Huang et al., 2003). It has been found that paxillin band is shifted to higher molecular weight in SDS-PAGE when PC-12 cells are stimulated with NGF (Rhee et al., 2000), suggesting that serine phosphorylation of paxillin also increases upon NGF treatment, but the signaling pathways involved and the physiological role are unknown. In this paper, we demonstrate that paxillin is phosphorylated at Ser 85 by p38MAPK and cdk5/p35 *in vitro*, and p38MAPK is the major kinase responsible for the phosphorylation of Ser 85 on paxillin in NGF-stimulated PC-12 cells. Furthermore, p38MAPK-mediated phosphorylation of Ser 85 on paxillin is involved in NGF-induced neurite outgrowth of PC-12 cells.

Results

NGF induces paxillin phosphorylation in PC-12 cells

To confirm NGF-stimulated paxillin phosphorylation in PC-12 cells, the cells were metabolically labeled with [^{32}P]orthophosphate and treated with NGF. The endogenous paxillin was immunoprecipitated with antipaxillin antibodies. The samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The phosphorylation of paxillin was detected by autoradiography. As shown in Fig. 1 A, phosphorylation of paxillin was significantly enhanced by NGF stimulation and was partially inhibited by PD98059, an Erk pathway inhibitor. The paxillin bands from Fig. 1 A were excised, digested with trypsin, and subjected to 2-dimensional (2-D) phosphopeptide mapping analysis. One major spot (Fig. 1 B, arrow) was observed on the 2-D maps of paxillin from NGF-treated cells, and stimulation with NGF caused a dramatic increase in the intensity

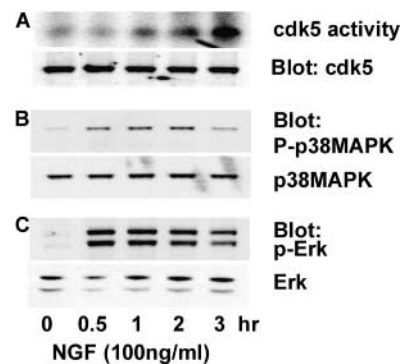


Figure 2. NGF-activated cdk5, p38MAPK, and erk/MAPK in PC-12 cells. (A) Cells were treated with NGF for different times as indicated and lysed. cdk5 was immunoprecipitated from cleared cell lysates and cdk5 activities were measured by *in vitro* kinase assays using $5 \mu\text{g}$ paxillin as substrates. (B and C) Cells were treated with NGF for different times as indicated and lysed in SDS sample buffer. The samples were applied to SDS-PAGE, transferred to nitrocellulose membrane, probed with (B) anti-phospho-p38MAPK or (C) anti-phospho-erk/MAPK antibodies to detect the activation of the kinases.

of the spot (Fig. 1 B). The increase was partially inhibited by PD98059, indicating that Erk pathway may be involved in the phosphorylation of paxillin in PC-12 cells.

cdk5, p38MAPK, and Erk are activated upon NGF stimulation

To dissect the signaling pathway involved in the NGF-stimulated phosphorylation of paxillin, PC-12 cells were challenged with NGF for different times and the activation of several candidate kinases were examined. To determine the activation of cdk5, the cells were lysed with a Triton lysis buffer and cdk5 was immunoprecipitated from cell lysates. The cdk5 activities in the immunoprecipitates were measured in an *in vitro* kinase assay using His-tagged paxillin as substrate. As shown in Fig. 2 A, cdk5 activity slowly increased with a similar time course as the NGF-induced paxillin phosphorylation. The activation of p38MAPK and Erk was examined by Western blot using phospho-specific antibodies. The phosphorylation of p38MAPK exhibited a sustained increase from 30 min after NGF stimulation and dropped after 3 h (Fig. 2 B), whereas the activation of Erk reached a peak as early as 30 min and slowly went down (Fig. 2 C). In a similar experiment, the activation of JNK was not detected upon NGF stimulation (unpublished data). Thus, cdk5, p38MAPK and Erk are the potential protein kinases responsible for the NGF-stimulated paxillin phosphorylation in PC-12 cells.

cdk5 and p38MAPK phosphorylates Ser 85 on paxillin *in vitro*

To learn which protein kinase may be responsible for the NGF-induced paxillin phosphorylation, three kinases were tested for their ability to phosphorylate paxillin *in vitro*. Therefore, recombinant paxillin β was phosphorylated with cdk5/p35, p38MAPK, and erk/MAPK *in vitro* in the presence of γ -[^{32}P]ATP, and the phosphorylated paxillin was digested with trypsin and analyzed by 2-D phosphopeptide mapping. As shown in Fig. 3 A, two major spots (a and b) were observed on the 2-D map of cdk5-phosphorylated

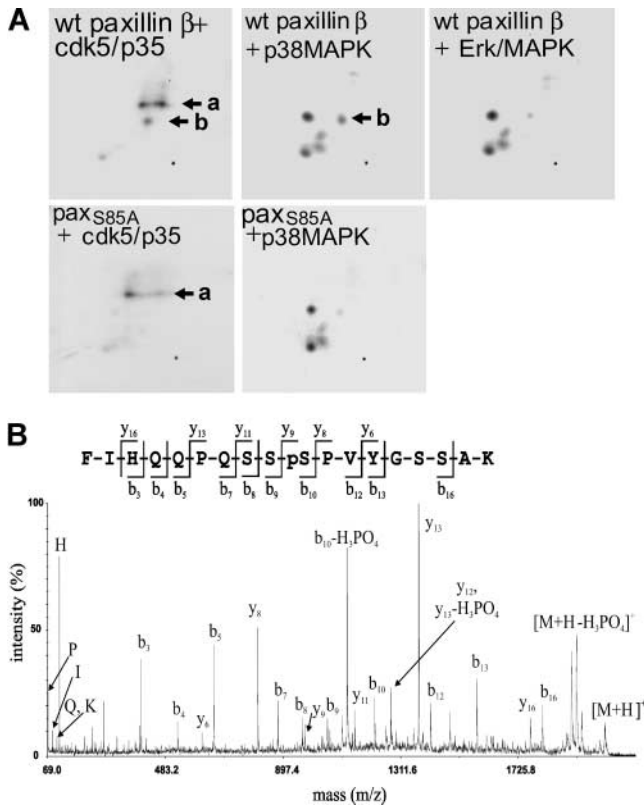


Figure 3. cdk5 and p38MAPK directly phosphorylate Ser 85 on paxillin in vitro. (A) His-tagged paxillin β or pax_{S85A} were incubated, respectively, with active cdk5/p35, p38MAPK, and erk/MAPK in 50 μ l of kinase buffer containing 40 μ M γ -[³²P]ATP (10 μ Ci) for 30 min. The phosphorylation of paxillin β and pax_{S85A} (not depicted) and 2-D phosphopeptide mapping were analyzed. The origins are marked by dots. Two major spots are pointed to by arrows a and b, respectively. (B) MS/MS analysis of a phosphopeptide recovered from spot b.

paxillin β . Substitution of Ser 244 (the consensus site for cdk5) with alanine resulted in the disappearance of spot a (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200307081/DC1>), indicating that Ser 244 is a phosphorylation site for cdk5 in vitro. The polymorphism of spot a is probably caused by the high molecular mass of the phosphopeptide (4249.59 D). Spot b is more interesting because it migrates to the same position as the major spot of in vivo phosphorylation (see Fig. 4 B). A similar spot also existed in the 2-D map of p38MAPK-phosphorylated paxillin, whereas a very weak spot was observed in the same position of the map of Erk-phosphorylated paxillin (Fig. 3 A).

