Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation

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The two MAP kinases JNK and ERK direct distinct cellular activities even though they share a number of common substrates, including several transcription factors. Here we have compared JNK and ERK signalling during PC12 cell differentiation and investigated how activation of c-Jun by the MAPKs contributes to this cellular response. Exposure to nerve growth factor, or expression of constitutively active MEK1—two treatments which cause differentiation of PC12 cells into a neuronal phenotype—result in activation of ERK-type MAP kinases and phosphorylation of c-Jun on several sites including Ser63 and Ser73. Constitutively activated c-Jun, which mimics the MAPK-phosphorylated form of the protein, can induce neuronal differentiation of PC12 cells independently of upstream signals. Conversely, expression of dominant-negative c-Jun^{bZIP} prevents neurite outgrowth induced by activated MEK1. Activation of MEKK1, which stimulates the JNK pathway, is not sufficient for PC12 cell differentiation but can induce apoptosis. However, neurite outgrowth is triggered when c-Jun is co-expressed with activated MEKK1 or SEK1. Consistently, MEK-induced ERK activation in PC12 cells induces c-Jun expression, while JNK signalling does not. Therefore, dual input of expression and phosphorylation of c-Jun provided by the ERK pathway is required to direct neuronal differentiation in PC12 cells.

Keywords: c-Jun/differentiation/MAP kinases/
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Introduction

MAP kinase cascades are universal signal transduction modules that are evolutionarily conserved and used in a wide variety of biological response mechanisms. In vertebrates, at least three such pathways have been identified, which activate different MAP kinase classes, known as ERK, JNK and p38 (Treisman, 1996; Robinson and Cobb, 1997). Even though these signalling systems are built from evolutionarily related protein kinases, they convey distinct biological responses. Whereas ERK signalling is generally involved in the control of cell proliferation and differentiation, JNK and p38 signal transduction pathways mediate responses to various forms of cellular stress, such as damage repair mechanisms, cell growth

arrest and cell death. The biological effects of MAP kinase signalling are executed by downstream phosphorylation substrates, most notably a number of signal-responsive transcription factors. A conceptual complication arose when it became evident that several of the MAP kinase-regulated transcription factors such as Elk1 and ATF-2 can serve as substrates for more than one MAP kinase and thus participate in different biological responses (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; Van Dam *et al.*, 1995; Whitmarsh *et al.*, 1995, 1997; Price *et al.*, 1996). This raised the question of how the specificity of signal response is maintained, i.e. how distinct biological responses are mounted after kinases with similar or overlapping substrate specificity are activated.

The transcription factor c-Jun provides a useful model to study the complexity and specificity of signalling. c-Jun is an inducible transcription factor which directs changes of gene expression in response to multiple extracellular stimuli (Angel and Karin, 1991; Karin *et al.*, 1997). Transcription of *c-jun* mRNA rises after exposure of cells to a number of treatments including exposure to mitogens and various forms of stress. In addition to this transcriptional mode of regulation, c-Jun activity can also be modulated directly at the protein level. Most notable in this regard are regulatory phosphorylations occurring on Ser63 and Ser73, and Thr91 and/or Thr93 within the *trans*-activation domain (Pulverer *et al.*, 1991; Smeal *et al.*, 1992; Papavassiliou *et al.*, 1995). Phosphorylation of these residues results in the stabilization of c-Jun, as well as enhanced *trans*-activation and DNA-binding activity (Devary *et al.*, 1992; Radler-Pohl *et al.*, 1993; Papavassiliou *et al.*, 1995; Musti *et al.*, 1997). In certain cells, these phosphorylation events have been attributed to the MAP kinases ERK1 and ERK2 (Binetruy *et al.*, 1991; Pulverer *et al.*, 1991, 1993; Smeal *et al.*, 1991). ERKs are regulated by growth factors, neurotrophins and phorbol esters. The signal for ERK activation is relayed from the cell surface to the nucleus through a well-characterized signal transduction pathway involving activation of the small GTP-binding protein Ras, and a kinase cascade comprised of Raf, MEK and ERKs (Marshall, 1995; Treisman, 1996).

c-Jun is also a substrate for a related group of MAPKs, called stress-activated protein kinases (SAPKs), or Jun N-terminal kinases (JNKs) (Kyriakis and Avruch, 1996; Treisman, 1996). Exposure of cells to certain cytokines, protein synthesis inhibitors, or various forms of stress, triggers a kinase cascade leading to the activation of JNK, which can bind directly to and phosphorylate c-Jun (Hibi *et al.*, 1993; Dérjard *et al.*, 1994; Kyriakis *et al.*, 1994; Sánchez *et al.*, 1994). Analogous to the ERK cascade, the JNK pathway involves the sequential activation of three kinases called MEKK, SEK and JNK, respectively (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997).

