The stress-activated protein kinase JNK plays an important role in the stability and activities of key regulatory proteins, including c-Jun, ATF2, and p53. To better understand mechanisms underlying the regulation of JNK activities, we studied the effect of expression of the amino-terminal JNK fragment (N-JNK; amino acids 1-206) on the stability and activities of JNK substrates under nonstressed growth conditions, as well as after exposure to hydrogen peroxide. Mouse fibroblasts that express N-JNK under tetracycline-off (tet-off) inducible promoter exhibited elevated expression of c-Jun, ATF2, and p53 upon tetracycline removal. This increased coincided with elevated transcriptional activities of p53, but not of c-Jun or ATF2, as reflected in luciferase activities of p21waf1/cip1-Luc, p21waf1/cip1-Luc, and Jun2-Luc, respectively. Expression of N-JNK in cells that were treated with H2O2 impaired transcriptional output as reflected in a delayed and lower level of c-Jun-, limited ATF2-, and reduced p53-transcriptional activities. N-JNK elicited an increase in H2O2-induced cell death, which is p53-dependent, because it was not seen in p53 null cells yet could be observed upon coexpression of p53 and N-JNK. The ability to alter the activity of ATF2, c-Jun, and p53 and the degree of stress-induced cell death by a JNK-derived fragment identifies new means to elucidate the nature of JNK regulation and to alter the cellular response to stress.

The family of mitogen-activated protein kinases (MAPK) consists of evolutionarily conserved proteins, which play a central role in development and growth as well as in the protection of cells from stress and DNA damage (1–5). Major components within the MAPK family are extracellular signal-regulated kinases (ERKs), stress-activated protein kinases (SAPK/JNK), and p38 (5, 6). Whereas ERKs are preferentially activated by mitogens (7, 8), the JNK and p38 pathways are triggered primarily by inflammatory cytokines and by a diverse array of cellular stresses, including UV light and hydrogen peroxide.

MAPKs phosphorylate proteins located at the plasma membrane, cytoplasm, and nucleus (16). In nonstimulated cells, MAPK are largely cytoplasmic. Upon activation, a portion of MAPK translocate to the nucleus (17–22). The duration of activation of MAPKs influences the extent of their nuclear translocation and, thus, their access to transcription factors (23–38). One of the modes of activation is dimerization, which was shown for ERK2. Whereas ERK2 is found as a monomer in resting cells, ERK2 exists primarily as a dimer in stimulated cells (39). Based on their structural homology, p38 and JNK/SAPK may also form homodimers. Although heterodimerization between different MAPK family members is unlikely, based on structural data (40–42), heterodimerization between different isoforms of JNK or within the MKK7 family (9, 30, 43) may take place.

To identify the function of respective domains within stress kinases, chimeric molecules have often been used. Chimeric p38-p44ERK was able to transduce stress and growth factor signals via a 40-amino acid fragment located within the amino-terminal region of p38, which determines the specificity of response to extracellular signals. However, the p44 (portion of the) chimera redirected stress signals into early mitogenic responses (44). ERK5/ERK2 chimeras served to identify c-Myc as one of the ERK5 substrates with V12 H-Ras acting as its upstream regulator (45). A JNK2-JNK1 chimera renders JNK a constitutively active kinase in nonstressed cells (46).

In normal growing cells, JNK activity is limited due to the inhibitory effect of GSTpi (47). Under the same conditions, JNK efficiently targets the ubiquitination and degradation of its associated proteins, as demonstrated for c-Jun, ATF2, and p53 (48–51). In response to stress, JNK phosphorylation on both Thr-183 and Thr-185 residues by MKK4/7 (9, 28) leads to the phosphorylation of JNK substrates, which include c-Jun (52, 53), ATM-p21waf1/cip1, and Jun2-Luc, respectively. Expression of N-JNK in cells that were treated with H2O2 impaired transcriptional output as reflected in a delayed and lower level of c-Jun-, limited ATF2-, and reduced p53-transcriptional activities. N-JNK elicited an increase in H2O2-induced cell death, which is p53-dependent, because it was not seen in p53 null cells yet could be observed upon coexpression of p53 and N-JNK. The ability to alter the activity of ATF2, c-Jun, and p53 and the degree of stress-induced cell death by a JNK-derived fragment identifies new means to elucidate the nature of JNK regulation and to alter the cellular response to stress.