To identify the phosphorylation site corresponding to spot b, the phosphopeptide at the spot was recovered from the cellulose plate and subjected to mass spectrometry. The parent ion was observed at m/z 2028.1656, corresponding to paxillin peptide 76-FIHQQPQSSSPVYGSSAK-93 with one phosphoryl group. A fragment ion was also detected at m/z 1930.2837, which is equivalent to the m/z of the same peptide minus 98. The coexistence of this precursor ion and its minus 98 counterpart ion indicates that a phosphorylation site is located on peptide F-I-H-Q-Q-P-Q-S-S-P-S-P-V-Y-G-S-S-A-K. Further MS/MS analysis of the parent ion revealed the presence of a phosphoryl group at Ser 85 because the additional mass of the phosphoryl group occurs at the b₁₀ ion and the

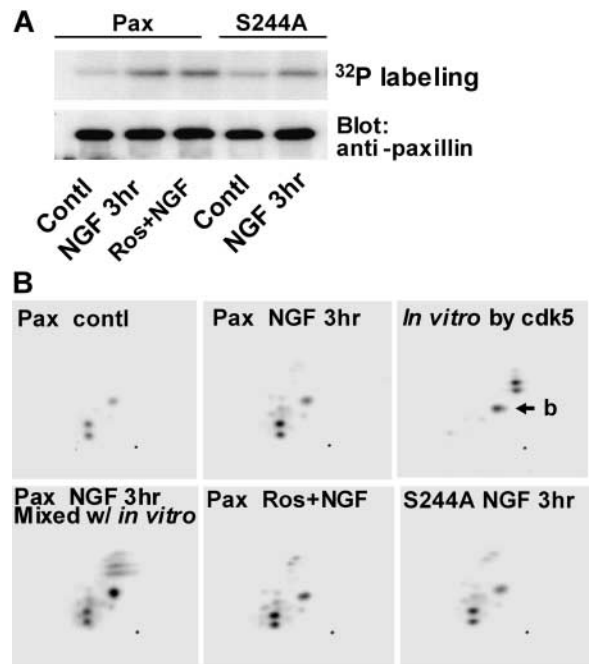


Figure 4. Paxillin was phosphorylated at the same site in vitro and in NGF-stimulated PC-12 cells, and the phosphorylation was not influenced by a cdk5 inhibitor, roscovitine. PC-12 cells stably expressing EGFP-paxillin β or -pax_{S244A} were cultured in collagen-coated dishes, labeled, treated with 100 μ M roscovitine (Ros) as indicated for 10 min, and stimulated with 100 ng/ml NGF for 3 h. Cells were then lysed and EGFP fusion proteins was immunoprecipitated from cell lysates with anti-GFP pAb. Total phosphorylation of EGFP-paxillin β or EGFP-pax_{S244A} (A) and the corresponding phosphopeptide mapping analysis (B) were analyzed as described in Materials and methods. A sample from in vitro phosphorylation and a mixed sample containing paxillin phosphopeptide from in vitro phosphorylation and NGF-stimulated PC-12 cells were also analyzed by 2-D phosphopeptide mapping. The origins are marked by dots.

y₉ ion and all subsequent b and y ions, but not at b₃-b₅, b₇-b₉, y₆, and y₈ ions that do not contain Ser 85 (Fig. 3 B). The observation of the loss of 98 of the b₁₀ ion (b₁₀-H₃PO₄) additionally confirms the phosphorylation of Ser 85 by cdk5 and p38MAPK in vitro. This conclusion was confirmed by site-directed mutagenesis. As shown in Fig. 3 A, the spot b is absent in the 2-D maps of paxillin phosphorylated by cdk5 and p38. Thus, Ser 85 of paxillin is one of the major residues targeted by cdk5 and p38MAPK in vitro.

Ser 85 is also phosphorylated in NGF-stimulated PC-12 cells

To learn whether Ser 85 on paxillin is also phosphorylated in NGF-stimulated PC-12 cells, the cells were transfected with EGFP-paxillin β and labeled with [³²P]orthophosphoric acid. EGFP-paxillin β was immunoprecipitated with an anti-GFP antibody. The samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The phosphorylation of EGFP-paxillin β was detected by autoradiography. As shown in Fig. 4 A, phosphorylation of paxillin β was significantly enhanced by NGF stimulation (Contl and NGF 3hr). The paxillin bands from Fig. 4 A were excised, digested with trypsin, and subjected to 2-D phosphopeptide mapping. The map of transfected EGFP-

paxillin is somewhat different from that of endogenous paxillin from PC-12 cells (Fig. 1 B), probably due to the species difference of paxillin. Three major spots were observed on the 2-D maps of EGFP-paxillin β , and treatment with NGF caused an increase in the intensity of the spots (Fig. 4 B). Furthermore, the spot b (Fig. 4 B, arrow) comigrated with the major spot from the 2-D map of *in vitro* phosphorylated paxillin β (Fig. 4 B). Therefore, paxillin is phosphorylated at the same site *in vitro* and in PC-12 cells.

NGF-induced phosphorylation of Ser 85 on paxillin is not inhibited by roscovitine and is only slightly impaired by purvalanol

Next, we asked whether paxillin is a substrate for cdk5 in NGF-stimulated PC-12 cells. The EGFP-paxillin β -transfected cells were labeled with [32 P]orthophosphoric acid, preincubated with 50 μ M roscovitine, a cdk5 inhibitor for 10 min, and then stimulated with NGF for 3 h. The phosphorylation of EGFP-paxillin β was examined as described in the previous paragraph. As shown in Fig. 4 A, the NGF-induced phosphorylation of paxillin β was not inhibited by roscovitine (NGF 3hr and Ros+NGF). Further 2-D phosphopeptide analysis confirmed that no major spot in the map is inhibited by roscovitine. Purvalanol, another cdk5 inhibitor, only slightly inhibited the phosphorylation of Ser 85 on paxillin in NGF-stimulated PC-12 cells (Fig. 5 B), whereas the total phosphorylation (Fig. 5 A, Purvalanol+NGF 3hr) and several other spots on the map (Fig. 5 B) were inhibited. Furthermore, substitution of Ser 244, the major cdk5-targeted site *in vitro*, with alanine had no any significant effect on either the total phosphorylation (Fig. 4 A, Contl and NGF 3hr under S244A) or the major spots on the phosphopeptide map (Fig. 4 B). Therefore, cdk5 is not the kinase responsible for the phosphorylation of Ser 85 on paxillin in NGF-stimulated cells.

