Specific Activation of a c-Jun NH$_2$-terminal Kinase Isoform and Induction of Neurite Outgrowth in PC-12 Cells by Staurosporine* 

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Staurosporine, a protein kinase inhibitor, is known to mimic the effect of nerve growth factor (NGF) in promoting neurite outgrowth. To elucidate the mechanism by which staurosporine induces neurite outgrowth in PC-12 cells, we performed an in-gel kinase assay using myelin basic protein as a substrate, and found that staurosporine induced the activation of a kinase with an apparent molecular mass of 57 kDa. The dose of staurosporine required to activate this kinase was consistent with that required to induce neurite outgrowth. Interestingly, the staurosporine-activated kinase was immunoprecipitated by anti-c-Jun NH$_2$-terminal kinase (JNK) isoforms antibody, but not by anti-JNK1-specific antibody or anti-ERK1 antibody, raising the possibility that this kinase is a novel JNK isoform. The substrate specificity of the kinase was distinct from those of osmotic shock-activated JNKs and NGF-activated ERK1. The kinase phosphorylates transcription factors including c-Jun, Elk-1, and ATF2, as well as myelin basic protein, suggesting that it plays a role in gene induction. Furthermore, staurosporine induced immediate-early genes including N-ras and fos, but not jun. The activation of the staurosporine-activated kinase, as well as the induction of neurite outgrowth, did not require Ras function, while Ras was required for the activation of ERKs and neurite outgrowth induced by NGF. Taken together, these results indicate staurosporine specifically activates a JNK isoform, which may contribute to biological activities including neurite outgrowth.

Neurotrophic factors play a key role in the normal development of the nervous system by regulating both differentiation and apoptosis of neurons (1). Nerve growth factor (NGF)$^1$ is the prototype of this family of neurotrophins and its intracellular signaling pathways have been intensely studied using rat pheochromocytoma PC-12 cells, which undergo neuronal differentiation in response to NGF stimulation (1, 2). NGF binds and activates its receptor, Trk, which leads to the activation of a guanine nucleotide-binding protein, Ras (3–5). Expression of oncogenic Ras in PC-12 cells induces neuronal differentiation (6), while introduction of anti-Ras antibody or dominant inhibitory Ras mutant into PC-12 cells blocks NGF-induced differentiation (7, 8), suggesting that Ras is necessary and sufficient for neuronal differentiation in PC-12 cells. The formation of GTP-Ras is followed first by the activation of Raf and then by activation of MEK (9, 10). MEK in turn activates the ERK family of MAP kinases, which then phosphorylate a variety of proteins including Elk-1 transcription factor (11–13). The expression of a dominant inhibitory mutant of Ras inhibits activation of the Raf/MEK/ERK kinase cascade (14–16). Furthermore, recent studies revealed that MEK and ERK play an important role in NGF-induced differentiation (17, 18). These results indicate the Ras/Raf/MEK/ERK signaling pathway plays a key role in NGF-induced differentiation in PC-12 cells.

The molecular cloning of c-Jun NH$_2$-terminal kinases (JNKs), also termed stress-activated protein kinases, led to the identification of JNKs as a member of the MAP kinase family of protein kinases (19–21). JNKs are activated by inflammatory cytokines and cellular stresses including ultraviolet irradiation, osmotic shock, and treatment with protein synthesis inhibitors, while ERKs are activated by growth factor stimulation (22–25). Activation of JNKs requires the phosphorylation of JNK on Thr and Tyr, which is mediated by a dual specificity protein kinase (19). One of the JNK activators, MKK4 is activated by phosphorylation by another protein kinase, MEKK1 (26, 27). Although the mechanism of MEKK1 activation is not fully understood, it appears that the signaling pathway leading to the activation of JNKs is distinct from the Ras/Raf/MEK signaling pathway, which is responsible for the activation of ERKs.

Recent studies identified 10 isoforms of JNKs (28). These isoforms arise from the alternative processing of transcripts from three different genes: those for JNK1, JNK2, and JNK3. Although similar in amino acid sequences, these JNK isoforms have distinct in vitro biochemical properties, suggesting they are not functionally redundant. Consistent with this, it has been shown that JNK1, but not JNK2, complements a defect in the expression of the MAP kinase, HOG1 in the yeast Saccharomyces cerevisiae (21).

