Molecular Cell *23*, 899–911, September 15, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.molcel.2006.07.028

JNK2 Is a Positive Regulator of the cJun Transcription Factor

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Summary

The cJun NH_2-terminal kinase (JNK) signal transduction pathway is established to be an important mechanism of regulation of the cJun transcription factor. Studies of *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ mice suggest that the JNK1 and JNK2 isoforms have opposite effects on cJun expression and proliferation. Here, we demonstrate, using a chemical genetic approach, that both JNK1 and JNK2 are positive regulators of these processes. We show that competition between JNK1 and JNK2 contributes to the opposite phenotypes caused by JNK1 and JNK2 deficiency. Our analysis illustrates the power of a chemical genetics approach for the analysis of signal transduction pathways and also highlights the limitations of single gene knockout strategies for the analysis of signaling pathways that are formed by a network of interacting proteins.

Introduction

The cJun NH_2-terminal kinase (JNK) signal transduction pathway is activated by numerous extracellular stimuli and represents a major physiological mechanism of regulation of cJun, a component of the AP1 transcription factor (Davis, 2000). Compound mutant mouse embryo fibroblasts (MEFs) that lack expression of both of the ubiquitously expressed *Jnk* genes exhibit markedly reduced cJun expression, no phosphorylation of cJun on the NH_2-terminal sites (Ser-63 and Ser-73) that increase transcription activity, low levels of AP1-dependent transcription activity, and very slow growth (Sabapathy et al., 2004; Tournier et al., 2000; Ventura et al., 2003). These data establish that the JNK signaling pathway is a key mediator of cell responses to environmental stimuli.

Functional dissection of the JNK pathway by analysis of the effects of targeted ablation of individual genes in mice has demonstrated nonredundant functions of several components of this signaling pathway. Thus, three genes encode JNK protein kinases, and mice lacking each of these genes exhibit a different phenotype (Davis, 2000). This observation suggests that individual JNK isoforms may serve different signaling functions. Support for this concept is derived from biochemical studies that indicate differences between the properties of different JNK isoforms in vitro (Gupta et al., 1996).

Studies of MEFs prepared from *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ embryos have also indicated that the JNK1 and JNK2 protein kinases may be functionally distinct (Sabapathy et al., 2004; Tournier et al., 2000). Thus, *Jnk1*$^{-/-}$ MEFs proliferate more slowly than wild-type MEFs, and *Jnk2*$^{-/-}$ MEFs proliferate more rapidly that wild-type MEFs. Furthermore, the expression of cJun is decreased in *Jnk1*$^{-/-}$ MEFs and is increased in *Jnk2*$^{-/-}$ MEFs compared with wild-type MEFs. These observations have led to the proposal that JNK1 is a positive regulator of cJun expression and cellular proliferation and that JNK2 is a negative regulator of cJun expression and proliferation (Ronai, 2004; Sabapathy and Wagner, 2004). This difference in function of JNK1 and JNK2 in vivo is intriguing because in vitro biochemical studies have demonstrated that both JNK1 and JNK2 can function as protein kinases that phosphorylate cJun and increase AP1-dependent gene expression (Davis, 2000).

The purpose of this study was to examine the proposed negative regulatory role of JNK2 on cJun expression and cellular proliferation (Ronai, 2004; Sabapathy and Wagner, 2004). Our approach was to employ a chemical genetic strategy (Bishop et al., 2001; Bishop et al., 2000) for the analysis of JNK2 function. This method involves the introduction of a mutation in the JNK2 protein kinase that expands the ATP pocket and results in sensitization to inactive derivatives of the general protein kinase inhibitor PP1. Treatment of wild-type cells with this small molecule inhibitor causes no inhibition of protein kinase activity. However, this small molecule drug does selectively inhibit JNK2 activity in cells that express the mutant JNK2 with an expanded ATP pocket. This chemical genetic approach has been successfully employed in studies of protein kinase signaling in yeast by using gene replacements to introduce specific protein kinases with an expanded ATP pocket (Bishop et al., 2000). In mammalian cells, this approach has been employed in studies where the mutated protein kinase has been overexpressed in wild-type cells (Wang et al., 2003). More recently, improved methods have been employed to selectively express protein kinases with ATP pocket mutations in mammalian cells in the absence of the wild-type protein kinase by using genetic complementation (Denzel et al., 2003; Ventura et al., 2006) and knockin approaches (Chen et al., 2005).

We used chemical genetic analysis of mice with a germ-line point mutation in the *Jnk2* gene that expands the ATP pocket to study the function of the JNK2 protein kinase. We show that JNK2 is a positive regulator of cJun expression and cellular proliferation. This conclusion contrasts with the results of previous

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studies of JNK2 based on the analysis of *Jnk2* gene disruption. Competition between JNK isoforms accounts for the misleading phenotype of $Jnk2^{-/-}$ cells. It is likely that similar competition between family members of other signaling proteins may confound the interpretation of single gene knockout studies. In addition, our study illustrates the power of the chemical genetic approach for the analysis of signal transduction pathways.

Results

Comparison of the Requirement of JNK1 and JNK2 for cJun Expression and Phosphorylation

We prepared primary MEFs from wild-type embryos and from JNK1- or JNK2- deficient embryos. Immunoblot analysis demonstrated that $Jnk1^{-/-}$ ($J1^{-/-}$) MEFs expressed 46 kDa and 55 kDa forms of JNK2, that $Jnk2^{-/-}$ ($J2^{-/-}$) MEFs expressed 46 kDa and 55 kDa forms of JNK1, and that wild-type MEFs expressed 46 kDa and 55 kDa forms of both JNK1 and JNK2 (Figure 1). Exposure of these MEFs to UV radiation caused JNK activation that was detected by immunoblot analysis using an antibody to phospho(Thr-183/Tyr-185)JNK and was also detected by in vitro protein kinase assays using cJun as the substrate (Figure 1). The in vitro kinase assay demonstrated that a much larger decrease in JNK activity was observed in $J1^{-/-}$ MEFs than $J2^{-/-}$ MEFs compared with wild-type MEFs (Figure 1). To test whether these differences in protein kinase activity in vitro are relevant to JNK activity in vivo, we examined the phosphorylation of the JNK substrate cJun by immunoblot analysis of cell lysates. This analysis confirmed that JNK1 deficiency caused a much larger decrease in cJun phosphorylation in vivo compared with that caused by JNK2 deficiency (Figure 1). Thus, loss of JNK1 expression causes a large decrease in JNK activity in vivo and in vitro. In contrast, markedly greater JNK activity in vitro and cJun phosphorylation in vivo were detected in JNK2-deficient MEFs compared with JNK1-deficient MEFs (Figure 1). These data confirm the conclusions of previous studies of JNK protein kinase activity in $J1^{-/-}$ and $J2^{-/-}$ MEFs (Tournier et al., 2000).

Comparison of cJun expression in MEFs that express only JNK1 or JNK2 demonstrated marked differences compared with wild-type cells; loss of JNK1 caused reduced cJun expression, whereas loss of JNK2 caused increased cJun expression (Figure 1). One interpretation of these data is that JNK1 is a positive regulator of cJun and that JNK2 is a negative regulator of cJun (Ronai, 2004; Sabapathy et al., 2004; Sabapathy and Wagner, 2004). The conclusion that JNK1 is a positive regulator of cJun in vivo was anticipated, but the conclusion that JNK2 might act as a negative regulator of cJun was unexpected because prior biochemical analyses of JNK1 and JNK2 indicate that both of these protein kinases can act as positive regulators of cJun in vitro (Davis, 2000).

Chemical Genetic Analysis of JNK2

To test the hypothesis that JNK2 is a negative regulator of cJun (Ronai, 2004; Sabapathy and Wagner, 2004), we employed a pharmacological approach to examine the effect of specific inhibition of JNK2 function. Small molecule inhibitors with an established specificity for JNK2

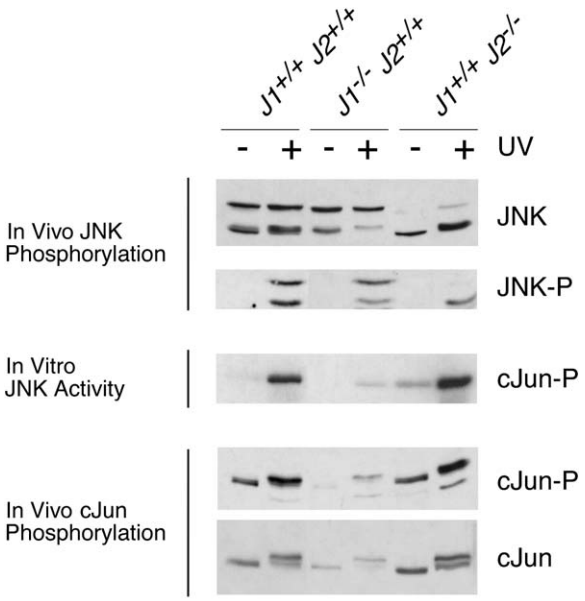

Figure 1. Comparison of cJun Expression and Phosphorylation in JNK1- and JNK2-Deficient MEFs

