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Chemical Genetic Analysis of the Time Course of Signal Transduction by JNK

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Summary

Exposure of primary murine embryonic fibroblasts to tumor necrosis factor (TNF) causes biphasic activation of the c-Jun NH_2-terminal kinase (JNK) signaling pathway. The early phase (30 min) of the response to TNF is a large and transient increase in JNK activity. This response is followed by a second and more sustained phase of JNK activation that lasts many hours. We employed a chemical genetic strategy to dissect the functional consequences of these two phases of JNK activation. We report that both the early and late phases of JNK activation contribute to TNF-induced gene expression. In contrast, the early transient phase of JNK activation (<1 hr) can signal cell survival, while the later and more sustained phase of JNK activation (1–6 hr) can mediate proapoptotic signaling. These data indicate that the time course of JNK signaling can influence the biological response to JNK activation.

Introduction

Tumor necrosis factor (TNF) is a proinflammatory cytokine that regulates cell differentiation, survival, and apoptosis. The response of cells to TNF represents a balance between the actions of opposing signal transduction pathways. Thus, TNF receptor 1 (TNF-R1) can assemble TRADD/TRAF2/RIP1 (complex 1) that initiates antiapoptotic responses mediated by NF-κB activation (Kelliher et al., 1998; Yeh et al., 1997). The TRADD/TRAF2/RIP1 complex at the receptor is transient. Following the release of complex I from the receptor, it associates with FADD and Caspase 8 to form complex II that can initiate apoptosis (Micheau and Tschopp, 2003). The balance between these opposing pathways that signal survival (complex I) and apoptosis (complex II) can therefore determine the consequences of the cellular response to TNF (Varfolomeev and Ashkenazi, 2004).

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The c-Jun NH_2-terminal kinase (JNK) signaling pathway is also activated by TNF (Kyriakis et al., 1994; Sluss et al., 1994). JNK is activated in a biphasic manner. The early phase of JNK activation (5–30 min) is robust but transient. The mechanism of receptor-mediated JNK activation during this early phase involves the adaptor protein TRAF2 (Yeh et al., 1997). The molecular events initiated by TRAF2 that mediate TNF-induced JNK activation are unclear; nevertheless, it is known that TNF causes activation of MAP3K isoforms, including MLK3 (Brancho et al., 2005; Sathyanarayana et al., 2002), TPL2/COT (Das et al., 2005), and TAK1 (Sato et al., 2005; Shim et al., 2005). The activation of these MAP3K isoforms then leads to signaling by the canonical protein kinase cascade that leads to JNK activation (Tournier et al., 2001).

The early transient phase of TNF-stimulated JNK activation is followed by a late phase of sustained JNK activation (Kyriakis et al., 1994; Sluss et al., 1994). This late phase of TNF-stimulated JNK activation does not require TRAF2 and appears to be mediated by TNF-stimulated production of reactive oxygen species (ROS) (Sakon et al., 2003). The effect of ROS to activate JNK is mediated, in part, by phosphatase inhibition (Kamata et al., 2005) and also by the activation of the MAP3K isoform ASK1 (Tobiume et al., 2001). The extent of ROS production is negatively regulated by NF-κB (Pham et al., 2004; Sakon et al., 2003) and is positively regulated in an amplification loop by JNK (Ventura et al., 2004).

The observation that TNF causes an immediate and transient activation of JNK by a TRAF2-dependent mechanism and a sustained late phase of JNK activation that is mediated by TNF-induced ROS production raises questions concerning the role of these two phases of TNF-stimulated JNK activation. What is the function of JNK in the cellular response to TNF? It is established that JNK activation is critical for TNF-stimulated AP-1-dependent gene expression (Ventura et al., 2003). However, the role of JNK in TNF-stimulated cell death is controversial. It appears that the role of JNK in TNF-stimulated death depends upon the physiological context (Davis, 2000).

JNK is not essential for TNF-stimulated cell death (Lamb et al., 2003; Liu et al., 1996; Natoli et al., 1997; Sakon et al., 2003; Yeh et al., 1997) and may signal a survival response in TNF-treated cells (Lamb et al., 2003; Lee et al., 1997; Reuther-Madrid et al., 2002; Roulston et al., 1998). However JNK activation, especially that observed following inhibition of the NF-κB pathway, can contribute to death signaling (De Smaele et al., 2001; Deng et al., 2003; Guo et al., 1998; Lamb et al., 2003; Liu et al., 2004; Papa et al., 2004; Tang et al., 2001, 2002; Ventura et al., 2004). Together, these studies indicate that JNK may contribute to both cell death and survival responses in TNF-stimulated cells (Varfolomeev and Ashkenazi, 2004).

An understanding of the function of JNK in the TNF response requires an explanation for the dual role of this protein kinase in cell survival and death. One possible explanation is that JNK-induced AP-1 activity increases