Phosphorylation of Ser 85 is abolished by SB203580, a p38MAPK inhibitor

Next, we examined whether SB203580, a p38MAPK inhibitor, influenced the phosphorylation of Ser 85 on paxillin. As shown in Fig. 5 A, the phosphorylation of EGFP-paxillin β was significantly inhibited by SB203580 (Fig. 5 A), and 2-D phosphopeptide mapping analysis showed that the phosphorylation of Ser 85 was completely abolished and several other spots on the map were inhibited by SB203580 (Fig. 5 B). This result suggests that the phosphorylation of Ser 85 on paxillin is largely mediated by p38MAPK in NGF-stimulated PC-12 cells.

Endogenous paxillin is phosphorylated at Ser 83 in NGF-stimulated PC-12 cells

To identify the major p38 phosphorylation sites on rat paxillin, the protein was isolated from a rat bladder tumor cell line (NBT-II), and phosphorylated with p38MAPK *in vitro*. The phosphorylated paxillin was subjected to 2-D mapping analysis. As shown in Fig. 6 A, there was one major phosphorylation spot on the map. To identify the phosphorylation site, phosphopeptides were recovered from that spot and analyzed by mass spectrometry. The parent ion was detected at m/z 2054.9556, which is identical to the calculated m/z of pep-

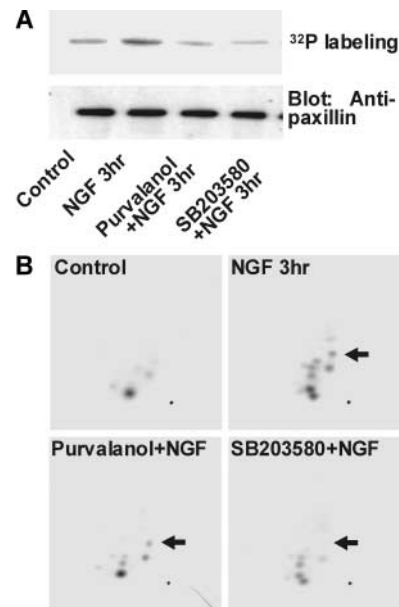


Figure 5. Phosphorylation of Ser 85 on paxillin was abolished by a p38MAPK inhibitor SB203580. PC-12 cells stably expressing EGFP-paxillin β was cultured in collagen-coated dishes, labeled, treated with 50 μ M purvalanol or 20 μ M SB203580 as indicated for 10 min, and stimulated with 100 ng/ml NGF for 3 h. Cells were then lysed and EGFP-paxillin β was immunoprecipitated from cell lysates with anti-GFP antibody. Total phosphorylation of EGFP-paxillin β (A) and the corresponding phosphopeptide mapping analysis (B) were analyzed as described in Materials and methods. The origins are marked by dots. The phosphorylation of Ser 85 is pointed to by arrows.

tide 76-YAHQQPPSPSPYSSSTK-93 with one phosphoryl group. This peptide is homologous to human paxillin peptide 76-FIHQQPQSSSPVYGSSAKA-93 (Fig. 6 C). A fragment ion was also detected at m/z 1956.9556, which is equivalent to the m/z of the same peptide minus 98, indicating that peptide YAHQQPPSPSPYSSSTK contains a phosphorylation site. The observation of the loss of 98 of the b_8 ion (b_8 -H $_3$ PO $_4$) indicates that a phosphorylation site is located on fragment 76-YAHQQPPS-83, whereas the additional mass of the phosphoryl group was not detected at the b_3 - b_7 ions (Fig. 6 B). Thus, Ser 83 on rat paxillin is phosphorylated by p38MAPK *in vitro*.

To learn whether endogenous paxillin is also phosphorylated at Ser 83 in NGF-induced PC-12 cells, endogenous paxillin was immunoprecipitated from [32 P]orthophosphoric acid-labeled, NGF-stimulated PC-12 cells and separated by SDS-PAGE. The paxillin band was isolated, digested, and subjected to 2-D peptide mapping analysis. The endogenous paxillin sample from PC-12 cells was also mixed with the *in vitro* paxillin sample and subjected to 2-D peptide mapping analysis. As shown in Fig. 6 A, a spot on the map of paxillin from PC-12 cells comigrated with a phosphopeptide from paxillin phosphorylated *in vitro*, indicating that endogenous paxillin was also phosphorylated at Ser 83 in NGF-stimulated PC-12 cells.

p38MAPK-mediated phosphorylation of paxillin is involved in NGF-induced neurite extension

To explore the role of p38MAPK in neurite extension, the effect of SB203580 on NGF-induced neurite extension in