A number of chemical compounds have been reported to induce neurite outgrowth. In this study, we investigated staurosporine-induced neurite outgrowth, and found staurosporine activated a protein kinase with an apparent molecular mass of 57 kDa. Interestingly, the staurosporine-activated kinase was recognized by anti-JNK isoforms antibody, but not by anti-JNK1-specific or anti-ERK1-specific antibody, suggesting this kinase is a JNK. However, the apparent molecular mass of this kinase was different from those of osmotic shock-activated JNKs whose molecular masses were 46 and 55 kDa. Interestingly, staurosporine did not activate osmotic shock-activated

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$ The abbreviations used are: NGF, nerve growth factor; JNK, c-Jun NH$_2$-terminal kinase; MBP, myelin basic protein; MEK, MAP kinase/ERK kinase; ERK, extracellular signal-regulated protein kinase; MAP, mitogen-activated protein; GST, glutathione S-transferase; kb, kilobase pair(s).
kinases, and conversely, osmotic shock did not induce the staurosporine-activated kinase activation. Furthermore, the substrate specificity of the staurosporine-activated kinase was different from that of JNK1 and ERK1. Taken together these results indicate that staurosporine specifically activates a JNK-related kinase, which is different from well characterized stress-activated JNKs. The staurosporine-activated kinase has a distinct substrate specificity, raising the possibility that it is involved in distinct biological activity including differentiation.

MATERIALS AND METHODS

Cell Culture—PC-12 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 5% calf serum. PC-12 cells expressing RasN17 (M-M17-26 cells, provided by Geoffrey M. Cooper (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA) (8) were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 5% calf serum, and 400 μg/ml G418.

Preparation of Recombinant Protein—The GST-c-Jun, GST-Elk-1, and GST-ATF2 expression plasmids were provided by Roger J. Davis (University of Massachusetts, Worcester). These plasmids were expressed in a bacteria strain, BL21(DE3), and GST fusion proteins were purified by glutathione chromatography as described (29).

In-gel Kinase Assay—In-gel kinase assay was performed essentially as described previously (30). Subconfluent dishes of PC-12 cells or PC-12 cells expressing RasN17 were lysed in MAP kinase extraction buffer, and the protein concentration was determined by Bio-Rad protein assay. To assay total cell lysate, equal amounts of protein (50–100 μg) were electrophoresed in 10% SDS-polyacrylamide gels containing either myelin basic protein (MBP) (0.5 mg/ml), GST-c-Jun (1–79) (0.1 mg/ml), GST-ATF2 (0.1 mg/ml), or GST-Elk-1 (0.1 mg/ml) as a substrate. For immunoprecipitation, the NaCl concentration of the cell extract was adjusted to 150 mM, 2 μg of each antibody was added to 100 μl of cell extract containing 50 μg of protein, and incubation carried out for 1 h at 4 °C. Anti-JNK isoforms antibody and anti-ERK1 antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-JNK1-specific antibody was purchased from PharMingen (San Diego, CA). Next, 50 μl of 10% protein A-Sepharose was added and the mixture incubated for 1 h at 4 °C. Protein A-Sepharose beads were collected by centrifugation and washed four times with MAP kinase extraction buffer. SDS-polyacrylamide gel electrophoresis sample buffer was added to protein A-Sepharose beads and samples were electrophoresed in SDS-polyacrylamide gels containing various substrates as indicated. Following electrophoresis, SDS were removed from the gel, protein was renatured, and a kinase assay was carried out by incubating the gel in buffer containing [γ-32P]ATP. Gels were washed, and incorporated radioactivity was quantified using an image analyzer (BAS2000, Fuji Film Co. Ltd., Japan).

Northern Blot Analysis—Total cytoplasmic RNA was isolated as described (31). RNAs (15 μg) were electrophoresed in 1% formaldehyde-agarose gels and transferred to Biodyne-B membrane (East Hills, NY). Filters were prehybridized, hybridized, and washed according to the manufacturer's instructions. The following DNA fragments were 32P-labeled with a random priming kit (Life Technologies, Inc.) for use as probes: fos, a 1-kb PstI fragment from pfos-1 (32); jun, a 0.9-kb PstI fragment of human jun (33); Nur77, a 1.9-kb EcoRI-KpnI fragment from a pGEM-Nur77 construct (34).