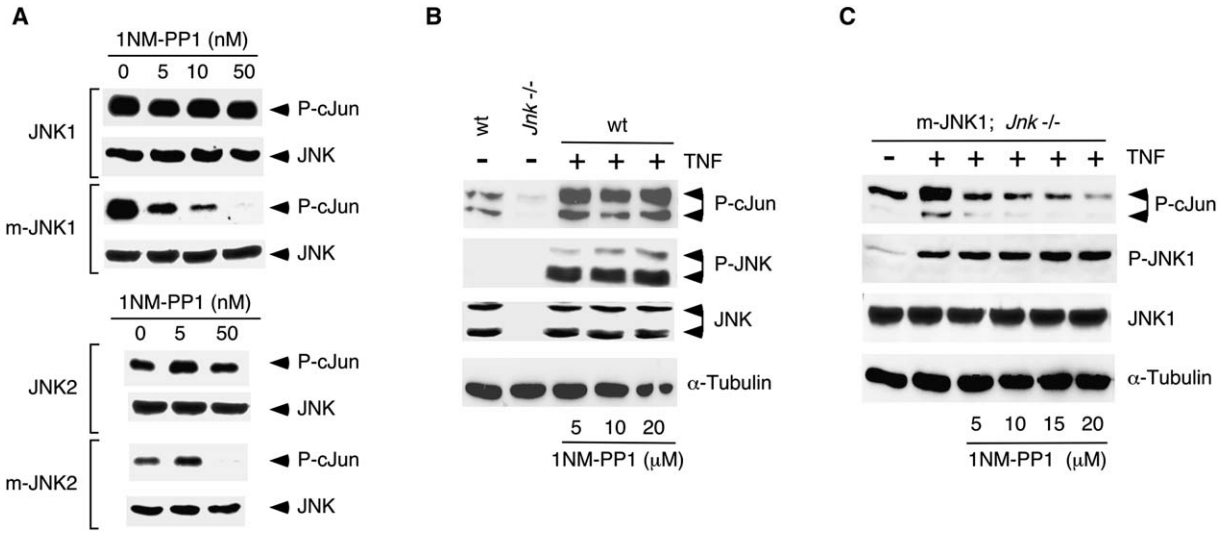

Figure 1. A Chemical Genetic Strategy for the Analysis of JNK Signaling

(A) The drug 1NM-PP1 selectively inhibits mutated JNK (m-JNK; [M108G, L168A]JNK) in vitro. Flag-tagged JNK proteins expressed in COS cells were activated by exposing the cells to 60 J/m^2 UV-C radiation. The activity of JNK was examined in an in vitro immune complex protein kinase assay using c-Jun and ATP[γ-^{32}P] as substrates. Phosphorylated c-Jun was identified by autoradiography following analysis by SDS-PAGE. The amount of JNK was examined by immunoblot analysis. The effect of the addition of the drug 1NM-PP1 to the in vitro kinase assay was examined. Assays using wild-type JNK1 and m-JNK1 (upper panel) and wild-type JNK2 and m-JNK2 (lower panel) are shown.

(B) The drug 1NM-PP1 does not inhibit JNK in vivo. Wild-type (wt) and *Jnk1*$^{-/-}$*Jnk2*$^{-/-}$ (*Jnk*$^{-/-}$) MEFs were incubated without and with 10 ng/ml TNF (15 min). Cell lysates were prepared and probed for tubulin, JNK, phospho-JNK, and phospho-cJun by immunoblot analysis.

(C) The drug 1NM-PP1 inhibits m-JNK1 in vivo. MEFs expressing m-JNK1 were prepared by transduction of *Jnk*$^{-/-}$ MEF with a recombinant retrovirus that expresses m-JNK1. Wild-type and *Jnk*$^{-/-}$ MEFs were incubated without and with 10 ng/ml TNF (15 min). Cell lysates were prepared and probed for Tubulin, JNK, phospho-JNK, and phospho-cJun by immunoblot analysis.

antiapoptotic signaling mediated by the NF-κB and Akt signaling pathways and that cell death signaling by JNK requires inhibition of one of these survival signaling pathways (Lamb et al., 2003). Thus, TNF-stimulated JNK activation antagonizes apoptosis but can promote cell death in cells with reduced NF-κB function (Ventura et al., 2004). This interpretation suggests that it is the interaction of the JNK and NF-κB signaling pathways that controls the effects of JNK on cell survival and death. One component of this interaction is the combinatorial actions of these signaling pathways on gene expression (Lamb et al., 2003). However, a second component of the interaction between these signaling pathways is the effect of NF-κB to repress TNF-stimulated ROS production and consequently the late phase of TNF-stimulated JNK activation (De Smaele et al., 2001; Reuther-Madrid et al., 2002; Sakon et al., 2003; Tang et al., 2001). These considerations indicate that the time course and biphasic nature of TNF-stimulated JNK activation may be critical for determining the biological function of JNK. This is consistent with the results of earlier studies that have implicated the time course of protein kinase signaling as a determining factor in biological specificity (Marshall, 1995).

The purpose of this study was to test the hypothesis that the time course of JNK activation is mechanistically relevant to the cellular response.

Results

Chemical Genetic Analysis of JNK Signaling

TNF causes an immediate and transient phase of JNK activation that is followed by a sustained phase of low-level JNK activation (Kyriakis et al., 1994; Sluss et al., 1994). Previous studies using cells derived from mice with compound mutations in the *Jnk* genes have addressed the requirement of JNK for signaling (Tournier et al., 2000). However, this approach does not allow the examination of the temporal requirement of JNK. An alternative approach is therefore required. Pharmacological inhibition of JNK represents one possible approach. However, small molecule inhibitors with an established specificity for JNK have not been described (Manning and Davis, 2003). We therefore employed a chemical genetic approach (Bishop et al., 2000) using JNK protein kinases that have been engineered to enlarge the ATP pocket (Habelhah et al., 2001), resulting in sensitization to inactive derivatives of the general protein kinase inhibitor PP1.

Wild-type JNK1 or JNK1 with mutations that increase the size of the ATP pocket (replacement of Met-108 with Gly and Leu-168 with Ala) were expressed in *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ (*Jnk*$^{-/-}$) MEFs that lack expression of endogenous JNK (Figure 1A). Cells expressing wild-type and mutated JNK2 were also prepared. In vitro protein kinase assays demonstrated that the drug 1-naphthylmethyl-4-amino-1-*tert*-butyl-3-(*p*-methylphenyl)pyrazolo [3,4-*d*]pyrimidine (1NM-PP1) (Shokat and Velleca, 2002) potently inhibited mutated JNK1 (m-JNK1) but did not inhibit wild-type JNK1 (Figure 1A). Similarly, the drug 1NM-PP1 inhibited mutated JNK2 (m-JNK2) but not wild-type JNK2 in vitro (Figure 1A).

The drug 1NM-PP1 was designed to act as a competitive inhibitor of the protein kinase activity of enzymes with an expanded ATP pocket (Bishop et al., 2000). We therefore expected that 1NM-PP1 would block the