Fig. 1. Induction of neurite outgrowth in PC12 cells expressing c-Jun variants in which MAPK phosphorylation sites have been mutated. (A) Morphology of PC12 cells expressing c-Jun mutants. PC12 cells were microinjected with expression vectors for nuclear β -galactosidase, HA-tagged wild-type c-Jun, HA-c-Jun^{Ala} or HA-c-Jun^{Asp} as indicated. After 48 h, the cells were fixed and stained with anti- β -galactosidase or anti-HA antibodies. Injected cells were detected using FITC-labelled secondary antibodies (green), and the morphology of the cells was visualized by actin staining (red). Cells were examined under confocal microscopy. (B) Quantification of neurite outgrowth. The percentage of the cells with neurites exceeding twice the cell length among the microinjected (FITC-positive) cells is shown. (C) Length distribution of c-Jun-induced neurites. The percentage of cells with neurites exceeding 2-, 3-, 4- or 5-fold the cell length among the microinjected (FITC-positive) cells is shown. The data represent mean values $\pm$ SE of three separate experiments.

With the discovery of JNKs, it became apparent that these enzymes phosphorylate c-Jun more efficiently than ERKs *in vitro* (Dérjard *et al.*, 1994; Minden *et al.*, 1994a,b). Thus, the role of ERKs in the regulation of c-Jun activity has come into question.

The diversity of signals and signalling pathways that are directed toward c-Jun is also reflected in the biological responses, in which the transcription factors have been implicated. It was thought earlier that the main function of c-Jun is to transmit proliferative signals in a cell (Schütte *et al.*, 1989; Bos *et al.*, 1990; Castellazzi *et al.*, 1991; Lloyd *et al.*, 1991; Johnson *et al.*, 1993). Paradoxically, it has also been reported that c-Jun is involved in certain types of apoptotic cell death. For example, dominant-negative mutants of c-Jun, or antibodies against c-Jun were shown to protect neuronal cells from apoptosis induced by nerve growth factor (NGF) withdrawal, a treatment which activates the JNK and p38 kinase cascades (Estus *et al.*, 1994; Ham *et al.*, 1995; Xia *et al.*, 1995). Finally, Jun signalling can positively or negatively regulate differentiation in a number of systems (Bengal *et al.*, 1992; Treier *et al.*, 1995; Hou *et al.*, 1997; Kockel *et al.*, 1997; Riesgo-Escovar and Hafen, 1997). The exact molecular role that c-Jun plays in these different situations awaits clarification.

To examine the role of c-Jun as a target for ERK and JNK signalling, we performed studies in PC12 cells, a well-established experimental system in which the choice between a range of different biological signal responses, proliferation, neuronal differentiation and cell death, can be studied in tissue culture. NGF treatment causes differen-

tiation into a sympathetic neuron-like cell. This coincides with the cessation of cell proliferation, neurite outgrowth and expression of immediate early genes, including *c-jun* and *c-fos*, as well as late response genes believed to function as determinants of neuronal differentiation (Sheng and Greenberg, 1990). The NGF response in PC12 cells requires activation of ERKs, since blocking the kinase cascade either by a specific inhibitor or by expression of dominant interfering mutants or antibodies against Ras or MEK1 inhibits the differentiation (Kremer *et al.*, 1991; Thomas *et al.*, 1992; Cowley *et al.*, 1994; Pang *et al.*, 1995). Conversely, constitutive activation of the ERKs by activated Raf, Ras or MEK1 induces differentiation (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985; Wood *et al.*, 1993; Cowley *et al.*, 1994). Here, we used the PC12 cell system to investigate if and how activation of c-Jun by phosphorylation contributes to neuronal differentiation of PC12 cells, and how the differential response to JNK and ERK activation might be mediated.

Results

Phosphorylated c-Jun induces neurite outgrowth in PC12 cells

To study the functional role of c-Jun phosphorylation in PC12 cell differentiation, plasmids encoding various c-Jun derivatives in which previously identified MAPK substrate residues had been modified, were introduced into PC12 cells by microinjection. The expression of the HA-epitope-tagged c-Jun derivatives in recipient cells was monitored by immunostaining with anti-HA antibodies (Figure 1A).

