

The Critical Features and the Mechanism of Inhibition of a Kinase Interaction Motif-based Peptide Inhibitor of JNK*

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We previously reported that a small peptide based on amino acids 143–153 of the c-Jun N-terminal kinase (JNK)-binding domain of JIP-1 functioned as an *in vitro* inhibitor of JNK activity. This peptide (TI-JIP: RP-KRPTTLNLF) resembles the kinase-interaction motif (KIM = (K/R)₂₋₃X₁₋₆(L/I)X(L/I)), which is common to upstream activators, downstream substrates, phosphatases, and scaffold proteins present in MAPK cascades. In this study, we characterized the mechanism of JNK inhibition by this peptide and further investigated the biochemical features of this peptide resulting in potent JNK inhibition. We also tested various KIM-based peptides for their ability to inhibit JNK activity. TI-JIP was found to be competitive with respect to the phosphoacceptor substrate c-Jun ($K_i = 0.39 \pm 0.08 \mu\text{M}$), and exhibit mixed (non-competitive) inhibition with respect to ATP. All seven substitutions of Pro-5 we tested significantly reduced the JNK inhibition, as did altering the Pro-5 to Leu-8 spacing. When we independently tested eight substitutions of either Thr-6 or Thr-7, only one substitution in each position was well tolerated. Furthermore, peptides based on the KIMs from other proteins were significantly less potent JNK inhibitors than TI-JIP, including a peptide from the JNK interactor Sab that contained all critical inhibitory residues present in TI-JIP. Therefore, despite having previously identified Arg-4, Pro-5, Leu-8, and Leu-10 in TI-JIP as independently critical for mediating JNK inhibition, we find their presence in other 11-mer peptides is not sufficient for JNK inhibition. TI-JIP is therefore a unique KIM-based inhibitor of JNK activity.

The c-Jun N-terminal kinases (JNKs)¹/stress-activated protein kinases (SAPKs) are a subfamily of mitogen-activated

protein kinases (MAPKs). In mammals, these Ser/Thr protein kinases are activated by various stimuli including growth factors, cytokines, and cellular stresses (1–5). JNK activation requires the phosphorylation of Thr-183 and Tyr-185 in its activation loop by the upstream kinases MKK4/SEK1/JNKK1 and MKK7/SEK2/JNKK2 (6). Following their activation, JNKs phosphorylate nuclear substrates including c-Jun, ATF-2, and Elk-1, and non-nuclear substrates such as Bcl-2 family members. This allows JNK to contribute to diverse biological processes including cell proliferation, differentiation, survival, and death (7).

Selective JNK or JNK pathway inhibitors have been developed following the implication of JNK signaling in various disease states. CEP-1347 is an orally active inhibitor of the mixed lineage kinases (MLKs) that function upstream in the JNK pathway (8). It has been used to inhibit JNK activation both *in vitro* and *in vivo* (9–12) and is currently in Phase II clinical trials for the treatment of Parkinson's disease, but is not commercially available for use as a tool to further investigate the JNK pathway. SP600125 was reported to be a reversible ATP-competitive JNK inhibitor with greater than 20-fold selectivity for JNK *versus* a range of kinases and enzymes tested (13). However, Bain *et al.* (14) recently reported that SP600125 was a relatively weak inhibitor of JNK isoforms, inhibiting JNK1 α 1 and JNK2 α 2 with IC₅₀ values of 5.8 and 6.1 μM , respectively. Moreover, 13 other protein kinases in the panel were inhibited with similar or greater potency by SP600125 (14). Given the limited availability of CEP-1347, and the relatively poor specificity of SP600125, there is a need for the development of potent, selective JNK inhibitors to investigate the intracellular actions of JNK.

Numerous groups have identified a conserved kinase-interaction motif (KIM) in the primary sequences of MAPK-interacting molecules, whether they be from the JNK, p38, or ERK MAPK pathways (15–18). The consensus KIM sequence is (K/R)₂₋₃X₁₋₆(L/I)X(L/I) and therefore consists of a cluster of basic amino acids followed by hydrophobic amino acids. Both the basic and hydrophobic residues are important for the MAPK docking interaction, and the KIM has been demonstrated to be both necessary and sufficient for binding to MAPKs (19).

Of particular interest is the interaction between JNKs and

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¹ The abbreviations used are: JNK, c-Jun N-terminal kinase; JIP,

JNK-interacting protein; TI-JIP, truncated inhibitory region of JIP; KIM, kinase interaction motif; MAPK, mitogen-activated protein kinase; IL-1 β , interleukin-1 β ; K_i , dissociation constant for the JNK-TI-JIP complex; K_{IS} , dissociation constant for the JNK-S-TI-JIP complex; MKK, MAPK kinase; CEP-1347, Cephalon Incorporated library compound number 1347; SP600125, Signal Pharmaceuticals library compound number 600125; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside.

TABLE I
Peptides based on naturally occurring KIMs

Peptides were synthesized commercially according to the sequences from the appropriate parent protein as indicated. Each peptide was examined experimentally for its ability to inhibit JNK activity significantly in an *in vitro* assay. NCBI Protein Database accession numbers for the parent proteins are given at the right of the table.