RESULTS

Staurosporine Induces Neurite Outgrowth in PC-12 Cells—We studied differentiation in PC-12 cells induced by chemical compounds and found that staurosporine was a potent inducer of neurite outgrowth. As reported (35, 36), staurosporine induced neurite outgrowth at concentrations of as low as 4–20 ng/ml, with maximal induction obtained at 100 ng/ml (Fig. 1). Staurosporine induced neurite outgrowth faster than NGF. After 6 h of staurosporine treatment, small neurites were observed. Maximal neurite outgrowth was obtained after 24 h of treatment, and was comparable to that obtained by 3 days of treatment with NGF. We also tested K-252a, a staurosporine-related compound that exhibits similar effects on a variety of kinases, and found that K-252a did not induce neurite outgrowth at a similar range of concentration (data not shown).

Staurosporine Activates a Kinase with an Apparent Molecular Mass of 57 kDa—Since ERK1 and ERK2 of the MAP kinase family have been shown to play an important role in NGF-induced differentiation (18), we tested whether staurosporine can induce MAP kinases. To this end, we conducted an in-gel kinase assay using MBP as a substrate (Fig. 2). Consistent with previous reports (30), NGF strongly induced ERK1 and ERK2 at 5 min after stimulation and the activity decreased with time. Interestingly, staurosporine induced a kinase with an apparent molecular mass of 57 kDa (Fig. 2A). When MBP was omitted from the gel, this band was not detected, indicating the phosphorylation was not due to autophosphorylation (data not shown). In contrast to the rapid and transient NGF-induced activation of ERKs, staurosporine induced a prolonged activation of the 57-kDa kinase (Fig. 2B). Weak activation was observed at 5 min after stimulation, and maximal activation was obtained at 120 min after stimulation. Weak activation of the kinase was induced with staurosporine treatment as low as 4 ng/ml, while maximal activation was obtained at a concentration of 100 ng/ml (Fig. 2C). Differences in molecular mass indicate that the staurosporine activated kinase is distinct from ERK1 and ERK2, both of which are thought to be involved in NGF-induced differentiation. However, the concentration of staurosporine required for the activation of the 57-kDa kinase was similar to that required for the induction of neurite outgrowth, raising the possibility that the 57-kDa kinase plays a role in staurosporine-induced neurite outgrowth. Consistent with this, K-252a, a compound similar to staurosporine in its structure and biological activities in inhibiting kinases, did not induce neurite outgrowth and did not induce the activation of the 57-kDa kinase (data not shown).

The Staurosporine-activated Kinase Was Recognized by Anti-JNK Isoforms Antibody—The MAP kinase family is part of a large family of serine-threonine protein kinases (19–21). Since JNKs are a subfamily of the MAP kinase family, we tested whether the staurosporine-activated kinase belongs to the JNK subfamily. To this end, we used three antibodies. 1) Anti-JNK isoforms antibody was raised against a full length recombinant protein of JNK2, and recognizes JNK1 and JNK2. 2) Anti-JNK1-specific antibody recognizes only JNK1. 3) Anti-ERK1 antibody recognizes ERK1 specifically. Using these antibodies, we immunoprecipitated JNKs and ERK1 from cells treated with either NGF, staurosporine or osmotic shock and tested for their kinase activities using an in-gel kinase assay (Fig. 3). As expected, NGF induced kinases with molecular masses of 42 and 44 kDa, and the 44-kDa kinase was recognized by anti-ERK1 antibody, but not by anti-JNK isoforms antibody or

