

# JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation

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**The transcriptional activity of c-Jun is augmented through phosphorylation at two sites by a c-Jun amino-terminal kinase (JNK). All cells express two distinct JNK activities, 46 and 55 kD in size. It is not clear which of them is the more important c-Jun kinase and how they specifically recognize c-Jun. The 46-kD form of JNK was identified as a new member of the MAP kinase group of signal-transducing enzymes, JNK1. Here, we report the molecular cloning of the 55-kD form of JNK, JNK2, which exhibits 83% identity and similar regulation to JNK1. Despite this close similarity, the two JNKs differ greatly in their ability to interact with c-Jun. JNK2 binds c-Jun ~25 times more efficiently than JNK1, and as a result has a lower  $K_m$  toward c-Jun than JNK1. The structural basis for this difference was investigated and traced to a small  $\beta$ -strand-like region near the catalytic pocket of the enzyme. Modeling suggests that this region is solvent exposed and therefore is likely to serve as a docking site that increases the effective concentration of c-Jun near JNK2. These results explain how two closely related MAP kinases can differ in their ability to recognize specific substrates and thereby elicit different biological responses.**

[Key Words: JNK; MAP kinase; phosphorylation; substrate specificity]

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The *trans*-activation potential of the c-Jun proto-oncogene protein is augmented by phosphorylation at Ser-63 and -73 (Binétruy et al. 1991; Pulverer et al. 1991; Smeal et al. 1991). Phosphorylation of these residues is stimulated by transforming oncoproteins (Binétruy et al. 1991; Smeal et al. 1991, 1992), growth factors (Smeal et al. 1992), UV irradiation (Devary et al. 1992), and antigenic activation of T cells (Su et al. 1994). In vitro, Ser-63 and -73 are specifically phosphorylated by two protein kinases, 46 and 55 kD in size, that bind to the amino-terminal activation domain of c-Jun, termed JNKs (Hibi et al. 1993; Minden et al. 1994). No other protein kinases were found to phosphorylate these sites (Hibi et al. 1993; Minden et al. 1994). The JNKs are strongly activated following UV irradiation and more modestly by Ha-Ras expression (Hibi et al. 1993; Dérijard et al. 1994). Molecular cloning identified the 46-kD form of JNK as a novel MAP kinase, termed JNK1, displaying ~40% sequence identity to other MAP kinases, including ERK1 and ERK2 (Dérijard et al. 1994). Despite their ability to use phosphoacceptor sites with primary structure similar to the JNK phospho-

rylation sites (Davis 1993), ERK1 and ERK2 do not phosphorylate Ser-63 and -73 and instead phosphorylate c-Jun at Ser-243 near its carboxyl terminus (Alvarez et al. 1991; Minden et al. 1994). c-Jun provides a good example for the distinct substrate specificity of MAP kinases, whose minimal consensus phosphorylation site is either Ser-Pro or Thr-Pro (Davis 1993). This group of protein kinases occupies an important and critical role in cellular signal transduction, as they convert extracellular stimuli to specific regulatory events affecting the pattern of gene expression, most likely through phosphorylation of specific transcription factors (Ammerer 1994; Karin 1994). Despite the rapidly accumulating knowledge regarding the regulatory pathways responsible for MAP kinase activation and their phenotypic effects on genetically dissectable organisms, such as yeast (Ammerer 1994) and *Drosophila* (Brunner et al. 1994; O'Neill et al. 1994), the mechanisms responsible for their substrate specificity are currently unknown.

More recently, a murine homolog of JNK1, termed SAPK $\gamma$ , was cloned as well as three related proteins SAPK $\alpha$ I, SAPK $\alpha$ II and SAPK $\beta$  (Kyriakis et al. 1994). However, the involvement of the individual SAPKs in

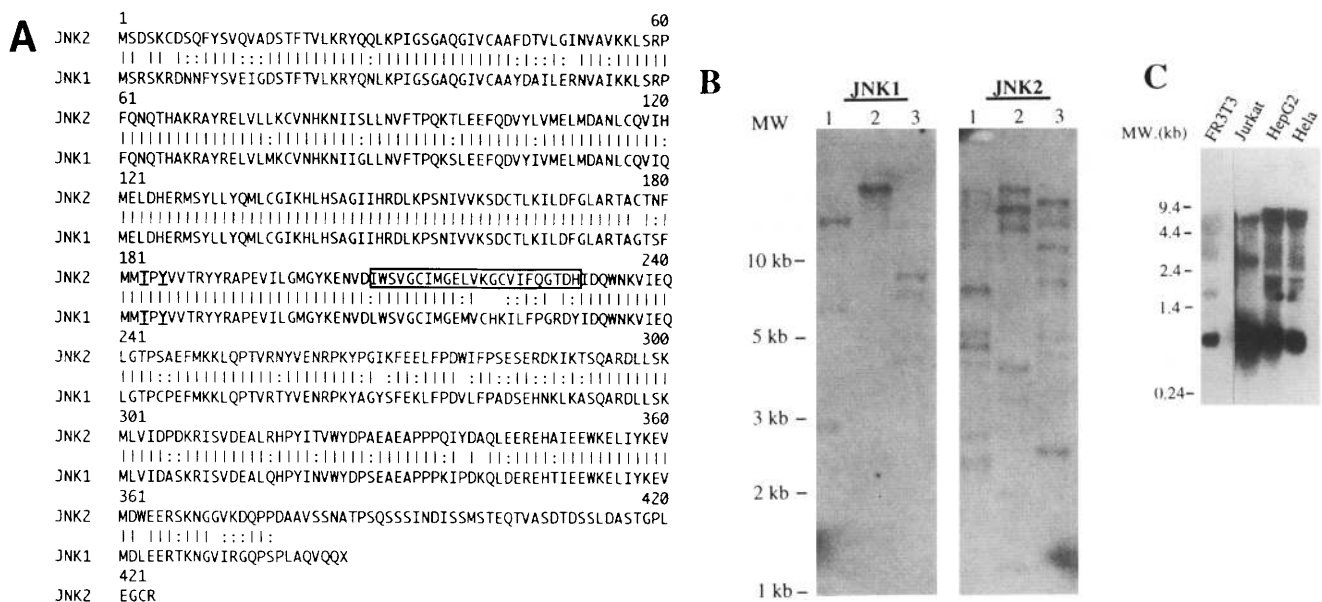
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c-Jun phosphorylation or their regulation was not determined. It is not clear which of the JNKs is directly involved in regulation of c-Jun activity and whether individual enzymes exhibit any functional or regulatory differences. Here, we describe the molecular cloning of the 55-kD form of human JNK, JNK2, which is probably the human homolog of SAPK $\alpha$ II. The two JNKs are closely related and similarly regulated. Despite the close similarity, JNK2 displays considerably higher affinity toward c-Jun than JNK1 does. The molecular basis for this difference was investigated through generation of JNK1/JNK2 chimeras and was traced to a small region located next to the catalytic pocket of the enzyme. Modeling suggests that this region is solvent exposed and thus may serve as a substrate docking site responsible for increasing the effective substrate concentration next to the JNK2 catalytic site. Consistent with this difference, overexpression of wild-type JNK2 but not JNK1 potentiates expression of a c-Jun-dependent reporter gene. Therefore, JNK2 is likely to be responsible for c-Jun activation, whereas JNK1 may be involved in regulation of other substrates. These structural and functional differences between two otherwise similar MAP kinases can explain why members of this group exhibit biological specificity even though their primary sequence specificities for phosphate transfer are very similar to one another.