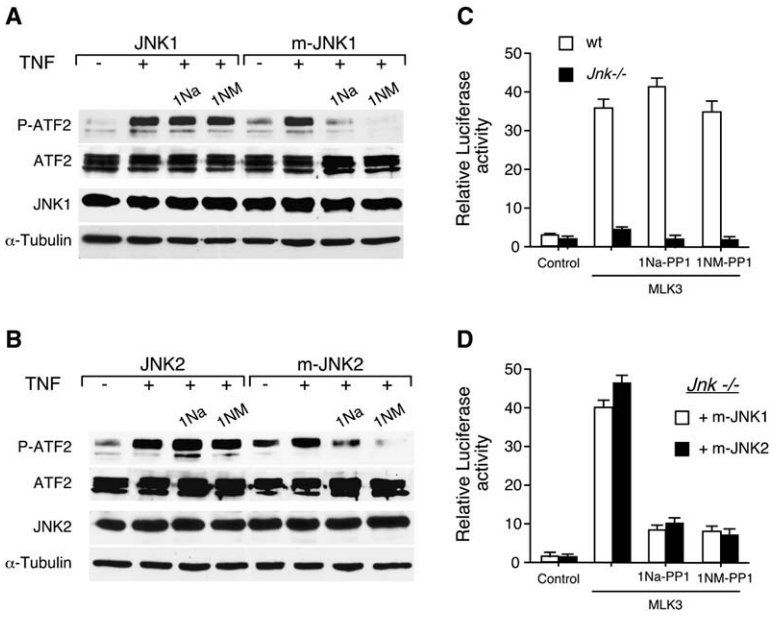

Figure 2. Chemical Genetic Analysis of JNK-Dependent ATF2 Transcription Activity

(A and B) The drug 1NM-PP1 is a selective inhibitor of m-JNK in vivo. MEFs expressing wild-type JNK1 or m-JNK1 (A) and wild-type JNK2 or m-JNK2 (B) were prepared by transduction of $Jnk^{-/-}$ MEF with recombinant retroviruses. The cells were incubated with 10 ng/ml TNF (15 min). The effect of preincubation (30 min) of the cells with 5 μM 1Na-PP1 (1Na) or 1NM-PP1 (1NM) is shown. Cell lysates were prepared and probed for Tubulin, JNK1, ATF2, and phospho-ATF2 by immunoblot analysis.

(C) Wild-type and $Jnk^{-/-}$ MEFs were examined in a transient transfection assay using a GAL4-dependent luciferase reporter gene assay and a recombinant transcription factor composed of the activation domain of ATF2 fused to the DNA binding domain of GAL4. JNK was activated by coexpression with the MAP3K isoform MLK3. The effect of the addition of 20 μM 1Na-PP1 or 1NM-PP1 is shown. The data presented represent the mean ± SD of triplicate determinations obtained in one experiment. Similar results were obtained in three independent experiments.

(D) ATF2 transcription activity is inhibited by 1Na-PP1 and 1NM-PP1 in cells that express m-JNK. MEFs expressing m-JNK1 or m-JNK2 were prepared by transduction of $Jnk^{-/-}$ MEF with recombinant retroviruses. ATF2 transcription activity was examined using a GAL4-dependent luciferase reporter gene assay and a recombinant transcription factor composed of the activation domain of ATF2 fused to the DNA binding domain of GAL4. The effect of the addition of 20 μM 1Na-PP1 or 1NM-PP1 is shown. The data presented represent the mean ± SD of triplicate determinations obtained in one experiment. Similar results were obtained in three independent experiments.

activity of m-JNK but not the activation of m-JNK. To test this hypothesis, we examined the activation of m-JNK and the phosphorylation of a JNK substrate in vivo. It is established that JNK is essential for TNF-stimulated phosphorylation of c-Jun within the NH_2-terminal activation domain (Ventura et al., 2003). We therefore examined c-Jun phosphorylation to test whether 1NM-PP1 inhibited JNK in vivo. Treatment of wild-type MEF with the drug 1NM-PP1 caused no change in c-Jun phosphorylation (Figure 1B). However, this drug caused a dose-dependent decrease in c-Jun phosphorylation in $Jnk^{-/-}$ MEFs that express m-JNK (Figure 1C). Immunoblot analysis with an antibody to activated (phosphorylated) JNK demonstrated that the decrease in c-Jun phosphorylation was not caused by reduced TNF-stimulated JNK activation (Figure 1C). Thus, the drug 1NM-PP1 functions as a selective inhibitor of the protein kinase activity of JNK with an expanded ATP pocket.

The concentration of the drug 1NM-PP1 required to inhibit m-JNK activity in vitro (Figure 1A) was lower than that required to inhibit JNK activity in vivo (Figure 1C). This difference most likely reflects the function of 1NM-PP1 as an ATP competitive inhibitor of protein kinases with pocket mutations and the approximately 100-fold higher concentration of ATP in vivo compared with in vitro kinase assays (Knight and Shokat, 2005).

Since the conclusion that 1NM-PP1 can cause selective inhibition of m-JNK was based on an analysis of c-Jun phosphorylation (Figure 1), we sought to confirm this finding by examining the effect of the drug 1NM-PP1 on a different JNK substrate, the transcription factor ATF2 (Gupta et al., 1995; Livingstone et al., 1995). 1NM-PP1 caused marked inhibition of TNF-stimulated phosphorylation of the NH_2-terminal activation domain of ATF2 in $Jnk^{-/-}$ MEFs that express m-JNK1 or m-JNK2 but not in cells expressing wild-type JNK1 or JNK2 (Figures 2A and 2B). The drug 1NM-PP1 was a more effective inhibitor of ATF2 phosphorylation than the related molecule 1Na-PP1 (Figures 2A and 2B). JNK phosphorylation of ATF2 causes increased transcription activity (Gupta et al., 1995; Livingstone et al., 1995). We therefore investigated the effect of the altered specificity protein kinase inhibitors 1NM-PP1 and 1Na-PP1 on ATF2-dependent reporter gene expression. JNK activation induced by the MAP3K isoform MLK3 caused increased ATF2 transcription activity in wild-type MEF but did not increase reporter gene expression in $Jnk^{-/-}$ MEF (Figure 2C). The drugs 1NM-PP1 and 1Na-PP1 caused no changes in ATF2-dependent reporter gene expression in assays using wild-type or $Jnk^{-/-}$ MEF (Figure 2C). However, these drugs markedly inhibited ATF2 transcription activity in $Jnk^{-/-}$ MEFs that express m-JNK1 or m-JNK2 (Figure 2D). Together, these data establish that a chemical genetics strategy can be successfully applied to the experimental manipulation of JNK function.

Requirement of JNK for TNF-Induced Gene Expression

The regulation of gene expression represents an important function of JNK (Davis, 2000). The *interleukin-6* (*Il-6*) gene is one target of the TNF-stimulated JNK signaling pathway that contributes to inflammatory responses (Ventura et al., 2003). Indeed, we found that exposure of wild-type MEF to TNF caused a marked increase in both JNK activation and *Il-6* gene expression (Figure 3). Interestingly, the induction of IL-6 mRNA was biphasic with a large and transient increase in IL-6 mRNA within 30–60 min that was followed by a second and sustained