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ERK2, is expected to mediate association with JNK substrates. Furthermore, due to the lack of a carboxyl-terminal domain, N-JNK is not expected to be subject to intramolecular inhibition, which is a common mode of regulation for stress kinases (59). By analogy to ERK2, dimerization of JNK molecules may be impaired upon expression of N-JNK.

Two-hybrid screening using the amino-terminal domain of JNK as bait has identified multiple clones that possess the carboxyl-terminal domain of JNK, thereby indicating association between the amino- and carboxyl-terminal regions of this protein.\(^2\) This result suggests that JNK may be found either as a dimer or that JNK may exhibit intramolecular inhibition in which the carboxyl-terminal region loops back onto the amino-terminal domain. Either possibility was shown to exist for other MAPK family members (39), and is expected to play an important role in the regulation of JNK activities. Using a tetracycline (tet)-off-inducible system we demonstrate the effect of N-JNK on its substrates (c-Jun, ATF2, and P53) under normal growing conditions and after exposure to external stress. The nature of N-JNK effects before and after stress and the possible use of N-JNK to further elucidate the regulation of JNK activities are discussed.

**Regulation of p53 by Amino-terminal JNK Fragment**

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**MATERIALS AND METHODS**

**Cells and Protein Preparation**—The mouse fibroblast cell line NIH 3T3 cells that stably express the pSV40-Hyg plasmid (CLONTECH) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (Life Technologies, Inc.). Cells were grown at 37 °C with 5% CO\(_2\). The pTet-N-JNK was constructed by subcloning the cDNA of the amino-terminal 206 amino acids of wild type JNK1 into the tet-regulated promoter of the pUHD-10-3 vector. Cell clones that stably express both constructs were selected in 600 \(\mu\)g/ml geneticin in the presence of hygromycin (100 \(\mu\)g/ml) 24 h after DOTAP transfection with the pTet-N-JNK-UHD-10-3 construct. Cells inducible with N-JNK-tet were maintained in Dulbecco’s modified Eagle’s medium containing fetal bovine serum (10%), hygromycin (100 \(\mu\)g/ml), and geneticin (400 \(\mu\)g/ml). To maintain suppression of N-JNK expression, tet was added to the medium every 3 days (to a final concentration of 1 \(\mu\)M). Proteins were prepared from cells as described previously (60). In all cases, buffer contained a mixture of proteases (1 \(\mu\)g/ml pepstatin, leupeptin, and aprotinin) and the phospha- tase inhibitors (sodium vanadate 1 mM; sodium fluoride 5 mM).

**Antibodies, Immunoprecipitations, and Immunoblots**—Antibodies to c-Jun, phospho-c-Jun, ATF2, phospho-ATF2, and phospho-JNK were purchased from New England BioLabs. Polyclonal antibodies to JNK or p53 were generated using bacterially expressed JNK or p53 as antigen. Monoclonal JNK antibodies (clone 333) (PharMingen) and monoclonal antibodies to p53, clone pAb421 (Oncogene Science) were purchased.

Immunoprecipitations were carried out using 1 mg of whole cell extracts (WCE) and 2 \(\mu\)g of the respective antibodies, for 16 h at 4 °C. Protein G beads (Life Technologies, Inc.) were added (15 \(\mu\)l) for 30 min at room temperature before washes were carried out in phosphate-buffered saline (0.5% L-C\(_{14}\)) supplemented with Tween 100 (0.5%). Immunoprecipitated material was subjected to Western blot analysis.

Immunoblot analysis was performed using 100 \(\mu\)g of WCE separated on SDS-polyacrylamide gel electrophoresis (PAGE) followed by electrotransfer to a nitrocellulose membrane. Ponceau staining was carried out to confirm equal loading followed by blocking (5% non-fat milk) and reaction with the respective antibodies. Reactions were visualized using chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech).

**H\(_2\)O\(_2\) Treatment**—Transfected cells in the growing phase were treated for 1 h with 100 \(\mu\)M H\(_2\)O\(_2\) by taking the medium from the culture dish and mixing it with freshly diluted H\(_2\)O\(_2\). Medium containing H\(_2\)O\(_2\) was immediately applied to the fibroblasts (10\(^7\) cells).

