Stress- and cell type-dependent regulation of transfected c-Jun N-terminal kinase and mitogen-activated protein kinase kinase isoforms

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INTRODUCTION

Members of the cJun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) family of mitogen-activated protein (MAP) kinases are strongly stimulated by numerous environmental stresses, but also more modestly by mitogens, oncogenes and inducers of cell differentiation and morphogenesis [1,2]. In fact, stimulation of JNK activity resulting in the induction of cell death, growth and differentiation indicates a broad role for the JNKs in cell biology. In a manner parallel to the regulation of the related extracellular signal-regulated kinases, the JNKs are activated after their phosphorylation on threonine and tyrosine by the dual-specificity MAP kinase kinases (MKKs), MKK4 and MKK7, which are specific activators of the JNK enzymes, as similarly expressed. Reverse transcription and PCR revealed that JNK3 is endogenously expressed in SCLC cells, but not in either chromaffin or neuronally differentiated PC12 cells. MKK4 and MKK7 were endogenously expressed in both PC12 cells and SHP77 cells. Immunoprecipitation and analysis of the JNKs expressed in SCLC cells revealed strong stimulation of all five JNK isoforms by UV radiation. Hypertonic stress, elicited by mannitol, also significantly stimulated these same JNKs, although the JNK3 isoforms were most strongly activated. In PC12 cell transfectants, however, selective and equal activation of p54JNK2/a and p54JNK3 by UV and osmotic stress was observed, with little or no activation of JNK1/a or JNK2/p. In contrast with the broad activation of the JNK enzymes by UV in SCLC cells, only HA-MKK4 was stimulated by UV exposure in these cells, whereas osmotic stress stimulated both HA-MKK4 and HA-MKK7. These findings indicate selective activation of JNK and MKK isoforms in a manner that is dependent upon the specific cell stress and the cell type.

Key words: gene transfer, PC12 cells, signal transduction, small-cell lung cancer.

MATERIALS AND METHODS

Materials

Recombinant glutathione S-transferase (GST)-cJun (1–79), GST–p54JNK3 (K–A) and His<sub>6</sub>-ATF2-NT (1–254) (ATF2-NT is the recombinant N-terminal domain of activating transcription factor 2) were expressed in bacteria and purified using glutathione-agarose (Sigma Chemical Co., St. Louis, MO, U.S.A.) and Ni<sup>2+</sup>-nitrilotriacetic acid-agarose (Qiagen, Studio City, CA, U.S.A.). Sera and powdered growth media were from Gibco-BRL. A mouse monoclonal antibody directed against the influenza haemagglutinin (HA) epitope (12CA5) was purchased from Boehringer Mannheim and polyclonal antibodies to MKK4 (C–20) and MKK7 (T–19) were purchased from Santa Cruz.

Abbreviations used: HA, haemagglutinin; SCLC, small-cell lung cancer; MAP kinase, mitogen-activated protein kinase; MKK, MAP kinase kinase; MKKK, MAP kinase kinase kinase; JNK, cJun N-terminal kinase; SAPK, stress-activated protein kinase.

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Expression plasmids encoding JNK and MKK isoforms

The cDNAs encoding the HA-tagged (SAPK) homologues of p46 and p54 JNK3 and p54 JNK2a and JNK2β were generously provided by Dr. James Woodgett (Ontario Cancer Institute, Toronto, Canada). The cDNAs for the HA-tagged rat JNK3 constructs as well as human HA-p65JNK1α [12] were ligated downstream of the cytomegalovirus promoter of the retrovirus expression vector pLNCX [13]. Coding sequences encompassing the Thr-Pro-Tyr phosphorylation motif and the z or β alternative splices were excised from the untagged rat JNK2 cDNAs with BamHI and BglII and ligated into a previously described [10] LNCX-HA-p54JNK2a-APF construct [APF refers to the JNK mutant in which the Thr-Pro-Tyr (TPY) phosphorylation motif within the JNK molecule is mutated to Ala-Pro-Phe (APF), rendering the kinase non-phosphorylatable], cut with the same enzymes, thereby generating LNCX expression vectors encoding wild-type forms of HA-p54JNK2α and HA-p54JNK2β. The predicted amino acid sequences of the rat MKK4 and rat MKK7 coding sequences encompassing the coding sequence for the HA epitope (YPYDVPDYA) was inserted by PCR at the N-terminus instead of Ile at codon 312. The coding sequence for the HA-p54JNK2α and HA-p54JNK2β were predicted amino acid sequences of the...