Fig. 2. Phosphorylation of c-Jun in response to NGF and activated MEK1. **(A)** Phosphorylation of c-Jun on Ser63 in PC12 cells. PC12 cells were injected with expression vectors for myc-tagged c-Jun alone, or together with vectors coding for activated MEK1 (MEK^{EE}) and ERK, or activated MEKK (Δ MEKK). NGF treatment was carried out for 60 min at 24 h post-injection. Doublestaining was performed with anti-myc antibody to detect the injected cells, and with anti-phospho-c-Jun to stain c-Jun phosphorylated on Ser63. These antibodies were chosen because the anti-c-Jun Ser73 phosphate antibodies used in the immunoblots were not suitable for immunostaining. Nuclei expressing myc-c-Jun appear red (left panel) and the phosphorylated form of c-Jun is visualized in green (middle panel). Note that a yellow colour in the overlay (right panel) indicates a high stoichiometry of c-Jun phosphorylation. **(B)** c-Jun is phosphorylated in response to ERK activation in PC12 and NIH 3T3 cells. HA-tagged c-Jun was expressed in PC12 or NIH 3T3 cells alone or together with MEK^{EE} and ERK2, or Δ MEKK as indicated. Cells were harvested 36 h post-transfection, and whole-cell extracts were analysed by SDS-PAGE and immunoblotting using antibodies against phosphorylated forms of c-Jun on Ser63 (top), Ser73 (middle), or an antibody against HA-epitope (bottom). **(C)** Specificity of JNK and ERK activation by Δ MEKK and MEK^{EE}, respectively. HA-tagged ERK or JNK was expressed in PC12 cells alone or together with MEK^{EE} or Δ MEKK (as a positive control) as indicated. The cells were harvested 36 h post-transfection, and the lysates were immunoprecipitated using anti-HA antibody. Immunocomplex kinase assay was performed using GST-c-Jun (amino acids 5–105; top) or myelin basic protein (MBP; bottom) as a substrate. The positions of GST-c-Jun and MBP are shown. The numbers below each lane indicate fold induction of kinase activity relative to the value measured in mock-transfected cells, as determined by PhosphorImager scanning.

In c-Jun^{Asp}, potential MAPK phosphorylation sites, including Ser63 and Ser73, and Thr91 and Thr93, have been replaced by phosphate-mimicking aspartic acid residues (Treier *et al.*, 1995). This 'gain of function' mutant acts like the active phosphoprotein in several assays (Papavassiliou *et al.*, 1995; Treier *et al.*, 1995; Musti *et al.*, 1997). Expression of c-Jun^{Asp} caused the development of long neurites in >50% of microinjected PC12 cells (Figure 1B). If the concentration of the Jun^{Asp} expression vector was titrated down from the standard concentration of 50 μ g/ml in the injected solution to 2 μ g/ml, significant neurite outgrowth was still detected (data not shown), indicating that moderate overexpression was sufficient to elicit the described effects. In control experiments, c-Jun^{Ala} (a mutant which cannot be phosphorylated by MAPKs) and c-Jun^{wt} caused flattening of the cells, increased cell diameter and only moderate neurite formation (Figure 1B). The cells developed fewer and notably shorter neurites as compared with those injected with c-Jun^{Asp} (Figure 1C). Control cells injected with an expression vector for

nuclear β -galactosidase were not induced to differentiate. We conclude that phosphorylation of c-Jun plays an important role in directing PC12 cells towards a neuronal differentiation pathway.

c-Jun is a downstream target of the ERK pathway in PC12 cells

Since the results shown in Figure 1 imply that activation of c-Jun by phosphorylation, as mimicked by c-Jun^{Asp} expression, is sufficient to cause PC12 cell differentiation, we next examined whether differentiation in response to NGF involves the same mechanism. Thus, the phosphorylation state of c-Jun upon NGF treatment was determined. PC12 cells expressing c-Jun^{wt} were treated with NGF, and immunostained with an antibody (anti-c-Jun Ser₆₃P) that specifically recognizes the Ser63-phosphorylated form of c-Jun but not c-Jun that is unphosphorylated at this site. Figure 2A shows that Ser63-phosphorylated c-Jun was hardly detectable in unstimulated cells. However, NGF treatment induced prominent c-Jun phosphorylation. Thus,