Wild-type ($J1^{+/+}$ $J2^{+/+}$) MEFs and JNK-deficient MEFs ($J1^{-/-}$ $J2^{+/+}$ and $J1^{+/+}$ $J2^{-/-}$) were exposed to UV radiation (50 J/m^2). The cells were incubated (30 min), and extracts in TLB buffer were prepared. The expression of JNK, phospho(Thr-183/Tyr-185)-JNK, cJun, and phospho(Ser-63)cJun was examined by immunoblot analysis. JNK protein kinase activity was measured in an in vitro kinase assay with [γ-^{32}P]ATP and cJun as substrates.

have not yet been described (Manning and Davis, 2003). We therefore employed a chemical genetic approach (Bishop et al., 2000) by using JNK protein kinases that have been engineered to enlarge the ATP pocket (Habelhah et al., 2001; Ventura et al., 2006), resulting in sensitization to inactive derivatives of the general protein kinase inhibitor PP1 (e.g., 1-naphthylmethyl-4-amino-1-*tert*-butyl-3-[*p*-methylphenyl]pyrazolo(3,4-*d*)pyrimidine [1NM-PP1]). This is a well-established method for the examination of protein kinase function (Shokat and Velleca, 2002). The method has also been validated in studies of recombinant JNK in vitro and in studies of JNK ectopically expressed in cultured cells (Habelhah et al., 2001; Ventura et al., 2006).

To examine the physiological function of JNK2, we constructed mice with a germ-line mutation in codon 108 (methionine replaced by glycine) that expands the ATP pocket of JNK2 (Figure 2). The $Jnk2^{+/MG}$ mice were backcrossed to the C57BL/6J strain (ten generations) and were intercrossed to generate homozygous $Jnk1^{+/+}$ $Jnk2^{MG/MG}$ mice ($J1^{+/+}$ $J2^{MG/MG}$) that express JNK1 and JNK2^{MG}. The mice were also crossed with $Jnk1^{-/-}$ mice to obtain homozygous $Jnk1^{-/-}$ $Jnk2^{MG/MG}$ mice ($J1^{-/-}$ $J2^{MG/MG}$) that express JNK2^{MG}, but not JNK1. No differences were detected between wild-type and $J1^{+/+}$ $J2^{MG/MG}$ mice or between $J1^{-/-}$ $J2^{+/+}$ and $J1^{-/-}$ $J2^{MG/MG}$ mice, suggesting that the $Jnk2^{MG}$ allele can fully replace the function of the wild-type *Jnk2* allele.

JNK2^{MG} Is Selectively Inhibited by the Drug 1NM-PP1

To study the effect of expanding the ATP pocket of JNK2 by replacing methionine-108 with glycine, we compared

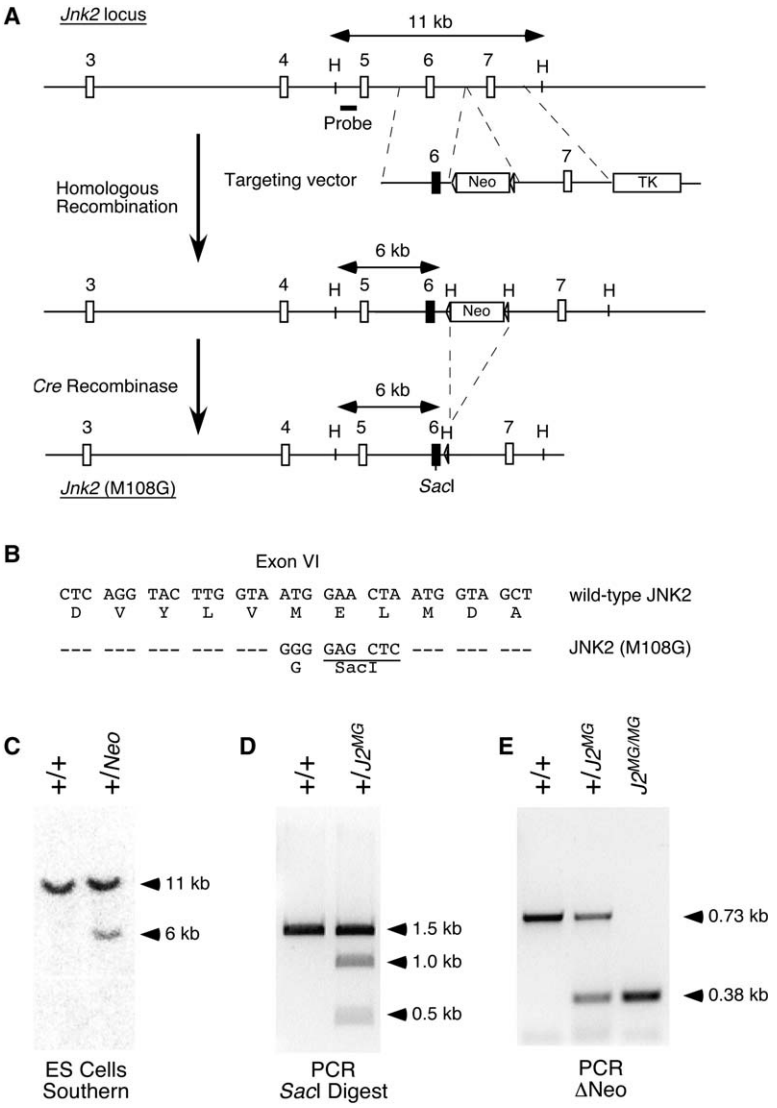

Figure 2. Creation of Mice with a Germ-Line Point Mutation in the *Jnk2* Gene

(A and B) Strategy for mutating the ATP binding pocket of JNK2. The targeting vector was designed to replace methionine 108 with glycine and to introduce silent mutations that create a SacI restriction site in exon VI. The wild-type allele was replaced with the mutant allele by homologous recombination. Restriction sites are indicated (H, HindIII). The *floxed-Neo*^{R} cassette used for selection was deleted by using *Cre* recombinase.

(C and D) Genomic DNA isolated from wild-type and targeted ES cells was restricted with HindIII and was examined by Southern blot analysis to demonstrate the correct insertion of the *floxed-Neo*^{R} cassette into the *Jnk2* locus. PCR analysis and restriction with SacI demonstrate the presence of the *Jnk2*^{MG} allele with a mutation in exon VI.

(E) Genomic DNA isolated from wild-type (+/+), heterozygous (+/*J2*^{MG}), and homozygous (*J2*$^{MG/MG}$) knockin mice was examined by PCR.

the protein kinase activity of JNK2 and JNK2^{MG}. No difference in the phosphorylation of cJun by JNK2 and JNK2^{MG} was detected in an in vitro protein kinase assay (Figure S1 in the Supplemental Data available with this article online). In contrast, addition of the drug 1NM-PP1 caused potent inhibition of the mutated JNK2^{MG} protein kinase but did not affect the protein kinase activity of wild-type JNK2 (Figure S1). These data are consistent with the results of previous studies of JNK proteins that indicate that the expansion of the ATP pocket does not cause obvious deleterious effects on JNK protein kinase activity (Habelhah et al., 2001; Ventura et al., 2006).

To establish the effective dose of 1NM-PP1 for inhibition of JNK2^{MG} activity in vivo, we examined the phosphorylation of cJun on Ser-63 by immunoblot analysis. Previous studies have established that JNK is essential for the phosphorylation of cJun on this site since this phosphorylation is not observed in primary mouse embryo fibroblasts (MEFs) derived from compound *Jnk* mutant embryos that lack expression of all JNK isoforms (Ventura et al., 2003). Pretreatment of *J1*$^{-/-}$ *J2*$^{MG/MG}$ MEFs with increasing doses of 1NM-PP1 prior to exposure to UV radiation demonstrated a dose-dependent decrease in cJun phosphorylation on Ser-63 in vivo (Figure 3A). Maximal inhibition of cJun phosphorylation was observed when the MEFs were incubated with 10 μM 1NM-PP1.

We examined the time course and reversibility of JNK2^{MG} inhibition in vivo by investigating cJun phosphorylation in *J1*$^{-/-}$ *J2*$^{MG/MG}$ MEFs treated with 10 μM 1NM-PP1. Drug wash-out studies demonstrated that JNK2^{MG} activity can be fully recovered within 15 min of removal of the drug from the culture medium (Figure 3B). Similarly, drug addition studies indicated that JNK2^{MG} activity was suppressed within 15 min of drug addition to the culture medium (Figure 3D). Control studies demonstrated that the addition of 1NM-PP1 to MEFs that express wild-type JNK2 caused no changes in cJun phosphorylation (Figure 3C). Together, these data demonstrate that the addition of the drug 1NM-PP1 to the culture medium causes the rapid and reversible inhibition of JNK2^{MG} activity in vivo.