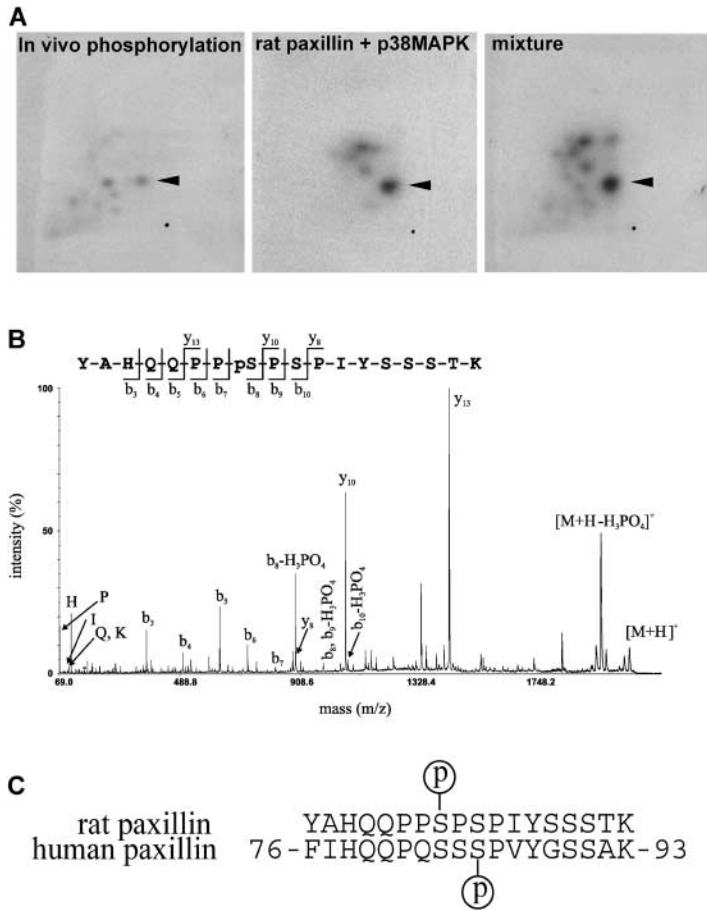


Figure 6. Phosphorylation of endogenous paxillin at Ser 83 in NGF-stimulated PC-12 cells. (A) Endogenous paxillin in PC-12 cells is phosphorylated at the same site where p38MAPK targeted paxillin in vitro. Rat bladder tumor cells (NBT-II) was lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin. Rat paxillin was pulled down by GST-FAT immobilized to glutathione beads (Thomas et al., 1999). Bound proteins were eluted from beads with glutathione and phosphorylated with p38MAPK. Paxillin was subjected to 2-D phosphopeptide mapping analysis (middle). The phosphorylation of endogenous paxillin from NGF-stimulated PC-12 cells was also analyzed (left). A mixed sample containing paxillin phosphopeptide from in vitro phosphorylation and NGF-stimulated PC-12 cells were also analyzed by 2-D phosphopeptide mapping (right). The spots that comigrated are pointed by arrowheads. The origins are marked by dots. (B) Rat paxillin is phosphorylated at Ser 83 by p38MAPK. MS/MS analysis of a phosphopeptide recovered from the major spot (arrow) in middle panel of A. (C) The major phosphopeptide from rat paxillin is homologous to the phosphopeptide from human paxillin.

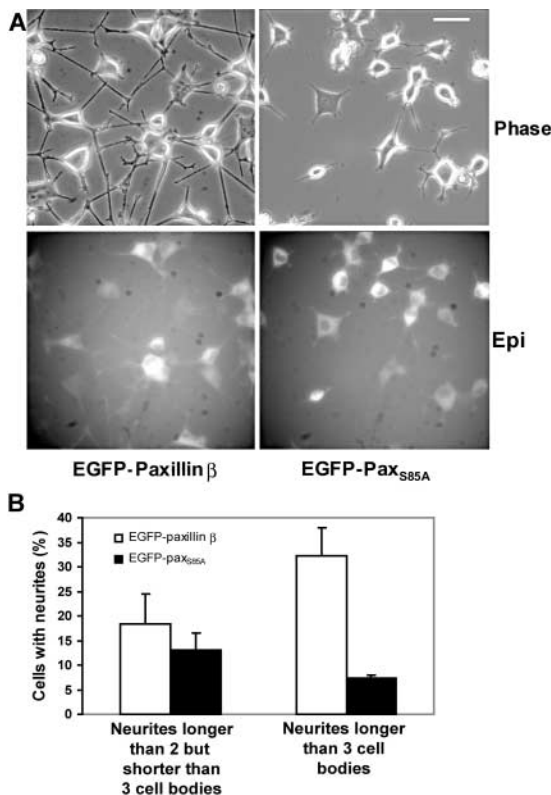


Figure 7. Expression of pax_{S85A} inhibited NGF-induced neurite extension in PC-12 cells. Cells stably expressing EGFP-paxillin β

PC-12 cells was examined. About 46% of control cells had neurites longer than two cell bodies and 28% of the cells had neurites longer than three cell bodies. Treatment with SB203580 inhibited neurite outgrowth. In the presence of 10 μ M SB203580 only 19% of cells extended neurites longer than two cell bodies and 7% extended neurites longer than three cell body lengths. Treatment with 20 μ M of SB203580 almost abolished the NGF-induced neurite extension of PC-12 cells (Fig. S2, A and B, available at <http://www.jcb.org/cgi/content/full/jcb.200307081/DC1>).

To assess the role of p38MAPK-mediated paxillin phosphorylation in neurite extension, PC-12 cells were transfected with EGFP-paxillin β or -pax_{S85A}, and the NGF-induced neurite extension of these cells was examined. As shown in Fig. 7, whereas cells expressing EGFP-pax_{S85A} exhibited ~20% of cells bearing neurites longer than two cell bodies and 7% of cells producing neurites longer than three cell bodies, >50% of cells expressing wild-type (wt) EGFP-paxillin β had neurites longer than two cell bodies and ~32% of cells had neurites longer than three cell bodies. Ex-

or -pax_{S85A} were plated on collagen-coated petri dishes and treated with 100 ng/ml NGF as described in Materials and methods. The neurite outgrowth was recorded after 96 h of NGF stimulation. (A) Typical inhibition of NGF-induced neurite extension by expression of pax_{S85A}. Bar, 40 μ m. (B) Quantitative analysis of neurite outgrowth from >300 cells. The data was plotted as the mean \pm SD from three experiments.

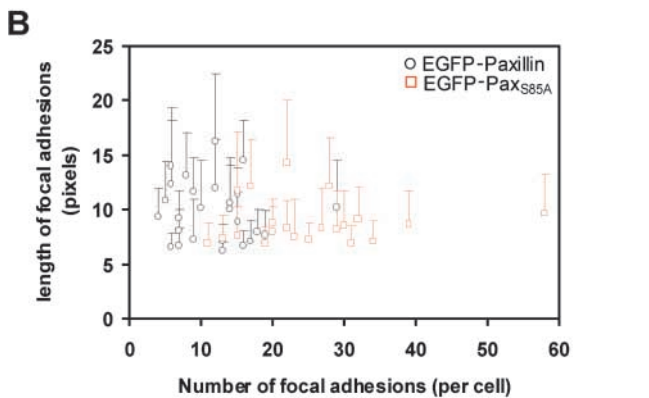
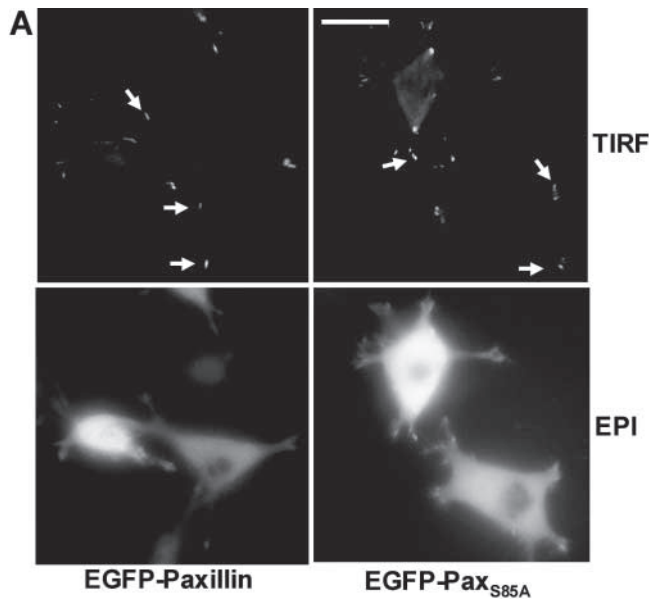