Fig. 3. NGF-mediated phosphorylation of c-Jun, ERKs and JNKs in PC12 cells. (A) Expression and phosphorylation of c-Jun in response to NGF. Top: nuclear extracts from PC12 cells treated with NGF for the indicated periods of time, with TPA for 1 h, or with anisomycin (ani) for 30 min, were subjected to SDS-PAGE and immunoblot analysis. Endogenous c-Jun was detected using an antibody against phosphorylated forms of c-Jun on Ser73 or an antibody against bacterially expressed c-Jun. Bottom: HA-tagged c-Jun was expressed in PC12 cells. At 36 h post-transfection, cells were starved for 6 h, and subsequently stimulated with NGF, anisomycin (ani) or TPA. Whole-cell extracts were prepared and analysed by SDS-PAGE and immunoblotting using anti-HA antibody. The position of phosphorylated and non-phosphorylated c-Jun is indicated by open and closed arrows, respectively. (B) Activation of ERKs in response to NGF. Top: whole-cell extracts from PC12 cells treated with NGF for the indicated periods of time, with anisomycin for 30 min, or with TPA for 1 h were subjected to SDS-PAGE and immunoblot analysis using an antibody against dual-phosphorylated ERKs. The lower panel shows an identical filter probed with anti-ERK2 antibody. The positions of ERK1 (upper band) and ERK2 (lower band) are indicated by arrowheads. Bottom: lysates from control cells (C) and cells treated with NGF for the indicated periods of time were immunoprecipitated using anti-ERK2 antibody. Immunocomplex kinase assay was performed using MBP as a substrate. The position of MBP is shown. The numbers below each lane indicate fold induction of ERK activity relative to the control level. (C) Activation of JNKs in response to NGF. Top: JNK activation upon NGF treatment was analysed using an antibody against activated JNKs, as described for (B). The lower panel shows an identical filter probed with anti-JNK1 antibody. The positions of JNK1 (lower band) and JNK2 (upper band) are shown. Bottom: lysates from control and NGF-treated cells were immunoprecipitated using anti-JNK1 antibody. Immunocomplex kinase assay was performed using GST-c-Jun (amino acids 5–105) as a substrate. The position of GST-c-Jun is shown. The numbers below each lane indicate fold induction of JNK activity relative to the control level.

NGF induces phosphorylation of c-Jun in PC12 cells which, according to the data shown in Figure 1, would be sufficient to initiate neuronal differentiation.

We next investigated the pathway through which the NGF signal is transmitted to the PC12 cell nucleus to induce c-Jun phosphorylation. It has previously been shown that NGF stimulates a signal transduction pathway that culminates in the activation of ERKs. Furthermore, PC12 cells differentiate in response to expression of activated components of this pathway, such as Ras, Raf and MEK1 (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985; Wood *et al.*, 1993; Cowley *et al.*, 1994). Hence, we first tested whether MEK1, a MAPK kinase specific for ERKs, could be a mediator between NGF and c-Jun phosphorylation. When c-Jun was co-expressed with a constitutively activated form of MEK1 (MEK^{EE}; see Materials and methods) and ERK2, intense nuclear immunostaining was detected with the anti-c-Jun Ser₆₃P antibody (Figure 2A). Under our experimental conditions, expression of MEK^{EE} or ERK2 alone did not result in strong phosphorylation (Figure 2B and C; data not shown), indicating that ERK protein concentration is a limiting factor for c-Jun phosphorylation in the cells. Prominent c-Jun phosphorylation was also detected in cells in which c-Jun was co-expressed with an activated JNK pathway component, such as the constitutively active MEKK1

(ΔMEKK), comprised of a 672-residue C-terminal fragment of the molecule (Whitmarsh *et al.*, 1995) (Figure 2A), or activated SEK plus JNK (data not shown). To corroborate these findings, we performed immunoblotting experiments with extracts from transiently transfected PC12 cells using phospho-specific antibodies or antibodies that are not sensitive to the phosphorylation state of c-Jun. In agreement with the immunostaining results shown in Figure 2A, we find that co-expression with MEK^{EE} and ERK2 results in significant phosphorylation of c-Jun on Ser63 and Ser73. Qualitatively similar results were obtained when the same experiment was performed in NIH 3T3 cells, and after co-transfection with ΔMEKK. Taken together, these results indicate that, in both PC12 and NIH 3T3 cells, c-Jun can be phosphorylated on Ser63 and Ser73 in an ERK- or a JNK-dependent manner. To monitor directly the specificity of MAPK activation under our assay conditions we performed immunocomplex kinase assays (Figure 2C). This experiment shows that, as expected, MEK^{EE} stimulated the kinase activity of HA-tagged ERK2 measured on MBP as a substrate. Importantly, no significant ERK activation was observed after co-expression with ΔMEKK, indicating that under our experimental conditions there is no cross-talk between the JNK and ERK signalling pathway. This finding further supports our conclusion that c-Jun is a target to ERK

Fig. 4. Dominant-negative Jun inhibits MEK-induced neurite outgrowth in PC12 cells. (A) Morphology of PC12 cells expressing activated MEK and c-Jun. PC12 cells were injected with expression vectors for MEK^{EE} alone, MEK^{EE} together with c-Jun^{wt}, or MEK^{EE} with c-Jun^{bZIP}, as indicated. Nuclear β -galactosidase was co-expressed to mark the injected cells. After 48 h, the cells were fixed and stained with anti- β -galactosidase (green) and TRITC-phalloidin (red). NGF-treated cells stained with TRITC-phalloidin are shown as a control. (B) Quantification of neurite outgrowth. The percentage of the cells with neurites exceeding twice the cell length among the microinjected (FITC-positive) cells is shown. The data shown are the mean values $\pm$ SE of two separate experiments.

phosphorylation in PC12 cells. In contrast, Δ MEKK specifically activated JNK and had no effect on ERK activity. Like others (Minden *et al.*, 1994a,b) we observed no phosphorylation of c-Jun by ERK *in vitro*. This negative result may be explained by a requirement for further factors in the cell that facilitate the phosphorylation of c-Jun by ERK.