## Results

### Molecular cloning of JNK2

By in-gel kinase assays we have identified two protein kinases, 46 and 55 kD in size, termed JNKs, that bind to the c-Jun activation domain and phosphorylate it on Ser-63 and -73 (the latter being the major phosphoacceptor site; Hibi et al. 1993). The 46-kD form of JNK was shown to be a novel MAP kinase, termed JNK1 (Dérjard et al. 1994). Because the two JNK activities are similarly regulated (Hibi et al. 1993; Su et al. 1994), they are likely to be structurally related. Thus, we screened a Jurkat T-cell lymphoma cDNA library with a JNK1 cDNA probe to identify a related species that may encode the 55-kD form of JNK. One of the cDNA clones thus identified, with an insert size of 3.3 kb, has turned out to encode a protein kinase that is 83% identical in its primary structure to JNK1 but only 39% identical to ERK2 (Fig. 1A). A cDNA clone encoding the same protein was also isolated from a HeLa cell library. No other cDNAs that exhibit considerable similarity to JNK1 were isolated despite extensive screening of both libraries. Importantly, the open reading frame of the newly identified cDNA specifies a 424 amino acid polypeptide that is larger than JNK1 (385 amino acids in length). Like JNK1, the new protein, termed JNK2, contains a Thr-Pro-Tyr sequence at position 183–185. This sequence is functionally and struc-



**Figure 1.** Molecular characterization of JNK2. (A) Comparison of JNK2 and JNK1 amino acid sequences. The Thr and Tyr residues that are required for activation of JNK1 and are conserved in JNK2 are underlined, and the region that determines the efficient binding of JNK2 to c-Jun (see below) is boxed. The JNK2 nucleotide sequence has been deposited to GenBank and is available under accession number U09759. (B) Southern blot analysis. Human genomic DNA (10  $\mu$ g) was digested with either *Eco*RI (lane 1), *Bam*HI (lane 2), or *Hind*III (lane 3), separated on an agarose gel, and transferred to a Nytran-nylon membrane (Schleicher & Schuell). The membranes were hybridized at 65°C for 16 hr with full-length JNK1 and JNK2 cDNA probes, washed extensively at room temperature with 1 $\times$  SSC, 0.1% SDS, followed by 0.2 $\times$  SSC, 0.1% SDS, at 65°C. The migration positions of molecular mass markers are shown. (C) Northern blot analysis of JNK2 expression. Samples of total cellular RNA (5  $\mu$ g) isolated from FR3T3, Jurkat, HepG2, and HeLa cells were separated in a 1.5% agarose gel transferred to Zetabind membrane (Cuno), hybridized with JNK2 cDNA probe for 16 hr at 65°C, and washed extensively at 65°C with 0.2 $\times$  SSC, 0.1% SDS.

turally equivalent (Dérijard et al. 1994) to the Thr-Glu-Tyr sequence, which contains the activating phosphorylation sites of ERK1 and ERK2 (Ahn et al. 1992). Southern blot analysis (Fig. 1B), as well as the scattered sequence differences between JNK1 and JNK2, indicated that they are products of different genes. Northern hybridization (Fig. 1C; data not shown) indicates that JNK2 is expressed in many cell types. As observed for JNK1 (Dérijard et al. 1994), several JNK2 transcripts were detected. The same transcript heterogeneity was revealed by analysis of poly(A)<sup>+</sup> RNA (data not shown). The abundant transcript that is <1 kb in length is likely to represent an alternatively spliced form of JNK2 that can potentially code for truncated protein of unknown functional significance (K. Yoshioka, unpubl.).

#### *JNK1 and JNK2 are identically regulated*

To examine whether JNK2 is a functional c-Jun amino-terminal kinase, we transfected Jurkat cells with an expression vector encoding JNK2 tagged with the HA epitope (Wilson et al. 1984) at its amino terminus. Because both forms of JNK are strongly activated following UV irradiation (Hibi et al. 1993), the transfected cells were UV irradiated prior to preparation of lysates. Immune complex kinase assays, using GST-c-Jun(1-79) as a substrate, revealed that the anti-HA antibody precipitated a 55-kD c-Jun kinase only from cells transfected with the HA-JNK2 vector (Fig. 2A). No such activity was present in control immune complexes generated with an anti-CD3 antibody. To determine whether JNK2 specifically phosphorylated the amino-terminal sites of c-Jun, we phosphorylated full-length recombinant c-Jun with immunopurified HA-JNK2 and subjected it to two-dimensional tryptic phosphopeptide mapping (Boyle et al. 1991). The resulting phosphopeptides (Fig. 2B) were identical in their mobilities to the X and Y phosphopeptides, whose abundance is increased following *in vivo* JNK activation (Devary et al. 1992; Su et al. 1994), and phosphopeptides T1 and T2, which correspond to secondary JNK phosphorylation sites (Hibi et al. 1993; Dérijard et al. 1994). The same phosphopeptides were generated by phosphorylation of c-Jun with HA-JNK1. As shown previously (Smeal et al. 1991), phosphopeptides X and Y correspond to phosphorylation of c-Jun at Ser-73 and Ser-63, respectively.

We used the immune complex kinase assay to compare the regulation of transiently expressed JNK1 and JNK2 to the endogenous 46- and 55-kD forms of JNK, probed by an *in-gel* kinase assay (Kameshita and Fujisawa 1989). The transiently expressed JNK1 and JNK2 were regulated very similarly to the endogenous forms of JNK in both HeLa and Jurkat cells (Fig. 2C). Both enzymes were rapidly activated in response to a variety of physiological (growth factors and cytokines) and stressful stimuli. The most potent activators of JNK1 and JNK2 were UV irradiation (50- to 90-fold) and a low concentration (50 ng/ml) of the protein synthesis inhibitor anisomycin (50- to 200-fold). At this concentration, anisomycin does not inhibit protein synthesis but induces

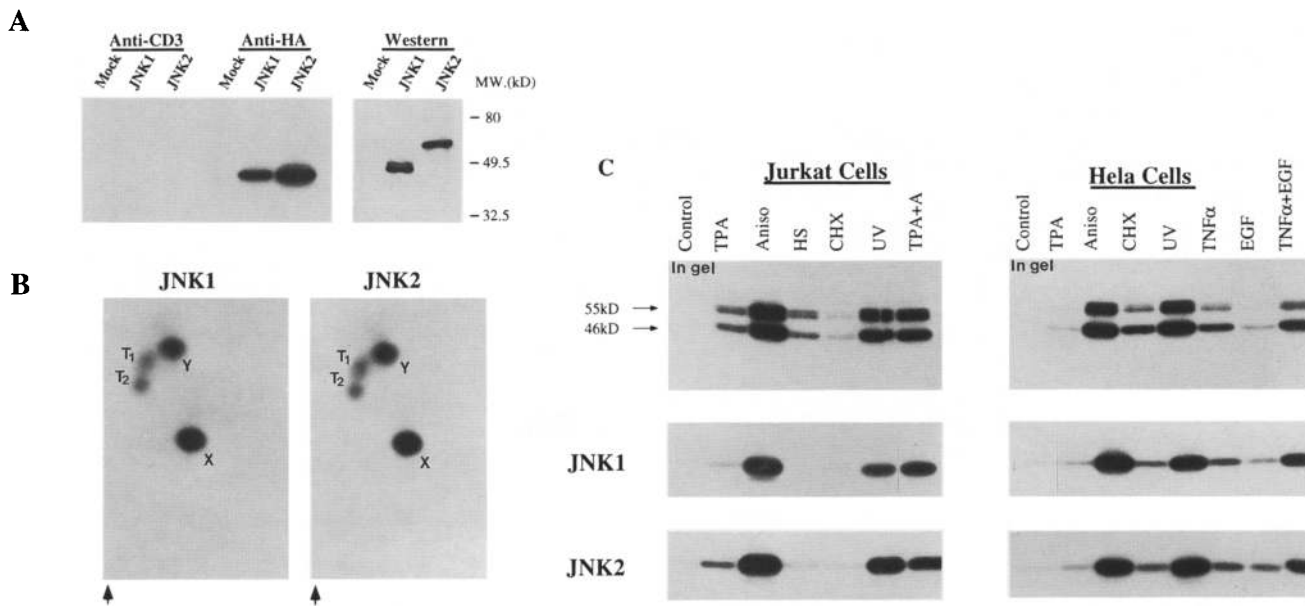
immediate early genes, including *c-jun* (Edwards and Mahadevan 1992) and activates protein kinases with sizes identical to those of JNK1 and JNK2 (Cano et al. 1994). This effect was not seen with another protein synthesis inhibitor, cycloheximide, which is a less effective activator of JNK1 and JNK2 (7- to 14-fold), even at fully inhibitory concentrations. Another stressful stimulus, heat shock, led to weak activation (three- to fourfold) of these enzymes. Modest activation of JNK1 and JNK2 was observed following treatment of HeLa cells with epidermal growth factor (EGF; 3- to 10-fold) or tumor necrosis factor- $\alpha$  (TNF $\alpha$ ; 6- to 16-fold). Together, the two factors synergistically activated both JNK1 and JNK2 (25- to 60-fold). As described (Su et al. 1994), JNK1 and JNK2 were synergistically activated (30- to 60-fold) by costimulation of Jurkat cells with the phorbol ester TPA and the calcium ionophore A23187. Whereas treatment with TPA alone gave rise to weak JNK1 and JNK2 activation (five- to sevenfold), treatment with A23187 alone did not activate either enzyme (data not shown).