Figure 8. Expression of pax_{S85A} enhanced focal adhesion formation in NGF-treated PC-12 cells. Cells stably expressing EGFP-paxillin β or EGFP-pax_{S85A} were plated on collagen-coated MatTek dishes and treated with 100 ng/ml NGF for 36 h. TIRF and epifluorescence images were taken as described in Materials and methods. (A) Expression of pax_{S85A} induced clustered focal adhesions. Arrows point to focal adhesions. Bar, 20 μ m. (B) Quantitative analysis of focal adhesions in PC-12 cells expressing EGFP-paxillin β or EGFP-pax_{S85A}. The data was analyzed using Metamorph software and focal contacts that were longer than five pixels (1 μ m) were scored.

pression of wt EGFP-paxillin had no effect on neurite extension of PC-12 cells, which is consistent with a recent report by Ivankovic-Dikic et al. (2000). Therefore, phosphorylation of paxillin is involved in NGF-induced neurite extension.

p38MAPK-mediated phosphorylation of paxillin is involved in the regulation of focal adhesions

To understand the role of phosphorylation of paxillin at Ser 85 in focal adhesion organization, the focal adhesions in PC-12 cells expressing EGFP-paxillin β or EGFP-pax_{S85A} were examined with TIRF and epifluorescence microscopy. EGFP-paxillin β localized to focal adhesions at the tips of cell processes (arrows), whereas EGFP-pax_{S85A} was more clustered in focal adhesions at the tips (arrows) and there appeared to be an increase in the number of focal adhesions (Fig. 8, A and B; Fig. 9). Staining these cells with Alexa 568-labeled antivinculin

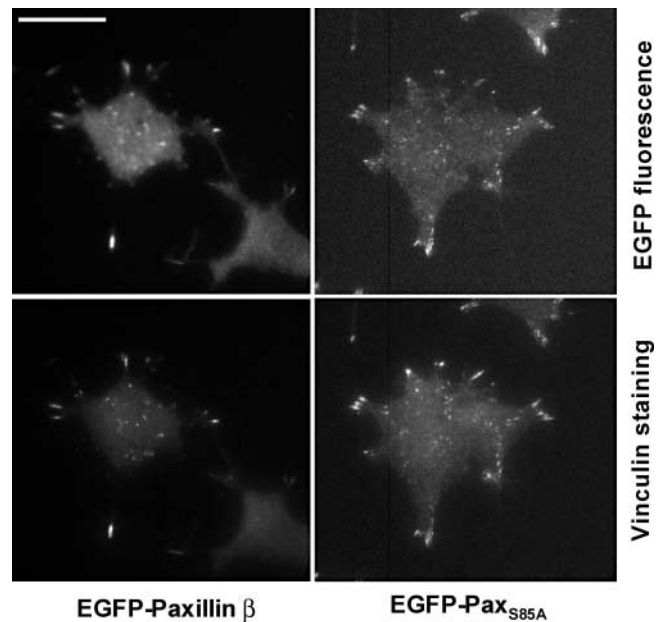


Figure 9. Vinculin was colocalized with paxillin at the tips of neurites. Cells stably expressing EGFP-paxillin β or EGFP-pax_{S85A} were plated on collagen-coated MatTek dishes and treated with 100 ng/ml NGF for 48 h. The cells were fixed and stained with Alexa 568-labeled antivinculin antibodies. TIRF images of EGFP-paxillin β or EGFP-pax_{S85A} (top) and vinculin (bottom) were taken as described in Materials and methods. Bar, 20 μ m.

antibodies gave a similar result and showed that vinculin colocalized with paxillin at the tips of cell processes (Fig. 9). These results also demonstrate that changes in EGFP-paxillin fluorescence reflect changes in focal adhesion structure rather than simply changes in paxillin localization. Expression of a p38MAPK dominant negative mutant (p38 α AF) also induced the formation of more clustered focal adhesions, whereas expression of MKK3bE, a constitutively active MAPK kinase for p38MAPK, induces focal adhesion disassembly in PC-12 cells (Fig. S3, arrows, available at <http://www.jcb.org/cgi/content/full/jcb.200307081/DC1>). Thus, phosphorylation of paxillin by p38MAPK plays an important role in focal adhesion organization in NGF-stimulated PC-12 cells.

p38MAPK-mediated phosphorylation of paxillin influences the binding of Pyk2 to paxillin

To explore the possible mechanism by which paxillin phosphorylation regulates neurite extension and cell adhesions, tyrosine phosphorylation of EGFP-paxillin β , EGFP-pax_{S85A}, FAK, and Src, in NGF-treated PC-12 cells expressing EGFP-paxillin β or EGFP-pax_{S85A} were examined using antiphosphotyrosine or phospho-specific antibodies. As shown in Fig. 10 (A and B), the tyrosine phosphorylation of these molecules was not changed by expressing EGFP-pax_{S85A}, suggesting that phosphorylation of paxillin by p38MAPK is not involved in regulating the tyrosine phosphorylation of these proteins.

Because paxillin is a focal adhesion adaptor interacting with a number of signaling and adhesion proteins, such as vinculin, FAK, and Pyk2, we tested the interaction of EGFP-paxillin and EGFP-pax_{S85A} with these molecules in NGF-induced cells, respectively. Although EGFP-paxillin and

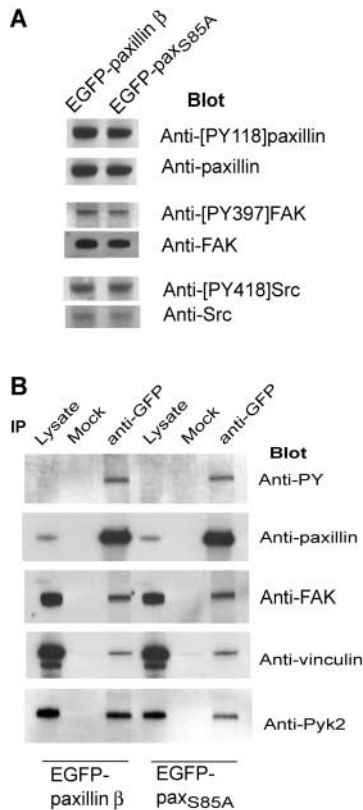


Figure 10. p38MAPK-mediated phosphorylation of paxillin influences the binding of Pyk2 to paxillin. PC-12 cells expressing EGFP-paxillin β or EGFP-pax_{S85A} were induced with 100 ng/ml NGF for 2 d. Cells were lysed and lysates were directly mixed with SDS sample buffer. Samples were analyzed by SDS-PAGE and transferred to nitrocellulose membrane for probing tyrosine phosphorylation of paxillin, FAK, and Src with phospho-specific antibodies (A). Alternatively, the lysates were immunoprecipitated with anti-GFP antibodies. The immune complexes were solubilized in SDS sample buffer. Samples were analyzed for tyrosine phosphorylation of paxillin and paxillin binding of vinculin, FAK, and Pyk2 (B).