To gain further insight into the relationship between ERK, JNK and c-Jun activation in PC12 cell differentiation, we analysed the phosphorylation state and hence the activity of these proteins over a time course following NGF addition. The top panel of Figure 3A shows that both the expression levels and the phosphorylation of endogenous c-Jun protein is increased after NGF treatment of PC12 cells. The expression of c-Jun directed from a

transfected CMV vector is not stimulated by NGF (Figure 3A, bottom panel) and thus permits assessment of NGF-dependent c-Jun phosphorylation over time at constant protein levels. This experiment reveals a kinase activity present in PC12 cells for hours after NGF induction that can specifically phosphorylate c-Jun. ERKs are good candidates for such an activity, as the strong and sustained activation of ERK1 and ERK2 (Figure 3B) correlates well with the persistence of phosphorylated forms of c-Jun (Figure 3A). In contrast, we observed only a weak and transient JNK1 activation after 15 min of NGF treatment, whereas JNK2 activity was not increased or even slightly reduced (Figure 3C). Consistently, when we measured JNK activation by immunocomplex kinase assay, we found that the basal levels of JNK activity were reduced upon exposure of cells to NGF (Figure 3C, bottom panel). Thus, while a contribution of JNK to c-Jun phosphorylation cannot be completely excluded, the kinetics and amplitude of ERK activity is more compatible with a major function of the latter class of MAPKs in the phosphorylation of c-Jun upon NGF treatment.

If c-Jun is an essential downstream component of the MEK1/ERK-mediated differentiation in PC12 cells, a dominant-negative form of c-Jun would be expected to interfere with this process. Conversely, wild-type c-Jun might be anticipated to enhance neurite outgrowth. Figure 4 shows that co-expression of c-Jun^{wt} with MEK^{EE} did not markedly increase the number of cells forming neurites, yet the neurites were longer, as compared with cells expressing MEK^{EE} alone. Similar results were obtained after co-expressing MEK^{EE} and ERK2 (data not shown). In contrast, co-expression of c-Jun^{bZIP}, a truncated, dominant-negative form of Jun, caused marked inhibition of MEK1 and ERK2-induced neurite outgrowth. Expression of c-Jun^{bZIP} alone did not result in any cellular responses (Figure 4B). These results therefore provide evidence that the MAPK/ERK pathway can trigger c-Jun phosphorylation, and that this event is critical for PC12 cell differentiation in response to NGF.

JNK can induce neuronal differentiation only if c-Jun is co-expressed

Next, we asked whether JNK, which can also phosphorylate the Ser63 and Ser73 residues of c-Jun, may elicit PC12 cell differentiation when activated. In the JNK signalling pathway, MEKK1 phosphorylates and activates SEK1, also called MKK4, which in turn activates JNK by phosphorylating its regulatory Tyr and Thr residues (Lange-Carter *et al.*, 1993; Dériard *et al.*, 1994; Kyriakis *et al.*, 1994; Sánchez *et al.*, 1994; Yan *et al.*, 1994; Lin *et al.*, 1995). Two components of the JNK pathway, Δ MEKK and partially active SEK1 (SEK^{ED}), in which the regulatory phosphorylation sites have been substituted by glutamic and aspartic acid residues (J. Woodgett, personal communication) were used in the experiments. Immunoblot analysis of transiently transfected NIH 3T3 cells (Figure 5A) indicated that SEK^{ED} could induce phosphorylation of c-Jun^{wt} *in vivo*, albeit to a lesser extent than MEK^{EE}. Phosphorylation of c-Jun was strongest when c-Jun was co-expressed with Δ MEKK. The same assay was also performed using c-Jun^{Ala} as a substrate for these kinases. c-Jun^{Ala} was not recognized by anti-c-Jun Ser63