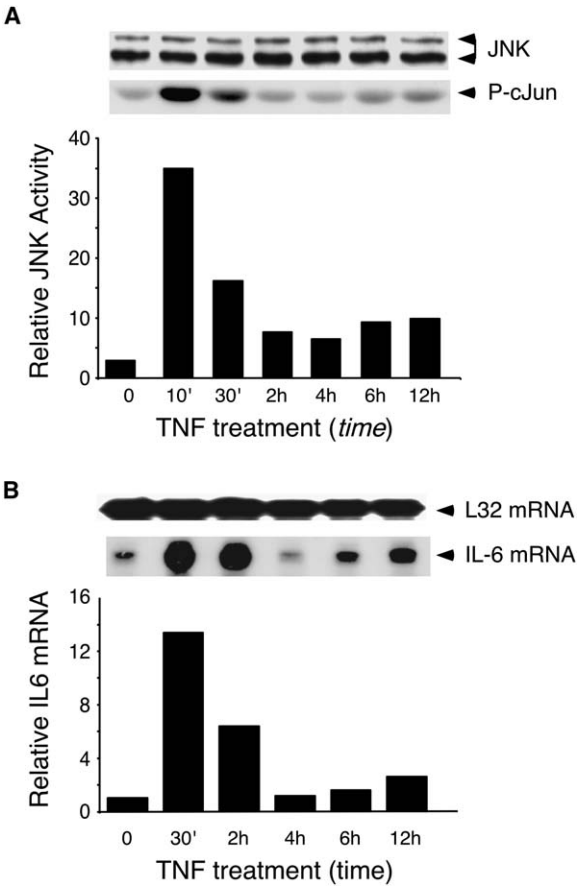

Figure 3. TNF Causes a Biphasic Increase in Both JNK Activity and IL-6 Expression

(A) Wild-type MEFs were incubated with TNF (10 ng/ml). Extracts were prepared, and the amount of JNK was examined by immunoblot analysis. JNK activity was measured in an immune complex kinase assay using cJun as the substrate and was quantitated by Phosphorimager analysis.

(B) Total RNA was isolated, and the expression of IL-6 mRNA was examined using a RNase protection assay, detected by autoradiography, and quantitated by Phosphorimager analysis. The expression of ribosomal protein L32 mRNA was employed as a loading control. The effect of treatment of wild-type MEF with TNF (10 ng/ml) is presented.

phase of increased IL-6 mRNA at later times (Figure 3A). These changes in *Il-6* gene expression correlate with the changes in JNK activity caused by TNF (Figure 3B).

To test the temporal requirement of JNK for *Il-6* gene expression, we examined the effect of TNF in experiments using $Jnk^{-/-}$ MEF complemented with wild-type JNK or m-JNK. Control studies demonstrated that TNF caused increased expression of IL-6 mRNA in wild-type cells but not in $Jnk^{-/-}$ MEF (Figures 4A and 4B). TNF-induced IL-6 expression was restored in $Jnk^{-/-}$ MEFs that express wild-type JNK or m-JNK. No changes in *Il-6* gene expression were detected when the drug 1NM-PP1 was added to wild-type MEF, $Jnk^{-/-}$ MEF, or $Jnk^{-/-}$ MEFs that express wild-type JNK (Figures 4A and 4B). However, treatment of $Jnk^{-/-}$ MEF expressing m-JNK1 or m-JNK2 with 1NM-PP1 before the addition of TNF strongly suppressed the TNF-induced increase in *Il-6* gene expression at 30 min (Figure 4A) and at 24 hr (Figure 4B). These data confirm that JNK activity is required for TNF-induced IL-6 expression.

To further examine the requirement of JNK for TNF-induced *Il-6* gene expression, we examined the *Il-6* promoter using ChIP assays. Control studies using an antibody to histone H3 demonstrated a similar amount of *Il-6* gene chromatin in each sample (Figure 4C). TNF induced the recruitment of RNA polymerase II (Pol II) to the *Il-6* promoter in wild-type MEFs and $Jnk^{-/-}$ MEFs that express m-JNK1 or m-JNK2 (but not in $Jnk^{-/-}$ MEF). The drug 1NM-PP1 caused decreased Pol II recruitment only in $Jnk^{-/-}$ MEFs that express m-JNK1 or m-JNK2 (Figure 4C). These changes in Pol II recruitment (Figure 4C) are consistent with the observed changes in IL-6 mRNA expression (Figure 3A). The pattern of TNF-induced recruitment of c-Jun to the *Il-6* promoter and the TNF-induced modification of histone H3 bound to the promoter on phospho(Ser^{10})/acetyl-Lys^{9} similarly reflected these changes in *Il-6* gene expression (Figure 4C). However, the recruitment of p65/NF-κB to the promoter displayed a different pattern, since TNF-induced recruitment of p65/NF-κB to the *Il-6* promoter was detected in all of the cell types examined; this is consistent with the observations that TNF-induced p65/NF-κB activation in wild-type and $Jnk^{-/-}$ MEF is similar (Lamb et al., 2003) and that the drug 1NM-PP1 does not affect p65/NF-κB activation in $Jnk^{-/-}$ cells that express m-JNK1 or m-JNK2. Interestingly, the drug 1NM-PP1 did reduce, but did not eliminate, the recruitment of p65/NF-κB to the *Il-6* promoter in $Jnk^{-/-}$ cells that express m-JNK1 or m-JNK2 (Figure 4C). These data indicate that JNK contributes to the recruitment of p65/NF-κB to the *Il-6* promoter. It is possible that the reduced recruitment of p65/NF-κB caused by loss of JNK function is related to the observation that the *Il-6* gene is transcriptionally inactive in JNK-deficient cells (Figures 4A and 4B).

To examine the role of the different phases of JNK activation in *Il-6* gene expression, we investigated the effect of changing the time of addition of the drug 1NM-PP1 (Figure 4D). These data indicated that the early transient phase of JNK activation is not sufficient for sustained TNF-induced *Il-6* gene expression because the drug 1NM-PP1 was equally capable of inhibiting IL-6 expression when it was added to cultures after the initial transient phase of JNK activation (60 min) or prior to the addition of TNF. The simplest interpretation of these data is that JNK activity is continuously required for TNF-induced *Il-6* gene expression and that JNK contributes to both the early and late phases of TNF-induced gene expression.