EGFP-pax_{S85A} had the same ability to bind vinculin and FAK, EGFP-paxillin more efficiently associated with Pyk2 than EGFP-pax_{S85A} (Fig. 10 B). Because phosphorylation of paxillin by p38MAPK in vitro had no significant effect on its binding to Pyk2, it is unclear how phosphorylation of paxillin influences the interaction between paxillin and Pyk2. Nevertheless, because Pyk2 is involved in regulating neurite extension and focal adhesions (Ivankovic-Dikic et al., 2000; Park et al., 2000; Taniyama et al., 2003), the interaction of paxillin with Pyk2 may play a role in these processes.

Discussion

p38MAPK plays an important role in neurite extension

It has been demonstrated that sustained activation of Erk and the consequent stimulation of cdk5 are involved in neurite outgrowth in many systems (Qui and Green, 1992; Harada et al., 2001; Desbarats et al., 2003). However, it has been reported that inhibition of sustained phase of Erk activation does not block neurite outgrowth (York et al., 1998). Also, c-Src and bone morphogenetic protein 2 are unable to induce the activation of Erk but still induce neurite outgrowth

(Iwasaki et al., 1999; Yang et al., 2002), indicating that multiple signaling pathways are involved in neurite outgrowth.

Several lines of evidence indicate that p38MAPK is essential for neurite outgrowth. First, SB203580, an inhibitor of p38MAPK, blocks neurite outgrowth in several systems (Morooka and Nishida, 1998; Iwasaki et al., 1999; Hansen et al., 2000; Ishii et al., 2001). Second, a dominant negative mutant of p38MAPK also inhibits neurite outgrowth induced by NGF and forskolin (Morooka and Nishida, 1998; Hansen et al., 2000). Third, activation of p38MAPK by expressing constitutively active MKK3/MKK6, the kinases that phosphorylate and activate p38, induces neurite outgrowth in the absence of growth factor stimulation (Iwasaki et al., 1999).

Paxillin is a substrate for p38MAPK in vitro and in NGF-stimulated cells

It has been suggested that paxillin is a substrate for Erk in hepatocyte growth factor-stimulated epithelial and PMA-induced thymoma cells (Ku and Meier, 2000; Liu et al., 2002). However, it is unclear whether paxillin is a direct substrate for Erk in these cells. Here, we show that Erk2 phosphorylates paxillin in vitro at two major sites and that Ser 85 is a minor phosphorylation site for Erk in vitro (Fig. 3), whereas Ser 85 of exogenously expressed human paxillin (Ser 83 for the endogenous rat paxillin) is major site in NGF-stimulated PC-12 cells (Fig. 4). Also, treatment of PC-12 cells with PD98058 only partially inhibits NGF-induced phosphorylation of Ser 83 (Fig. 1). The partial inhibition could be due to the indirect inhibition of p38MAPK because it has reported that constitutive activation of MEK1, the upstream kinase of Erk and the target for PD98058, up-regulates p38MAPK activity in PC-12 cells (Morooka and Nishida, 1998). Thus, Erk may be upstream of paxillin, but may not be directly responsible for Ser 83 phosphorylation in NGF-stimulated PC-12 cells.

There is a consensus site (Ser 244) for cdk5 on paxillin. Also, NGF induces paxillin phosphorylation with a similar time course as cdk5 activation (Figs. 1 and 2). This had led us to suspect that paxillin could be a substrate for cdk5 in NGF-stimulated PC-12 cells. However, our experiments indicate that Ser 244, the consensus and the major phosphorylation site for cdk5 in vitro, is not phosphorylated in NGF-stimulated PC-12 cells (Fig. 4). Also, the phosphorylation of Ser 85 is not significantly inhibited by two cdk5 inhibitors (Figs. 4 and 5). Therefore, cdk5 is not involved in the phosphorylation of Ser 85 in PC-12 cells.

p38MAPK has been implicated in heregulin β 1-induced paxillin phosphorylation in breast cancer cells (Vadlamudi et al., 1999). Paxillin phosphorylation induced by heregulin β 1 was partially inhibited by a p38 inhibitor SB203580 and a dominant negative mutant of p38MAPK, but not by PD98058 (Vadlamudi et al., 1999). Here, we show that p38MAPK phosphorylates human paxillin at three major sites in vitro (Fig. 3) and rat paxillin at one major site (Fig. 6). Ser 85 was unambiguously identified as one of the major p38MAPK phosphorylation sites on human paxillin in vitro and a major NGF-induced phosphorylation site of exogenous paxillin in PC-12 cells (Figs. 3 and 4). Also, Ser 83 was identified as the major p38MAPK phosphorylation site on rat paxillin in vitro and a major phosphorylation site of en-

ogenous paxillin in PC-12 cells (Figs. 1 and 6). Because the sequence of rat paxillin around Ser 83 is homologous to that of human paxillin around Ser 85, and the Ser 83 and Ser 85 sites are so close (Fig. 6 C), suggesting that the two sites are likely comparable. Furthermore, the phosphorylation of Ser 85 is completely abolished by a p38MAPK inhibitor SB203580 in NGF-stimulated PC-12 cells (Fig. 5). Thus, p38MAPK is a kinase that directly phosphorylates paxillin in NGF-stimulated PC-12 cells.

Phosphorylation of paxillin is involved in neurite extension of PC-12 cells

The role of cell adhesions in neurite extension has been established previously (Ivankovic-Dikic et al., 2000; Rhee et al., 2000; Vogelesang et al., 2001). As an important component of adhesions, paxillin is also involved in neurite outgrowth (Ivankovic-Dikic et al., 2000). Although expression of wt paxillin does not influence the neurite outgrowth, expression of a LD4-deleted paxillin mutant significantly inhibits the neurite outgrowth of PC-12 cells (Ivankovic-Dikic et al., 2000). Expression of v-Crk, an adaptor protein that binds to tyrosine phosphorylated paxillin, promotes neurite formation (Hempstead et al., 1994). Surprisingly, expression of paxillin mutant (Y31F, Y118F, Y187F), which is deficient in tyrosine phosphorylation and unable to bind Crk, has no effect on neurite extension (Ivankovic-Dikic et al., 2000). This result indicates that tyrosine phosphorylation of paxillin is not essential for neurite outgrowth in PC-12 cells.