Fig. 5. Activation of MEK or SEK can mediate PC12 cell differentiation in the presence of c-Jun. (A) c-Jun is phosphorylated in response to activated MEK, SEK and MEKK in NIH 3T3 cells. c-Jun^{wt} or c-Jun^{Ala} were expressed in NIH 3T3 cells alone or with MEK^{EE}, ERK, SEK^{ED}, JNK or ΔMEKK, as indicated. Cells were harvested 24 h post-transfection. Whole-cell extracts were assayed for c-Jun phosphorylation using SDS-PAGE and immunoblotting with antibodies against c-Jun phosphorylated on Ser63 (top) or Ser73 (middle), or anti-HA antibody (bottom). The arrowhead indicates JNK that is also HA-tagged. (B) Morphology of PC12 cells expressing activated components of the JNK pathway. PC12 cells were injected with expression vectors for SEK^{ED} or ΔMEKK in the absence or presence of a plasmid coding for c-Jun^{wt}, as indicated. Nuclear β-galactosidase was co-expressed to mark the injected cells. The cells were fixed after 40 h and stained with anti-β-galactosidase (green) and TRITC-phalloidin (red). (C, D) Quantification of neurite outgrowth and length distribution of neurites were performed as in Figure 1B and C, respectively. The data shown are mean values ± SE of two separate experiments.

or Ser73 phosphate antibodies, verifying the specificity of these antibodies.

The ability of the activated components of the JNK pathway to elicit PC12 cell differentiation responses was examined using the microinjection assay (Figure 5B). In contrast to MEK^{EE} (Figure 4), neither SEK^{ED} nor ΔMEKK induced neurite outgrowth when expressed in PC12 cells, which further indicates that activation of JNK pathway is not sufficient to induce PC12 cell differentiation. However, consistent with previously published results (Xia *et al.*, 1995), we found that ΔMEKK-expressing cells underwent apoptosis. PC12 cells expressing SEK^{ED} displayed neither differentiation nor apoptosis. If, however, SEK^{ED} or ΔMEKK were expressed along with c-Jun^{wt}, marked neurite outgrowth ensued (Figure 5B and C). The fraction of differentiating cells, as well as the average length of the appearing neurites, was significantly increased as compared with cells that had received c-Jun^{wt} alone (Figure 5C and D). Interestingly, the apoptotic effect of ΔMEKK

was suppressed when c-Jun was co-expressed. The basis for this is unclear and subject to further investigation. The cooperation between JNK and c-Jun in the induction of neurite outgrowth suggests that c-Jun phosphorylation is sufficient to induce PC12 cell differentiation, regardless of whether it is mediated by MEK-induced ERK activity or through activation of JNK.

ERK- but not JNK-activation induces c-Jun expression in PC12 cells

An attractive hypothesis to explain the above results poses that PC12 cell differentiation requires two events, namely the induction of c-Jun synthesis and c-Jun phosphorylation. Whereas both ERK and JNK can catalyse phosphorylation of the relevant sites in c-Jun, only the former can stimulate c-Jun expression in PC12 cells efficiently (Figure 7). According to this model, JNK activation would thus not be sufficient to stimulate differentiation, unless c-Jun is provided *in trans*. To test this idea, we investigated

Fig. 6. c-Jun expression is induced by NGF and activated MEK, but not by activated MEKK and SEK. Left: expression of endogenous c-Jun in PC12 cells in response to activated MEK, MEKK and SEK. PC12 cells were injected with expression vectors coding for MEK^{EE}, ΔMEKK or SEK^{ED}. Nuclear β-galactosidase was co-expressed to mark the injected cells. The cells were fixed after 16 h and double stained with anti-β-galactosidase (red) and anti-c-Jun (green) antibodies. Note that a yellow colour in the overlay indicates prominent c-Jun immunoreactivity. Right: expression of endogenous c-Jun in response to NGF. Cells were starved for 16 h and subsequently treated with NGF and TPA (as a positive control) for 1 h. After fixation, the cells were stained with anti-Jun antibody.

Fig. 7. Model of signalling to c-Jun in undifferentiated PC12 cells. Two MAPK cascades converge on c-Jun. The ERK pathway regulates c-Jun expression and phosphorylation during neuronal differentiation of PC12 cell. If c-Jun expression levels are increased JNK pathway can also mediate differentiation response.

whether endogenous c-Jun expression can be induced by specifically activating either the ERK or the JNK pathway. MEK^{EE}, SEK^{ED} or ΔMEKK were expressed in PC12 cells, and endogenous c-Jun expression was examined by immunostaining (Figure 6). Interestingly, expression of MEK^{EE} caused a significant increase in c-Jun expression

similar to the one seen in NGF- or TPA-treated PC12 cells (Figure 6, right panel). In contrast, ΔMEKK, which effectively activates JNK, resulted in only marginal c-Jun induction. When PC12 cells were injected with expression plasmid for SEK^{ED}, c-Jun expression was not detectably induced. Taken together, these results establish a biologically relevant difference between the ERK and the JNK-signalling in the regulation of c-Jun activation.