Requirement of JNK for Apoptotic Signal Transduction

It is established that JNK can contribute to cell death signaling (Tournier et al., 2000). However, JNK-deficient MEFs do not exhibit defects in TNF-stimulated cell death unless the NF-κB pathway is inhibited (Ventura et al., 2004). To test the requirement of JNK for apoptosis, we therefore examined the effect of a different form of stress (UV radiation) on apoptosis that causes JNK-dependent death in cells with a functional NF-κB signaling pathway. Our approach was to investigate the effect

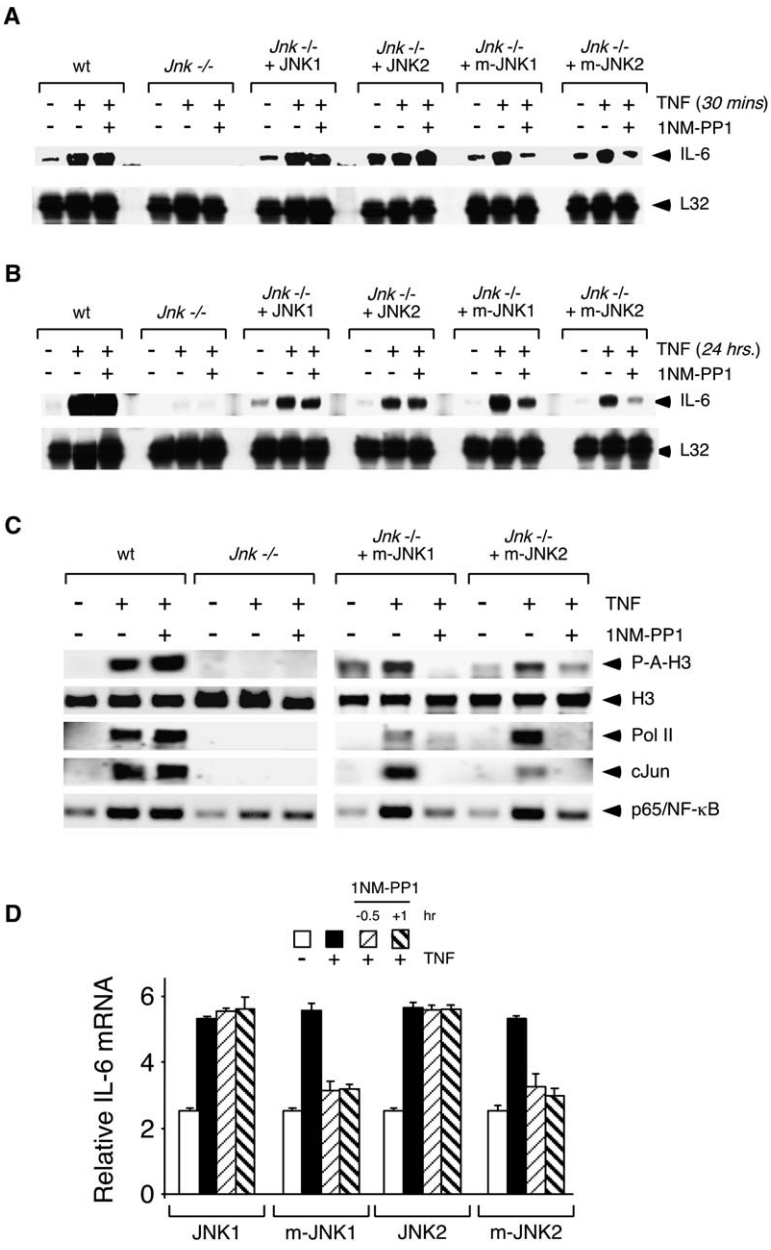

Figure 4. JNK Activity Is Required for TNF-Stimulated IL-6 Expression

(A and B) MEFs expressing wild-type JNK or mutated-JNK were prepared by transduction of *Jnk*$^{-/-}$ MEF with recombinant retroviruses. Control experiments were performed using wild-type (wt) and *Jnk*$^{-/-}$ MEF. The cells were incubated with 10 ng/ml TNF for 30 min (A) or 24 hr (B). The effect of preincubation (30 min) of the cells with 5 μM 1NM-PP1 is shown. The expression of IL-6 and L32 mRNA was examined using RNase protection assays.

(C) ChIP assays were performed to detect histone H3 (H3), phospho-acetyl histone H3 (P-A-H3), Pol II, cJun, and p65 NF-κB associated with the *Il-6* gene promoter. The effect of addition of 10 ng/ml TNF (30 min) or pretreatment with 5 μM 1NM-PP1 (5 min) to the culture medium was examined.

(D) MEFs expressing wild-type JNK or mutated-JNK were prepared by transduction of *Jnk*$^{-/-}$ MEF with recombinant retroviruses. The effect of addition of the drug 1NM-PP1 (5 μM) at different times on the TNF (10 ng/ml)-stimulated expression of IL-6 mRNA at 24 hr was examined using RNase protection assays. The expression of ribosomal protein L32 mRNA was employed as a loading control. The data presented represent the mean ± SD of triplicate determinations obtained in one experiment. Similar results were obtained in three independent experiments.

of 1NM-PP1 on cells expressing drug-sensitized forms of JNK (m-JNK). Control studies demonstrated that *Jnk*$^{-/-}$ MEF exhibited reduced UV-stimulated apoptotic DNA fragmentation compared with wild-type MEF (Figure 5A). Complementation studies using *Jnk*$^{-/-}$ MEFs that express m-JNK1 or m-JNK2 demonstrated that the reduced UV-induced DNA fragmentation was caused by JNK deficiency (Figure 5A). To test whether JNK protein kinase activity contributed to the proapoptotic response, we examined the effect of 1NM-PP1. Treatment of wild-type or *Jnk*$^{-/-}$ MEF with 1NM-PP1 caused no changes in apoptotic DNA fragmentation. In contrast, treatment of *Jnk*$^{-/-}$ MEFs that express m-JNK1 or m-JNK2 with 1NM-PP1 caused a dose-dependent suppression of apoptotic DNA fragmentation (Figure 5A). These data demonstrate that JNK protein kinase activity contributes to the apoptotic response. To confirm this conclusion, we investigated UV-stimulated death of *Jnk*$^{-/-}$ MEFs that express JNK1 or m-JNK1 by propidium iodide (PI) staining and analysis by flow cytometry (see Figure S1 in the Supplemental Data available with this article online). Cells expressing either JNK1 or m-JNK1 exhibited increased PI staining following exposure to UV (Figure S1). Analysis of PI-positive cell size by measurement of light scattering by flow cytometry (Kamata et al., 2005) demonstrated that most of the dead PI-positive cells were small, consistent with apoptotic death. However, a small number of large PI-positive cells, consistent with necrotic death, were detected. These data indicate that UV caused increased apoptosis and necrosis, but the majority of cell death was apoptotic. Treatment with the drug 1NM-PP1 suppressed UV-stimulated PI staining in cells that expressed m-JNK1, but not in cells that express