KIM	Peptide sequence	Parent protein / Description	
MKK4	MQGKRKALKLN	MKK4 (37–47)	#NP_003001
MKK7	EARRRIDLNLD	MKK7 (23–33)	#AAH3295
MKK1	PKKKPTPIQLN	MEK1 (2–12)	#NP_002746
MKK3	KSKRKKDLRIS	MKK3 (18–28)	#AAH32478
MKK6	GKKRNPGLKIP	MKK6 (6–16)	#AAH12009
MEF2A/C	MNNRKPDLRVV	MEF2A (266–276)	#NP_005578
		MEF2C (248–258)	#NP_002388
NFAT4	FLERPSRDHLY	Sequence from Ref. 30	
NFAT4ii	ERPSRDHLYLP	Sequence from Ref. 30	
PTP-SL	RRGSNVSLTLD	Sequence from Ref. 30	
Sab K1	AVVRPGSLDLR	Sab (310–320)	#BAA25922
Sab K2	LENRMKQLSLQ	Sab (397–407)	#BAA25922
Altered KIM	Peptide sequence	Parent protein / Description	
MKK4R5P	MQGKPKALKLN	MKK4 (37–47)	#NP_003001
MKK7R5P	EARRPIDLNLD	MKK7 (23–33)	#AAH3295
MKK1P7A	PKKKPTAIQLN	MEK1 (2–12)	#NP_002746
MKK3K5P	KSKRPKDLRIS	MKK3 (18–28)	#AAH32478
MKK65P6A	GKKRPAGLKIP	MKK6 (6–16)	#AAH12009

the KIM of the JNK-interacting protein-1 (JIP-1) scaffold protein. We demonstrated that an 11-mer peptide, TI-JIP, corresponding to residues 153–163 of murine JIP-1 potently inhibited JNK activity *in vitro* (20). In addition, cell-permeable peptides based on the KIM of JIP-1 effectively inhibit JNK activity *in vivo*. For example, Bonny *et al.* (21) engineered bioactive cell-permeable peptide inhibitors of JNK by linking 20-amino acid inhibitory domains of the JIP-1/IB1 proteins to the 10-amino acid HIV-TAT cell-permeable sequence. The peptides were found to protect pancreatic β TC-3 cells against apoptosis induced by IL-1 β . In addition, equivalent D retro-inverso peptides penetrated cells as efficiently as the L-enantiomers, decreased c-Jun phosphorylation by JNK, and remained stable inside cells. These D retro-inverso peptides conferred full protection against apoptosis during 2 weeks of continual treatment with IL-1 β (21).

The success of these inhibitors is further highlighted by the use of the cell-permeable, protease-resistant, IB1/JIP-1-based peptide inhibitor to protect against excitotoxicity and cerebral ischemia *in vivo* (22) and the use of this peptide to protect against both aminoglycoside and acoustic trauma-induced auditory hair cell death and hearing loss (23). This peptide has high specificity for inhibiting JNK because 10 μ M peptide did not inhibit the activity of 40 different kinases tested, and when 500 μ M peptide was tested, it reduced substrate phosphorylation by six different kinases by no more than 1% (22).

Although the IB1/JIP-1-based peptide inhibitors have proved successful in a number of instances, their mode of JNK inhibition remains undefined. Therefore, we used kinetic assays to characterize the mechanism of JNK inhibition by TI-JIP, and we used a series of substituted TI-JIP peptides to clarify the biochemical features mediating its potent JNK inhibition. We show that the TI-JIP peptide is competitive with respect to the protein substrate c-Jun, and non-competitive with respect to ATP. In addition, Pro-5 could not be replaced by 7 tested substitutions, and the Pro-5 to Leu-8 spacing was critical for potent JNK inhibition. Thr-6 and Thr-7 demonstrated a limited tolerance for change. In addition, the presence of all critical inhibitory residues in a peptide based on the KIM of Sab (24) was not sufficient for potent JNK inhibition. Taken together, these results suggest that TI-JIP is a KIM that is a unique and potent inhibitor of JNK activity.

EXPERIMENTAL PROCEDURES

Cell Transfection and *in Vitro* JNK Activity Assays—Procedures for cell transfection and *in vitro* JNK activity assays have previously been described in detail (20). Briefly, COS cells were transfected with pCMV-FLAG-JNK1 and then hyperosmotically shocked by incubation in 0.5 M sorbitol for 30 min. Following cell lysis, activated FLAG-JNK1 was immunoprecipitated and pretreated with inhibitor peptides for 10 min at 30 °C. JNK activity toward a GST-c-Jun-(1–135) substrate was then assayed in the presence of [γ - 32 P]ATP for 30 min at 30 °C. The phosphorylated substrate was separated by SDS-PAGE, visualized by autoradiography, and quantitated by Cerenkov counting. For all assays, incubations in the absence of inhibitor peptides and incubations with the TI-JIP peptide were routinely performed as controls. Data from each experiment were expressed relative to the uninhibited control. In some figures, images have been spliced to simplify viewing, but all lanes come from a single experiment.