FIG. 1. Induction of neurite outgrowth induced by staurosporine. PC-12 cells were plated at a density of 105 cells/well in 24-well plates treated with poly-L-lysine, and incubated for 6–24 h. For NGF treatment (NGF), fresh media containing 0.5% fetal bovine serum and 100 ng/ml NGF were used. For staurosporine treatment (SSP), staurosporine was added to the media at concentrations indicated. Cells were photographed after 3 days (NGF) or 1 day (SSP) of treatment.
anti-JNK1-specific antibody, indicating NGF specifically activates ERK1 and ERK2. Osmotic shock, induced by treatment of cells with 0.5 M sorbitol for 30 min, induced the activation of kinases with molecular masses of 46 and 55 kDa, which probably represent mixtures of JNK1 and JNK2 (28). Consistent with this, these kinases were recognized by anti-JNK isoforms antibody and anti-JNK1-specific antibody, but not by anti-ERK1 antibody. Anti-JNK isoforms antibody only recognized the 55-kDa isoform of JNKs, which is probably due to the preferential recognition of the COOH-terminal region of JNKs by this antibody. The kinase activated by staurosporine was recognized by anti-JNK isoforms antibody, but not by anti-JNK1-specific antibody. Anti-JNK isoforms antibody only recognized the 55-kDa isoform of JNKs, which is probably due to the preferential recognition of the COOH-terminal region of JNKs by this antibody. The kinase activated by staurosporine was recognized by anti-JNK isoforms antibody, but not by anti-JNK1-specific antibody. Anti-JNK isoforms antibody only recognized the 55-kDa isoform of JNKs, which is probably due to the preferential recognition of the COOH-terminal region of JNKs by this antibody. The kinase activated by staurosporine was recognized by anti-JNK isoforms antibody, but not by anti-JNK1-specific antibody. Anti-JNK isoforms antibody only recognized the 55-kDa isoform of JNKs, which is probably due to the preferential recognition of the COOH-terminal region of JNKs by this antibody.

Although the staurosporine-activated kinase was recognized by anti-JNK isoforms antibody, it is possible that the signal detected by an in-gel kinase assay represented multiple kinase activities. To test this possibility, we analyzed the staurosporine-treated cell extract by chromatography using a Mono Q column. Since only a single peak of 57-kDa kinase activity was identified (data not shown), it is therefore unlikely that the 57-kDa kinase activity detected by an in-gel kinase assay contains multiple kinase activities.

Taken together, these results indicate that staurosporine specifically activates a kinase that is immunologically related to JNKs, but is distinct from JNK1 and JNK2.

The Staurosporine-activated Kinase Has Different Substrate Specificity—It has been reported that ERKs and JNKs have a different substrate specificity, which is thought to contribute to their distinct biological activities. We therefore determined the substrate specificity of the staurosporine-activated kinase (Fig. 4). As expected, ERK1 that was activated by NGF efficiently phosphorylated MBP and Elk-1. In contrast, JNK1 activated by osmotic shock strongly phosphorylated ATF2, and, to a lesser extent, Elk-1 and c-Jun. However, the staurosporine-activated kinase phosphorylated MBP, Elk-1, ATP2, and c-Jun. These results indicate that, although the staurosporine-activated kinase is related to JNKs, it has a different substrate specificity to JNK1 and ERK1.

Staurosporine Induces Transcription of Nur77 and fos, but Not jun Genes—Both ERKs and JNKs are believed to regulate gene expression by phosphorylating transcription factors (37).
Interestingly, staurosporine induced Nur77 transcription. Quantitation of the induction indicated that the in- 
duction of early response genes including Nur77, fos, and jun (Fig. 5). Interestingly, staurosporine induced Nur77 and fos 
transcription. Quantitation of the induction indicated that the induction of Nur77 by staurosporine was 2.9-fold at 3 h after 
stimulation, while that by NGF was 12.8-fold at 45 min after stimulation. Similarly, staurosporine induced fos transcription 
by 1.9-fold after 2 h of stimulation, while NGF induced fos transcription by 8.0-fold after 45 min of stimulation. Although 
the induction of Nur77 and fos by staurosporine was smaller than that of NGF, the induction of these genes by staurosporine 
is significant, because jun was not induced by staurosporine. It is worthy to note that staurosporine induced prolonged 
transcriptional activation with a maximal activation at 180 min after treatment, while NGF-induced transcriptional activation 
was more rapid and transient. Thus it appears staurosporine induces transcriptional activation of early response genes, but 
the induction was different from that by NGF with respect to species of genes and time course of the induction.