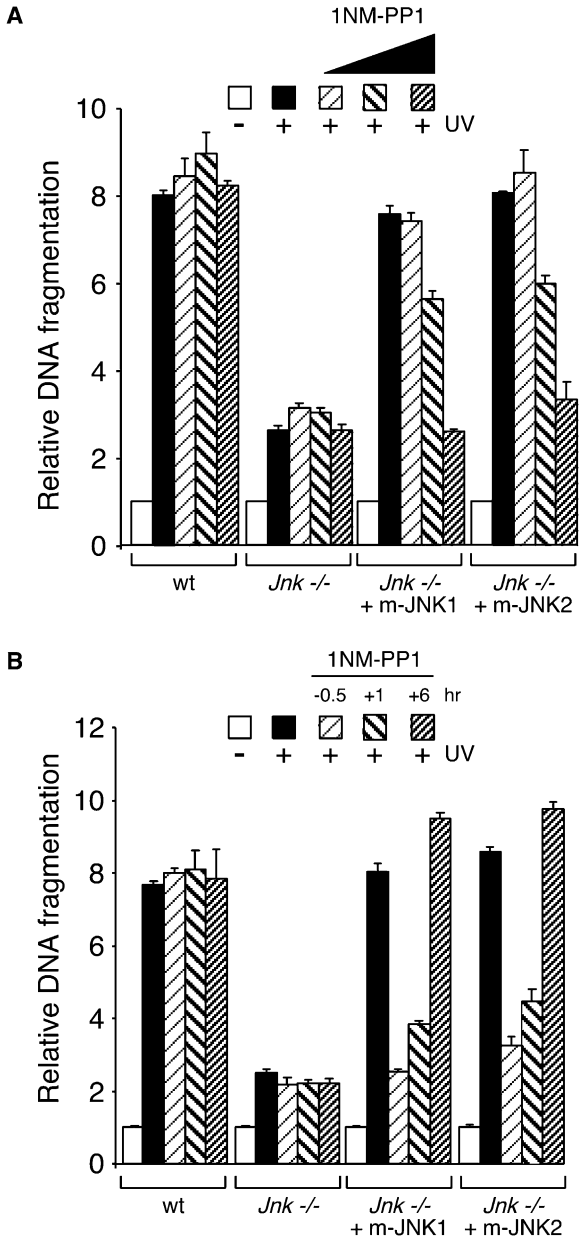

Figure 5. Sustained JNK Activity Is Required for Proapoptotic Signal Transduction

(A) MEFs expressing mutated JNK were prepared by transduction of $Jnk^{-/-}$ MEF with recombinant retroviruses. Control experiments were performed using wild-type (wt) and $Jnk^{-/-}$ MEF. The cells were treated without and with 60 J/m^2 UV and incubated for 24 hr. The effect of pretreatment (30 min) of the cells with increasing concentrations of the drug 1NM-PP1 (5, 7.5, 10 μM) was examined. Apoptotic DNA fragmentation was examined by ELISA. The data presented represent the mean ± SD of triplicate determinations obtained in one experiment. Similar results were obtained in three independent experiments.

(B) The effect of the time of the addition of the drug 1NM-PP1 (10 μM) on UV-stimulated apoptotic DNA fragmentation was examined. The data presented represent the mean ± SD of triplicate determinations obtained in one experiment. Similar results were obtained in three independent experiments.

wild-type JNK1 (Figure S1). Together, these data demonstrate that JNK activity contributes to UV-stimulated cell death.

To test the temporal requirement of JNK activity for UV-stimulated apoptosis, we examined the effect of the time of addition of 1NM-PP1 (Figure 5B). Pretreatment (−30 min) or posttreatment (+60 min) of MEFs that express m-JNK with 1NM-PP1 inhibited apoptotic DNA fragmentation, but 1NM-PP1 did not inhibit DNA fragmentation when added 6 hr after the apoptotic insult (Figure 5B).

These data demonstrate that, although JNK is required for apoptosis, the early transient activation of JNK (<1 hr) is not sufficient for apoptotic signaling and that JNK activation after 6 hr is not required for apoptosis. We conclude that JNK activity during the late and more sustained phase of JNK signaling after 1 hr and before 6 hr mediates proapoptotic signaling.

Requirement of JNK for Survival Signal Transduction

We have previously reported that JNK-deficient fibroblasts exhibit an accelerated apoptotic response compared with wild-type fibroblasts when incubated with TNF in the presence of inhibitors of macromolecular synthesis (Lamb et al., 2003). These data suggest that JNK has an antiapoptotic role in this paradigm of TNF-induced apoptosis (Lamb et al., 2003). To investigate whether this antiapoptotic role of JNK is mediated by one or more phases of TNF-induced JNK activation, we tested the effect of the addition of the drug 1NM-PP1. Control studies using immunoblot analysis demonstrated that the level of expression of m-JNK in $Jnk^{-/-}$ MEF was similar to the level of JNK expression wild-type MEF (Figure 6A). As expected, TNF caused death of both wild-type MEF and $Jnk^{-/-}$ MEF within 24 hr (Lamb et al., 2003), but apoptotic DNA fragmentation was markedly increased in $Jnk^{-/-}$ MEF compared with wild-type cells at earlier time points, including 6 hr following addition of TNF (Figure 6B). Complementation studies using wild-type JNK (Lamb et al., 2003) and m-JNK (Figure 6B) in $Jnk^{-/-}$ MEF demonstrated that the accelerated apoptosis of $Jnk^{-/-}$ MEF is mediated by JNK deficiency. These data confirm the conclusion that JNK can suppress apoptosis by signaling cell survival in this paradigm of TNF-induced apoptosis (Lamb et al., 2003). To test whether JNK protein kinase activity is required for survival signaling, we examined the effect of the addition of the drug 1NM-PP1. Treatment of wild-type MEF or $Jnk^{-/-}$ MEF with 1NM-PP1 caused no apoptotic changes in assays designed to measure DNA fragmentation (Figure 6B). In contrast, pretreatment of $Jnk^{-/-}$ MEF expressing m-JNK1 or m-JNK2 with 1NM-PP1 caused accelerated TNF-induced apoptosis compared with cells incubated without the drug (Figure 6B). This effect of the drug 1NM-PP1 was not observed if the drug treatment was initiated 30 min after the addition of TNF (Figure 6B). Together, these data indicate that the early phase of TNF-induced JNK activation is critical for survival signaling.