Kinetic Assays—Assays to determine the kinetics of JNK inhibition by TI-JIP were carried out in 30- μ l volumes containing the final concentrations of the following: 20 mM HEPES pH 7.6, 20 mM MgCl₂, 20 mM β -glycerophosphate, 500 μ M dithiothreitol, 100 μ M sodium orthovanadate, 2.9 nM active JNK1 α 1 (Upstate Biotechnology Inc.), and 0.7–31.5 μ M GST-c-Jun-(1–135). The reactions were pretreated with TI-JIP inhibitor (0–4 μ M, as indicated) in the absence of ATP for 10 min at 30 °C. Reactions were initiated with the addition of 2 μ Ci [γ - 32 P]ATP (3000 Ci/mmol) and 0.5–100 μ M ATP (final concentration) and incubated for 15 min at 30 °C. Where the concentration of GST-c-Jun-(1–135) was varied, the ATP concentration was held constant at 50 μ M. Where the ATP concentration was varied, the GST-c-Jun-(1–135) concentration was held constant at 8 μ M. Under these conditions, less than 10% of substrate was converted to product. Reactions were stopped with 15 μ l of 3 \times SDS sample buffer, and then the phosphorylated product was separated by SDS-PAGE and quantitated by Cerenkov counting.

Peptide Synthesis—All peptides were commercially synthesized by Mimotopes, with a free amine at the N terminus and an amide group at the C terminus. The TI-JIP peptide used for determination of kinetic parameters was synthesized individually, whereas other peptides were synthesized as a “PEPSET” using multipin technology, which allowed parallel synthesis of multiple peptides with replacement of amino acids within each peptide sequence to assess the JNK inhibition afforded by variant TI-JIP peptides and other KIM-based peptides. Peptides were prepared in Me₂SO and solubilized by brief sonication, then incubation at 37 °C for 30–60 min. All peptides remained soluble under the assay conditions. TI-JIP variant sequences are specified in individual figures, and Table I lists the sequences of peptides not based on the TI-JIP sequence including those based on the KIMs of MKK4, MKK7, MKK1, MKK3, MKK6, MEF2A/C, NFAT4, PTP-SL, and Sab.

Plasmids and Yeast Strains—All bait constructs for yeast two-hybrid analysis were made by first cloning the sequence encoding TI-JIP into

the pGILDA vector (Clontech). These bait sequences were expressed as fusions with the 202-residue LexA protein, which acts as a DNA-binding domain in the two-hybrid system. The pGILDA plasmid carries the *HIS3* selectable marker gene. TI-JIP variants (P5A, L8A) were made by performing site-directed mutagenesis of the pGILDA-TI-JIP construct according to the Stratagene QuikChange protocol. Similarly, the TI-JIP sequence was altered to become the first KIM sequence from the Sab protein by performing three successive rounds of site-directed mutagenesis on the pGILDA-TI-JIP plasmid. A second yeast expression plasmid, pJG4-5/pB42AD (Clontech), was modified to contain the coding sequence of human JNK1 (25). The JNK1 prey protein was expressed as a fusion with the B42 domain, which acts as a transcriptional activation domain in the two-hybrid system. The pJG4-5 plasmid carries the *TRP1* selectable marker gene. All hybrid proteins were expressed in yeast cells under the control of the tightly regulated *GAL1* promoter, and transcription was repressed in the presence of glucose, but induced in the presence of galactose.

For two-hybrid screening, the yeast strain RFY 206 (*MATa*, *trp1*, *ura3-52*, *his3-200*, *leu2-3*, *lys2-Δ201*, *trp1::hisG*) was transformed with the pSH18-34 *lacZ* reporter plasmid, which contains the *URA3* selectable marker gene. These yeast were then further transformed with pGILDA-TI-JIP or related bait constructs. These transformants were then mated overnight on YPD pH 6.0 agar to the yeast strain PRT 49 (PRT 49 is a derivative of the SKY 48 strain (*MATa*, *trp1*, *ura3*, *his3*, *6-lexAop-LEU2*, *3-cIop-LYS2*, *ade2*)), which had been transformed with pJG4-5-JNK. Diploids were selected using synthetic complete medium lacking uracil, histidine, and tryptophan (UHW⁻).

β-Galactosidase Overlay Assays—For qualitative analysis of β-galactosidase activity, RFY 206/PRT 49 diploids transformed with pSH18-34, pGILDA-TI-JIP (or variants), and pJG4-5-JNK were replica plated onto UHW⁻ agar (or where *LEU2* reporter activity was monitored, this medium also lacked leucine (UHWL⁻)) containing either 2% glucose or 2% raffinose/0.05% galactose. Following incubation at 30 °C for 48 h, protein-protein interactions were assessed using the chloroform overlay assay technique (adopted from Ref. 26). Yeast grown on agar plates were overlaid with chloroform and incubated at room temperature for 5 min. Plates were then rinsed with chloroform, dried upside down for 5 min, then overlaid with a solution of 1% low melting agarose in 100 mM potassium phosphate buffer, pH 7.0, containing X-gal at a concentration of 1 mg/ml. Once the agarose solidified, plates were incubated at 30 °C and monitored for 20 min to 3 h for color changes. Protein-protein interactions were monitored via *lacZ* reporter activity converting the colorless X-gal substrate to a colored product.