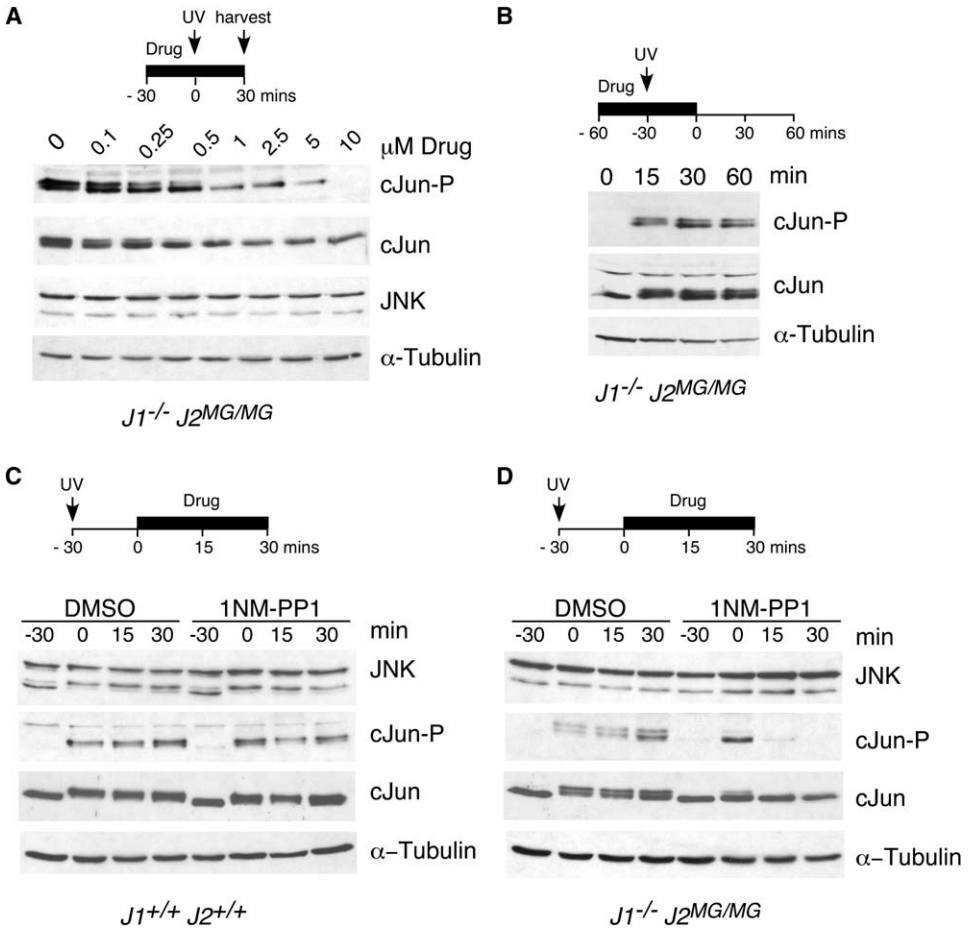

Figure 3. Characterization of JNK2^{MG} Inhibition by 1NM-PP1

(A) Primary MEFs prepared from *J1*$^{-/-}$ *J2*$^{MG/MG}$ embryos were incubated in medium supplemented with different concentrations of the drug 1NM-PP1 (30 min). The cells were exposed to 50 J/m^2 UV radiation and incubated (30 min) with medium supplemented with the drug. The expression of phospho(Ser-63)cJun, cJun, JNK, and tubulin was examined by immunoblot analysis.

(B) The inhibition of JNK2^{MG} is reversible. Primary MEFs prepared from *J1*$^{-/-}$ *J2*$^{MG/MG}$ embryos were incubated (30 min) with medium supplemented with 10 μM 1NM-PP1. The cells were exposed to 50 J/m^2 UV radiation and incubated (30 min) with medium supplemented with the drug. The cells were then washed twice and then incubated in fresh medium without the drug. The cells were harvested immediately (time = 0) or at the indicated time points. The expression of phospho(Ser-63)cJun, cJun, and tubulin was examined by immunoblot analysis.

(C and D) 1NM-PP1 is a rapid inhibitor of JNK2^{MG}. Primary MEFs prepared from wild-type embryos (C) or *J1*$^{-/-}$ *J2*$^{MG/MG}$ embryos (D) were exposed to 50 J/m^2 UV radiation and then incubated in medium for 30 min before the drug 1NM-PP1 (10 μM) or solvent (DMSO) was added to the medium. The cells were harvested immediately after UV treatment (time = −30 min) or at the indicated times (0, 15, and 30 min) after the addition of the drug. The expression of phospho(Ser-63)cJun, cJun, JNK, and tubulin was examined by immunoblot analysis.

JNK2 Activity Increases cJun Phosphorylation In Vivo

The drug 1NM-PP1 causes the rapid and reversible inhibition of JNK2^{MG} (but not JNK2) activity in vivo (Figure 3). We therefore used this drug to evaluate the role of the JNK2 protein kinase in the regulation of cJun phosphorylation. Primary MEFs were prepared from wild-type embryos, *J1*$^{+/+}$ *J2*$^{MG/MG}$ embryos, and from *J1*$^{-/-}$ *J2*$^{MG/MG}$ embryos. These MEFs were treated with the drug 1NM-PP1 or with the solvent (DMSO) and then exposed to 50 J/m^2 UV radiation. The cells were harvested at different times after exposure to UV light. Phosphorylation of JNK and cJun was examined by immunoblot analysis. We found that the drug 1NM-PP1 caused no change in cJun or JNK phosphorylation in experiments using wild-type MEF or *J1*$^{+/+}$ *J2*$^{MG/MG}$ MEFs (Figure 4). The absence of changes in JNK phosphorylation in these studies was anticipated because 1NM-PP1 is an inhibitor of JNK^{MG} activity and is not an inhibitor of JNK activation (Figure 3). Similarly, the absence of changes in cJun phosphorylation in wild-type MEFs was anticipated because 1NM-PP1 is not an inhibitor of wild-type JNK (Figure 3). In contrast, we did anticipate that 1NM-PP1 would inhibit JNK2^{MG} in *J1*$^{+/+}$ *J2*$^{MG/MG}$ MEFs. Consequently, the lack of an effect of 1NM-PP1 on cJun phosphorylation in *J1*$^{+/+}$ *J2*$^{MG/MG}$ MEFs was unexpected (Figure 4). This finding suggests that JNK2 either has no role in cJun phosphorylation in vivo or has a role that is redundant with JNK1.

To test whether JNK2 has no role or a redundant role in cJun phosphorylation in vivo, we examined the effect of 1NM-PP1 on cJun phosphorylation in *J1*$^{-/-}$ *J2*$^{MG/MG}$ MEFs (Figure 4). We found that 1NM-PP1 potently inhibited cJun phosphorylation in these cells. Together, these data indicate that JNK2 does cause cJun phosphorylation in wild-type cells exposed to UV

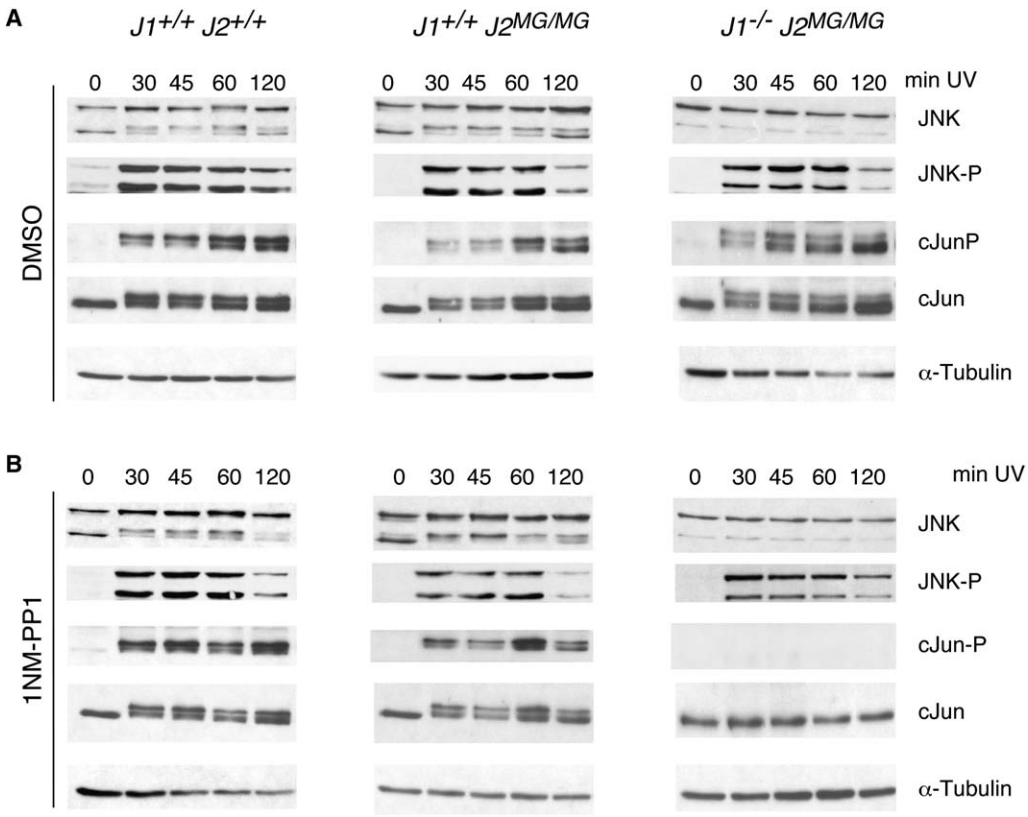

Figure 4. JNK1 and JNK2 Protein Kinase Activity Contributes to cJun Phosphorylation