**Protein Kinase Assays**—Protein kinase assays were carried out using fusion proteins, GST-Jun or GST-ATF2 (60), as substrates. Purity of bacterially produced c-Jun and ATF-2 was confirmed via silver-stained SDS-PAGE. Immunokinase reactions were carried out on immunoprecipitated kinase using antibodies to JNK, which was then incubated with the respective substrate, in a soluble form, in the presence of kinase buffer (20 mM HEPES, pH 7.6, 1 mM EGTA, 1 mM dithiothreitol, 2 mM MgCl\(_2\), 2 mM MnCl\(_2\), 5 mM NaF, 1 mM NaVO\(_3\), 50 mM CaCl\(_2\)) at 37 °C for 15 min. The protein G beads were pelleted and washed extensively with PBST (150 mM NaCl, 16 mM sodium phosphate, pH 7.5, 0.01% Triton X-100, 2 mM EDTA, 0.1% β-mercaptoethanol, 8 mM sodium fluoride, and 5 mM benzamidine) and then incubated with \(\gamma\)\(^32\)P\(_{-}\)ATP (50 cpm/m\(_\mu\)l) in the presence of kinase buffer. Following extensive washing, the phosphorylated substrate was boiled in SDS sample buffer and the eluted proteins were run on a 10% SDS-polyacrylamide gel. The gel was dried, and phosphorylation of the c-Jun or ATF-2 substrate was determined by autoradiography. In all cases the buffer used contained a mixture of protease and phosphatase inhibitors (10, 47, 60).

**In Vitro Ubiquitination Assay**—The in vitro ubiquitination assay (see Ref. 49 for details) was performed using 50 \(\mu\)g of whole cell lysates prepared from N-JNK-expressing cells (maintained with or without tetracycline) that were incubated on ice with bacterially expressed (6x)his-tagged human p53 (5 \(\mu\)g) bound to NTA beads for 45 min. After extensive washes (four times with 1 ml of kinase buffer (49)), the substrate-bound beads were equilibrated with 1× ubiquitination buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl\(_2\), 0.5 mM dithiothreitol, 2 mM NaF, and 5 mM okadaic acid) and incubated in the same buffer supplemented with 2 mM ATP, 10 mM creatine phosphate, 0.02 unit of creatine phosphokinase, 2 \(\mu\)g of hemagglutinin (HA)-tagged ubiquitin (49), 1.5 mM ATP\(_5\)S (Sigma; a reagent included to block degradation, thus enabling monitoring of the degree of ubiquitination) and 33% reticulocyte ubiquitase (v/v) in a total volume of 30 \(\mu\)l at 37 °C for 5 min. The reaction was stopped by adding 0.5 ml of 8 M urea in sodium phosphate buffer (pH 6.3) with 0.1% Nonidet P-40. The beads were washed, and the protein moiety was eluted and resolved on 8% SDS-PAGE followed by electrotransfer onto nitrocellulose filters. Nitrocellulose filters were blocked with 5% non-fat milk and probed with HA11 antibody (BabCo).

**Transcription Assays**—Respective luciferase constructs were co-transfected with \(\beta\)Gal vector controlled by the same promoter. Transcriptional analysis was carried out on proteins prepared at time points indicated under “Results.” Transcriptional analysis of Jun/ATF2 was carried out using the 5xJun2 or AP1-driven luciferase construct as previously reported (61). Analysis of p53 transcription was carried out using the p21-luc construct. In all cases, values were normalized with respect to transfection efficiency based on levels of \(\beta\)Gal activities. Data shown represent three independent experiments performed in duplicate.

**Analysis of Cell Death**—Analysis of cell death was carried out as described by Kumar et al. (62). Briefly, triplicates of >5,000 cells per measurement, at the time points indicated under “Results,” were subjected to blue or blue-green stain which identifies cells with apoptotic nuclei. Asass, all cases, analysis was performed at least three times. Analysis of cell death in transfected 10.1 cells (constructs: p53-HA in pcDNA3, JNK1–206 in pcDNA3) was carried out using PI staining. Flow cytometric analysis (20,000 cells per assay) was performed on a fluorescence-activated cell sorter (FACS; Calibur flow cytometer, Becton Dickinson). Data analysis was carried out using the FACS Desk software.