Liquid β-Galactosidase Assays—The protocol for liquid β-galactosidase assays was modified from Ref. 27. For quantitative analysis, RFY 206/PRT 49 diploids transformed with pSH18-34, pGILDA-TI-JIP (or variants), and pJG4-5-JNK were incubated at 30 °C at 300 rpm for 48 h in UHW⁻ selective medium containing 2% raffinose/0.05% galactose. A control sample with repressed bait and prey expression was grown in UHW⁻ medium containing 2% glucose, to use as a blank for OD_{420 nm} measurements in the β-galactosidase assay. From each culture, 1.4 ml of suspension were centrifuged at 20,800 × *g* for 30 s to pellet the yeast. Yeast pellets were resuspended in 0.8 ml of Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM Mg₂SO₄, pH 7.0) containing 0.27% (v/v) 2-mercaptoethanol. Following addition of 50 μl of CHCl₃ and 50 μl of 0.1% SDS, samples were vortexed for 30 s. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG, 160 μl of a 4 mg/ml solution in H₂O, prepared fresh on the day) was added to each sample and following thorough mixing, samples were incubated at 30 °C for 35 min. At this time a yellow color was apparent, and reactions were quenched by addition of 0.4 ml of 1 M Na₂CO₃ with thorough vortexing. Samples were centrifuged at 20,800 × *g* for 10 min, then supernatants were removed, and their absorbance at 420 nm was measured. β-Galactosidase activity was calculated using Equation 1,

$$\text{Activity (Miller Units)} = 1000 \times ((\text{OD}_{420\text{nm}})/(T \times V \times \text{OD}_{600\text{nm}})) \quad (\text{Eq. 1})$$

where *V* = volume of yeast added, and *T* = time incubated at 30 °C.

Data Analysis—StatView 3.0 was used to perform one way analysis of variance, and levels of inhibition that were found to be significantly different from the TI-JIP inhibition are denoted in the figures by asterisks (**, *p* ≤ 0.01; *, *p* ≤ 0.05). Analysis of kinetic data for the inhibition of GST-c-Jun-(1-135) phosphorylation was performed by fitting Equations 2 and 3 to the data, using the non-linear least squares regression analysis facility of the Scientist™ program,

$$\text{competitive inhibition: } v = V[S]/([S] + K_m(1 + [\text{TI-JIP}]/K_i)) \quad (\text{Eq. 2})$$

$$\text{mixed inhibition: } v = V[S]/([S](1 + [\text{TI-JIP}]/K_{IS}) + K_m(1 + [\text{TI-JIP}]/K_i)) \quad (\text{Eq. 3})$$

where *V* is the maximum velocity, [*S*] is the concentration of the varied substrate (either ATP or GST-c-Jun-(1-135)), *K_m* is the apparent *K_m* of the varied substrate, *K_i* is the dissociation constant for the JNK-TI-JIP complex and *K_{IS}* is the dissociation constant for the JNK-S-TI-JIP complex. From these fits, estimates of *K_i* and *K_{IS}* and standard deviations of these estimates were obtained.

RESULTS

JNK Inhibition by TI-JIP Is Competitive with Respect to the Protein Substrate, and Non-competitive with Respect to ATP—We performed kinetic assays using activated JNK1α1 where either GST-c-Jun-(1-135) or ATP concentrations were varied at a fixed concentration of the other substrate in the presence or absence of TI-JIP. Data obtained from experiments performed at fixed ATP concentration, where GST-c-Jun-(1-135) concentrations were varied in the presence of increasing concentrations of TI-JIP, were indicative of competitive inhibition (Fig. 1A). A fit of Equation 2 to the data gave a value of *K_i* of 0.39 ± 0.08 μM. When a fit of Equation 3 was attempted, the estimate of *K_{IS}* obtained was many orders of magnitude greater than *K_i*, which indicates that the affinity of TI-JIP for the JNK-GST-c-Jun-(1-135) complex is negligible. Similar experiments were performed with constant GST-c-Jun-(1-135) concentration and varied ATP concentrations, at several concentrations of TI-JIP (Fig. 1B). When Equation 3 was fitted to the data from these experiments, the estimates of *K_i* and *K_{IS}* obtained were 1.7 ± 0.7 and 1.8 ± 0.2 μM, respectively, which indicates that TI-JIP has similar affinity for both JNK and JNK-ATP. Therefore, it appears that TI-JIP competes with phosphoacceptor substrate for binding to JNK, and that the interaction between TI-JIP and JNK occurs independently of binding between ATP and JNK.