**DISCUSSION**

ERK1 and ERK2 of the MAP kinase family play a key role in NGF-induced neuronal differentiation in PC-12 cells. Since 
staurosporine is a potent inhibitor of protein kinases and also an inducer of neurite outgrowth in PC-12 cells, in this study, 
we first investigated whether staurosporine activates the signaling pathway that leads to the activation of members of the 
MAP kinase family. Interestingly, we found that staurosporine activated a kinase detected by an in-gel kinase assay using 
MBP as a substrate. This kinase differed from known ERKs in molecular mass, and in that it was not recognized by anti-
ERK1-specific antibody. However, it was recognized by anti-JNK isoforms antibody, which recognizes JNK1, JNK2, and 
probably other isoforms of JNKs. Therefore it is most likely that this staurosporine-activated kinase is a JNK. The finding 
that staurosporine activates a member of the JNK family is consistent with a previous report (38). However, the molecular 
mass of the kinase is 57 kDa, which is distinct from those of well characterized JNKs activated by various stimuli including 
neotoxic shock (Figs. 3 and 4). Furthermore, the substrate specificity of the kinase was also distinct (Fig. 4). Recent detailed 
analysis led to the identification of 10 different JNK isoforms that arise from the alternative processing of transcripts from 
three different genes (28). It turned out that the JNKs with the molecular mass of 46 and 55 kDa represented a mixture of JNK 
isoforms. Thus it is possible that the staurosporine-activated kinase is a novel JNK isoform. Consistent with this, anti-

**FIG. 5. Induction of early response genes.** Cells were plated at a 
density of $2 \times 10^5$ cells/100-mm plate, cultured for 3 days, and treated 
with 100 ng/ml NGF (NGF), 100 ng/ml staurosporine (SSP), or 0.5 M 
sorbitol (SRB) for the times indicated. Total cytoplasmic RNAs (15 µg) 
were analyzed by Northern blot analysis with Nur77, fos, or jun probes. 
Staining of the gels with ethidium bromide prior to blotting revealed 
comparable amounts of 28 and 18 S rRNAs in each lane.

**FIG. 4. The substrate specificity of the staurosporine-activated 
kinase.** Cells were left untreated (−) or incubated with 100 ng/ml NGF for 5 min (NGF), 100 ng/ml staurosporine for 120 min (SSP), 
or 0.5 M sorbitol for 30 min (SRB). Immunoprecipitation was performed 
as described under “Materials and Methods.” The kinase activity was 
determined by an in-gel kinase assay using MBP (NGF, SSP) or c-Jun (SRB) as a substrate. The positions of kinases activated 
by each treatment are indicated by arrows.
Previous results suggest the action of staurosporine to induce the 57-kDa kinase activation and neurite outgrowth is not due to the inhibition of kinases. Staurosporine did not activate ERK1 and ERK2 of MAP kinases, which play an important role in NGF-induced differentiation. However, the 57-kDa kinase activated by staurosporine phosphorylated MBP and ERK1, which suggests the 57-kDa kinase can phosphorylate and activate common downstream target(s) to ERKs. Furthermore, the concentrations required for the activation of the 57-kDa kinase and for the induction of neurite outgrowth are almost the same, which suggest the involvement of the 57-kDa kinase in the induction of neurite outgrowth. This conclusion is supported by experiments using a compound, K-252a, which is structurally similar to staurosporine and also has similar inhibition profile on a variety of kinases. Despite these similarities, K-252a did not induce neurite outgrowth, and importantly, failed to activate the 57-kDa kinase. The MAP kinases are part of a large family of serine-threonine protein kinases. However, their physiological roles are thought to be distinct. For example, ERKs are thought to play a key role in the differentiation of PC-12 cells, while JNKs may play a role in induction of apoptosis (18, 38). Consistent with this, the substrate specificities of these kinases are different. MBP is effectively phosphorylated by ERK1, while ATF2 was strongly phosphorylated by JNK1. Interestingly, the staurosporine-activated 57-kDa kinase phosphorylated both MBP and ATF2 as well as Elk-1 and c-Jun, suggesting the staurosporine-activated kinase may have similar but distinct biological roles. Consistent with this, staurosporine induced transcriptional activation of Nur77 and fos, but not jun. Furthermore, although staurosporine induces neurite outgrowth in PC-12 cells, it also has been reported that apoptosis was induced by staurosporine under certain conditions (38). In Drosophila, recent studies revealed that a JNK homolog mediates cell morphogenesis and an immune response (44, 45). Further studies will be necessary to identify the biological significance of the staurosporine-activated kinase.

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