#### *JNK2 has higher affinity to c-Jun*

The two forms of JNK were identified by their ability to bind the amino-terminal activation domain of c-Jun (Hibi et al. 1993). The binding site is located between amino acids 30 and 60 of c-Jun and its integrity is important not only for JNK binding *in vitro* but also for phosphorylation of c-Jun and enhancement of its transcriptional activity *in vivo* (Hibi et al. 1993). To investigate whether JNK2 binds c-Jun through the same site, we incubated immunopurified HA-JNK2 with glutathione-agarose beads coated with different GST-c-Jun fusion proteins that either contain or lack an intact JNK-binding site (Hibi et al. 1993). As described (Hibi et al. 1993; Dérijard et al. 1994), the incubations were carried out in the absence of ATP, the beads were washed extensively, and JNK binding was assayed by addition of [ $\gamma$ -<sup>32</sup>P]ATP and phosphorylation of the c-Jun moiety of the immobilized fusion protein (Fig. 3A). Like JNK1, JNK2 bound to GSTc-Jun(1-223), GSTc-Jun(1-79), and GSTc-Jun(1-223; Ala-63/73). [The latter is a mutant in which Ser-63 and -73, which are not required for JNK binding, were substituted by Ala residues. This mutant is phosphorylated on secondary sites (Hibi et al. 1993)]. JNK2 also bound to the chicken (ch) c-Jun amino-terminal domain [GSTch.c-Jun(1-144)]. JNK2 did not bind to GSTc-Jun(43-223) or GSTch.v-Jun. The latter two proteins lack intact JNK-binding sites (Hibi et al. 1993).

To examine the binding of JNK1 and JNK2 to c-Jun by a more conventional assay, we passed lysates of HA-JNK1- or HA-JNK2-expressing Jurkat cells, containing similar amounts of either enzyme, through glutathione-agarose-GSTc-Jun(1-79) columns and, after extensive washing, eluted the bound proteins with buffer containing SDS. The input and bound fractions were separated on a denaturing gel and analyzed by immunoblotting with anti-HA antibody (Fig. 3B). Approximately 10% of the HA-JNK2 input bound the affinity resin, whereas <1% of the HA-JNK1 input was retained on the column.





**Figure 2.** JNK2 corresponds to the 55-kD form of JNK. (A) JNK2 is a 55-kD c-Jun kinase. Jurkat cells were transfected with either empty expression vector (pSR $\alpha$ 3), HA-JNK1 or HA-JNK2 expression vectors. Forty hours later the cells were exposed to 40 J/m<sup>2</sup> of UV-C. After 20 min the cells were washed with PBS and lysed in the presence of proteinase inhibitors. Half of the lysates were incubated with monoclonal anti-CD3 antibody and the other half with monoclonal anti-HA antibody. The immune complexes were suspended in 30  $\mu$ l of kinase buffer containing 1  $\mu$ M cold ATP, 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, and 0.5  $\mu$ g of GSTc-Jun(1-79), as a substrate. After 20 min at 30°C, the reactions were terminated by boiling in SDS-sample buffer; they were then separated on a 10% SDS-polyacrylamide gel and visualized by autoradiography. These results are shown in the *left-hand* panel. Expression of HA-JNK1 and HA-JNK2 was monitored by Western blot analysis of the same lysates using the anti-HA antibody and the ECL detection system (Amersham). These results are shown in the *right-hand* panel. (B) JNK2 phosphorylates the same sites on c-Jun as JNK1. HA-JNK1 and HA-JNK2 were immunoprecipitated from transiently transfected Jurkat cells following UV irradiation as described above. The immune complexes were washed thoroughly and used to phosphorylate recombinant full-length c-Jun protein *in vitro*. After separation by SDS-PAGE, the phosphorylated c-Jun bands were transferred to a nitrocellulose membrane and subjected to two-dimensional tryptic peptide mapping. The phosphorylated peptides (X, Y, T1, and T2) are marked. (C) Regulation of transiently expressed JNK1 and JNK2 and the endogenous 46- and 55-kD forms of JNK. Jurkat and HeLa cells were transfected with either HA-JNK1 or HA-JNK2 expression vectors. Forty hours after transfection, the cells were exposed to 10 ng/ml of TPA, 50 ng/ml of anisomycin (Aniso), 150  $\mu$ g/ml of cycloheximide (CHX), 40 J/m<sup>2</sup> UV-C, 10 ng/ml of TPA + 1  $\mu$ g/ml of A23187, 10 ng/ml of TNF $\alpha$ , 15 ng/ml of EGF, 10 ng/ml of TNF $\alpha$  + 15 ng/ml of EGF or heat shock (HS) at 42°C. After 20 min, lysates were prepared and immune complex kinase assays were performed as described above. Phosphorylation of GSTc-Jun(1-79) by immunoprecipitated HA-JNK1 and HA-JNK2 in both Jurkat and HeLa cells is shown in the two *bottom* panels. To examine the effect of these treatments on endogenous JNK activities, nontransfected Jurkat and HeLa cells were subjected to the same treatments and analyzed by an *in-gel* kinase assay using GSTc-Jun(1-79) as a substrate. These results are shown in the *top* panel.

Quantitation of the binding of the two JNKs to GSTc-Jun(1-79) revealed that JNK2 bound c-Jun 25-fold more efficiently than JNK1 (Fig. 4). A similar difference was found when the binding of <sup>35</sup>S-labeled c-Jun to GST-JNK1 and GST-JNK2 was measured (data not shown). No effect of activation by anisomycin on binding of either JNK1 or JNK2 was detected (Fig. 3B). Binding was specific, as no binding of JNK2 (or JNK1; data not shown) to a glutathione-agarose-GSTch.v-Jun column could be detected.