Investigating the Importance of Pro-5 and the Pro-5 to Leu-8 Spacing within TI-JIP for JNK Inhibition—In our previous alanine-scanning replacement of the TI-JIP sequence, we identified Arg-4, Pro-5, Leu-8, and Leu-10 as independently critical for mediating JNK inhibition (20). Arg-4, Leu-8, and Leu-10 reside within the highly conserved basic and hydrophobic regions of the KIM that are important for docking to MAPKs, so their replacement with alanine most likely disrupts the interaction between TI-JIP and JNK. However, we found that Pro-5 was also critical for mediating the inhibition by TI-JIP, although it is not a conserved feature of the reported KIMs. Therefore, we investigated the tolerance for change at this position within the 11-amino acid peptide, to assess the importance of Pro-5 for JNK inhibition by TI-JIP. Using JNK activity assays, we assessed the inhibition by a series of TI-JIP Pro-5 variants, with either hydrophobic (small/bulky) or basic substitutions at this position. We found that these seven substitutions at position 5 resulted in peptides that produced significantly less JNK inhibition than TI-JIP (Fig. 2A). Therefore, despite its lack of conservation within the KIM consensus sequence, Pro-5 appeared critical for the inhibition of JNK by TI-JIP.

Within the KIM consensus sequence, there is also variability in the spacing between the conserved basic N-terminal residue and the first conserved hydrophobic C-terminal residue. This prompted an investigation into whether the spacing between these residues affected the ability of TI-JIP-based peptides to inhibit JNK activity. Given the critical nature of Pro-5 for JNK inhibition, we varied the Pro-5 to Leu-8 spacing, rather than the Arg-4 to Leu-8 spacing. Using JNK activity assays, we tested a series of TI-JIP-based peptides with 0–4 residues separating Pro-5 and Leu-8. Like the Pro-5 variant peptides, these peptides resulted in significantly less JNK inhibition

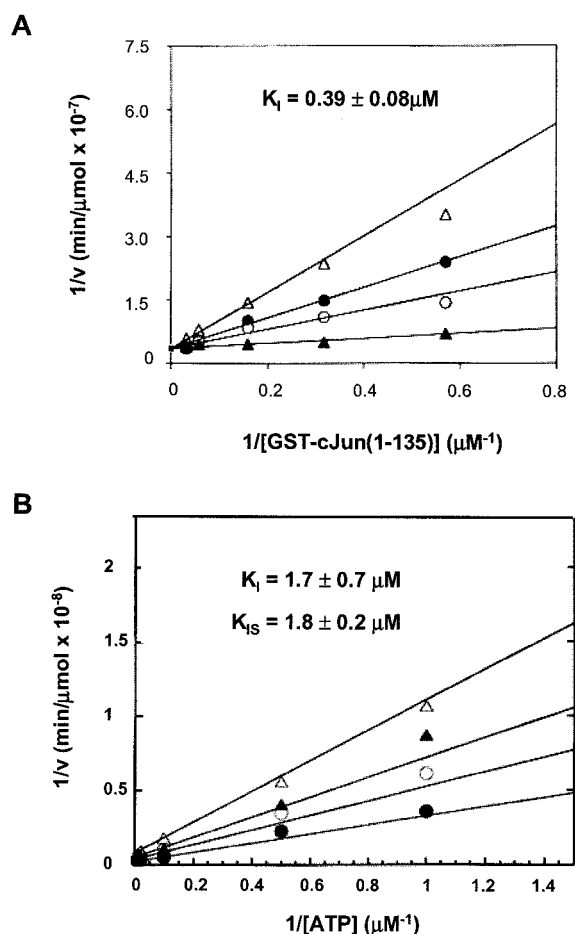


FIG. 1. TI-JIP is competitive with respect to the phosphoacceptor substrate c-Jun, and non-competitive with respect to ATP, for binding JNK. *A*, active JNK1 α 1 (2.9 nM) and GST-c-Jun-(1–135) (0.7–31.5 μ M) were preincubated with TI-JIP inhibitor (\blacktriangle , 0 μ M; \circ , 1 μ M; \bullet , 2 μ M; \triangle , 4 μ M) in the absence of ATP for 10 min at 30 $^{\circ}$ C. Reactions were initiated with the addition of 2 μ Ci [γ - 32 P]ATP (3000 Ci/mmol) and 50 μ M ATP (final concentration) and incubated for 15 min at 30 $^{\circ}$ C. Reactions were stopped with 3 \times SDS sample buffer and then the phosphorylated substrate was separated by SDS-PAGE and quantitated by Cerenkov counting. The lines shown and the estimate of K_i were derived from a non-linear least squares fit of Equation 2 to the data. *B*, active JNK1 α 1 (2.9 nM) and GST-c-Jun-(1–135) (8 μ M) were preincubated with TI-JIP inhibitor (\bullet , 0 μ M; \circ , 1 μ M; \blacktriangle , 2 μ M; \triangle , 4 μ M) in the absence of ATP for 10 min at 30 $^{\circ}$ C. Reactions were initiated with the addition of 2 μ Ci [γ - 32 P]ATP (3000 Ci/mmol) and 0.5–100 μ M ATP (final concentration) and incubated for 15 min at 30 $^{\circ}$ C. Reactions were stopped with 3 \times SDS sample buffer and then the phosphorylated substrate was separated by SDS-PAGE and quantitated by Cerenkov counting. The lines shown were derived from a non-linear least squares fit of Equation 3 to the data.

than the TI-JIP peptide (Fig. 2*B*). Therefore, we concluded that in addition to the presence of Pro-5, the spacing between Pro-5 and Leu-8 was an essential feature allowing potent inhibition of JNK by TI-JIP.