**RESULTS**

**Expression and Association of N-JNK in NIH 3T3 Cells**—To elucidate the possible contribution of JNK domains to its cellular activities, we analyzed the effects of a JNK fragment, which represents the amino-terminal portion (amino acids 1–206) of JNK (N-JNK), and includes the kinase domain. The N-JNK fragment was cloned into the tet-off-inducible expression vector, which was transfected into mouse fibroblasts. Following drug selection, we identified cell clones in which expression of amino-terminal JNK is induced upon tetracycline removal. Immunoblot analysis allows detection of N-JNK expression as early as 1 h after tet removal followed by a time-dependent increase, which reaches its maximal expression levels after 12 h (Fig. 1a). Expression of N-JNK increased the level of endogenous JNK expression (compare time 0 in tet– versus tet+ in Fig. 1b) but not the degree of JNK phosphorylation (Fig. 1b). Expression of N-JNK did not cause major changes in JNK expression and activity following exposure to H\(_2\)O\(_2\) (Fig. 1c).

**Effect of N-JNK on Phosphorylation and Transcriptional Activation of Jun/ATF2**—To test the effect of the N-JNK fragment on JNK substrates, we first monitored changes in the...
Fig. 1. a, expression of N-JNK in Swiss 3T3 cells after removal of tetracycline. HA-tagged N-JNK (1–206 amino acids) was used to establish 3T3 cells that are tet-regulated. Mixed populations of cells were selected based on drug resistance and analyzed for level of N-JNK expression at the indicated time points (hours) after tet removal. Western blot analysis revealed the expression of a 28-kDa protein detected by antibodies to HA. b, expression and phosphorylation of JNK1 and JNK2 in the presence or absence of N-JNK expression and H$_2$O$_2$ treatment. Cells were maintained in the presence or absence of tet (for 12 h) and harvested at the indicated time points after H$_2$O$_2$ treatment. Western blot analysis was carried out using specific polyclonal antibodies against either JNK or the phosphorylated protein. Western blot shown is representative of three independent experiments.

expression, phosphorylation, and activity of c-Jun and ATF2. Under nonstressed growth conditions, expression of N-JNK led to increased expression of c-Jun (4-fold; compare time 0 in tet$^-$ and tet$^+$ lanes of Fig. 2a, upper panel) and ATF2 (2-fold; Fig. 2b, upper panel) based on densitometric analysis of three experiments. Increased expression of N-JNK also caused elevated basal phosphorylation levels of c-Jun (23-fold) and ATF2 (40-fold) (Fig. 2a, compare time 0 in tet$^+$ with tet$^-$ lanes). Because JNK targets the ubiquitination of nonphosphorylated forms of its associated proteins (48), elevated phosphorylation is expected to confer respective increases in the expression levels of this protein.

Next, we assessed the changes in the expression and activities of c-Jun and ATF2 in cells exposed to stress, using hydrogen peroxide as a model. Treatment with H$_2$O$_2$ increased phosphorylation of c-Jun and ATF2. Although phosphorylation of c-Jun was 9-fold higher 1 h after H$_2$O$_2$ treatment, it was otherwise similar to that seen in the absence of N-JNK expression. Duration of ATF2 phosphorylation is shorter upon N-JNK expression, because ATF2 phosphorylation is three times lower 4 h after H$_2$O$_2$ treatment (Fig. 2b, compare tet$^+$ and tet$^-$ lanes at the 4-h time point). These data suggest that the effects of N-JNK are more pronounced in nonstressed cells where N-JNK increases expression and phosphorylation levels of both ATF2 and c-Jun. Importantly, immunoprecipitation of N-JNK using HA antibodies from hydrogen peroxide tet$^-$ cells followed by solid phase kinase reaction using GST-Jun as a substrate did not reveal Jun phosphorylation, whereas immunoprecipitation using antibodies to JNK (allowing detection of endogenous JNK activity) as a positive control revealed such activity (data not shown). This observation suggests that, although the N-JNK fragment contains the kinase domain, it does not elicit kinase activities.