One mechanism that has been proposed to contribute to JNK-mediated survival signaling responses is the induced expression of the ubiquitin ligase cIAP2 (Lamb et al., 2003). Indeed, TNF-stimulated expression of cIAP2 is not detected in $Jnk^{-/-}$ MEF (Lamb et al., 2003). Complementation analysis demonstrates that the loss of cIAP2 observed in $Jnk^{-/-}$ MEF is caused by JNK deficiency because the expression of JNK1 in

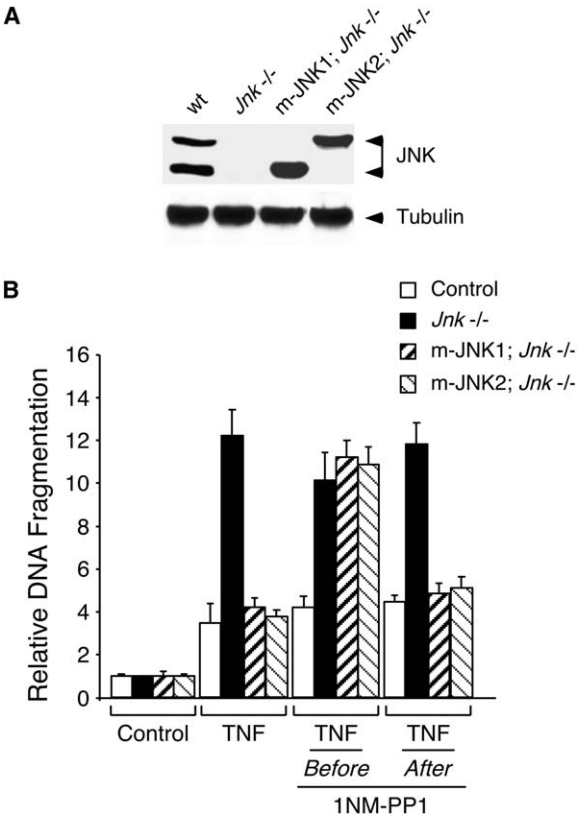

Figure 6. The Early Phase of TNF-Stimulated JNK Activation Is Required for Survival Signaling

(A) MEFs expressing mutated JNK were prepared by transduction of $Jnk^{-/-}$ MEF with recombinant retroviruses. Control experiments were performed using wild-type (wt) and $Jnk^{-/-}$ MEF. The amount of JNK and tubulin was examined by immunoblot analysis.

(B) MEFs were treated without and with 10 ng/ml TNF plus 2 μM emetine (TNF) for 6 hr. The effect of addition of the drug 1NM-PP1 (10 μM) to the culture medium 30 min before or 30 min after the addition of TNF was examined. Apoptotic DNA fragmentation was investigated after 6 hr of incubation with TNF by ELISA. The data presented represent the mean ± SD of triplicate determinations obtained in one experiment. Similar results were obtained in four independent experiments.

$Jnk^{-/-}$ MEF restores the ability of TNF to induce cIAP2 (Lamb et al., 2003). Similarly, the expression of JNK1 with an expanded ATP pocket (m-JNK1) in $Jnk^{-/-}$ MEF also restored TNF-stimulated cIAP2 expression (data not shown). These data demonstrate that JNK is required for TNF-stimulated cIAP2 expression.

The late phase of JNK activation does not appear to contribute to TNF-mediated survival signaling (Figure 6B). We therefore tested whether the late phase of JNK activation may contribute to cIAP2 expression using the chemical genetic approach. These studies demonstrated that 1NM-PP1 treatment of $Jnk^{-/-}$ MEFs that express m-JNK1 caused only a modest decrease (<10%) in cIAP2 mRNA expression (data not shown). Together, these data demonstrate that the late phase of TNF-stimulated JNK activation does not contribute to survival signaling or cIAP2 expression; nevertheless, our data establish that the early phase of JNK activation is important for signaling TNF-mediated survival.

Discussion

The results of this study indicate that the time course of JNK activation is critically important for signal transduction; for example, apoptotic signaling by JNK requires sustained activation (Figure 5), while transient activation can signal cell survival (Figure 6). This role of the time course of JNK activation provides an explanation, in part, for the opposing biological consequences of JNK activation under different physiological conditions.

Our conclusions concerning the role of the time course of JNK signaling are consistent with the results of earlier studies that have implicated the time course of protein kinase signaling as a determining factor in biological specificity (Marshall, 1995). Thus, sustained activation, but not transient activation, of the extracellular signal-regulated group of MAP kinases (ERK) in neuronlike cells (Cowley et al., 1994) and megakaryocytic precursor cells (Whalen et al., 1997) can promote growth arrest and differentiation. In contrast, sustained activation of ERK in fibroblasts causes proliferative responses (Cowley et al., 1994; Mansour et al., 1994) and is required for normal cell cycle progression (Pouyssegur et al., 2002).

An important question for future studies is the identification of the mechanism that accounts for the role of the time course of signaling to define biological specificity. Previous studies of the ERK signal transduction pathway have indicated a requirement for nuclear accumulation and the existence of thresholds for responses to signaling pathway activation (Marshall, 1995; Pouyssegur et al., 2002). One example of a molecular mechanism for the generation of a signaling threshold during ERK signaling is the function of the transcription factor c-Fos as a sensor for the duration of ERK activation (Murphy et al., 2002). We anticipate that similar mechanisms may contribute to the ability of cells to monitor the time course of JNK signaling.

The ability of JNK to initiate distinct biological responses in the same cell type following treatment with TNF is intriguing. The survival response requires transient JNK activation (Figure 6) and is dependent on JunD and the cooperation of JNK signaling with the NF-κB and Akt signal transduction pathways (Lamb et al., 2003). It has been proposed that JNK-mediated survival signaling involves the cooperative effects of JNK-induced AP-1 activity to increase the expression of antiapoptotic genes by the NF-κB and Akt signaling pathways (Lamb et al., 2003). This mechanism implies that the requirement of early JNK activation for the survival response is a consequence of the need for the expression of antiapoptotic genes at an early time during the response to an inducer of apoptosis. However, it is possible that nontranscriptional responses may also contribute to JNK-mediated survival responses. Thus, it has been proposed that the ubquitin ligase cIAP2 may play a role in survival signaling (Lamb et al., 2003). Similarly, other ubiquitin ligases may contribute to TNF-mediated survival responses. One example is represented by the ubiquitin ligase AIP4/Itch that is phosphorylated and activated by JNK (Gao et al., 2004).

The mechanism of cell death signaling by JNK is unclear, in part, because several different possible mechanisms have been proposed. JNK is required for

TNF-induced cell death when the NF-κB pathway is inhibited (Varfolomeev and Ashkenazi, 2004). This form of cell death involves the generation of ROS (Sakon et al., 2003), and JNK acts as a positive regulator of TNF-induced ROS production (Ventura et al., 2004). In this case, the requirement of sustained JNK activation for cell death may reflect the need to generate sufficient ROS to overcome a threshold to achieve cell death.