Investigating the Tolerance for Change at Thr-6 and Thr-7—We had previously shown that in contrast to alanine replacement of Pro-5, substitution of either Thr-6 or Thr-7 with alanine did not significantly reduce the potency of the TI-JIP peptide (20). We therefore investigated the tolerance for change in these residues in an attempt to better understand the requirements for JNK binding. Using JNK activity assays, we tested the inhibition produced by TI-JIP variant peptides where either Thr-6 or Thr-7 was replaced by acidic, basic or hydrophobic amino acids. We also investigated the effect of substituting Pro at this position, which would be expected to alter the conformation of the amino acid backbone, or phospho-Thr, because the basic amino acids situated N-terminal to

these threonine residues suggests that they might represent a phosphorylation site (28). For both Thr-6 and Thr-7 variants, a Thr to Pro substitution virtually abolished the JNK inhibition relative to wild-type TI-JIP (Fig. 3, *A* and *B*). Most of the other substitutions of Thr-6 resulted in significantly less JNK inhibition than TI-JIP, with the exception of a Thr-6 to Lys substitution (Fig. 3*A*). Most substitutions of Thr-7 also resulted in peptides with significantly less JNK inhibitory activity, and like the Thr-7 to Pro substitution, a Thr-7 to Tyr substitution abolished the JNK inhibition (Fig. 3*B*). The only substitution at position 7 that did not significantly decrease the JNK inhibition was Thr-7 to Phe (Fig. 3*B*). Therefore, changes to both Thr-6 and Thr-7 were sufficient to significantly reduce JNK inhibition by the peptides and hence these residues contribute to the potent inhibition of JNK by TI-JIP.

Assessing JNK Inhibition by other KIM-based Peptides—As mentioned previously, the KIM consensus sequence is present in MAPK interactors including upstream activators, substrates, phosphatases, and scaffolds (as reviewed in Ref. 19). Indeed, the TI-JIP sequence is directly derived from the KIM present in the JNK pathway scaffold protein, JIP-1. Given the homology between TI-JIP and other KIM sequences, we hypothesized that similar KIM-based peptides might function as inhibitors of JNK activity. This hypothesis is supported by recent work by Ho *et al.* (29), who reported that a docking site in MKK4 mediates high affinity binding to JNKs and competes with similar docking sites in JNK substrates. A peptide version of this docking site was found to inhibit MKK4/JNK binding, MKK4-mediated phosphorylation of JNK1, and JNK2-mediated phosphorylation of c-Jun and ATF-2 when used at final concentrations of 25, 200, and 10 μ M, respectively (29).

We used JNK activity assays to test the potency of peptides based on the KIMs of MKK4 and MKK7 from the JNK cascade. In addition, we tested peptides based on the KIMs of MKK proteins from the related ERK and p38 MAPK cascades. We found that all of these KIM-based peptides produced significantly less JNK inhibition than the TI-JIP peptide, and that the KIMs from the JNK cascade proteins were not better inhibitors of JNK when compared with KIMs from proteins from the ERK or p38 MAPK cascades (Fig. 4*A*, *black bars*). We hypothesized that this might be due to the lack of Pro-5 in the sequences, or the presence of a Pro residue at either position 6 or 7, which is poorly tolerated (Fig. 3, *A* and *B*). Therefore, we tested similar KIM-based peptides, but with the addition of Pro-5 to the sequences and the substitution of central Pro residues with Ala. Despite these changes, the KIM-based peptides still produced significantly less JNK inhibition than TI-JIP (Fig. 4*A*, *gray bars*).

In a recent report by Chang *et al.* (30), the KIM sequences present in various MAPK-interacting proteins, including JIP-1, were aligned. In addition to the MKK proteins and well-characterized transcription factor substrates of the MAPKs (c-Jun, Elk-1, ATF-2), proteins such as NFAT4, MEF2A/C, and PTP-SL were also included in the alignment. These proteins conformed to the KIM consensus sequence, but did not contain the critical Pro-5 residue and had variable spacing between the basic cluster and the first hydrophobic residue in the KIM consensus. Given their relatively low homology to TI-JIP, we predicted that these proteins would function as relatively poor JNK inhibitors. This was confirmed by JNK activity assays where the proteins reduced JNK activity by a maximum of 25%, compared with 86% reduction by TI-JIP (Fig. 4*B*). Therefore, although various KIMs allow docking to the JNK protein, this interaction is unlikely to inhibit JNK activity *in vivo*. This confirms our previous observations (20) that peptides based on the KIMs of the JNK substrates c-Jun (ILKQSMITLNL) or ATF2 (KHKHEMTLKF) were also poor inhibitors of JNK activity.