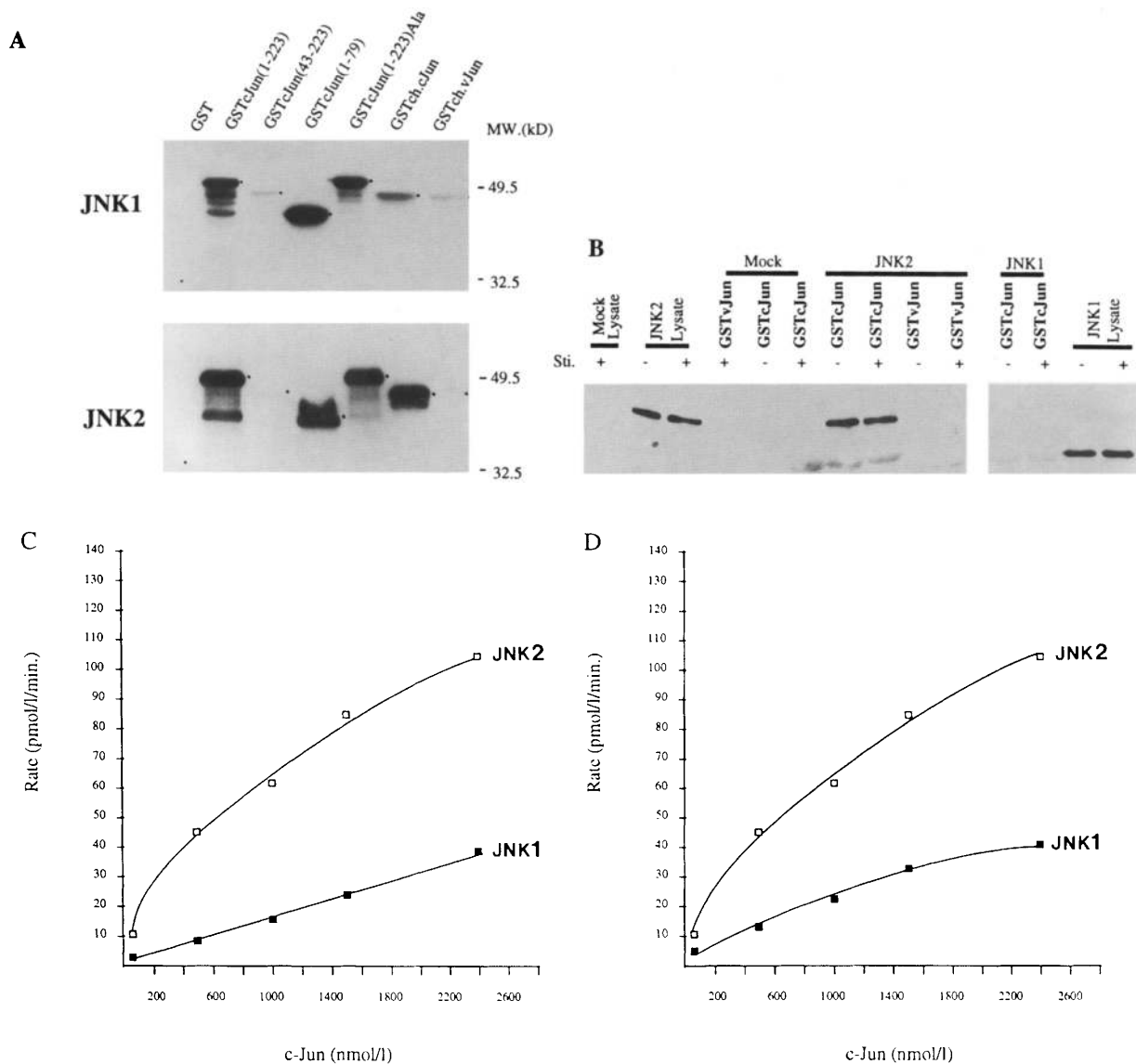
The more efficient binding of JNK2 to c-Jun suggested that it may be a more effective c-Jun kinase than JNK1, especially at low substrate concentrations. To examine this point, we have immunopurified transiently expressed HA-JNK1 and HA-JNK2 from anisomycin-stimulated HeLa cells or UV-irradiated COS 1 cells and eluted both enzymes from the immune complex using a

peptide corresponding to the HA epitope. Equal amounts of each enzyme were incubated with various concentrations of c-Jun, and the rate of c-Jun phosphorylation was quantitated. Either preparation of JNK2 was a more effective c-Jun kinase than the equivalent preparation of JNK1 (Fig. 3C,D). The Michaelis constants ( $K_m$ ) of JNK1 and JNK2 toward c-Jun determined by double reciprocal plots (Lineweaver-Burke), were 2500  $\pm$  500 nM and 350  $\pm$  50 nM, respectively. Thus, at low substrate concentrations JNK2 is likely to be the predominant c-Jun amino-terminal kinase.

#### *The structural basis for preferential c-Jun phosphorylation by JNK2*

The marked differences between the abilities of JNK2 and JNK1 to bind and phosphorylate c-Jun provided an

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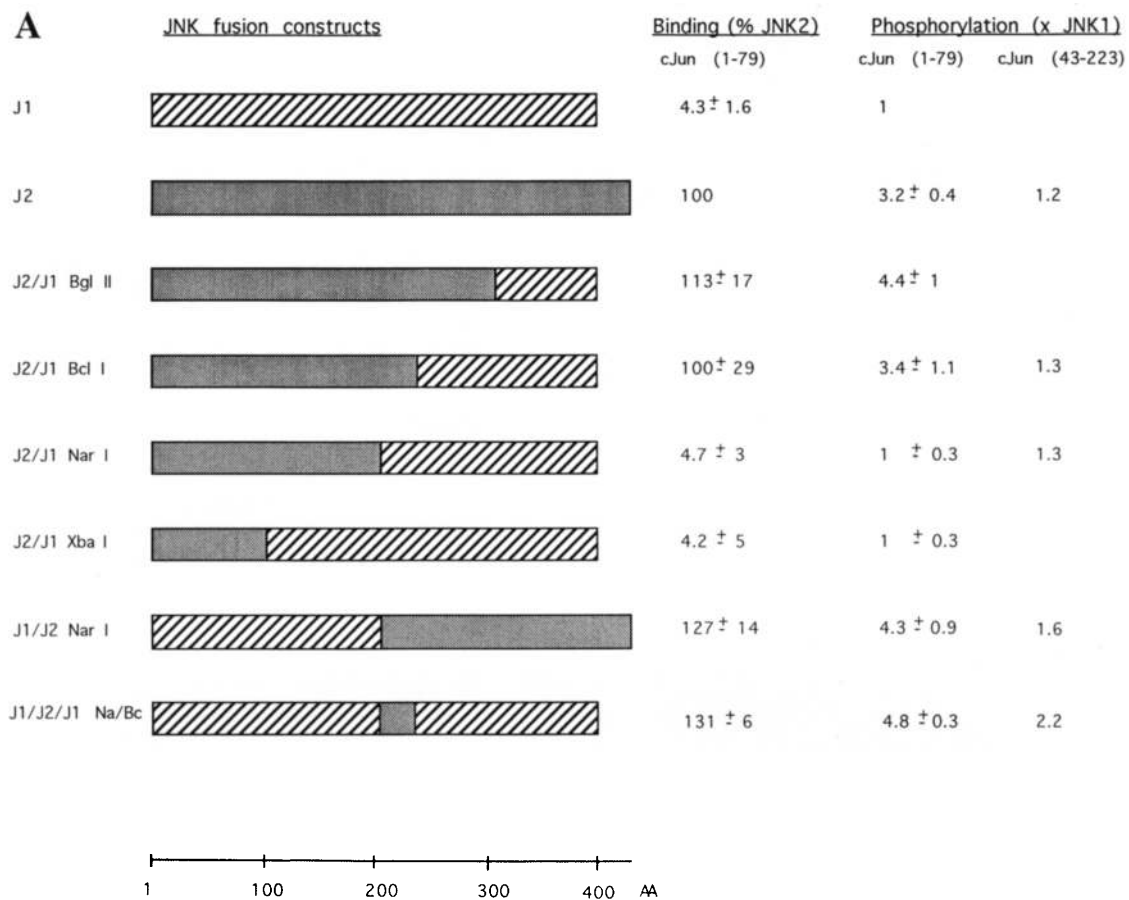
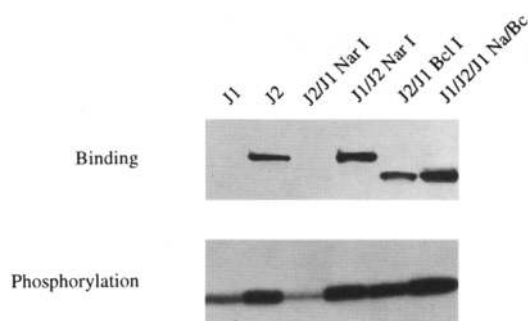


**Figure 3.** Binding and phosphorylation of c-Jun by JNK1 and JNK2. (A) JNK2 recognizes the same binding site as JNK1. HA-JNK1 and HA-JNK2 were isolated from transiently transfected Jurkat cells treated with 50 ng/ml of anisomycin. The immunopurified kinases were incubated with glutathione-agarose beads coated with 3  $\mu$ g of GST, GSTc-Jun(1-223), GSTc-Jun(43-223), GSTc-Jun(1-79), GSTc-Jun(1-223)Ala, GSTch.c-Jun(1-144), or GSTch.v-Jun. After extensive washing, the beads were incubated with [ $\gamma$ - $^{32}$ P]ATP in kinase buffer for 20 min at 30°C. The phosphorylation reaction was terminated by incubation in SDS-sample buffer and boiling. The phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography. (B) JNK2 binds c-Jun more efficiently than JNK1. Jurkat T-antigen cells ( $10^7$ ) were transfected with 10  $\mu$ g of empty expression vector (pSRa3; mock), or HA-JNK1 or HA-JNK2 expression vector. Forty hours later cells were either not treated (-) or treated (+) with 50 ng/ml of anisomycin to activate the JNKs. After 20 min, lysates were prepared and 90% of the material was incubated with glutathione-agarose beads coated with 40  $\mu$ g of either GSTc-Jun(1-79) or GSTch.v-Jun. After extensive washing the bound proteins were eluted by boiling in SDS-sample buffer and separated by SDS-PAGE. Ten percent of the unfractionated lysates were separated on the same gel. The proteins were transferred to Immobilon-P membranes (Millipore), and the bound HA-JNK proteins were detected by Western blotting with anti-HA antibody and the ECL detection system. (C) Effect of c-Jun concentration on its phosphorylation by JNK1 or JNK2 isolated from HA-JNK1 and HA-JNK2-transfected UV-irradiated (UV-C 40 J/m $^2$ ) COS 1 cells. The rate of c-Jun phosphorylation (determined as described in Materials and methods) by equal amounts of either HA-JNK1 or HA-JNK2 is plotted as a function of substrate concentration. The values shown are averages of two different experiments. (D) Effect of c-Jun concentration on its phosphorylation by JNK1 and JNK2 isolated from anisomycin (50 ng/ml)-induced HeLa cell extracts. The rate of c-Jun phosphorylation by equal amounts of HA-JNK1 and HA-JNK2 is plotted as averages of two different experiments as in C.