Cell culture and retrovirus-mediated gene transfer

SHP-77 SCLC cells were cultured in RPMI 1640 containing 10% (v/v) fetal bovine serum. PC12 cells were cultured in Dulbecco’s modified Eagle’s medium containing 5% (v/v) horse serum, 2.5% (v/v) calf serum and 2.5% (v/v) fetal bovine serum. The LNCX-JNK and MKK expression plasmids were packaged into a replication-defective retrovirus using 293T cells [13]. Immuno complex kinase assays

Cells expressing the HA-tagged MKKs and JNKs were lysed in MAP kinase lysis buffer and microfuged extracts containing 200 μg of protein were incubated (4 °C, 2 h) with 2 μg of the 12CA5 monoclonal antibody and 10 μl of packed Protein G-Sepharose in a total volume of 0.5 ml. The immune complexes were washed three times in lysis buffer and then suspended in 40 μl of 50 mM β-glycerophosphate (pH 7.2), 0.1 mM sodium vanadate, 10 mM MgCl2, 100 μM [γ-32P]ATP (5000 c.p.m./pmol), 1 mM EGTA and either 2 μg of His2-ATF2-NT for analysis of HA-JNKs or 1 μg of kinase-inactive GST-JNK3 for analysis of HA-MKK activity. After a 20 min incubation at 30 °C, the kinase reactions were terminated with 10 μl of SDS sample buffer and submitted to SDS/PAGE. The ATF2 and GST-JNK3 polypeptides were excised from the Coomassie-stained, dried gels and incorporated radioactivity was determined in a scintillation counter.

Immunoblot analyses

Samples were resolved by 10% SDS/PAGE and transferred to nitrocellulose. The filters were blocked in Tris-buffered saline [10 mM Tris/HCl (pH 7.4)/140 mM NaCl] containing 0.1% Tween-20 (TTBS) and 3% non-fat dry milk and then incubated with blocking solution containing the indicated antibodies at 1 μg/ml for 12–16 h. The filters were extensively washed in TTBS and bound antibodies were visualized with horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminescence (ECL; Amersham) according to the manufacturer’s directions.

Analysis of JNK3 mRNA expression by reverse-transcription PCR

Poly(A)⁺ RNA was prepared from whole rat brain, SCLC cell lines H345 and SHP-77 and PC12 cells exhibiting either the chromaffin phenotype or the neuronal phenotype (induced by culturing in the presence of nerve growth factor for 7 days) with the Promega PolyATtract system (Madison, WI, U.S.A.) according to the manufacturer’s protocols. Reverse transcription of the mRNA was performed with random hexamers and the components that are included in the GeneAmp RNA PCR kit from Perkin-Elmer (Branchburg, NJ, U.S.A.). A ~ 700 bp JNK3 cDNA fragment was amplified (35 cycles of 95 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min) with the forward primer GCCGCGTATGATGCTGTCCTTGAC and the reverse primer TGGGGAAGAGTTGGGAAAGGTGAG. To verify the integrity of the mRNA preparations, a β-actin cDNA fragment was amplified with the forward primer GACGATATCGCTGCGCTG and the reverse primer ACATGATCTGGGTACATCTTT.

RESULTS AND DISCUSSION

JNK activation is initiated by diverse cell stimuli that induce cytotoxic, mitogenic and differentiative actions in cells [2]. In the present study, we have characterized the activation of transfected JNK isoforms by UV radiation, which induces DNA and macromolecular damage, as well as by osmotic stress, initiated...
Selective activation of transfected cJun N-terminal kinases

Figure 1 Stimulation of endogenous JNK activity by UV and osmotic stress in SCLC and PC12 cells

(A) PC12 cells or SHP77 cells were irradiated (196 J/M²) with a UV-C source or incubated with the indicated concentrations of mannitol in culture medium. After a 30 min incubation at 37 ºC, the cells were rinsed with PBS, lysed in MAP kinase lysis buffer and clarified extracts were analysed for JNK activity with the GST–cJun adsorption assay (see the Materials and methods section). The experiment shown is representative of two independent experiments. (B) The indicated cells lines were UV-irradiated as in (A) or incubated in medium containing 300 mM mannitol for 30 min. Cell extracts were prepared and assayed for JNK activity with the GST–cJun adsorption assay. The results are means ± S.E.M. of three to four independent experiments where * indicates significant stimulation relative to untreated cells, P < 0.05.