A

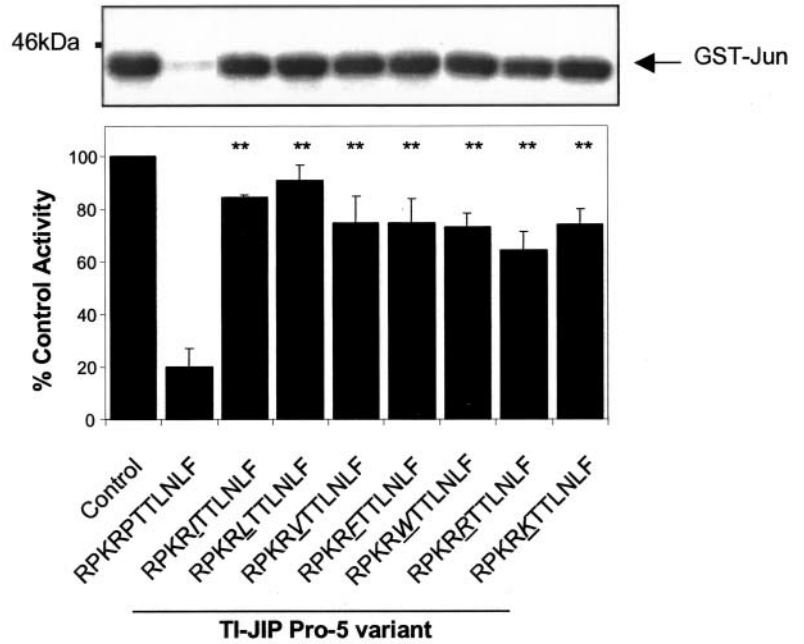
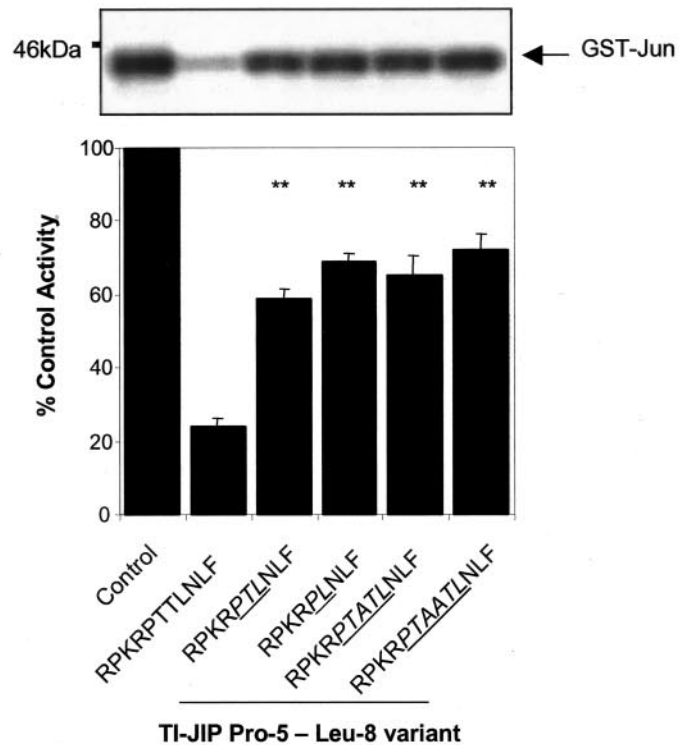


FIG. 2. Investigating the effects of altering Pro-5, or changing the Pro-5 to Leu-8 spacing within TI-JIP, on JNK inhibition. Activated JNK1 was preincubated for 10 min at 30 °C with either a buffer control, TI-JIP (1700 nM final assay concentration), or equivalent concentrations of peptides representing variants of TI-JIP at position 5 (A), or variants of TI-JIP with altered Pro-5 to Leu-8 spacing (B) before assaying its kinase activity toward GST-c-Jun-(1–135). Regions of TI-JIP that were altered in the variants are *underlined* and shown in *italics*. ³²P incorporated into the substrate (as indicated by the arrow to the right of the autoradiographs (*upper panel*)) was differentially inhibited by the TI-JIP variant peptides. The *lower panel* summarizes data from three independent experiments, normalized to the activity in the uninhibited reaction. *Error bars* represent the S.E. of the means, and *asterisks* indicate that values are significantly different (**, $p \leq 0.01$) from the TI-JIP inhibition.

B



Most TI-JIP/MKK7 and TI-JIP/MKK4 Hybrid Peptides Were Relatively Poor JNK Inhibitors—We were surprised that peptides based on the KIMs of MKK7 and MKK4, which are upstream activators of JNK, were not more effective JNK inhibitors than peptides based on the KIMs of proteins from other

signaling pathways. To further investigate the reason why these peptides functioned as weaker JNK inhibitors, we tested a series of hybrid peptides where the N-terminal, C-terminal, and central regions of the TI-JIP peptide were systematically altered to more closely resemble the MKK7 and MKK4 KIM