To test whether N-JNK expression would alter transcriptional activity of Jun/ATF2, cells were transfected with Jun2-Luciferase or AP1-Luciferase constructs, which contain the target sequence for Jun/ATF2 or c-Jun/c-Fos heterodimers, respectively. Expression of N-JNK caused a modest decrease (25%) in AP1-Luc activity (time 0 in Fig. 2c), and it increased (33%) activity of Jun2-Luc (Fig. 2d). Within 4 h after H$_2$O$_2$ treatment, there was a noticeable increase in both AP1-Luc (70%) and Jun2-Luc (110%) activities. Removal of tet, which enables the expression of N-JNK in hydrogen peroxide-treated cells, caused a delayed and a modest increase (40%) in AP1-Luc activities after 4 h (Fig. 2e). Furthermore, at this time point after treatment with hydrogen peroxide, the degree of AP1-driven transcription was 30% lower than the level seen in cells that do not express N-JNK (Fig. 2c). This observation suggests that expression of N-JNK attenuates the degree of AP1-mediated transcription under conditions that are known to increase c-Jun phosphorylation and transactivation. Analysis of Jun2-Luc activities in hydrogen peroxide treated cells revealed a 2-fold increase within the first 4 h, which was also attenuated in the presence of N-JNK expression. In fact, N-JNK-expressing cells maintained the same level of transactivation seen under nonstressed conditions, which was 30% higher than the basal levels of Jun2-Luc activity (Fig. 2d). This result indicates that, in N-JNK-expressing cells, activities of c-Jun and ATF2 are limited following exposure to stress in the form of hydrogen peroxide. Such change is expected to alter the cellular response to stress and damage.

To test whether N-JNK associates with c-Jun or ATF2, proteins prepared from control or hydrogen peroxide-treated cells that were maintained in the presence or absence of tet were subjected to immunoprecipitation using antibodies to HA (which recognizes the HA-tagged N-JNK) followed by immunoblot using antibodies to either c-Jun or ATF2. This analysis did not identify association between ATF2 or c-Jun and N-JNK (Fig. 2e).

N-JNK Associates with p53 in Vitro and in Vivo—To further explore cellular effects of the amino-terminal JNK fragment, we elucidated possible changes in the stability and activity of the tumor suppressor protein p53. Previous studies revealed that JNK contributes to the stability as well as the activity of p53 (50, 58). To elucidate the effect of N-JNK on p53 we first determined whether it associates with p53 in vitro. Bacterially expressed p53 was incubated with N-JNK peptide followed by immunoprecipitation using antibodies to either p53 or JNK. This experiment revealed that N-JNK-derived peptide bound to p53 in vitro (Fig. 3a). Because these reactions were performed in the absence of full length JNK, we conclude that JNK peptides can directly associate with p53. Association between the JNK fragment and p53 was confirmed in cells that were induced to express N-JNK. Pull down experiments using antibodies to p53 identified the association of N-JNK with p53 in the cells that were maintained in medium deprived of tetracycline, in which N-JNK expression was induced. Such association was identified in nonstressed cells as well as in cells that were treated with hydrogen peroxide (Fig. 3b).

We next determined whether N-JNK associates with p53 through the JNK docking site, which was previously mapped to amino acids 96–117 (63). Forced expression of p53 construct that was deleted of the JNK binding site followed by pull down and analysis of N-JNK association no longer identified the N-JNK fragment in complex with p53 (Fig. 3c). These data suggest that the N-JNK fragment associates with p53 through the JNK docking site.

N-JNK Fragment Increases p53 Stability—As JNK is among the proteins that actively target the ubiquitination and degradation of p53, we next studied whether the expression of N-JNK could alter p53 expression and stability under normal conditions and after stress. In the presence of tetracycline, when N-JNK expression is suppressed, p53 levels are low and exhibit a characteristic, time-dependent increase after treatment with H$_2$O$_2$ (Fig. 4a). Expression of N-JNK significantly increased basal levels of p53 expression (11-fold) under non-
stressed conditions (compare time 0 in tet + and tet − lanes, Fig. 4a). Levels of p53 did not increase further in response to treatment with H2O2 (Fig. 4a), probably because the expression of N-JNK caused maximal stabilization of p53 expression levels.