opportunity to determine the structural basis underlying the specificity of c-Jun-JNK2 interaction. Thus, we generated a series of JNK1/JNK2 chimeras and examined

their ability to bind c-Jun and phosphorylate it. Binding to c-Jun was measured by passing extracts of HeLa cells transiently transfected with the various HA-JNK1/JNK2

## The molecular basis for JNK-c-Jun interaction

**B**

chimeras. (Top) Western blot showing binding of the different constructs to a GSTc-Jun(1-79) affinity column after loading of equal amounts of each protein. (Bottom) The phosphorylation of soluble GSTc-Jun(1-79) by the different JNKs. For both assays, extracts of anisomycin-stimulated HeLa cells containing equal amounts of the different JNKs were used.

**Figure 4.** Mapping of the c-Jun binding site of JNK2. (A) A series of JNK1/JNK2 chimeras were generated using naturally occurring and artificially introduced restriction endonuclease cleavage sites in the JNK1 and JNK2 cDNAs. Schematic descriptions of the chimeras are shown at left. (Hatched bars) JNK1 sequences; (shaded bars) JNK2 sequences. The binding of each chimera to GSTc-Jun(1-79) affinity beads was determined as described in Materials and methods and is displayed relative to the binding efficiency of JNK2 that was chosen to be 100%. Averages and standard errors of three separate experiments are shown. The abilities of the different chimeras, as well as HA-JNK1 and HA-JNK2 isolated from anisomycin-treated HeLa cells to phosphorylate soluble GSTc-Jun(1-79) at 150–300 nM, was determined as described in Fig. 3. The relative JNK activities are expressed using the activity of HA-JNK1 as 1. Results shown are averages and standard errors of three separate experiments. (B) Binding and phosphorylation of GSTc-Jun(1-79) by selected JNK1/JNK2

constructs through a GSTc-Jun(1-79) affinity column. c-Jun phosphorylation was examined by using equal amounts of transiently expressed HA-JNK1/JNK2 constructs isolated from anisomycin-activated HeLa cells and low concentrations (150–300 nM) of a soluble GSTc-Jun(1-79) substrate. As summarized in Figure 4A, JNK2 bound GSTc-Jun(1-79) 25-fold more efficiently than JNK1 and phosphorylated c-Jun at least 3-fold more efficiently. The higher binding affinity and kinase activity

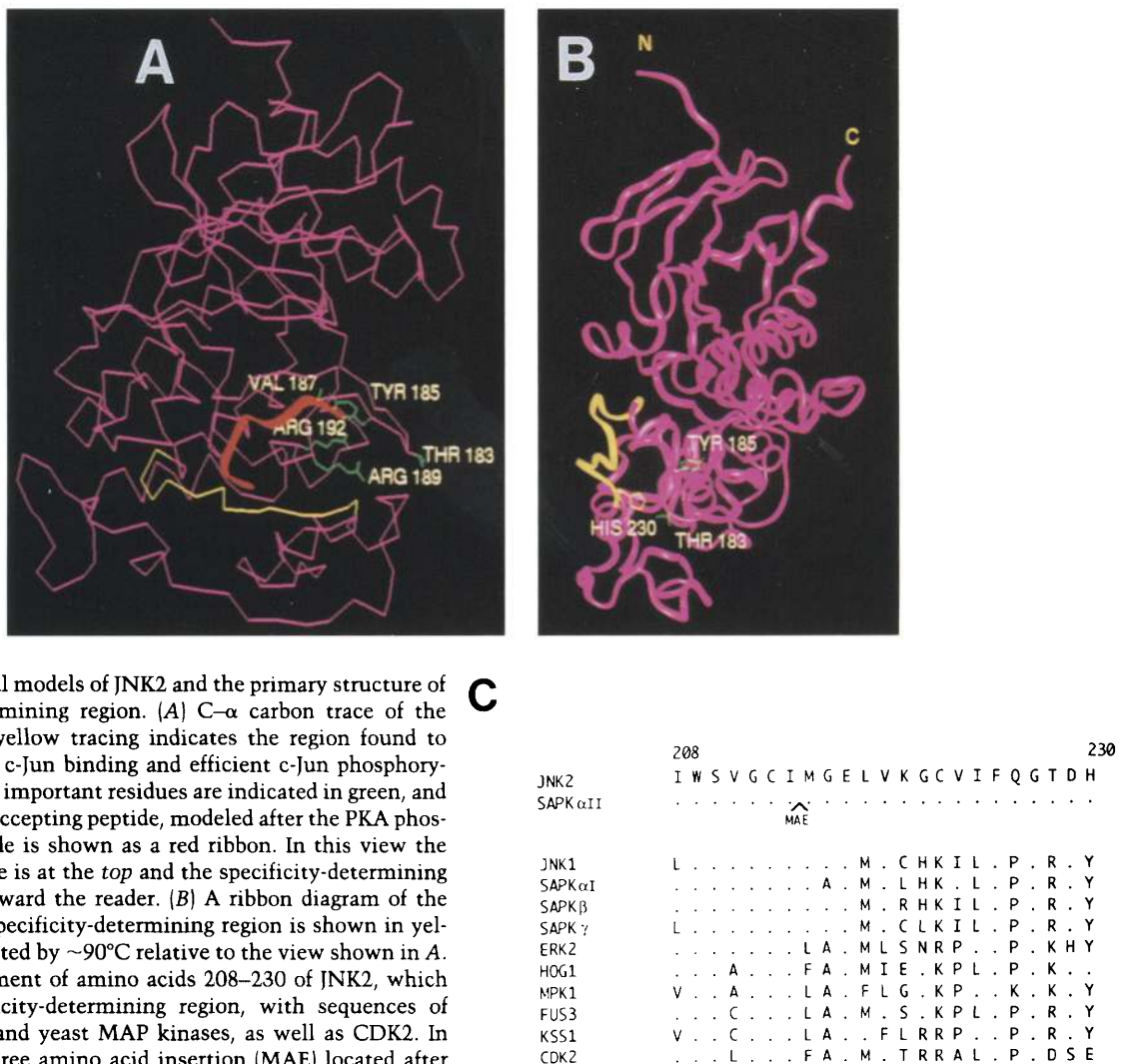
of JNK2 toward c-Jun were found to be predominantly determined by a small region in its carboxy-terminal domain between amino acids 208 and 230 (boxed in Fig. 1A). In this region, JNK1 and JNK2 differ by four highly conservative amino acid substitutions (such as Ile → Leu) and six less conservative and nonconservative changes. Introduction of the corresponding JNK2 sequences into this region of JNK1 increases its ability to bind c-Jun by 30-fold and its c-Jun kinase activity by

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4.8-fold. These differences are mediated by an interaction of JNK2 with the JNK-binding site of c-Jun because a GST fusion protein lacking this site, GSTc-Jun(43–223) is phosphorylated to nearly the same extent, albeit very inefficiently, by the different JNK constructs.