Primary MEFs prepared from wild-type embryos or mutated embryos ($J1^{+/+}$ $J2^{MG/MG}$ or $J1^{-/-}$ $J2^{MG/MG}$) were incubated in medium supplemented with solvent (DMSO) (A) or with 10 μM 1NM-PP1 (B). After 30 min, the cells were exposed to 50 J/m^2 UV radiation and then incubated in the same medium for 120 min. The cells were harvested immediately after UV treatment (time = 0 min) or at the indicated times (0, 30, 45, 60, and 120 min) after UV exposure. The expression of JNK, phospho(Thr-183/Tyr-185)-JNK, cJun, phospho(Ser-63)cJun, and tubulin was examined by immunoblot analysis.

radiation, but this function of JNK2 is largely redundant with JNK1.

JNK2 Activity Increases cJun Expression

Studies of $Jnk2^{-/-}$ MEFs have suggested that JNK2 is a negative regulator of cJun expression (Ronai, 2004; Sabapathy et al., 2004; Sabapathy and Wagner, 2004). To test this hypothesis, we employed a chemical genetic approach to examine the role of JNK2 activity in the regulation of cJun expression (Figure 5). Treatment of proliferating wild-type or $J1^{+/+}$ $J2^{MG/MG}$ MEFs with 1NM-PP1 caused no changes in the expression of cJun mRNA (Figures 5A and 5B). Control studies demonstrated that this drug also caused no changes in the expression of mRNAs that encode other members of the Jun transcription factor group, including JunB and JunD. In contrast, studies using $J1^{-/-}$ $J2^{MG/MG}$ MEFs demonstrated that the drug 1NM-PP1 caused major decreases in the expression of steady-state cJun mRNA (Figures 5A and 5B). These data are consistent with previous studies of $J1^{-/-}$ $J2^{-/-}$ fibroblasts that have established a role for JNK in the expression of cJun and JunD, but not JunB (Lamb et al., 2003). Indeed, decreased expression of JunD mRNA was also observed in $J1^{-/-}$ $J2^{MG/MG}$ MEFs treated with 1NM-PP1, but no changes in JunB mRNA expression were detected (Figures 5A and 5B). Together, these data indicate that JNK2 activity increases the expression of cJun and JunD mRNA, but this function of JNK2 is largely redundant with JNK1. This conclusion is consistent with the results of our analysis of the role of JNK2 in the regulation of cJun phosphorylation (Figure 4).

To test the role of JNK2 activity on the expression of the cJun protein, we examined the effect of the drug 1NM-PP1 on cJun by immunoblot analysis. Treatment of proliferating wild-type or $J1^{+/+}$ $J2^{MG/MG}$ MEFs with 1NM-PP1 caused no changes in cJun protein expression, but 1NM-PP1 did cause a marked decrease in cJun protein expression in $J1^{-/-}$ $J2^{MG/MG}$ MEFs (Figure 5C). These data confirm that JNK2 activity plays a positive role in cJun expression that is largely redundant with JNK1.

JNK2 Activity Increases Cellular Proliferation

Studies of $Jnk2^{-/-}$ MEFs demonstrate that the loss of JNK2 causes increased proliferation (Sabapathy et al., 2004; Tournier et al., 2000). This observation has led to the proposal that JNK2 is a negative regulator of cell proliferation (Ronai, 2004; Sabapathy and Wagner, 2004). To test this hypothesis we employed the chemical genetic approach to examine the role of JNK2 activity in the regulation of cellular proliferation (Figure 6). Treatment

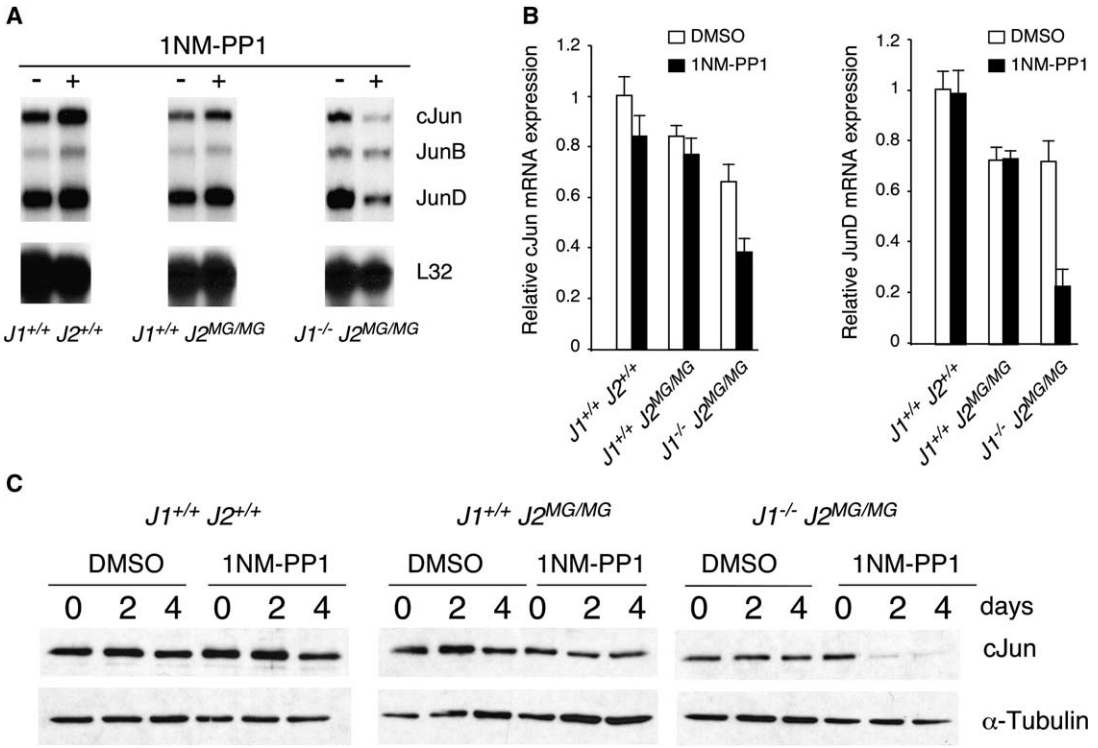

Figure 5. JNK1 and JNK2 Protein Kinase Activity Contributes to cJun Expression

(A) Primary MEFs prepared from wild-type embryos ($J1^{+/+}$ $J2^{+/+}$) and mutated embryos ($J1^{+/+}$ $J2^{MG/MG}$ or $J1^{-/-}$ $J2^{MG/MG}$) were incubated in medium supplemented with solvent (DMSO) or 10 μM 1NM-PP1 for 48 hr. The expression of cJun, JunB, and JunD mRNA was examined in a ribonuclease protection assay. Control studies were performed to measure the amount of ribosomal protein L32 mRNA.

(B) The ribonuclease protection assay data (A) were quantitated by Phosphorimager analysis, and the relative expression of cJun mRNA and JunD mRNA is presented as the normalized ratio [AP1 mRNA]:[L32 mRNA] (mean ± SD; n = 3).

(C) Primary MEFs prepared from wild-type embryos ($J1^{+/+}$ $J2^{+/+}$) or mutated embryos ($J1^{+/+}$ $J2^{MG/MG}$ or $J1^{-/-}$ $J2^{MG/MG}$) were incubated in medium supplemented with solvent (DMSO) or 10 μM 1NM-PP1. The cells were harvested, and the expression of cJun and tubulin was examined by immunoblot analysis.

of wild-type or $J1^{+/+}$ $J2^{MG/MG}$ MEFs with 1NM-PP1 caused no changes in proliferation (Figures 6A and 6B). Studies of $J1^{-/-}$ $J2^{MG/MG}$ MEFs demonstrated that the rate of proliferation of these cells was reduced compared with wild-type MEFs. This observation is consistent with the results of previous studies of $Jnk1^{-/-}$ MEFs that demonstrate a key role for the JNK1 protein kinase in proliferation (Sabapathy et al., 2004; Tournier et al., 2000). Nevertheless, treatment of $J1^{-/-}$ $J2^{MG/MG}$ MEFs with 1NM-PP1 caused a further decrease in the rate of proliferation (Figures 6A and 6B). Together, these data indicate that JNK2 activity increases cellular proliferation, but this function of JNK2 is largely redundant with JNK1.