Interestingly, PC12 cells and SHP77 cells exhibited different sensitivities to mannitol. Figure 1(A) reveals that maximal mannitol-stimulated JNK activity, measured by GST–cJun binding and phosphorylation, occurred with 300 mM mannitol in PC12 cells. SHP77 SCLC cells were reproducibly less sensitive to osmotic stress, as mannitol concentrations of at least 600 mM were required for maximal JNK activation (Figure 1A). The average response of SHP77 and PC12 cells to UV and mannitol is shown in Figure 1(B) and again demonstrates a greater JNK activation induced in PC12 cells, relative to SHP77 cells, by 300 mM mannitol, which is a sub-optimal concentration for the SCLC line. The existence of two proximal osmoregulatory mechanisms with distinct sensitivities to ranges of tonicity that impinge on common MAP kinase pathways has been described previously in yeast and mammalian cells [16,17]. The distinct sensitivities of JNK activation by mannitol in PC12 and SHP77 cells may reflect the variable dominance of the mammalian equivalents of these osmotic sensors in the two cell lines.

Figure 2 Regulation of HA-JNK isoforms expressed in SCLC cell lines and PC12 cells by UV and osmotic stress

The indicated JNK isoforms were expressed in the SCLC cell line SHP77 (A) and PC12 cells (B) as HA-tagged constructs using retrovirus-mediated gene transfer (see the Materials and methods section). The insets to (A) and (B) show anti-HA immunoblots of cell extracts from the various retrovirus-infected, G418-resistant cells that reveal similar expression levels of the different HA-tagged JNK isoforms. The cells expressing the individual HA-JNK isoforms were UV-irradiated (196 J/M²) or incubated with 300 mM mannitol. After a 30 min incubation, cell extracts were prepared and HA-JNKs were immunoprecipitated and assayed for kinase activity with recombinant ATF2. The results are representative of three to six independent experiments and are presented as the mean fold-stimulation (± S.E.M.) above the non-specific activity that adsorbs to Protein G beads in the LNCX control cells; * indicates P < 0.05 relative to control treatments. The SHP77 cells expressing HA-JNKs were pooled G418 resistant cultures, whereas (B) shows results from cloned HA-JNK-positive PC12 cell lines that are representative of at least two other independent clones for each HA-JNK isoform.

Owing to the high degree of homology among the products of the three jnk genes [6,7,18], antisera with sufficient specificity to assess the regulation of the different JNK isoforms are not available. As an alternative approach, cDNAs encoding HA-tagged p46JNK1a, p54JNK2a, p54JNK2b, p46JNK3 and p54JNK3 were ligated into the retroviral expression vector, pLNCX, packaged into retroviruses and transduced into SHP77 SCLC cells and PC12 phaeochromocytoma cells. After selection with G418, stable pooled populations of SCLC transfectants and independent clones of the PC12 transfectants were collected and analysed for HA-JNK expression by immunoblot analysis with an anti-HA antibody. The insets to Figures 2(A) and 2(B) verify expression of the predicted JNK polypeptides in SHP77 and PC12 cells respectively. Furthermore, note that the various HA-JNKs are expressed at approximately equivalent levels within a given cell line.

The cell lines expressing the panel of HA-JNK polypeptides were used to define the pattern of JNK activation achieved with...
UV irradiation and osmotic stress. After exposure to UV or mannitol, cell extracts were prepared and the HA-JNK polypeptides were collected by immunoprecipitation with anti-HA antibodies and assayed for ATF2 kinase activity. Strong activation of all of the five HA-JNK isoforms was observed in extracts from UV-irradiated SHP77 (Figure 2A) SCLC cells. Osmotic stress, initiated by addition of 300 mM mannitol, cell extracts were resolved and stained with ethidium bromide. No products were obtained when reverse transcriptase was excluded from the reactions (results not shown). The JNK3 products obtained from rat brain and H345 SCLC RNA were cloned into pT7 Blue (Novagen) and sequenced to verify that the JNK3 primers had amplified JNK3 mRNA present in these samples. MAP kinase lysis buffer extracts prepared from SHP77 SCLC cells and PC12 cells were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-MKK4 (C-20; Santa Cruz) or anti-MKK7 (T-19; Santa Cruz). The bound antibodies were visualized with secondary antibodies coupled to horseradish peroxidase and ECL.

UV, a similar stimulation of JNK activity was observed as in p46JNK1-expressing PC12 cells that had been stimulated with UV, a similar stimulation of JNK activity was observed as in extracts from LNCX controls (results not shown).