To better understand the structural basis for the efficient and specific interaction of JNK2 with c-Jun, a three-dimensional model of JNK2 was constructed based on the structural homologies between the catalytic domains of cAMP-dependent protein kinase (PKA; Zheng et al. 1993), cyclin-dependent kinase 2 (CDK2; De Bondt et al. 1993), and ERK2 (Zhang et al. 1994). First, the JNK2 sequence was aligned with the sequences of all three kinases, and then using the Homology program (Biosym, 1994) and crystallographically solved structures of these protein kinases, the JNK2 model shown in Figure 5 was derived. There was a significant match in the regions including amino acids 1–243 and 293–369 of JNK2 and the published ERK2 structure (Zhang et al. 1994). The

JNK2 region that corresponds to an insert and includes amino acids 243–280 was more consistent with the CDK2 structure based on sequence alignment. The position of the entire insert was modeled on the basis of the general position of the insert in CDK2. The region that corresponds to amino acids 280–293 was further modeled using the fragment generator feature of the Homology program and was chosen as the lowest energy structure. As highlighted in the models shown in Figure 5, A and B, the region responsible for the specific and efficient interaction between JNK2 and c-Jun appears to form an extended chain that protrudes below the region involved in binding of the phosphoacceptor peptide and its phosphorylation. In this model the segment containing His-230 is in close proximity to Thr-183 and Tyr-185, whose phosphorylation mediates JNK activation (Dérjard et al. 1994). Asp-229, Arg-189, and Arg-192 could be involved in strong electrostatic interactions and, on the basis of the PKA structure, are likely to be involved in binding of



**Figure 5.** Structural models of JNK2 and the primary structure of its specificity-determining region. (A) C- $\alpha$  carbon trace of the JNK2 model. The yellow tracing indicates the region found to confer high-affinity c-Jun binding and efficient c-Jun phosphorylation. Functionally important residues are indicated in green, and a putative phosphoaccepting peptide, modeled after the PKA phosphoaccepting peptide is shown as a red ribbon. In this view the amino-terminal lobe is at the top and the specificity-determining region protrudes toward the reader. (B) A ribbon diagram of the JNK2 model. The specificity-determining region is shown in yellow. This view is tilted by  $\sim 90^\circ$ C relative to the view shown in A. (C) Sequence alignment of amino acids 208–230 of JNK2, which include the specificity-determining region, with sequences of other mammalian and yeast MAP kinases, as well as CDK2. In this alignment a three amino acid insertion (MAE) located after Ile-214 in SAPK $\alpha$ II (Kyriakis et al. 1994) is marked by arrowhead. With the exception of this insert, the specificity-determining region of JNK2 is full conserved in SAPK $\alpha$ II and most likely comprises residues 208–230, as suggested by this alignment.



the phosphoaccepting peptide (Zheng et al. 1993). The proximity of His-230 to both the positively charged Arg-189 and Arg-192 and the negatively charged Asp-229 should make it very sensitive to pH changes or substitutions of these charged residues. Whereas the precise orientations of this region in the insert to the core obviously requires a crystal structure of JNK2 itself, the general proximity of the specificity-determining region to the active site cleft is nevertheless independent of whether CDK2 or MAPK structures are used for its modeling.

Sequence alignment of various MAP kinases, as well as CDK2, indicates that the specificity-determining region is unique to JNK2 and SAPK $\alpha$ II, which therefore is its murine homolog (Fig. 5C). Interestingly, in four of the six positions where it differs considerably from JNK2, JNK1 contains residues that are highly conserved among MAP kinases. With the exception of ERK1 and ERK2, no two MAP kinases exhibit complete sequence identity throughout this region.

#### *In vivo effects of JNK on c-Jun transcriptional activity*

To test which protein kinase is more likely to modulate c-Jun activity *in vivo*, we cotransfected the reporter plasmid -79jun-LUC, containing the c-Jun-responsive *c-jun* promoter (Angel et al. 1988), with expression vectors containing either wild-type or kinase-defective forms of JNK1 and JNK2 into Jurkat cells. As stimulation of Jurkat cells with TPA results only in partial JNK activation (Su et al. 1994), we reasoned that elevation of the intracellular level of the effective JNK should augment the weak activation of the *c-jun* promoter by TPA (Su et al. 1994). Activation of the *c-jun* promoter correlates well with changes in amino-terminal c-Jun phosphorylation (Devary et al. 1992; Su et al. 1994). Whereas cotransfection of -79jun-LUC with a JNK1 expression vector into Jurkat cells did not potentiate its weak induction (threefold above baseline) by TPA, coexpression of wild-type JNK2 resulted in twofold potentiation, leading to sixfold induction (Fig. 6A). Cotransfection of the inactive JNK1(T183A,Y185F) mutant [JNK1(APF)], whose activating phosphorylation sites were replaced by nonphosphorylatable residues (Dérillard et al. 1994), had an insignificant effect on -79jun-LUC expression. In contrast, cotransfection of the equivalent JNK2 mutant [JNK2(APF)] resulted in a 30% decrease in -79jun-LUC expression. An even greater decrease (twofold) in -79jun-LUC expression was produced by transfection of JNK2(G35E) mutant (TJ-1), in which a conserved Gly residue involved in ATP binding (Hanks et al. 1988), is substituted by a Glu residue. None of the JNK expression vectors affected expression of an ACT-LUC reporter, containing the  $\beta$ -actin promoter (Fig. 6A). Because the *c-jun* TRE appears to be recognized by a c-Jun:ATF2 heterodimer (van Dam et al. 1993) and recent evidence indicates that ATF2 activity is also stimulated by JNK-mediated phosphorylation (Gupta et al. 1994), we examined the effects of JNK1 and JNK2 on c-Jun transcriptional activity using a more specific assay. A GAL4

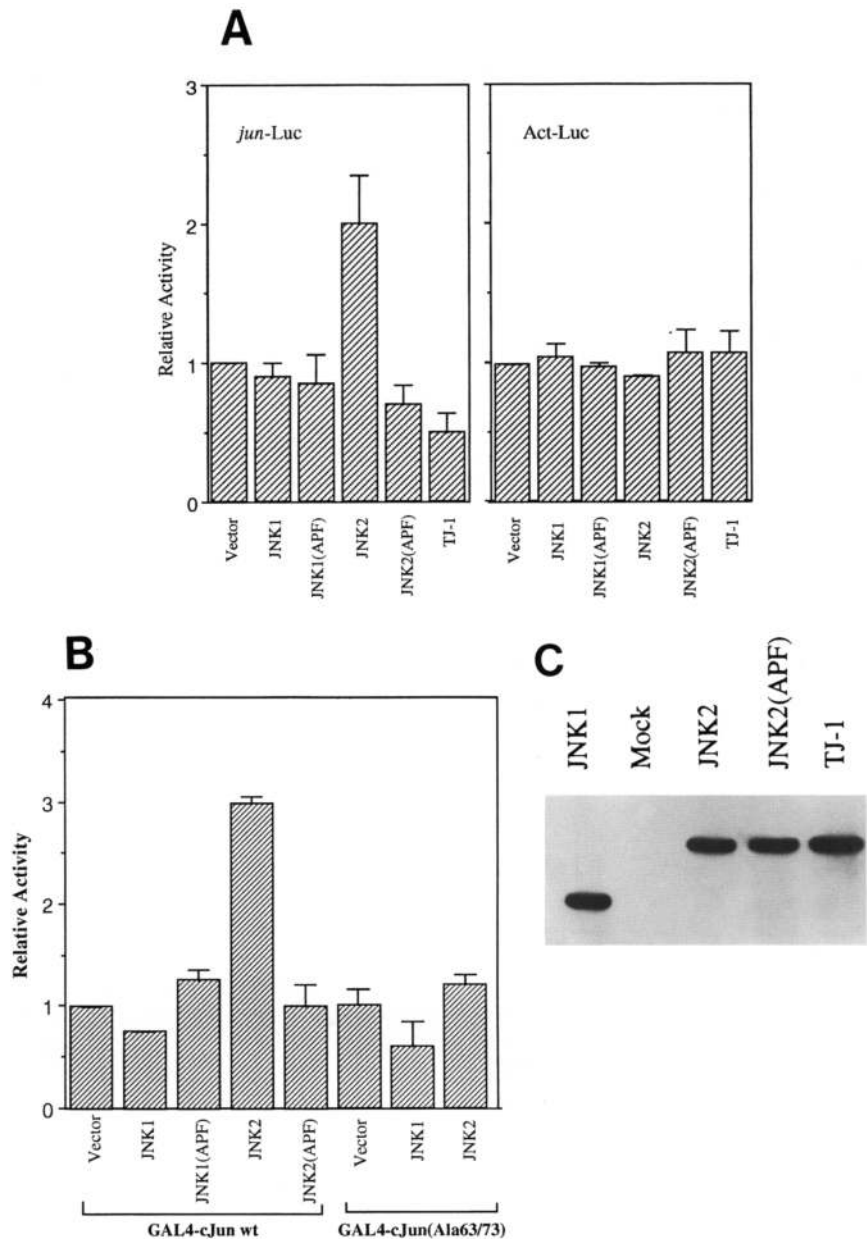
responsive reporter was cotransfected into HeLa cells with a GAL4-c-Jun (1-223) construct encoding the GAL4 DNA-binding domain fused to the c-Jun activation domain. When a JNK2 expression vector was included in the transfection the basal activity of the c-Jun activation domain was stimulated threefold. No enhancement of *trans*-activation was seen when a vector encoding JNK1 or the nonactivatable mutants of JNK1 and JNK2, JNK1 (APF) and JNK2 (APF), were included in the transfections (Fig. 6B). The enhancing effect of JNK2 was dependent on phosphorylation of the c-Jun activation domain, because the activity of a GAL4-c-Jun (1-223; Ala 63/73) construct, encoding a phosphorylation site-defective version of the c-Jun activation domain (Smeal et al. 1991), was not enhanced. The basal activity of GAL4-c-Jun (1-223; Ala 63/73) was similar to that of GAL4-c-Jun (1-223). All of the different JNK proteins (wild type and mutant) were expressed at similar levels (Fig. 6C; Dérillard et al. 1994). However, the JNK1(APF) and JNK2(APF) mutants were completely inactive, whereas the TJ-1 mutant had 5% of wild-type JNK2 activity (data not shown).