The effect of JNK inhibition to decrease cellular proliferation may be caused by JNK-dependent changes in cyclin expression. For example, it has been reported that JNK activity promotes the expression of cyclin D1 (Lee et al., 1999, 2000). We therefore examined cyclin mRNA expression by wild-type MEFs, $J1^{+/+}$ $J2^{MG/MG}$ MEFs, and $J1^{-/-}$ $J2^{MG/MG}$ MEFs. Cyclin mRNA expression levels were similar in wild-type and $J1^{+/+}$ $J2^{MG/MG}$ MEFs, but lower levels of cyclin mRNA were detected in $J1^{-/-}$ $J2^{MG/MG}$ MEFs, consistent with the reduced proliferation of these cells. However, studies of all three of these primary cell cultures demonstrated that the drug 1NM-PP1 caused no changes in cyclin mRNA expression (Figure 6C). These data suggest that changes in cyclin expression do not account for the effect of JNK2 activity to increase cellular proliferation. An alternative mechanism that may account for the observed JNK2-mediated proliferation (Figures 6A and 6B) is the marked decrease in cJun expression that is observed in $J1^{-/-}$ $J2^{MG/MG}$ MEFs treated with 1NM-PP1 (Figure 5C) because it is known that cJun-deficient MEFs proliferate extremely poorly (Johnson et al., 1993).

JNK2 Activity Regulates Cell Survival

It is established that JNK functions as a regulator of MEF survival. However, the specific role of JNK2 activity in cell survival signaling is unclear. Previous studies of compound knockout MEFs that lack JNK expression have demonstrated that JNK can mediate both prosurvival and proapoptotic signaling depending on the stimulus that is examined; the loss of JNK accelerates TNF-stimulated apoptosis but suppresses UV-stimulated apoptosis (Lamb et al., 2003; Tournier et al., 2000; Ventura et al., 2004). To test the role of JNK2 activity, we employed the chemical genetic approach to examine TNF and UV-stimulated apoptosis. Treatment of wild-type MEFs and $J1^{+/+}$ $J2^{MG/MG}$ MEFs with TNF or UV radiation caused increased apoptosis that was detected by

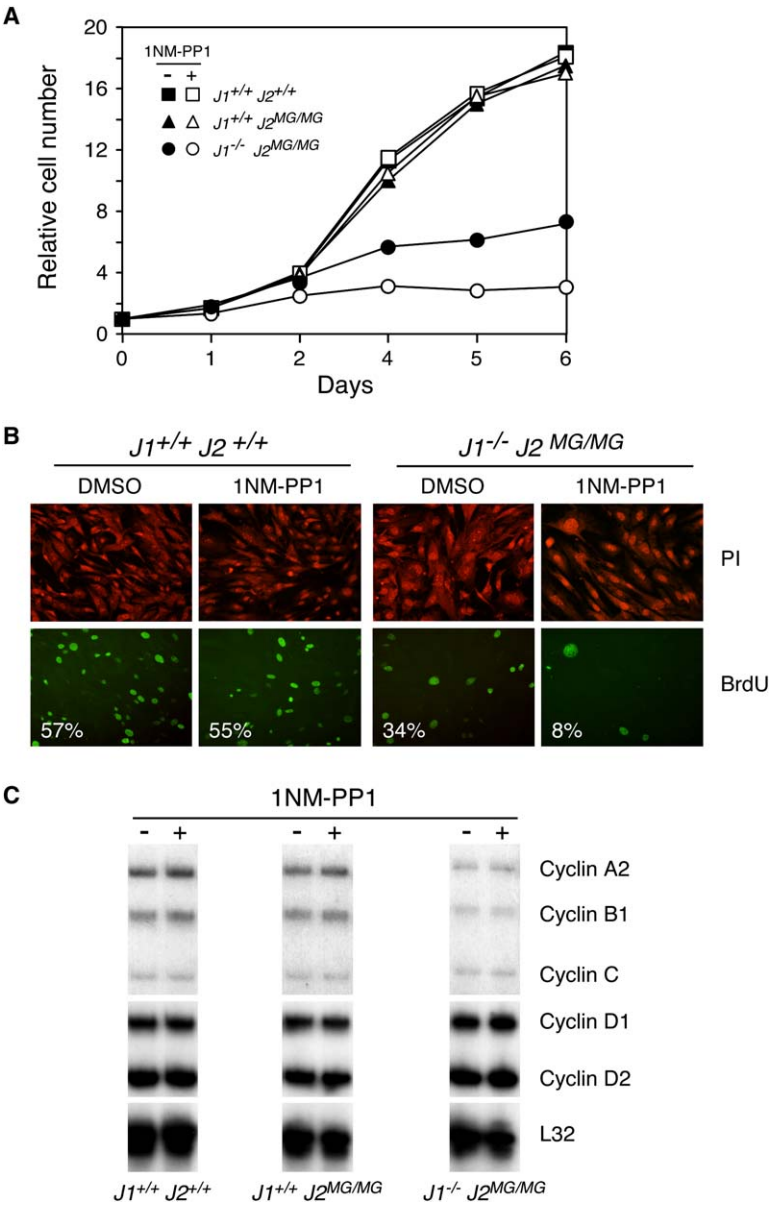

Figure 6. JNK1 and JNK2 Protein Kinase Activities Are Required for Cell Proliferation

(A) Primary MEFs prepared from wild-type embryos ($J1^{+/+}$ $J2^{+/+}$) or mutated embryos ($J1^{+/+}$ $J2^{MG/MG}$ or $J1^{-/-}$ $J2^{MG/MG}$) were incubated in medium supplemented with solvent (DMSO) or 10 μM 1NM-PP1. Relative cell numbers were measured by staining with crystal violet.

(B) Primary MEFs were cultured in medium supplemented with solvent (DMSO) or 10 μM 1NM-PP1 for 2 days and then pulse labeled (8 hr) with BrdU. The cells were fixed and stained with an antibody to BrdU (green) and with propidium iodide (red).

(C) The expression of cyclin mRNA and ribosomal protein L32 mRNA was examined by ribonuclease protection assay.

measurement of DNA fragmentation. Treatment with 1NM-PP1 caused no changes in TNF or UV-stimulated apoptosis in these cells (Figure S2). In contrast, treatment of $J1^{-/-}$ $J2^{MG/MG}$ MEFs with 1NM-PP1 increased TNF-stimulated apoptosis and decreased UV-stimulated apoptosis (Figure S2). These changes in apoptosis of $J1^{-/-}$ $J2^{MG/MG}$ MEFs caused by 1NM-PP1 phenocopy the apoptotic properties of compound mutant $Jnk1^{-/-}$ $Jnk2^{-/-}$ MEFs (Lamb et al., 2003; Tournier et al., 2000; Ventura et al., 2004). Together, these data indicate that JNK2 activity regulates cell survival and that this function of JNK2 is redundant with JNK1.

JNK2 Activity Promotes Cell Motility

JNK has been implicated in cell motility (Huang et al., 2004). Whether JNK2 activity might have a positive or a negative role in cell migration is unclear. To test whether JNK2 activity contributes to JNK-mediated cell migration, we examined confluent monolayers of primary MEFs (Figure 7). A scratch wound in monolayers of wild-type MEFs causes increased JNK activity in the cells immediately adjacent to the wound (Lallemand et al., 1998). This increased JNK activity was detected by immunofluorescence analysis using an antibody to phospho(Ser-63)cJun (Figure 7A, right panels). Treatment of $J1^{-/-}$ $J2^{MG/MG}$ MEFs, but not wild-type MEFs, with 1NM-PP1 blocked this increase in cJun phosphorylation (Figures 7A and 7B, right panels). Time-lapse microscopy demonstrated that wounds in monolayers of wild-type MEFs and $J1^{+/+}$ $J2^{MG/MG}$ MEFs were rapidly repaired in the presence and absence of the drug 1NM-PP1 (Figure 7A, left panels, and data not shown). Rapid wound repair was also observed in cultures of $J1^{-/-}$ $J2^{MG/MG}$ MEFs, but treatment of these MEFs with 1NM-PP1 dramatically slowed the wound repair (Figure 7B, left panels). Together, these data