Recently, we demonstrated that phosphorylation of Ser 178 on paxillin by JNK regulates cell migration in several cell types (Huang et al., 2003). However, JNK is not activated and Ser178 is not phosphorylated in NGF-stimulated PC-12 cells (unpublished data). Instead, the activity of p38MAPK is up-regulated and the phosphorylation level of Ser 85 increases. Moreover, expression of a paxillin mutant pax_{S85A} significantly retards NGF-induced neurite extension (Fig. 7), indicating the involvement of phosphorylation of Ser 85 by p38MAPK in NGF-induced neurite extension. Thus, two types of different cell motility are regulated by two related pathways through phosphorylating their common substrate-paxillin at distinct sites.

Paxillin phosphorylation by p38MAPK is also involved in cell adhesion reorganization

It has been reported that heregulin β 1 induces p38MAPK activation and serine phosphorylation on paxillin, resulting in focal adhesion disassembly (Vadlamudi et al., 1999). In another report, collagen I stimulates the activities of p38MAPK accompanying a decrease in focal adhesion in endothelial cells, without affecting MAPK kinase, focal adhesion kinase, or phosphatidylinositol 3-kinase (Sweeney et al., 2003). It is known that focal adhesions undergo reorganization when PC-12 cells are induced to differentiate by NGF (Rhee et al., 2000). Here, we show that either a p38MAPK dominant negative mutant or a paxillin mutant (pax_{S85A}) cause clustered focal adhesions, whereas MKK3bE, a constitutively active MAPK kinase for p38MAPK, induces focal adhesion disassembly when they are expressed in NGF-treated PC-12 (Figs. 8 and 9; Fig. S3), suggesting that paxillin phosphorylation by p38MAPK is involved in focal adhesion organization.

How p38MAPK-mediated phosphorylation of paxillin modulates focal adhesions and neurite extension remains to be elucidated. The phosphorylation of paxillin may facilitate the interaction of paxillin with Pyk2, a nonreceptor protein tyrosine kinase that is involved in regulating focal adhesion dynamics and neurite extension (Ivankovic-Dikic et al., 2000; Park et al., 2000; Taniyama et al., 2003). Pyk2 may in turn phosphorylate the Arf-GTPase-activating protein ASAP1 and 3-phosphoinositide-dependent protein kinase 1, or activate Rho pathway (Kruljac-Leticic et al., 2003; Okigaki et al., 2003; Taniyama et al., 2003). All these signaling events regulate focal adhesion dynamics and may modulate neurite extension. However, other currently unknown mechanisms may also mediate the role of paxillin phosphorylation. For example, we found that a phosphorylated degradation product of paxillin (\sim 50 kD) was generated after NGF stimulation in PC-12 cells (unpublished data), implying that paxillin phosphorylation may be involved in its degradation. Further studies are required to understand the focal adhesion dynamics during NGF-induced differentiation of PC-12 cells and to fully elucidate the role of serine phosphorylation of paxillin in the regulation of focal adhesion structure.

Materials and methods

Reagents

Active cdk5/p35 was purchased from Novagen; active p42MAPK was purchased from New England Biolabs, Inc.; Trypsin (sequencing grade) was purchased from Promega; and Flag-p38 α , -p38 α AF, and -MKK3bE in pcDNA3.1 vector and His-tagged MKK6E were gifts from J. Han (Scripps Research Institute, La Jolla, CA). Human paxillin cDNA was a gift from H. Sabe (Osaka Bioscience Institute, Osaka, Japan). Zip-Tips were purchased from Millipore; Ni-NTA-agarose and PQE-30 vector were purchased from QIAGEN; Pfu and Quick-Change mutation kit were purchased from Stratagene; PD98059 and SB203580 were purchased from Calbiochem; roscovitine, purvalanol, rat tail collagen I, antivinulin mAb, and NGF were purchased from Sigma-Aldrich; γ -[³²P]ATP was purchased from NEN Life Science Products; [³²P]orthophosphate was purchased from ICN Biomedicals; Alexa Fluor 568 protein labeling kit was purchased from Molecular Probes; antipaxillin mAbs were purchased from Upstate Biotechnology; anti-GFP pAbs were purchased from CLONTECH Laboratories, Inc.; and rat phaeochromocytoma PC-12 cells were obtained from W. Snider's lab and cultured in RPMI 1640 supplemented with 10% horse serum, 5% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Plasmid construction

To generate His-tagged p38 α , DNA fragments encoding p38 α were amplified by pfu-based PCR using Flag-p38 α as template and 5'-TTACTTG-GATCCCAGGAGAGGCCACGTTTC-3' and 5'-TTTTTCTGCAGTCAG-GACTCCATTTCTTCTGGTC-3' as primers, and subcloned into PQE-30 vector via BamHI and PstI sites. To create His-tagged paxillin β , DNA fragments encoding paxillin β were amplified by pfu-based PCR using 5'-CGACGACCTCGACGCCCTG-3' and 5'-AAAAAAGTCGACCTAGCA-GAAGAGCTTGAGGAAGCA-3' as primers, and subcloned into pQE-30 vector through Klenow-blunt BamHI site and Sall. His-tagged pax_{S85A} was created by pfu-based PCR using Quick-Change mutation kit and primer pair 5'-CCTCAGTCCTCAGCACCTGTGTACGGC-3' and 5'-GCCGTACA-CAGGTGCTGAGGACTGAGG-3'. To construct EGFP-paxillin β and -pax_{S85A}, DNA fragments encoding paxillin β and pax_{S85A} were amplified respectively by pfu-based PCR using primer pair 5'-AAAAAAGTAATCA-GACGACCTCGACGCCCT-3' and 5'-AAAAAAGTCGACCTAGCAGAA-GAGCTTGAGGAAGCA-3', and subcloned into pEGFP-C1 vector through EcoRI site and Sall. The DNA fragments encoding EGFP-paxillin β and EGFP-pax_{S85A} were cut out by sequential treatment with Sall, Klenow, and AgeI and inserted into a retroviral MGIN vector (Li et al., 2000) through AgeI and Klenow-blunt NotI sites.

Protein purification

His-tagged proteins were purified by affinity chromatography using Ni-NTA-agarose. In brief, expression was induced with 1 mM IPTG for 3.5 h,

bacteria harvested, and sonicated in a lysis buffer (50 mM Tris-HCl, pH 8.1, 300 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 20 mM imidazole). The cleared lysate was applied to Ni-NTA-agarose column. His-tagged protein was eluted with 250 mM imidazole in 20 mM Tris-HCl, pH 8.1, 300 mM NaCl.