#### Discussion

Biochemical characterization of JNK activity identified two distinct polypeptides, 46 kD and 55 kD in size, that phosphorylate the amino-terminal sites of c-Jun (Hibi et al. 1993). Both the 46-kD JNK1 (Dérillard et al. 1994) and the 55-kD JNK2 are members of the MAP kinase group. Whereas the two JNKs display 39-43% sequence identity to other MAP kinases, such as ERK2, they are 83% identical to each other. While this manuscript was in preparation, cDNAs encoding four JNK related proteins isolated from a rat brain cDNA library were described (Kyriakis et al. 1994). One of these enzymes, SAPK $\gamma$ , appears to be the rat homolog of JNK1, and SAPK $\alpha$ II is most similar to JNK2. Together, these enzymes define a new subgroup of MAP kinases that share the Thr-Pro-Tyr sequence, which harbors their activating phosphorylation sites (Dérillard et al. 1994), suggesting a common activation pathway. This sequence is equivalent to the Thr-Glu-Tyr sequence, which contains the activating phosphorylation sites of ERK1 and ERK2 (Ahn et al. 1992). The two JNKs are activated by diverse extracellular stimuli, including UV irradiation, protein synthesis inhibitors, cytokines, growth factors, and T-cell activators. So far no differences in the regulation of JNK1 and JNK2 have been detected. Although the regulation of individual SAPKs was not examined, studies with a polyclonal antiserum that recognizes all forms suggest that their collective activity is regulated similarly to JNK (Kyriakis et al. 1994). The similarity in regulation of JNK1 and JNK2 suggests that they have overlapping functions in the transduction of environmental signals to the transcriptional machinery, a function similar to those of other MAP kinases (Davis 1993; Ammerer 1994). However, despite the highly similar structure and essentially identical regulation, the two JNKs



**Figure 6.** Effect of wild-type and mutant JNK1 and JNK2 on *c-jun* and Gal4 promoter activities. (A) Jurkat cells were cotransfected with 5  $\mu$ g of either -79*jun*-LUC or ACT-LUC reporters and 1  $\mu$ g of ACT- $\beta$ Gal, as an internal control, together with 0.5  $\mu$ g of the indicated JNK1 and JNK2 expression vectors or an empty expression vector. Thirty hours after transfection the cells were stimulated with 10 ng/ml of TPA and, 10 hr later, harvested for measuring luciferase and  $\beta$ -galactosidase activities. Luciferase activity was normalized relative to  $\beta$ -galactosidase activity, and the level of expression in cells cotransfected with the empty vector was given a relative value of 1.0. This value represents threefold induction of -79*jun*-LUC by TPA above basal level. The values shown represent the averages of two separate experiments. (B) HeLa cells were cotransfected with 1.5  $\mu$ g of GAL4-LUC, 0.1  $\mu$ g of GAL4-c-Jun (wt), 0.1  $\mu$ g of GAL4-c-Jun (Ala-63/73), 1  $\mu$ g of ACT- $\beta$ Gal, and 0.25  $\mu$ g of the indicated JNK1 and JNK2 expression vectors or empty expression vector. The cells were harvested, and the data were processed as described in A. (C) Wild-type and mutant JNKs are expressed with similar efficiencies. Equal numbers of Jurkat cells transfected with the different JNK expression vectors were lysed in SDS sample buffer. The lysates were separated by SDS-PAGE, transferred to Immobilon P membrane, and analyzed by Western blotting with anti-HA antibody and the ECL detection system.



are considerably different in their ability to bind and phosphorylate *c-Jun*.

In vitro, JNK2 displays a considerably lower  $K_m$  toward *c-Jun* than JNK1 does. This is consistent with its more efficient binding to GST-*c-Jun* beads. Both efficient *c-Jun* binding and *c-Jun* phosphorylation are determined by a short solvent-exposed region on the catalytic carboxy-terminal lobe of the enzyme, which differs between JNK2 and JNK1. These results suggest that at low substrate concentrations, as probably exist in vivo, JNK2 is more likely to phosphorylate *c-Jun* than is JNK1. Previously, we estimated that most cells contain 1000–10,000 molecules of AP-1. Using a nuclear diameter of 5  $\mu$ m and an upper limit of 10,000 *c-Jun* molecules per nucleus, we obtain a concentration of 260 nM. This con-

centration is much lower than the  $K_m$  of JNK1 toward *c-Jun* and is within the range where JNK2 phosphorylated *c-Jun* three- to fourfold more efficiently than JNK1, in vitro. These assumptions are supported by cotransfection experiments. Cotransfection with a JNK2 expression vector potentiated activation of the *c-Jun*-responsive *c-jun* promoter, whereas cotransfection of a JNK1 expression vector had no effect on *c-jun* promoter activity. Similar results were obtained by using a more specific GAL4 responsive reporter recognized by a GAL4-*c-Jun* fusion protein, containing the *c-Jun* activation domain, whose activity was stimulated threefold by JNK2 but not by JNK1. Little or no inhibition, however, was observed upon cotransfection of inactivated JNK2 mutants. The reason for the limited effects of the wild-type

and kinase-defective JNK2 on promoter activity is a combination of the relatively higher level of JNK expression than c-Jun expression and the transient nature of the c-Jun:JNK interaction. So far no stable c-Jun:JNK complexes have been isolated from cell lysates.

Differential substrate recognition is an important property that can explain the multitude of MAP kinases and their biological functions. Most cell types coexpress both ERK1 and ERK2, which are highly similar to each other in both structure and regulation (Boulton et al. 1990, 1991). Yet no differences in the ability of ERK1 and ERK2 to recognize specific substrates have been described so far. As discussed below, this may be attributable to the presence of identical residues in their putative specificity determining region. On the other hand, differential substrate recognition is strongly implied by genetic analysis of two other MAP kinases, FUS3 and KSS1, in *Saccharomyces cerevisiae* (Elion et al. 1990). Although these enzymes are highly similar in their structure and response to pheromones, only some of their functions overlap and KSS1 cannot fully substitute for FUS3 (Kurjan 1992). Other MAP kinases in yeast have totally different functions (Ammerer 1994). As shown recently, the unique activities of FUS3 may be mediated by its ability to specifically interact with a particular set of substrates (Elion et al. 1993). It is expected, but not yet demonstrated, that KSS1 would be able to interact with only a subset of these substrates. Such differential interactions, as demonstrated for the two JNKs, are likely to play critical roles in conferring biological specificity to the actions of protein kinases that respond to either similar or distinct extracellular stimuli.