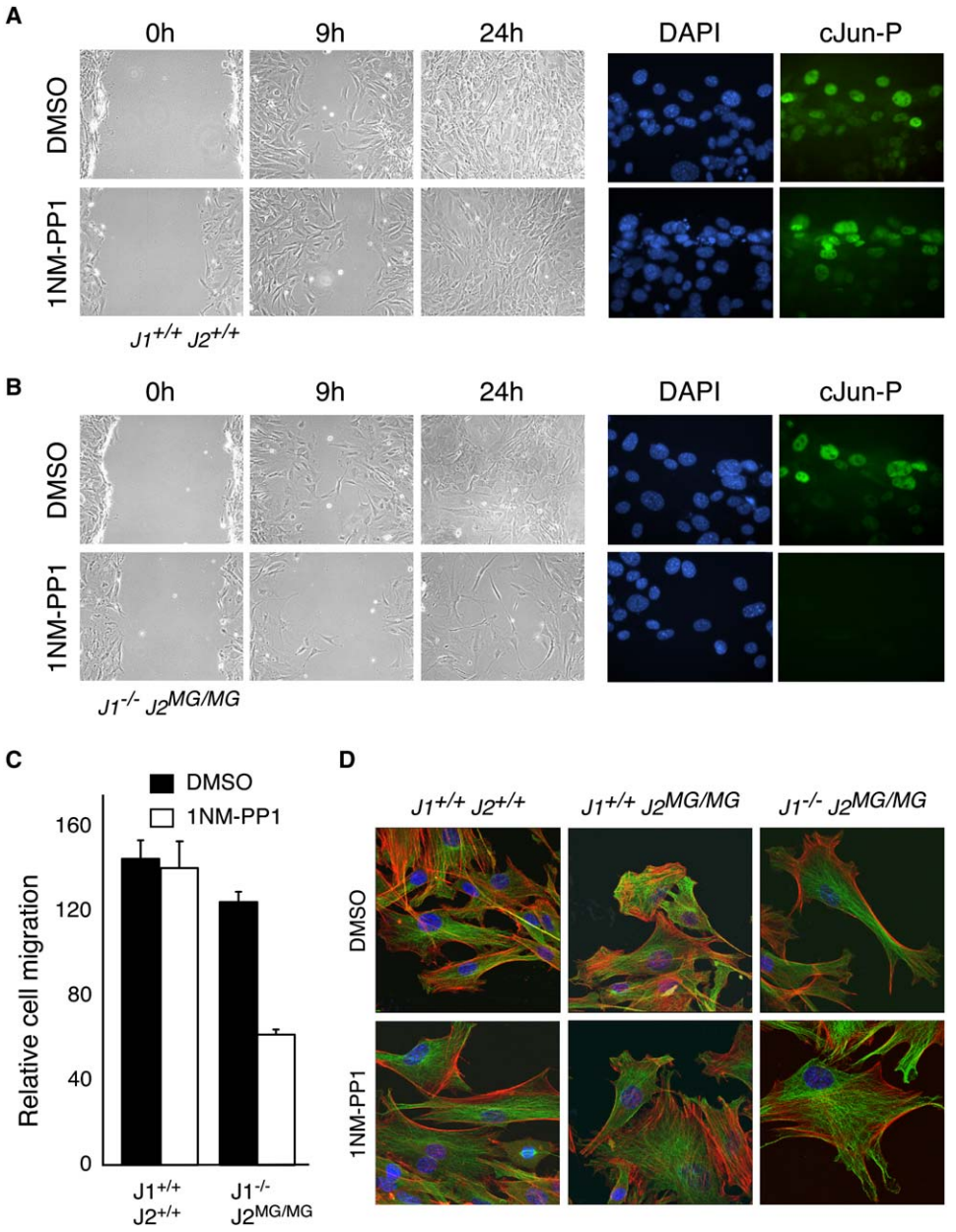

Figure 7. JNK2 Protein Kinase Activity Promotes Wound Closure In Vitro

(A and B) Confluent cultures of primary MEFs prepared from (A) wild-type embryos ($J1^{+/+}$ $J2^{+/+}$) or (B) mutated embryos ($J1^{-/-}$ $J2^{MG/MG}$) were scratched by using a pipette tip to create a wound. The effect of addition of solvent (DMSO) or 10 μM 1NM-PP1 to the culture medium prior to wounding was examined. The wound closure was monitored by video microscopy and representative images taken at 0, 9, and 24 hr are presented (left panels). The phosphorylation of cJun (at 30 min after wounding) at the leading edge of the closing wound was examined by immunofluorescence analysis with an antibody to phospho(Ser-63)cJun (green); nuclei were stained with DAPI (blue) (right panels).

(C) Boyden chamber assays were performed by using primary mouse keratinocytes to examine cell migration. The keratinocytes were pretreated (1 hr) and then incubated in Boyden chambers (24 hr) with or without 100 nM 1NM-PP1. The number of cells that migrated to the other side of the membrane was counted. The data represent the mean ± SD of triplicate determinations obtained in one experiment. Similar results were obtained in studies using three independent keratinocytes preparations.

(D) Primary MEFs were incubated with medium supplemented with solvent (DMSO) or 10 μM 1NM-PP1 for 48 hr and fixed. Microtubules were stained with an antibody to tubulin (green), filamentous actin was stained with phalloidin (red), and DNA was stained with DAPI (blue).

demonstrate that JNK2 activity contributes to in vitro wound repair and that this function of JNK2 is largely redundant with JNK1.

To test whether JNK2 might directly influence cell migration, we performed Boyden chamber assays with MEFs (data not shown) and also primary keratinocytes (Figure 7C). Treatment of wild-type keratinocytes with the drug 1NM-PP1 caused no change in cell migration. The extent of $J1^{-/-}$ $J2^{MG/MG}$ keratinocyte migration was slightly reduced compared to wild-type keratinocytes (Figure 7C). However, treatment of $J1^{-/-}$ $J2^{MG/MG}$ keratinocytes with 1NM-PP1 caused markedly

decreased cell migration. These data confirm the conclusion that JNK2 activity contributes to cell motility and that this function of JNK2 is redundant with JNK1.

Previous studies of JNK-mediated cell migration have implicated a role for JNK in the formation of actin stress fibers in migrating cells (Zhang et al., 2005). We therefore examined actin stress fibers in MEFs by immunofluorescence analysis. No differences between wild-type MEFs, $J1^{+/+}$ $J2^{MG/MG}$ MEFs, and $J1^{-/-}$ $J2^{MG/MG}$ MEFs were detected, and treatment of these cells with 1NM-PP1 caused no detected changes in actin stress fiber formation (Figure 7D). These data suggest that JNK-dependent changes in actin stress fibers may be relevant only to the motility of some cell types in response to specific stimuli. Our analysis is not consistent with a general role for JNK-mediated changes in actin stress fiber formation in cell motility.

JNK2 Deficiency Causes a Compensatory Increase in JNK1 Function

Chemical genetic analysis demonstrates that JNK2 is a positive regulator of cJun expression and cellular proliferation (Figures 5 and 6). This positive role of JNK2 contrasts with conclusions drawn from previous studies that have suggested a negative regulatory role of JNK2 (Ronai, 2004; Sabapathy and Wagner, 2004). This negative role of JNK2 depends upon the interpretation of studies of $Jnk2^{-/-}$ cells that exhibit increased expression of cJun and increased proliferation (Sabapathy et al., 2004; Tournier et al., 2000).

One major difference between the chemical genetic approach and the gene knockout approach to the analysis of JNK2 function is that $JNK2^{MG}$ is expressed in the cells employed for chemical genetic analysis, but no JNK2 protein is expressed in JNK2-deficient cells. This loss of JNK2 expression in the knockout cells provides an opportunity for compensatory adaptations to the loss of JNK2. In contrast, in the chemical genetic approach, loss of $JNK2^{MG}$ activity (but not expression) is acutely induced at the time of the experimental analysis when the cells are treated with the drug 1NM-PP1; the opportunities for compensatory adaptive changes in cells expressing $JNK2^{MG}$ are therefore limited. This reasoning led us to question whether compensatory adaptations in $Jnk2^{-/-}$ cells may contribute to their phenotype.

Because studies of compound mutant $Jnk1^{-/-}$ $Jnk2^{-/-}$ MEFs demonstrate that JNK is required for cJun expression and proliferation (Tournier et al., 2000; Ventura et al., 2003), the increased expression of cJun and increased proliferation observed in studies of $Jnk2^{-/-}$ MEFs are consistent with the possibility that these cells exhibit a gain of JNK function. The simplest hypothesis is that there is a compensatory increase in JNK1 activity in $Jnk2^{-/-}$ MEFs. We therefore examined JNK1 activity in $Jnk2^{-/-}$ MEFs by performing in vitro protein kinase assays using cJun as the substrate. This analysis demonstrated that JNK1 activity is indeed increased in nonstimulated $Jnk2^{-/-}$ MEFs compared with wild-type MEFs (Figure 1 and Figure S3C).

To confirm the conclusion that $Jnk2^{-/-}$ MEFs exhibit a compensatory increase in JNK1 function, we tested the effect of reducing JNK1 function in $Jnk2^{-/-}$ MEFs. First, we examined the effect of inhibiting JNK1 by using the drug SP600125 (Bennett et al., 2001). These studies demonstrated that the loss of JNK1 signaling caused by SP600125 prevented the increase in cJun expression observed in $Jnk2^{-/-}$ MEFs (Figure S3A). Second, we examined the effect of reducing *Jnk1* gene dosage in $Jnk2^{-/-}$ MEFs. Comparison of $Jnk1^{+/+}$ $Jnk2^{-/-}$ MEFs with $Jnk1^{-/+}$ $Jnk2^{-/-}$ MEFs demonstrated that reduced JNK1 function in $Jnk2^{-/-}$ MEFs decreased the expression of cJun (Figure S3B). Together, these data demonstrate that the increase in cJun expression observed in $Jnk2^{-/-}$ MEFs requires the expression and activity of JNK1.