Discussion

Several lines of evidence presented here suggest that c-Jun can act as a substrate for ERK phosphorylation in at least two different cell types, and that in the NGF response, ERK-mediated activation of c-Jun directs PC12 cells towards neuronal differentiation. First, analogous to activated components of the Ras/MAPK pathway such as Ras, Raf or MEK1 (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985; Wood *et al.*, 1993; Cowley *et al.*, 1994), expression of c-Jun^{ASP} in PC12 cells induced marked neurite outgrowth. Secondly, NGF treatment of PC12 cells induced sustained activation of ERKs and phosphorylation of c-Jun. A transient small increase in JNK1 activity after NGF exposure does not match the kinetics of c-Jun phosphorylation. Thirdly, expression of constitutively active MEK and ERK, which has been shown previously to lead to PC12 cell differentiation (Cowley *et al.*, 1994), resulted in prominent c-Jun expression and phosphorylation on Ser63 and Ser73, but not JNK activation. Fourthly,

expression of c-Jun potentiated differentiation of PC12 cells induced by MEK1, whereas dominant-negative mutants of c-Jun inhibited it.

Thus, while ERKs are less effective kinases of c-Jun *in vitro*, as compared with JNKs, phosphorylation of c-Jun by ERKs appears to mediate signal responses *in vivo*, at least during PC12 cell differentiation. This is consistent with our recent findings in *Drosophila*, which indicate that Jun can act as an effector of both JNK and ERK pathways during development of this organism (Peverali *et al.*, 1996; Kockel *et al.*, 1997). Nevertheless, JNKs can phosphorylate c-Jun more efficiently than ERKs on the sites which, according to our mutant analysis, are critical for PC12 cell differentiation. Considering this—and our finding that phosphorylation of c-Jun on these sites is sufficient to direct PC12 cells along a path of neuronal differentiation—one might predict that activation of the JNK pathway would also induce PC12 cells to differentiate. However, this is not the case. Stimulation of JNK activity either by activated forms of MEKK or SEK does not trigger neurite formation in the way that it was seen when the ERK pathway was stimulated (by MEK^{EE} or by NGF). Instead, consistent with observations by others (Xia *et al.*, 1995; Lassignal Johnson *et al.*, 1996), Δ MEKK induced apoptosis in undifferentiated PC12 cells. In this situation, c-Jun seems not to be involved, but conversely counteracts apoptosis and induces differentiation when provided in addition. Since c-Jun itself has also been implicated in apoptosis in some circumstances (Ham *et al.*, 1995; Xia *et al.*, 1995; Watson *et al.*, 1998), it seems clear that the effects of c-Jun on cellular responses depend on the cell type and the context of regulatory inputs that the cell is receiving.

Our results show that a JNK activating signal could promote neuronal differentiation in PC12 cells only when c-Jun was provided in addition. These data suggest a model in which activation of the ERK pathway in PC12 cells results in stimulation of both c-Jun synthesis and c-Jun phosphorylation, whereas the JNK pathway triggers phosphorylation only (Figure 7). Indeed, we could show that activation of ERK pathway, but not JNK, induced prominent c-Jun expression in PC12 cells. This is consistent with recent data reporting that activation of JNK is not sufficient to activate the *c-jun* promoter in fibroblasts (Hazzalin *et al.*, 1996). Providing c-Jun exogenously, however, will turn the JNK activation into a differentiation signal. According to this model, the specificity of signal response is not based on a qualitative or quantitative difference in the way ERKs or JNKs phosphorylate Jun (e.g. in terms of kinetics or phosphorylation site preference). Phosphorylation of c-Jun by either kinase can promote PC12 cell differentiation. Based on the data presented here, we favour a combinatorial model in which a dual input on the level of transcription and phosphorylation of c-Jun is required to start a programme of neuronal differentiation in PC12 cells. There may also be situations where simultaneous activation of ERK and JNK act synergistically to elicit a response that is distinct from the response to the activation of either pathway alone.

Materials and methods

Plasmids

Plasmids for mammalian cell expression of CMV-driven epitope-tagged c-Jun^{WT}, c-Jun^{Ala}, c-Jun^{Asp} and nuclear β -galactosidase have been

described (Treier *et al.*, 1994, 1995). CMV-driven expression vector for HA-tagged dominant-negative form of c-Jun (c-Jun^{bZIP}) was constructed by deleting amino acids 25–181 from the sequence. Bacterial pGEX2T expression vector containing GST–c-Jun (kindly provided by F.A.Peverali) was constructed by PCR amplification of sequences containing amino acids 5–105. Constructs for mammalian pEXV expression vectors for constitutively active MEK1 and ERK2myc (Cowley *et al.*, 1994) were provided by C.Marshall; the construct for mammalian expression of constitutively active MEKK1 (Whitmarsh *et al.*, 1995) was provided by R.J.Davis; the construct for mammalian expression for HA-tagged JNK (Coso *et al.*, 1995) was provided by S.Gutkind; the construct for mammalian expression for HA-tagged ERK2 was provided by C.J.Der; and the construct for mammalian expression for partially active SEK was provided by J.Woodgett.