JNK has also been implicated in apoptotic cell death because $Jnk^{-/-}$ MEFs exhibit defects in the release of mitochondrial cytochrome c (Tournier et al., 2000). The mechanism that accounts for this proapoptotic signaling by JNK has not been established, although it is known that this form of JNK-dependent death requires Bax and Bak (Lei et al., 2002). It is likely that the mechanism involves the induced expression or phosphorylation of one or more members of the Bcl2 protein family. Thus, JNK can induce the expression of the proapoptotic BH3-only family members Dp5/Hrk and Bim (Harris et al., 2002; Harris and Johnson, 2001; Putcha et al., 2001; Whitfield et al., 2001). Furthermore, JNK phosphorylates and inhibits multidomain antiapoptotic members of the Bcl2 protein family including Bcl2, Bcl-xL, and Mcl-1 (Inoshita et al., 2002; Kharbanda et al., 2000; Yamamoto et al., 1999). JNK also phosphorylates and regulates members of the BH3-only group of proapoptotic Bcl2-related protein family, including Bim, Bmf, and Bad (Becker et al., 2004; Donovan et al., 2002; Lei and Davis, 2003; Putcha et al., 2003; Yu et al., 2004). JNK may also phosphorylate and inhibit some members of the 14-3-3 protein family that bind and sequester the proapoptotic multidomain Bcl2 family member Bax and the BH3-only protein Bad (Sunayama et al., 2005; Tsuruta et al., 2004). Similarly, JNK-mediated phosphorylation of both 14-3-3 and Foxo proteins may contribute to cell survival and death responses (Essers et al., 2004; Oh et al., 2005; Sunayama et al., 2005). The requirement for sustained signaling by JNK for cell death may therefore be a consequence of the complex interactions of pro- and antiapoptotic members of the Bcl2 protein family. It is possible that the transient activation of JNK is insufficient to perturb the balance of these Bcl2 family proteins to commit a cell to apoptosis and that sustained JNK signaling is required for this commitment process.

In conclusion, the biological response of cells to JNK activation is determined by both the physiological context and the time course of JNK signaling. This understanding has critical implications for the molecular mechanism of JNK signaling and the use of this pathway as a target for therapeutic strategies for the treatment of disease (Manning and Davis, 2003).

Experimental Procedures

Materials

The drugs 1NM-PP1 and 1Na-PP1 were prepared and dissolved in DMSO (Bishop and Shokat, 1999; Bishop et al., 2000).

Plasmid Construction

Point mutations (replacement of Met-108 with Gly and Leu-168 with Ala) in the cDNA clones that encode Flag epitope-tagged JNK1 and JNK2 were obtained using a standard PCR-based method and were confirmed by sequencing. The wild-type and mutated JNK1 and JNK2 cDNAs were subcloned as blunt-ended fragments into the EcoRI site of the retroviral vector pMCSV-IRES-GFP (Zindy et al., 1998).

Tissue Culture

Wild-type and $Jnk1^{-/-}Jnk2^{-/-}$ ($Jnk^{-/-}$) fibroblasts were established from E13.5 mouse embryos (Kennedy et al., 2003; Tournier et al., 2000). The cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen) at 37ºC in a humidified atmosphere with 5% CO_2. Retroviral transduction experiments were performed using MSCV-IRES-GFP vectors, and the transduced cells were isolated by flow cytometry. Drugs dissolved in DMSO were added directly to the culture medium. Control experiments were performed using the solvent alone. The final concentration of DMSO in all assays was 0.1% (v/v).

Kinase Assays

JNK activity was measured using an in vitro kinase assay using GST-cJun (1–79) as the substrate (Whitmarsh and Davis, 2001).

Luciferase Reporter Gene Assays

Transfection assays were performed using Lipofectamine reagent (Invitrogen) according to manufacturer's recommendations. The cells were transfected with 0.5 μg of the luciferase reporter plasmid pG5Elb-Luc (Gupta et al., 1995), 0.2 μg of GAL4/ATF2 (1–109) (Gupta et al., 1995), 0.1 μg of pCMV-HA-MLK-3 (Whitmarsh et al., 1998), and 0.05 μg of the control vector pRL null (Promega). Control studies were performed using the empty expression vectors. Cell extracts and luciferase quantitation were performed using the Dual-Luciferase Reporter Assay System (Promega).

Apoptosis Assays

Cell death was examined by DNA fragmentation using the Cell Death Detection Elisa^{plus} Kit (Roche) following the manufacturer's recommendations (Lamb et al., 2003; Ventura et al., 2004). Cell death was also investigated by examining cell staining with PI and analysis of PI fluorescence and light scattering by flow cytometry (Kamata et al., 2005).

Immunoblot Analysis

Proteins were resolved by SDS-PAGE (10% gel) and electrophoretically transferred to an Immobilon-P membrane (Millipore, Inc.). The membranes were incubated with 5% nonfat dry milk (4ºC, 5 hr) and then probed with antibodies to α-tubulin (Sigma), ATF2 (N-96 and F2BR-1, Santa Cruz), phospho(Thr-71)-ATF2 (F1, Santa Cruz), cJun (H-79, Santa Cruz), phospho(Ser-63)-Jun (Upstate Biotech), JNK 1/2 (Pharmingen), and phospho-JNK (Cell Signaling). Immune complexes were detected by enhanced chemiluminescence (NEN).

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were performed using 10^7 cells (Weinmann et al., 2001). The cells were crosslinked with formaldehyde, and nuclear extracts were sonicated to fragment the genomic DNA (500 bp average size). The fragmented DNA was immunoprecipitated with antibodies to cJun (SantaCruz), p65 NF-κB (SantaCruz), Pol II (SantaCruz), histone H3 (Upstate), and phospho(Ser^{10})-acetyl-Lys^9 histone H3 (Upstate) together with salmon sperm/agarose beads. The presence of the mouse IL-6 promoter (−463/−22) in 2 μl of the eluate was detected by PCR using the amplimers 5′-TGTGTGTGTATGTGTGTCGTC-3′ and 5′-TATCGTT CTTGGTGGGCTCCAG-3′. The product (441 bp) was resolved in a 2% agarose gel and visualized with ethidium bromide.

Measurement of mRNA Expression

The expression of cIAP2 and GAPDH mRNA was measured by real-time PCR using Taqman probes (Applied Biosystems). IL-6 and L32 mRNA were measured using a ribonuclease protection assay. Total RNA (5 μg) was examined using the Multiprobe RNase Protection Assay (Pharmingen) with the template set mCK3 following the manufacturer's recommendations. The products were separated on a 5% sequencing gel, detected by autoradiography, and quantitated by Phosphorimager analysis (Molecular Dynamics).

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.molecule.org/cgi/content/full/21/5/701/DC1/.

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