Discussion

Dissections of protein kinase signaling pathways using gene disruption and RNAi-based approaches represent powerful methods for the study of mammalian signaling pathways that have led to important new insight into the physiological function and mechanism of signal transduction. Nevertheless, the interpretation of such studies is not straightforward. It is recognized that the long-term loss of gene function can lead to compensatory changes in the function of other genes (Sage et al., 2003). Here, we demonstrate that competition between isoforms of signaling proteins can lead to acute changes in the function of signaling pathways after selective loss of one isoform. Thus, studies using either acute knockdown or long-term disruption of protein expression can yield misleading information concerning the function of signaling pathways, particularly when one member of gene family is examined.

Chemical Genetic Analysis of Protein Kinase Signaling

Pharmacological methods to study signal transduction using small molecule inhibitors provide an approach that is complementary to the use of gene ablation and RNAi. The advantage of small molecules is that they can cause rapid (and rapidly reversible) dose-dependent inhibition of signal transduction. The major disadvantage of small molecule inhibitors is their functional selectivity. For example, small molecule inhibitors of JNK have been described, but these molecules are not specific inhibitors of JNK (Manning and Davis, 2003). Recently, a chemical genetic approach for the analysis of protein kinase signaling that overcomes the specificity limitations of available small molecule inhibitors has been reported (Bishop et al., 2000). This method involves the introduction of a mutation into the kinase active site that expands the ATP pocket and confers sensitivity to inactive derivatives of the general protein kinase inhibitor PP1. A major advantage of this chemical genetic approach is that cells expressing the wild-type protein kinase can be employed for control studies to document possible off-target effects of the PP1 derivative. It should be noted that one possible caveat of this approach is that it is possible that the expansion of the ATP pocket might confer a mutant phenotype in the absence of the small molecule inhibitor.

The use of the chemical genetic approach has been widely employed in studies of yeast protein kinases (Knight and Shokat, 2005). Interestingly, phenotypes detected in response to acute inhibition by a small

molecule can markedly differ from phenotypes detected after gene ablation (Knight and Shokat, 2005). Thus, whereas a *cdc28-ts* allele can arrest yeast in G1 at the restrictive temperature, small molecule inhibition of CDC28 with an expanded ATP pocket causes G2/M arrest in the presence of low concentrations of inhibitor and G1 arrest at high concentrations of inhibitor; these data are consistent with the hypothesis that the mitotic checkpoint is more sensitive to CDC28 activity than the G1 checkpoint (Bishop et al., 2000). Differences between small molecule inhibition and gene ablation phenotypes have also been noted for Ire1 (Papa et al., 2003), Apg1 (Abeliovich et al., 2003), Cla4 (Weiss et al., 2000), Elm1 (Sreenivasan et al., 2003), and Pho85 (Carroll et al., 2001). In each of these studies, the phenotype caused by small molecule inhibition provides insight into normal protein kinase function beyond that gained from gene ablation studies (Knight and Shokat, 2005). Together, these studies of yeast protein kinase signaling demonstrate that the chemical genetic approach represents a powerful tool for the analysis of signal transduction.

Chemical Genetic Analysis of the JNK2 Protein Kinase

We employed a chemical genetic strategy (Bishop et al., 2000) to examine the function of JNK2 by using mice with a knockin mutation in the *Jnk2* gene that expands the ATP pocket of the JNK2 protein kinase and confers sensitivity to inhibition by inactive derivatives of the general protein kinase inhibitor PP1. Comparison of the effect of the loss of JNK2 function in studies using small molecule inhibition of JNK2 activity and targeted ablation of the *Jnk2* gene demonstrated marked differences. Specifically, ablation of the *Jnk2* gene in $Jnk1^{+/+}$ cells caused increased expression of cJun and increased cellular proliferation (Sabapathy et al., 2004; Tournier et al., 2000). In contrast, small molecule inhibition of JNK2 in $Jnk1^{+/+}$ cells caused no decrease in cJun expression (Figure 5C) or proliferation (Figure 6A). Although these different methods (gene ablation and chemical genetic analysis) that cause loss of JNK2 function result in distinct phenotypes in $Jnk1^{+/+}$ cells, it is striking that both methods of blocking JNK2 function cause the same phenotype in $Jnk1^{-/-}$ cells. Thus, both small molecule inhibition of JNK2 in $Jnk1^{-/-}$ cells and ablation of the *Jnk2* gene in $Jnk1^{-/-}$ cells cause reduced expression of cJun and reduced cellular proliferation. These data indicate that the presence of an intact *Jnk1* gene is required for the divergent effects of loss of JNK2 function caused by a small molecule inhibition and by targeted gene ablation. Indeed, reduced JNK1 function caused by pharmacological inhibition (Figure S3A) or reduced *Jnk1* gene dosage (Figure S3B) suppressed the effects of *Jnk2* gene ablation. The simplest explanation of these data is that *Jnk2* gene ablation causes a gain of function of the JNK1 protein kinase. Indeed, a compensatory increase in JNK1 activity is observed in nonstimulated $Jnk2^{-/-}$ cells (Figure 1 and Figure S3C). Together, these observations suggest that the phenotype of $Jnk2^{-/-}$ MEFs is misleading and that previous conclusions, based on these data, indicating that JNK2 functions as a negative regulator of cJun and cellular proliferation (Ronai, 2004; Sabapathy et al., 2004; Sabapathy and Wagner, 2004) may not be correct. Indeed, the increased expression of cJun and increased proliferation of $Jnk2^{-/-}$ MEFs appear to be the result of a compensatory increase in JNK1 function rather than the proposed negative regulatory role of JNK2. The increased JNK1 function may be caused by increased activation by MAP2K or by decreased inactivation by phosphatases.

Our analysis demonstrates that JNK2 is a positive regulator of cJun and that this function of JNK2 is redundant with JNK1. This conclusion is fully consistent with previous biochemical analyses of JNK2 function (Davis, 2000).

JNK2 Deficiency Causes Increased JNK1 Function

The observation that JNK2 deficiency causes increased JNK1 function is intriguing. Similar compensatory changes in the function of individual members within a protein kinase family have been reported previously. One example is represented by the p38 subgroup of MAPK. Thus, mutation of the *p38*γ and *p38*δ genes results in a compensatory increase in the activity of p38α MAPK and also causes functional compensation of substrate phosphorylation (Sabio et al., 2005). It is likely that similar compensatory changes occur after the targeted ablation of genes that encode individual members of other protein kinase families and that this response may be quite common.

What mechanism could account for the increased JNK1 activity detected in JNK2-deficient cells ? One possibility is that JNK1 and JNK2 compete as substrates for the JNK activators MKK4 and MKK7. This hypothesis suggests that the loss of JNK2 enables increased JNK1 activity by reducing competition for the upstream MAPKK. The docking of JNK to the upstream MAPKK is mediated by the NH_2-terminal D domain of the MAPKK (Weston and Davis, 2002), and it is established that affinity of JNK2 for D-domain ligands is greater than JNK1 (Gupta et al., 1996; Kallunki et al., 1994; Sluss et al., 1994). These considerations suggest that the loss of JNK2 expression will have a marked effect on JNK1 activation. In contrast, small molecule inhibition of JNK2 activity does not block JNK2 activation by upstream MAPKK and therefore does not affect the competition between JNK1 and JNK2 for activation by the upstream MAPKK (Figure S4).

Regulation of cJun Degradation by JNK

It is established that the phosphorylation of cJun by JNK reduces the ubiquitin-mediated proteasomal degradation of cJun (Fuchs et al., 1997; Musti et al., 1997). Increased JNK1 activity in $Jnk2^{-/-}$ cells can therefore account for the observation that the half-life of endogenous cJun in $Jnk2^{-/-}$ MEFs (260 min), measured by pulse-chase analysis using [^{35}S]methionine, is longer than endogenous cJun in wild-type MEFs (135 min) (A.J., unpublished data). This increased half-life is consistent with previous studies of ectopically expressed cJun in $Jnk2^{-/-}$ MEFs (Sabapathy and Wagner, 2004). An alternative explanation for these data is that the JNK2 protein itself is a direct regulator of cJun ubiquitin conjugation and proteasomal degradation and that the loss of JNK2 in $Jnk2^{-/-}$ cells directly causes the observed increase in cJun half-life (Fuchs et al., 1997, 1998; Ronai, 2004; Sabapathy et al., 2004; Sabapathy

and Wagner, 2004). Indeed, it has been proposed that inactive JNK2 may directly act on cJun as a negative regulator by increasing ubiquitination (Ronai, 2004; Sabapathy and Wagner, 2004). Our observation that the drug 1NM-PP1 does not cause loss of cJun expression in $J1^{+/+}$ $J2^{MG/MG}$ MEFs (Figure 5C) is inconsistent with this proposal. We conclude that the simplest hypothesis that can account for the effect of JNK2 deficiency to increase cJun expression is that JNK2 deficiency causes increased JNK1 function (Figure 1 and Figure S3C) that leads to increased expression of cJun mRNA (Ventura et al., 2003) and an increased half-life of the cJun protein (Fuchs et al., 1997; Musti et al., 1997).