To determine whether increased p53 levels after N-JNK expression result from impaired ubiquitination of p53 we performed in vitro ubiquitination assays, which enabled us to monitor the amount of polyubiquitin chains on p53. Protein extracts prepared from N-JNK-expressing cells were incubated with bacterially expressed histagged p53 followed by addition of reticulocyte lysates that allowed p53 ubiquitination to be carried out. Changes in the levels of p53 ubiquitination upon N-JNK expression were monitored through the level of polyubiquitin chains that consist of HA-ubiquitin added to the reaction. Polyubiquitination of p53 under these conditions is monitored via the degree of smear above the p53-reacting band. Such analysis revealed that, although p53 exhibits extensive levels of ubiquitination under normal growth conditions, upon expression of the N-JNK fragment there is a marked decrease in the amount of polyubiquitin chains seen on p53 (Fig. 4b). These findings suggest that elevated levels of p53 expression under induced expression of N-JNK fragment could be attributed to inhibition of p53 ubiquitination.

Effect of N-JNK on p53 Transcriptional Activity—To determine whether elevated expression of p53 would be also reflected in its activities, we monitored changes in the transcriptional activation of p21Waf1/Cip1 promoter sequences, one of the transcriptional targets of p53. Transfection of the luciferase construct, which is regulated by p21 promoter sequences, revealed low basal levels of activities, which were induced in a time-dependent manner after exposure to H2O2 (6-fold increase after 4 h). Expression of N-JNK in nonstressed cells led to a 10-fold increase in p21Waf1/Cip1-Luc activity (Fig. 4c), suggesting that elevated expression of p53 due to N-JNK also contributes to increased transcriptional activities. Of interest, however, is that although exposure to hydrogen peroxide caused a time-dependent increase in p21Waf1/Cip1-Luc activities, there was a time-dependent decrease in levels of p21Waf1/Cip1-Luc activity in the presence of N-JNK expression and hydrogen peroxide treatment, with a maximal (20-fold) decrease seen after 4 h (Fig. 4c). These findings suggest that N-JNK attenuates p53 transcriptional activities in H2O2-treated cells.

Effect of N-JNK on H2O2-induced Cell Death—To further elucidate the possible cellular effects of N-JNK expression, we monitored the level of cell death in response to hydrogen peroxide treatment. The expression of N-JNK did not affect the
level of cell death in nonstressed cells. Exposure to H$_2$O$_2$ caused cell death, which increased in a time-dependent manner. Eight hours after hydrogen peroxide treatment, the level of cell death reached 43% (Fig. 5a). Removal of tetracycline, allowing the expression of N-JNK, further increased the amount of dead cells from 43 to 71% (Fig. 5a). These data suggest that expression of N-JNK increases level of H$_2$O$_2$-induced cell death.

Given the effect of the N-JNK fragment on other JNK substrates, as shown for c-Jun and ATF2, we next determined whether the changes seen in H$_2$O$_2$-induced cell death could be primarily attributed to the effect of N-JNK on p53. To this end, p53 null mouse fibroblasts (10.1 cells) were transfected with wild-type p53 and N-JNK expression vectors. Control cells transfected with empty expression vector exhibit 1.2% cell death, which increased to 1.7% within 4 h after H$_2$O$_2$ treatment (Fig. 5b). Forced expression of N-JNK increased level of cell death to 2.8%, which was not affected in the presence of hydrogen peroxide. Forced expression of p53 led to 6.9% of cell death, which doubled (12.6%) in p53-transfected cells that were exposed to H$_2$O$_2$. Coexpression of p53 and N-JNK did not cause noticeable change in basal levels of cell death (from 6.9 to 7.7%). H$_2$O$_2$ treatment of cells that were cotransfected with p53 and N-JNK led to a marked increase (from 7.7 to 35.4%) in the level of cell death (Fig. 5b). These findings suggest that hydrogen peroxide-mediated cell death is p53-dependent and that expression of N-JNK efficiently increases the degree of cell death in a p53- and stress-dependent manner.

**DISCUSSION**

Among mechanisms underlying the regulation of stress kinases is their dimerization and abrogation of intramolecular inhibition, which are dependent upon their phosphorylation by their respective upstream kinases (39). Although neither mechanism was shown to exist for JNK, studies with other members...
of the MAPK family, which share structural and functional similarities with JNK, imply that such changes may also take place in the JNK family of stress kinases.