Using a series of JNK1/JNK2 chimeras, the structural basis for the higher affinity of JNK2 toward c-Jun was determined. As we have shown, a short  $\beta$ -strand-like region, composed of a part of kinase domain IX and the sequence between domains IX and X, is largely responsible for increasing the efficiency of the JNK-c-Jun interaction. This region appears to interact with the JNK-binding site between amino acids 30–60 of c-Jun (Hibi et al. 1993). Interestingly, a part of the sequence of this short region is unique to JNK2 and its rat homolog SAPK $\alpha$ II and is variable among all known MAP kinases, with the exception of ERK1 and ERK2. Although, with the exception of the ERKs, no two MAP kinases share fully identical sequences in this region, most of the residues in which JNK1 differs most dramatically from the corresponding JNK2 sequence are well conserved among most family members (Fig. 5C). When switched to the corresponding JNK2 sequence, the resultant JNK1 mutant (J1/J2/J1 Na/Bc; Fig. 4) binds c-Jun and phosphorylates it very efficiently. Therefore, it is tempting to speculate that the same region is involved in conferring substrate selectivity in other MAP kinases. According to a three-dimensional model of JNK2, based on the structures of three other protein kinases, including ERK2, we suggest that this specificity-determining region may form an exposed shelf-like structure that is located next to the catalytic site of the enzyme. We suggest that the region forms a docking site recognized by the JNK-bind-

ing site of c-Jun, attracting c-Jun to the vicinity of the catalytic pocket of JNK2. This increases the likelihood that the phosphoaccepting peptide loops of c-Jun will interact with the catalytic pocket of JNK2. This suggestion is consistent with the kinetic analysis of c-Jun phosphorylation by the JNKs. As shown in Figure 3, JNK2 has a considerably lower  $K_m$  toward c-Jun as a substrate than JNK1 does. Both kinases, however, are similar in their ability to phosphorylate a substrate with which they fail to form a complex detectable by our *in vitro* binding assay. The bipartite interaction between a protein kinase and its substrate increases the selectivity of the recognition process by a significant factor and can explain why MAP kinases have distinct substrate specificities even though the primary structures of their phosphoacceptor sites are quite similar to each other. These results provide a molecular framework for understanding the basis for the different biological functions of closely related MAP kinases.

## Materials and methods

### Recombinant DNAs

A human T-cell Jurkat cDNA library (Stratagene) was screened using a full-length JNK1 cDNA (Dérjard et al. 1994) as a probe, and a 3.3 kb clone was obtained, whose nucleotide sequence revealed a high homology to JNK1 cDNA. No other clones exhibiting considerable similarity to JNK1 were found after screening  $2 \times 10^6$  phages. The JNK2-coding region was fused in its amino terminus to a synthetic oligonucleotide specifying the HA epitope (Wilson et al. 1984). The resulting fragment was subcloned into the pSR $\alpha$ 3 expression vector. JNK1/JNK2 chimeras were constructed by using a conserved *Bcl*I cleavage site and by introducing *Xba*I, *Nar*I, and *Bgl*II cleavage sites present in the JNK2 cDNA into the JNK1 cDNA using site-directed mutagenesis. JNK2 mutants were generated by PCR and were confirmed by DNA sequencing as described (Dérjard et al. 1994). -79jun-LUC, ACT-LUC, and GAL4 constructs were described earlier (Hibi et al. 1993; Su et al. 1994). ACT- $\beta$ Gal contains the  $\beta$ -galactosidase reporter gene under control of the same  $\beta$ -actin promoter. The GST-c-Jun constructs have been described previously (Hibi et al. 1993).

### Hybridization analyses

Southern blot analysis was performed as described earlier (Dérjard et al. 1994). Hybridization was performed at 65°C for 16 hr with full-length JNK1 and JNK2 cDNA probes. Northern blot analysis was carried out by standard protocols using the full-length JNK2 cDNA as a probe and hybridization for 16 hr at 65°C. After hybridization the membranes were washed extensively at 65°C, followed by autoradiography.

Western blot analysis was done according to standard procedures (Dérjard et al. 1994) using Immobilon P membranes (Millipore). The membranes were probed with anti-HA antibody, and the immune complexes were visualized by the ECL detection system (Amersham) and quantitated using a Molecular Imager GS-250 (Bio-Rad).

### Transfections

Jurkat T antigen cells (a gift from Dr. G. Crabtree, Stanford University, CA) were grown and transfected as described (Su et

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al. 1994). HeLa S3 and COS 1 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and transfected using lipofectamine (GIBCO-BRL). For immune complex kinase assays, cells were harvested 20 min following the different treatments, and for luciferase assays 10 hr after the treatments. Normally, all assays were done 40 hr after the initial transfection.

#### Immunoprecipitations

The cells were lysed in 20 mM Tris-HCl (pH 7.7), 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 0.5% NP-40, 1 mM *p*-nitrophenylphosphate (PNPP), 10 mM  $\beta$ -glycerophosphate, and 100  $\mu$ M Na-vanadate in the presence of proteinase inhibitors. The cell debris was spun down, the lysates were precleared, and the recombinant proteins were precipitated with monoclonal anti-HA antibody (Boehringer Mannheim) or affinity purified monoclonal anti-CD3 antibody OKT3, together with protein A-Sepharose as described (Dérjard et al. 1994).

#### Protein purification, binding, and kinase assays

GST-fusion proteins were purified, and JNK binding to c-Jun was assayed as described (Hibi et al. 1993; Dérjard et al. 1994). The amounts of cell extracts loaded onto the columns were normalized by Western blotting and ECL detection using a Molecular Imager (Bio-Rad) to contain equal amounts of HA-JNK1 and HA-JNK2. The total amount of protein was adjusted with extracts of mock transfected cells. GSTc-Jun(1-79) beads were incubated at 4°C for 12 hr with cell extracts followed by seven washes with lysis buffer containing 0.05% Tween 20 instead of NP-40. The proteins were eluted in SDS-PAGE loading buffer, separated by SDS-PAGE, transferred to a nylon membrane, immunoblotted with anti-HA antibody, visualized by ECL, and quantitated on a Molecular Imager (Bio-Rad) using a chemiluminescence screen.

Immune complex kinase assays were performed in 30  $\mu$ l of kinase buffer consisting of 20 mM HEPES at pH 7.6, 2 mM DTT, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM PNPP, 10 mM  $\beta$ -glycerophosphate and 100  $\mu$ M Na-vanadate, and 1  $\mu$ M of ATP supplemented with 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. To compare the kinetics of JNK1- and JNK2-catalyzed c-Jun phosphorylation, lysates from several HA-JNK1 and HA-JNK2 transfections were pooled, and after the immunoprecipitations and extensive washings the antibody-antigen complexes were disrupted by incubating them with 100-fold molar excess of HA-peptide in kinase buffer in the presence of 0.2 mg/ $\mu$ l of bovine serum albumin for 16 hr at 4°C. According to our pilot experiments, both albumin and HA-peptide when used in the above concentrations do not have any effect on kinase activity. The amounts of the eluted HA-JNK fusion proteins used in the phosphorylation reactions were adjusted following determination of their relative concentrations by Western blot analysis. Equal amounts of HA-JNK1 and HA-JNK2 were incubated with various amounts of GST c-Jun(1-79) for 10 min in kinase buffer. The kinase reactions were initiated by adding ATP and terminated by addition of SDS-PAGE sample buffer. Reaction rates were determined as the amount of phosphate incorporated versus reaction time (usually 10 min; the reactions were linear for 30 min). The phosphorylated GSTc-Jun bands were cut out after separation by SDS-PAGE and quantitated by liquid scintillation.

The in-gel kinase assay was done as described (Hibi et al. 1993) using GSTc-Jun(1-79) as a substrate. The incorporation of phosphate was detected by autoradiography and quantitated

with a PhosphorImager and ImageQuant software (Molecular Dynamics).

#### Molecular modeling

The JNK2 model was created using Silicon Graphic Indigo Station and the Homology program (Biosym, 1994). The structure of all insertions was constructed using the "Fragment Generator" Homology program. The side chains of JNK2 model were then minimized using the Discover program (Biosym, 1994) for 2000 interactions with steepest descents algorithm.

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## JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation.

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