Conclusions

Our analysis demonstrates that the JNK2 protein kinase is a positive regulator of cJun expression and cellular proliferation. This conclusion is fully consistent with the results of previous biochemical studies that have established that both JNK1 and JNK2 function as positive regulators of cJun (Davis, 2000). This conclusion markedly contrasts with proposals based on an analysis of $Jnk2^{-/-}$ cells that suggest that JNK2 has a direct negative regulatory role on cellular proliferation and cJun expression (Ronai, 2004; Sabapathy et al., 2004; Sabapathy and Wagner, 2004). In conclusion, we suggest that gene ablation studies alone are not sufficient for understanding protein kinase function and that conclusions concerning physiological functions should be based upon multiple experimental approaches, including mutational analysis and pharmacology. This study indicates that a chemical genetic approach is a particularly powerful method that can provide important insight into the function of protein kinases in vivo.

Experimental Procedures

Materials

The drugs 1NM-PP1 (Bishop and Shokat, 1999; Bishop et al., 2000) and SP600125 (Calbiochem) were dissolved in DMSO.

Animal Studies

Mice with targeted disruptions of the *Jnk1* and *Jnk2* genes have been described previously (Dong et al., 1998; Yang et al., 1998). Mice with a germ-line mutation in the *Jnk2* gene that replaces methionine 108 with glycine were constructed by using homologous recombination in embryonic stem (ES) cells. Mouse strain 129/Svev genomic BAC clones containing the *Jnk2* gene were employed to create a targeting vector that was designed to mutate codon 108 (ATG replaced with GGG), to introduce silent mutations in codons 109 and 110 that create a SacI restriction site, and to insert a floxed Neo^R cassette (Figures 2A and 2B). This vector was constructed by using standard techniques. Mouse strain 129/Svev ES cells were electroporated with this vector and selected with 200 μg/ml G418 and 2 μM gangcyclovir. Six correctly targeted ES cell clones were identified by Southern blot analysis and PCR analysis. Two ES cell clones were injected into C57BL/6J blastocysts to create chimeric mice that transmitted the mutated *Jnk2* allele through the germ line. The floxed Neo^R cassette was excised by using Cre recombinase. The mice were backcrossed (ten generations) to the C57BL/6J strain (Jackson Laboratories) and were housed in a facility accredited by the American Association for Laboratory Animal Care (AALAC). The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School.

Genotype Analysis

The genotype at the *Jnk2* locus was examined by Southern blot analysis of HindIII-restricted genomic DNA by probing with a random-primed ^{32}P-labeled probe (260 bp) that was isolated by PCR using a *Jnk2* genomic clone as the template and the amplimers 5′-CGATGCTAGATGTGAGAGAG-3′ and 5′-GCTATCCAGGTTGCTG TGCC-3′. The wild-type and mutated alleles of *Jnk2* were also detected by PCR amplification of genomic DNA using the primers 5′-GGGTAGAGAGGAAAGAACCTGGTG-3′ and 5′-AGACTGCCTTG GGAAAAGCG-3′ and incubation of the 1.5 kb PCR product with the restriction enzyme SacI. Agarose gel electrophoresis indicates the presence of the wild-type *Jnk2* allele that lacks a SacI site (1.5 kb) or the mutated *Jnk2* allele (1.0 kb and 0.5 kb) with a SacI site.

Primary Cell Culture

Primary murine embryo fibroblasts (MEFs) were isolated and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen) at 37°C in a humidified atmosphere with 5% CO_2. Drugs dissolved in DMSO were added directly to the culture medium. Control experiments were performed by using the solvent alone. The final concentration of DMSO in all assays was 0.1% (v/v). All experiments were performed by using MEFs between passage 2 and passage 5. Similar data were obtained in experiments using independently isolated MEF cultures. Proliferation assays were performed by staining with crystal violet (Tournier et al., 2000).

Primary mouse keratinocytes were prepared as previously described (Hakkinen et al., 2001) with some modifications. Briefly, skins from 3-day-old mice were isolated and floated overnight (4°C) on 1 ml of enzyme solution containing trypsin, collagenases I, II, and IV, and dispase (Skin Dissociation System I, CHI Scientific). The epidermis was separated from the dermis, and epidermi from each group were pooled in a sterile dish containing 1 ml medium A. Skins were macerated with surgical scissors, transferred to 9 ml medium A containing 0.5 ml dissociation enzymes, and pipeted vigorously. The isolated cells were strained through a 100 μm mesh, collected by centrifugation, resuspended in medium A, and plated on collagen-coated dishes (Vitrogen 30 μg/ml, Cohesion). The keratinocytes were allowed to attach for 3–5 hr, and medium A was replaced with medium B. Media A/B and supplements were purchased from CHI Scientific.

Immunoblot Analysis

Cell extracts were prepared using Triton lysis buffer (20 mM Tris [pH 7.4], 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL of aprotinin and leupeptin). Extracts (50 μg of protein) were examined by protein immunoblot analysis by probing with antibodies to cJun (H-79, Santa Cruz), phospho(Ser-63)-Jun (Upstate Biotech.), JNK (Pharmingen), phospho-JNK (Cell Signaling), and α-tubulin (Sigma). Immunecomplexes were detected by enhanced chemiluminescence (NEN).

Protein Kinase Assays

The activity of JNK was measured by using in vitro kinase assays using the substrates cJun and [γ-^{32}P]ATP (Whitmarsh and Davis, 2001).

Cell Migration Assays

Boyden chamber assays were performed with using 5×10^4 keratinocytes in 0.5 ml medium B placed in each insert of a 24 multiwell plate (BIOCOAT, Becton Dickinson). Medium B supplemented with 10% FBS was placed on the other side of the membrane. Migration assays were performed by incubating the cells at 37°C (24 hr). The inserts were placed in methanol (−20°C) and stained with 4′-6′-diamino-2-phenylindole (Vector Laboratories). The cells were visualized with an Axioplan 2 microscope with a MicroImager CCD camera (Carl Zeiss).

Apoptosis Assays

Cell death was examined by measurement of DNA fragmentation using the Cell Death Detection Elisa^{plus} kit (Roche) according to the manufacturer's recommendations (Lamb et al., 2003).

Protein Half-Life Determination

Primary MEFs were cultured in 6 cm dishes and were pulse labeled by incubation in methionine-free DMEM containing 5% dialyzed

FBS (1 hr) prior to the addition of [^{35}S]methionine (100 μCi/ml) for 2 hr. The cells were washed two times with PBS and then incubated in DMEM supplemented with 5% FBS. Lysates were prepared at different times after the pulse labeling with [^{35}S]methionine. The cJun protein was isolated by incubation of the lysates with an antibody to cJun conjugated to agarose (Santa Cruz). The immunoprecipitates were examined by SDS-PAGE, and the amount of radioactive cJun was quantitated by Phosphorimager analysis (Molecular Dynamics).

RNase Protection Assays

Total RNA (5 μg) was examined by using the "Multi-probe RNase protection assay" (Pharmingen) with the template sets mFos/Jun, mCR-4, and mCK-3b according to the manufacturer's recommendations. The products were separated on a 5% sequencing gel, detected by autoradiography, and quantitated by Phosphorimager analysis (Molecular Dynamics).

Immunofluorescence Analysis

Cells were grown on coverslips and fixed in 4% paraformaldehyde. The coverslips were washed in PBS, incubated (30 min at 22°C) in BPT buffer (3% BSA/PBS/0.2% Tween-20), and stained with a mouse monoclonal antibody to α-tubulin (Sigma) in BPT buffer (60 min at 22°C) or with a rabbit antibody to phospho(Ser-63)cJun (Cell Signaling). Immunecomplexes were visualized by using an Alexa488-conjugated goat anti-mouse Ig secondary antibody or an Alexa488-conjugated goat anti-rabbit Ig secondary antibody (Molecular Probes). Actin was stained with Alexa546-conjugated phalloidin (Molecular Probes). Slides were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Inc). Fluorescence microscopy was performed by using a confocal laser scanning microscope (Leica TCS SP2).

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.molecule.org/cgi/content/full/23/6/899/DC1/.

Acknowledgments

We thank Dr Stephen Jones for assistance with blastocyst injections, Judy Reilly and Jian-Hua Liu for expert technical assistance, and Kathy Gemme for administrative assistance. These studies were supported by grants from the National Institutes of Health. K.M.S., R.A.F, and R.J.D. are Investigators of the Howard Hughes Medical Institute.

Received: January 13, 2006
Revised: June 14, 2006
Accepted: July 21, 2006
Published: September 14, 2006

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