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 (paxS85A) 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 paxS85A exhibit small, clustered focal adhesions which are not normally seen in cells expressing wt paxillin. Although wt paxillin and paxS85A have the same ability to bind vinculin and focal adhesion kinase, wt paxillin more efficiently associates with Pyk2 than paxS85A. 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; Vogelezang 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 role in neurite outgrowth (Hempstead et al., 1994), but due to the potential lack of specificity of these inhibitors, the results remain to be confirmed more directly.

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.
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 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 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. As shown in Fig. 2 B and C, the activation of p38MAPK and 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 1. NGF-stimulated paxillin phosphorylation in PC-12 cells and PD98059 partially inhibited the phosphorylation. Cells were cultured in tissue culture dishes, labeled, treated with 25 μM 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.

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 μg 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.
paxillin. 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-FIHQQPQSSPVYGSSAK-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 FIHQQPQSSPVYGSSAK.

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 b<sub>10</sub> ion and all subsequent b and y ions, but not at b<sub>1</sub>-b<sub>9</sub>, y<sub>6</sub> and y<sub>8</sub> ions that do not contain Ser 85 (Fig. 3 A). The observation of the loss of 98 of the b<sub>10</sub> ion (b<sub>10</sub>-H<sub>3</sub>P<sub>4</sub>) 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-EGFP 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 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 analysis. 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 [32P]orthophosphoric acid, preincubated with 50 μM 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 3 hr 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 3 hr) 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 3 hr 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 peptide 76-YAHQQPPSPSPIYSSSTK-93 with one phosphorylation group. This peptide is homologous to human paxillin peptide 76-FIHQQPQSSIPYYGSSAKA-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 YAHQQPPSPIYSSSTK contains a phosphorylation site. The observation of the loss of 98 of the b3 ion (b3-H3PO4) 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 b3 ion (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 [32P]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...
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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 μM 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 μM 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 -paxS85A, and the NGF-induced neurite extension of these cells was examined. As shown in Fig. 7, whereas cells expressing EGFP-paxS85A 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-
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-paxS85A were examined with TIRF and epifluorescence microscopy. EGFP-paxillin β localized to focal adhesions at the tips of cell processes (arrows), whereas EGFP-paxS85A 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 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 (p38AF) 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-paxS85A, FAK, and Src, in NGF-treated PC-12 cells expressing EGFP-paxillin β or EGFP-paxS85A 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-paxS85A, 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-paxS85A with these molecules in NGF-induced cells, respectively. Although EGFP-paxillin and
paxillin with Pyk2 may play a role in these processes. Park et al., 2000; Taniyama et al., 2003), the interaction of extension and focal adhesions (Ivankovic-Dikic et al., 2000; Nevertheless, because Pyk2 is involved in regulating neurite lin influences the interaction between paxillin and Pyk2. paxillin by p38MAPK in vitro had no significant effect on its phosphorylation of Ser 85 in PC-12 cells. Also, the phosphorylation of Ser 85 is not significantly inhibited by two cdk5 inhibitors (Morooka and Nishida, 1998; Ishii et al., 2001). Thus, Erk may be upstream of paxillin, but may not be directly responsible for Ser 85 phosphorylation in NGF-stimulated PC-12 cells. There is a consensus site (Ser 244) for cdk5 on paxillin. 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 phosphorylates human paxillin at three major phosphorylation sites on human paxillin in vitro (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 PD98059 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 PD98059, 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 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). Ser 83 was identified as the major p38MAPK phosphorylation site on rat paxillin in vitro and a major phosphorylation site of en-
dogenous 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; Vogezeang 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 paxS85A 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 β1 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 (paxS85A) 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 (Kruiljac-Letunic 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 (~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, -p38AF, and -MKK3bE in pDNA3.1 vector and His-tagged MKK6Eb 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; Ptu and Quick-Change mutation kit were purchased from Stratagene; PD98059 and SB203580 were purchased from Calbiochem; roscovitine, purvalanol, rat tail collagen I, antivinculin mAb, and NGF were purchased from Sigma-Aldrich; γ-(32)P]ATP was purchased from NEN Life Science Products; 10 PM p38 MAPK kinase 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. Svider’s lab and cultured in RPMI 1640 supplemented with 10% horse serum, 5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

**Plasmid construction**

To generate His-tagged p38α, DNA fragments encoding p38α were amplified by pfu-based PCR using Flag-p38α as template and 5’-TACTTGG-GATCCCAAGGAGGCCAGTCCG-3’ and 5’-TTTTTCTGACGCTAGCGAGTTTCTTCTTGTC-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, DNA fragments encoding paxillin β were amplified by pfu-based PCR using Flag-p38α as template and 5’-CAGCAAGCTTCAAGCCCTG-3’ and 5’-AAAAATGCCTAGCTACGAGAGCTTGAGAAGCA-3’ as primers, and subcloned into pQE-30 vector through Klenow-blunt BamHI site and SalI site. His-tagged paxS85A was created by pfu-based PCR using Quick-Change mutation kit and primer pair 5’-CCTAGTCCTAGGCCCTG-3’ and 5’-CGCCGTCGACGACCTAGCGAGTAGG-3’. To construct EGFP-paxillin β and -paxS85A, DNA fragments encoding paxillin β and paxS85A were amplified respectively by pfu-based PCR using primer pair 5’-AAAAAAGATCAGCGGCACTGCTAGCTACGAGAGCTTGAGAAGCA-3’ and 5’-AAAAGATCCCAAGGAGGCCAGTCCG-3’. EGFP-paxS85A was cut out by sequential treatment with SalI, Klenow, and Agel and inserted into a retroviral MGIN vector (Li et al., 2000) through Agel and Klenow-blunt Ncol sites.

**Protein purification**

His-tagged proteins were purified by affinity chromatography using Ni-NNTA-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 (293GFP) were maintained in DMEM supplemented with 10% FBS, 100 µM 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 DMEM supplemented with 10% FBS, 100 µM penicillin, and 100 µg/ml streptomycin. The medium of transfectants was collected at 48 and 72 h, filtered though 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/11.6 µCi/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-labeled phosphoric 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% IGE-GAL, 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 mM okadaic acid, 1 mM α-naphthyl acid phosphate). The lysates were immunoprecipitated with nonphospho 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 DMEM 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 2 d. Cells were lysed with a buffer containing 50 mM Tris-HCl, pH 7.4, 1% IGE-GAL, 100 mM NaCl, 5% glycerol, 5 mM EDTA, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 500 mM okadaic acid, 1 mM α-naphthyl acid phosphate, and 1 mM NaVO3. 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-p38AαE + EGFP vector, and Flag-MKK3βE + 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 S68-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.

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