Previous findings by others have demonstrated that JNK1 and JNK2 are ubiquitously expressed, whereas expression of JNK3 is restricted largely to brain, heart and testis [2,7,19]. To determine whether the regulation of the HA-JNK3 isoforms transfected into the SCLC cells and PC12 cells is strictly an example of activation of an ectopically expressed enzyme, we used reverse transcription and PCR to test for expression of JNK3 mRNA in these cell lines. The findings in Figure 3(A) reveal a JNK3 cDNA product that is amplified from rat brain mRNA, as well as mRNA isolated from the two SCLC cell lines that co-migrate with the cDNA product amplified from an authentic JNK3 cDNA. Direct sequence analysis verified that the amplified products from brain and SCLC cells were rat and human JNK3 respectively. In contrast, the assay failed to detect JNK3 mRNA in PC12 cells, regardless of whether they exhibited the chromaffin cell phenotype or the neuronal phenotype induced by incubation with nerve growth factor for 7 days. The amplification of a β-actin cDNA product in these samples verified the integrity of the mRNA preparations. Thus, JNK3 is endogenously expressed in the SHP77 SCLC cells, but not PC12 cells, despite the fact that stress. The experiments performed in the SCLC transfectants indicated that the HA-JNK1 and HA-JNK2β expression vectors encode functional protein kinases that are significantly stimulated by UV irradiation and osmotic stress (Figure 2A). A possible mechanism for the lack of regulation of JNK1 and JNK2β in PC12 cells is the induction or activation of a phosphatase activity by over-expression of these specific JNK isoforms, which then inhibits or reduces the activation of JNKs. However, when the GST-cJun binding assay was performed on extracts from HA-p46JNK1-expressing PC12 cells that had been stimulated with UV, a similar stimulation of JNK activity was observed as in extracts from LNCX controls (results not shown).

The cDNAs encoding HA-tagged MKK4 and MKK7 were expressed in SHP77 cells by retrovirus-mediated gene transfer. Extracts from pooled populations of the G418-resistant cells were immunoblotted with anti-HA antibodies to verify expression of the HA-MKK4 and HA-MKK7 polypeptides (inset). SHP77 cells expressing the empty vector (LNCX) or the HA-MKKs were UV irradiated or incubated with 300 mM mannitol as described in Figures 1 and 2. Extracts were prepared and HA-MKKs were collected by anti-HA immunoprecipitation and assayed for protein kinase activity with recombinant kinase-inactive GST–JNK3. The upper panel shows a representative autoradiogram of the phosphorylated GST–JNK3 resolved by SDS-PAGE and the lower panel shows the mean activity±S.E.M. of three independent experiments.

Figure 3 Expression of JNK and MKK isoforms in SCLC cells and PC12 cells

(A) Samples of poly(A)+ RNA prepared from rat brain as a positive control and the indicated cell lines were reverse transcribed and submitted to PCR with primers specific for JNK3 or β-actin as a control for RNA integrity. Rat JNK3 cDNA was used as a positive control for the PCR reaction and as an electrophoresis mobility standard. DNA products in the reverse-transcription PCR reactions were resolved by electrophoresis through a 1% agarose gel and visualized by staining with ethidium bromide. No products were obtained when reverse transcriptase was excluded from the reactions (results not shown). The JNK3 products obtained from rat brain and H345 SCLC RNA were cloned into pT7 Blue (Novagen) and sequenced to verify that the JNK3 primers had amplified JNK3 mRNA present in these samples. (B) MAP kinase lysis buffer extracts prepared from SHP77 SCLC cells and PC12 cells were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-MKK4 (C-20; Santa Cruz) or anti-MKK7 (T-19; Santa Cruz). The bound antibodies were visualized with secondary antibodies coupled to horseradish peroxidase and ECL.

Figure 4 Regulation of HA-MKK4 and HA-MKK7 expressed in SHP77 SCLC cells by UV and osmotic stress

The experiments performed in the SCLC transfectants indicated that the HA-JNK1 and HA-JNK2β expression vectors encode functional protein kinases that are significantly stimulated by UV irradiation and osmotic stress (Figure 2A). A possible mechanism for the lack of regulation of JNK1 and JNK2β in PC12 cells is the induction or activation of a phosphatase activity by over-expression of these specific JNK isoforms, which then inhibits or reduces the activation of JNKs. However, when the GST-cJun binding assay was performed on extracts from HA-p46JNK1-expressing PC12 cells that had been stimulated with UV, a similar stimulation of JNK activity was observed as in extracts from LNCX controls (results not shown).

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both cell types exhibit a neuroendocrine phenotype. Because the HA-JNK3 expression must be considered ectopic in PC12 cells, p54NKJκα is probably the only endogenous JNK isoform of the five considered in this study that is regulated by UV and osmotic stress in these cells. In contrast, the HA-JNK activation profiles observed in Figures 2(A) and 2(B) are likely to be representative of the regulation of the endogenous JNK isoform equivalents in SCLC cells.