Cell culture and transfections

Rat pheochromocytoma PC12 cells were routinely cultured on collagen-coated dishes in a humidified 7.5% CO₂ atmosphere at 37°C in DMEM, supplemented with 10% horse serum (HS) and 5% fetal calf serum (FCS). Transient transfection into PC12 cells was done with Lipofectamine according to the manufacturer's instructions (Gibco-BRL). Mouse NIH 3T3 cells were cultured in DMEM with 10% calf serum (CS). Transfection was performed using the calcium phosphate method (Graham and van der Eb, 1973). Cells were harvested 24–36 h after transfection.

Microinjection

For microinjection, cells were seeded on laminin-coated plastic plates (20 μ g/ml mouse EHS-laminin; Boehringer) to provide better adhesion and facilitate neurite outgrowth. Microinjections were performed on an automated injection system using a Zeiss inverted microscope. All plasmids were injected into the nucleus at a concentration of 50 μ g/ml unless otherwise stated. 100–150 cells were injected per experiment.

Immunostaining

Cells were fixed with 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), washed with PBS and permeabilized with 0.1% Triton X-100 in PBS on ice. Blocking with 1% bovine serum albumin (BSA) in PBS for 30 min, and incubations with primary antibodies in 1% BSA–PBS for 1 h, were done at room temperature (RT). Antibodies included a monoclonal antibody (mAb) against β -galactosidase (Promega), a mAb against HA-epitope (clone 12CA5), a mAb against myc-epitope (clone 9E10), a polyclonal antibody against c-Jun (Bohmann and Tjian, 1989), and a polyclonal antibody against phosphorylated c-Jun at position Ser63 (New England Biolabs). After several washes, bound antibodies were detected using FITC- and Texas red-conjugated secondary antibodies (Dianova) for 1 h at RT. The morphology of the cells was visualized using TRITC-labelled phalloidin (Sigma). Cells were further washed extensively with PBS, and Hoechst dye 33258 (Sigma) was included in the last wash to visualize the nuclei. Finally, the cells were mounted under a coverslip using Mowiol. Samples were examined using a Zeiss LSM410 confocal imaging system. For quantification of neurite outgrowth, the cells forming neurites longer than twice the diameter of the cell body were defined as positive.

Western blot analysis

Whole-cell extracts were prepared by lysing the cells directly in SDS sample buffer. Nuclear extracts were obtained by extracting the cells with hypotonic lysis buffer (10 mM Tris pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 0.2 mM Na₃VO₄, 50 μ M NaF, 2 mM DTT, 0.5% NP-40), followed by solubilization of nuclei into SDS sample buffer. After sonication, protein samples (10–20 μ g) were separated on SDS–polyacrylamide gels and electroblotted onto nitrocellulose filters. Immunoblotting was performed using a mAb against HA-epitope, polyclonal antibodies against phospho-c-Jun (anti-c-Jun phosphorylated on Ser63 or Ser73; New England Biolabs), polyclonal antibody against c-Jun (Bohmann and Tjian, 1989), and polyclonal antibodies against activated phospho-ERKs and phospho-JNKs (Promega). ERK1/2 and JNK1/2 were detected by polyclonal antibodies C-14 and C-17, respectively (Santa Cruz). HRP-conjugated secondary antibodies were purchased from Dianova. The blots were developed with an enhanced chemiluminescence method (ECL, Amersham).

In vitro kinase assays

The cells were washed with PBS and solubilized in lysis buffer (25 mM HEPES–NaOH pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 5 mM EGTA, 25 mM β -glycerophosphate, 0.1 mM

Na₃VO₄). Transiently transfected HA-tagged JNK1 was immunoprecipitated for 1 h at 4°C using monoclonal anti-HA (12CA5) antibody. Immunocomplexes were coupled to protein-A-Sepharose beads for an additional 1 h at 4°C and washed four times with dilution buffer (25 mM HEPES–NaOH pH 7.5, 5 mM EDTA, 5 mM EGTA, 25 mM β-glycerophosphate, 0.1 mM Na₃VO₄), followed by one wash with kinase buffer (50 mM HEPES–NaOH pH 7.5, 10 mM MgCl₂, 1 mM DTT, 25 mM β-glycerophosphate, 1 mM Na₃VO₄). Kinase reactions were performed in the presence of 2 μCi of [γ-³²P]ATP for 20 min at 30°C using myelin basic protein (MBP) or GST–c-Jun (5–105) as a substrate. Phosphorylated proteins were analysed by SDS–polyacrylamide gel electrophoresis and autoradiography. The intensities of the radioactive signals were quantitated with a PhosphorImager (Molecular Dynamics).

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