Dimerization or interference with intramolecular inhibition is expected to require intact JNK molecules. On this premise, we tested the effect of the JNK fragment derived from the amino-terminal domain. Because the amino-terminal region of MAPK is similar in structure to JNK and is implicated in mediating the association between MAPK and its substrates (39, 64), it was expected that the N-JNK fragment would efficiently associate with its substrates. However, our data indicate that such association could be found for p53 but not for c-Jun or ATF2, suggesting that N-JNK association is conformation-dependent. The increased expression of JNK substrates seen under normal growth coincides with their increased phosphorylation as shown for c-Jun and ATF2 and is expected to decrease the degree of ubiquitination, as demonstrated here for p53. Decreased targeting for ubiquitination is expected in light of elevated basal phosphorylation, which protects JNK substrates from degradation (48–50). The nature of increased phosphorylation seen upon elevated N-JNK expression could be attributed to a lack of intramolecular inhibition, thereby allowing constitutive activities of JNK, or to a different form of heterodimerization under which a full length JNK molecule could associate with the amino-terminal JNK. Our data do not allow us to distinguish between these two possibilities.

Interestingly, increased stability caused a respective increase in the transcriptional output of p53, as reflected in a 10-fold increase in p21-Luc promoter activities. Conversely, the changes seen in AP1-Luc or Jun2-Luc were marginal; the nature of these differences may relate to N-JNK association with p53 but not with c-Jun or ATF2. One can not exclude the possibility that N-JNK may increase p53 tetramerization but not heterodimerization of ATF2 and c-Jun, which may be tempered upon N-JNK association.

Of further interest is the ability of N-JNK to reduce transcriptional output from JNK substrates in response to hydrogen peroxide. The three scenarios observed in our studies were: (a) N-JNK expression delayed and reduced the degree of transcriptional output as shown for c-Jun; (b) N-JNK increased basal transcriptional activities, which were maintained at the same levels after exposure to stress, thereby not affected by stress per se, as shown for ATF2/Jun output; (c) Expression of N-JNK in hydrogen peroxide-treated cells abrogated transcriptional activities, as shown for the effect of p53 on p21<sup>Waf1/Cip1</sup> promoter sequences. The changes seen upon N-JNK expression suggest that the nature of the substrate affected and the subsequent heterodimerization of the substrate with other transcription factors greatly impacts the degree of change seen due to N-JNK expression. Overall, N-JNK expression caused delayed/reduced transcriptional output of JNK substrates in response to hydrogen peroxide, suggesting that N-JNK association interferes with additional changes that are required for eliciting H<sub>2</sub>O<sub>2</sub>-induced transcriptional activities. Alternatively, expression of N-JNK may alter protection from protein phosphatases that are activated in response to H<sub>2</sub>O<sub>2</sub> (65), thereby reducing the overall signal seen upon N-JNK expression in nonstressed cells. Additionally, N-JNK expression may increase association of Hsp72 with JNK, which attenuates JNK activities in response to stress (66).

The implications of reduced transcriptional output from JNK substrates in hydrogen peroxide-treated cells are clearly seen via elevated degree of cell death. Of interest is that increased cell death is p53-dependent, because it is not seen in p53 null cells unless p53 is transfected. The latter observation suggests that the effect seen on ATF2 and c-Jun can also be channeled through p53, as part of the cellular response to the type of stress studied here.

The role of JNK in promoting or protecting from cell death has been widely demonstrated and appears to depend on the nature of the cells, the degree of damage, and the availability of other stress kinases. Here we demonstrate that, via blocking JNK substrates, the protection from cell death is impaired, as implicated in the increased degree of H<sub>2</sub>O<sub>2</sub>-mediated cell death. Under these conditions, JNK appear to elicit protection from hydrogen peroxide-induced cell death.

The JNK fragment that has been used in the present studies consists of the first 206 amino acids, which contain the kinase domain of JNK. It is, therefore, of interest to explore the effects of additional amino-terminal fragments that are shorter and lack the kinase domain. Preliminary studies revealed that the JNK fragment, which consists of the first 88 amino acids, exhibits a substantially greater affinity for p53 and is, there-
fore, a good candidate for subsequent analysis of biological output.