Preparation of viruses and cell infection

Retrovirus packaging cells (293GPG) were maintained in DME supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 µg/ml tetracycline, 2 µg/ml puromycin, and 0.3 mg/ml G418. The cells were transfected with MGIN plasmids using Lipofectamine Plus transfection reagent (Invitrogen) according to the manufacturer's protocol. To harvest viruses, the transfectants were grown in DME supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. The medium of transfectants was collected at 48 and 72 h, filtered through 0.2-µm filter and applied to overnight cultures of PC-12 cells for infection. Cells stably expressing recombinant proteins were obtained by growing infected PC-12 cells in the presence of 0.7 mg/ml G418 for 1 wk.

In vitro phosphorylation

5 µg His-tagged paxillin β was incubated with ~0.5 µg of kinase in 50 µl of the kinase buffer (20 mM MOPS, pH 7.2, 10 mM MgCl₂, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT) containing 40 µM ATP (10 µCi γ-[³²P]ATP) for 30 min at RT. The reactions were terminated by adding SDS-sample buffer. The samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane for autoradiography.

In vivo phosphorylation

Cells were incubated with [³²P]orthophosphoric acid in sodium phosphate-deficient MEM (Sigma-Aldrich). After 12 h, cells were harvested and lysed with a RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% IPEGAL, 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 500 nM okadaic acid, 1 mM α-naphthyl acid phosphate). The lysates were immunoprecipitated with antipaxillin or anti-GFP antibodies. The immune complexes were analyzed by SDS-PAGE and transferred to nitrocellulose membrane for detection of phosphorylation by autoradiography.

Peptide mapping

Peptide mapping was performed as described previously (Boyle et al., 1991; Huang et al., 2003). In brief, protein bands were cut from nitrocellulose membrane and digested with trypsin (sequencing grade; Promega) in NH₄HCO₃, pH 7.8, with shaking at 37°C for 8 h. The samples were dried and washed in a speed-vac, and spotted onto cellulose plate for 2-D phosphopeptide mapping, using pH 8.9 buffer for electrophoresis and phosphochromatography buffer for TLC.

Mass spectrometry

Phosphopeptide was recovered from cellulose plates, cleaned up with a Zip-Tip (Millipore) as described previously (Raska et al., 2002) and analyzed by a MALDI-TOF/TOF (Voyager 4700) instrument purchased from Applied Biosystems with α-cyano-4-hydroxy-cinnamic acid as matrix. The instrument was calibrated by external calibration using the ABI 4700 calibration mixture.

Neurite outgrowth

PC-12 cells were plated on 35-mm petri dishes precoated with 10 µg/ml collagen I and cultured in RPMI 1640 containing 10% horse serum, 5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin for overnight, and cultured in DME supplemented with 1% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin for additional 6 h. The cells were then challenged with 100 ng/ml NGF and cultured for 4 d. The neurite outgrowth was photographed using a microscope (model IX81; Olympus) equipped with a 40× objective and a CCD camera (model C4880; Hamamatsu). Scoring for neurite outgrowth of PC-12 cells was performed as described by Dikic et al. (1994).

EGFP fluorescence, immunofluorescence, and microscopy

PC-12 cells expressing EGFP-paxillin β or -pax_{S85A} were plated on MatTek dishes (with a glass coverslip at the bottom) precoated with collagen and treated with 100 ng/ml NGF for 48 h. EGFP epifluorescence and TIRF images were taken at 37°C using a microscope (model IX81; Olympus) equipped with a 60×, 1.45 NA objective, a CCD camera (model C4880; Hamamatsu), and a Metamorph software (Universal Imaging Corp.). For immunofluorescence staining, the cells were fixed with 3% PFA and stained for vinculin with antivinculin mAb pre-labeled with Alexa 568. Vin-

culin staining and EGFP fluorescence were observed and photographed at RT using the same system. The brightness of images were adjusted using Photoshop software.

Protein interaction and tyrosine phosphorylation

PC-12 cells expressing EGFP-paxillin β or EGFP-pax_{S85A} were plated on 100-mm petri dishes precoated with 10 µg/ml collagen I and cultured in normal RPMI 1640 culture medium overnight and cultured in DME supplemented with 1% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin for 12 h. The cells were then induced with 100 ng/ml NGF and cultured for 2 d. Cells were lysed with a buffer containing 50 mM Tris-HCl, pH 7.4, 1% IPEGAL, 100 mM NaCl, 5% glycerol, 5 mM EDTA, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 500 nM okadaic acid, 1 mM α-naphthyl acid phosphate, and 1 mM Na₂VO₄. The lysates were directly mixed with SDS sample buffer, or were immunoprecipitated with anti-GFP antibodies. Immune complexes were solubilized in SDS sample buffer. Samples were analyzed by SDS-PAGE and transferred to nitrocellulose membrane to detect tyrosine phosphorylation and various protein interactions.

Online supplemental material

Fig. S1 shows that cdk5 directly phosphorylated Ser 244 on paxillin in vitro. To demonstrate that Ser 244 on paxillin is a phosphorylation site for cdk5, wt His-tagged paxillin β or pax_{S244A} were phosphorylated, respectively, with active cdk5/p35 and subjected to 2-D phosphopeptide mapping analysis as described in Peptide mapping section. Fig. S2 demonstrates that NGF-induced neurite extension was inhibited by a p38MAPK inhibitor, SB203580. To test the effect of SB203580 on NGF-induced neurite extension, PC-12 cells were plated on collagen-coated petri dishes, treated with SB203580 as indicated for 10 min, and stimulated with 100 ng/ml NGF without removing the inhibitor. Neurite outgrowth was analyzed after 96 h of NGF stimulation. Fig. S3 suggests that the p38MAPK pathway is involved in regulating focal adhesion reorganization. To explore the role of the p38MAPK pathway in focal adhesion dynamics, PC-12 cells were transiently transfected with EGFP vector, Flag-p38αAF + EGFP vector, and Flag-MKK3bE + EGFP vector for 24 h, and plated on MatTek dishes precoated with 10 µg/ml collagen and treated with 100 ng/ml NGF for 36 h. The cells were fixed and stained with Alexa 568-labeled antivinculin antibodies. Vinculin staining and EGFP fluorescence were viewed by epifluorescence microscopy as described above (EGFP...and microscopy). Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200307081/DC1>.

We thank Dr. Z. Rajfur for his assistance in microscopic images, Dr. A. Markus for her help in PC-12 cell culture, Drs. P. Elms and R. M. Pope for their help in mass spectrometric analysis, Dr. J. Han for Flag-p38α, -p38αAF and -MKK3bE plasmids, and Dr. H. Sabe for paxillin β plasmid, respectively.

This work was supported by National Institutes of Health grant to K. Jacobson and M.D. Schaller; the Cell Migration Consortium to K. Jacobson; and National Institute for Dental and Cranial Research grant to K. Jacobson and M.D. Schaller.

Submitted: 14 July 2003

Accepted: 13 January 2004

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