To date, two distinct MKKs specific for the JNK pathway have been identified, MKK4 and MKK7 [3–5]. The failure of UV and mannitol to stimulate JNK1 and JNK2β in PC12 cells could be due to the lack of expression of one of these MKK isoforms. However, immunoblot analysis of cell extracts from SHP77 SCLC cells and PC12 cells with antisera specific for MKK4 and MKK7 revealed a similar level of expression of each of these MKKs in the two cell types (Figure 3B). Thus, the failure of JNK1 and JNK2β to be regulated in PC12 cells is likely to be a result of differential expression of a still uncharacterized MKK isoform or, more likely, differential expression or activation of one of the many MKK kinases (MKKks) [20] that integrate proximal signals into the JNK pathway.

To assess the possibility of differential regulation of MKK4 and MKK7 by UV and osmotic stress, HA-tagged MKK4 and MKK7 were expressed in SHP77 cells by retrovirus-mediated gene transfer (see the Materials and methods section). Figure 4 (inset) reveals equivalent expression of HA-MKK4 and HA-MKK7 in SHP77 SCLC cells as assessed by anti-HA immunoblotting. Analysis of the protein kinase activity of HA-MKK4 and HA-MKK7 immunoprecipitated from extracts from control and stimulated cells indicated that HA-MKK4 was equally stimulated by UV and 300 mM mannitol, whereas HA-MKK7 was regulated only by osmotic stress and not UV irradiation. A similar pattern of HA-MKK4 and HA-MKK7 regulation by UV and mannitol was also observed in PC12 cell transfectants expressing HA-MKK4 and HA-MKK7 (results not shown).

The selective regulation of JNK3 and JNK2α, but not JNK1α and JNK2β, after expression in PC12 cells is unexpected. All five isoforms were strongly activated when expressed in SHP77 cells and a previous report demonstrated regulation of all ten known JNK isoforms by interleukin 1 after transient expression in Chinese hamster ovary cells [6], p54NKJκα and p54NKJκβ differ overall by only nine amino acids which are found within the alternative splice residing between protein kinase domains IX and X [6]. Interestingly, comparison of the primary sequences of the α splice region of JNK1α and the β splice region of JNK2 reveal that they are more similar (four differences) than are the α and β splices of JNK2 (nine differences). The alternative α/β splices have been previously shown to influence the affinity of the JNK isoforms for substrates [6,9], a finding supported by the recently reported crystal structure of JNK3 [21], which indicates that this region of the JNK polypeptide is located on the protein surface corresponding to the peptide substrate binding channel in CAM-dependent protein kinase. However, the failure of UV or osmotic stress to regulate JNK1α or JNK2β in PC12 cells indicates that the peptide sequences encoded by these alternative splices may also serve as binding sites that co-ordinate specific proximal MKKK and MKK regulatory inputs as well.

The selective JNK isoform activation observed in this study was not accounted for by differential expression of the known JNK-specific MKKs (Figure 3B). In fact, the differential regulation of MKK4 and MKK7 by UV and osmotic stress (Figure 4) indicates that stress and cell type-dependent influences on JNK isoform regulation is accomplished at more proximal points, perhaps at the level of the MKKks. Our findings are consistent with the emerging idea of MAP kinase signalling modules composed of specific MKKKs, MKKs and MAPKs [17,22]. Genetic and biochemical studies in yeast provide evidence that these three component MAPK modules are assembled by scaffolding proteins or by interacting domains within the protein kinases themselves [22–24]. Recent studies have identified two distinct proteins with putative scaffolding functions for MAP kinases in mammalian cells [25,26]. Also, direct binding of the N-terminal regulatory domains of MKKK, MKK1 and JNK has been observed [27]. Thus, it is interesting to speculate that cell-specific expression of particular MKKks, a still-emerging family of protein kinases [20], or scaffolding proteins, could account for the stress activation of JNK1α and JNK2β in SCLC cells, but not in PC12 cells.

In summary, analysis of the regulation of five distinct JNK isoforms and two MKKs stably transfected into PC12 cells and SCLC cells indicates that they are not equivalently regulated. From these findings, one may predict that the ten known JNK isoforms are unlikely to function equivalently in diverse cell programmes where activation of JNKs has been observed. In some instances, specific JNK regulation may be achieved through tissue-specific expression, as has been demonstrated for JNK3 polypeptides [19,28]. In other cases, we hypothesize that selective JNK regulation may be achieved through differential expression or utilization of proximal regulatory components, including MKKks [20].

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