In all, our studies have identified and characterized a reagent that is useful in our quest to better understand the regulation and function of JNK, one of the key stress kinases. The N-JNK fragment can thus assist in further characterization of JNK and its substrates under normal as well as stressed growth conditions. Changes in the regulation of JNK and respective substrates were identified in several human tumors, in most cases due to altered expression/activity of regulatory components (i.e. TRAF2, M KK4, GSTp). The ability to utilize a stress kinase-driven fragment to increase the degree of stress-induced cell death also identifies a new target for design of reagents that may assist in selective potentiation of cell death.

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REFERENCES
1. Errede, B., and Levin, B. E. (1993) Curr. Opin. Cell Biol. 5, 254–260
2. Davis, R. J. (1994) Trends Biochem. Sci. 19, 470–473
3. Marshall, C. J. (1994) Curr. Opin. Genet. Dev. 4, 82–89
4. Kyriakis, J. M., and Avruch, J. (1996) J. Biol. Chem. 271, 24313–24316
5. Schaeffer, H. J., and Weber, M. J. (1999) Mol. Cell. Biol. 19, 2435–2444
6. Widmann, C., Gibson, S., Jarbe, M. B., and Johnson, G. L. (1999) Physiol. Rev. 79, 143–180
7. Lopez-Iasaca, M. (1998) Biochem. Pharmacol. 56, 269–277
8. Kyriakis, J. M., and Avruch, J. (1996) Bioessays 18, 567–577
9. Derijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) Cell 76, 1025–1037
10. Adler, V., Schaffer, A., Kim, J., Dolan, L. R., and Ronai, Z. (1995) J. Biol. Chem. 270, 26071–26077
11. Karin, M. (1995) J. Biol. Chem. 270, 16483–16486
12. Minden, A., and Karin, M. (1997) Biochem. Biophys. Acta 1333, P85–P104
13. Adler, V., Yin, Z., Tew, K. D., and Ronai, Z. (1999) Oncogene 18, 6110–6111
14. Kamata, H., and Hirata, H. (1999) Cell Signalling 11, 1–14
15. Whitmarsh, A. J., Cavanagh, J., Tournier, C., Yasuda, J., and Davis, R. J. (1998) Science 281, 1671–1674
16. Moriguchi, T., Gotoh, Y., and Nishida, E. (1996) Adv. Pharmacol. 36, 121–137
17. Chen, R.-H., Sarnecki, C., and Blenis, J. (1992) Mol. Cell. Biol. 12, 915–927
18. Chen, Y. B., Wang, X., Templeton, D., Davis, R. J., Tan, T. H. (1996) J. Biol. Chem. 271, 31912–31936
19. Gonzalez, F. A., Seth, A., Raden, D. L., Bowman, D. S., Faye, F. S., and Davis, R. J. (1993) J. Cell Biol. 122, 1089–1101
20. Lenormand, P. S., Sardi, C., Pages, G., L’Allemain, G., Brunet, A., and Pouyssegur, J. (1993) J. Cell Biol. 122, 1079–1088
21. Reska, A. A., Sager, R., Diller, C. D., Krebs, E. G., and Fischer, E. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8881–8885
22. Fukuda, M., Gotoh, Y., and Nishida, E. (1997) EMBO J. 16, 1901–1908
23. Chou, S.-y., Baichwal, V., and Ferrell, J. E., Jr. (1992) Mol. Biol. Cell 3, 1117–1130
24. Gille, H., Sharrocks, A. D., and Shaw, P. E. (1992) Nature 358, 414–416
25. Hunter, T., and Karin, M. (1992) Cell 70, 375–387
26. Seth, A., Gonzalez, F. A., Gupta, S., Raden, D. L., and Davis, R. J. (1992) J. Biol. Chem. 267, 24796–24804
27. Cheng, J.-T., Cobb, M. H., and Baichwal, V. (1993) Mol. Cell. Biol. 13, 801–808
28. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Genes Dev. 7, 2135–2148
29. Brunner, D., Ullrich, A., Bhojwani, and Z. Ronai, unpublished data.
Amino-terminal-derived JNK Fragment Alters Expression and Activity of c-Jun, ATF2, and p53 and Increases H₂O₂-induced Cell Death
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