A

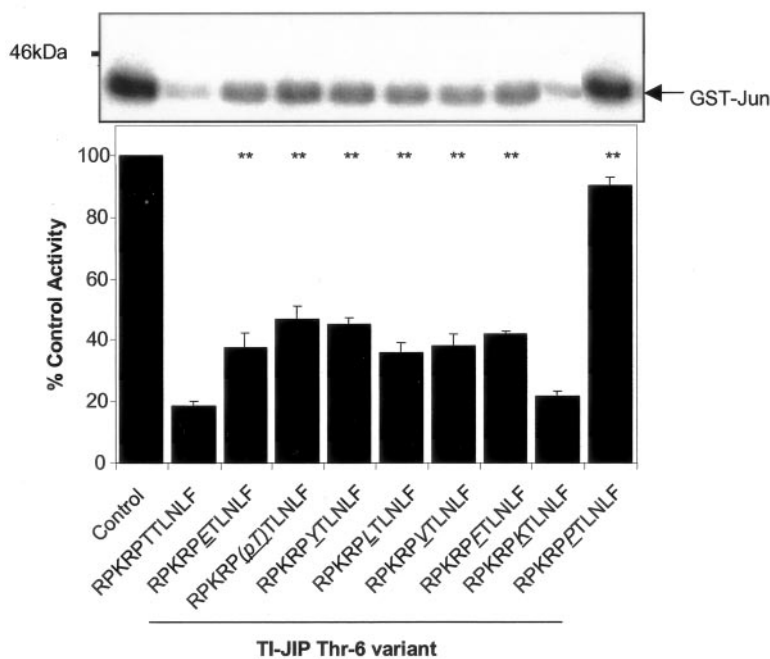
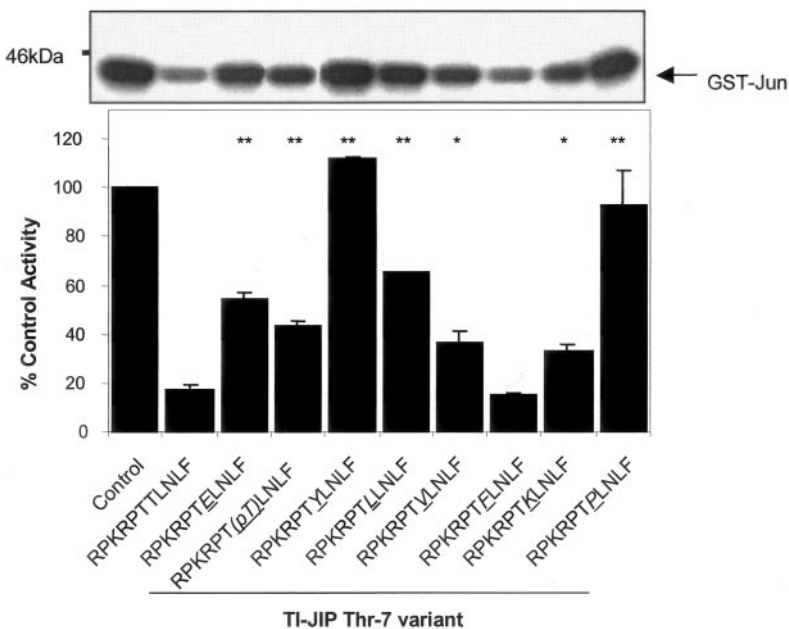


FIG. 3. Investigating the tolerance for change at Thr-6 and Thr-7. Activated JNK1 was preincubated for 10 min at 30 °C with either a buffer control, TI-JIP (1700 nM final assay concentration), or equivalent concentrations of peptides representing variants of TI-JIP at either position 6 (A) or position 7 (B) before assaying its kinase activity toward GST-c-Jun-(1–135). Altered residues within the TI-JIP sequence are *underlined* and shown in *italics*. Note that *pT* denotes phosphothreonine. ^{32}P incorporated into the substrate (as indicated by the arrow to the right of the autoradiographs (upper panel)) was differentially inhibited by the TI-JIP variant peptides. The lower panel summarizes data from three independent experiments, normalized to the activity in the uninhibited reaction. Error bars represent the S.E. of the means, and asterisks indicate that values are significantly different (**, $p \leq 0.01$; *, $p \leq 0.05$) from the TI-JIP inhibition.

B



sequences. In the case of MKK7, most hybrid peptides were significantly weaker JNK inhibitors than TI-JIP (Fig. 5A). The only hybrid peptide that inhibited JNK to a similar extent as TI-JIP was “RPKRPTTLNLD,” where the TI-JIP C terminus was replaced by the MKK7 KIM C terminus. This peptide represented a Phe-11 → Asp substitution of TI-JIP, and we previously demonstrated that the extreme C-terminal residue of TI-JIP could be removed from the sequence with no significant decrease in JNK inhibition (20). When the central residues of TI-JIP were changed to the corresponding MKK7 KIM

residues (*i.e.* TT to ID), the altered peptide produced significantly less JNK inhibition than TI-JIP (Fig. 5A). Similarly, the substitution of the TI-JIP N terminus with the MKK7 KIM N terminus (*i.e.* RPK to EAR) significantly reduced the JNK inhibition produced by TI-JIP (Fig. 5A). Therefore it appeared that the N-terminal and central residues within the MKK7 KIM contributed to its reduced ability to inhibit JNK activity.

When a similar set of hybrid peptides based on TI-JIP and the KIM of MKK4 were tested for their ability to inhibit JNK activity, all hybrid peptides produced significantly weaker JNK

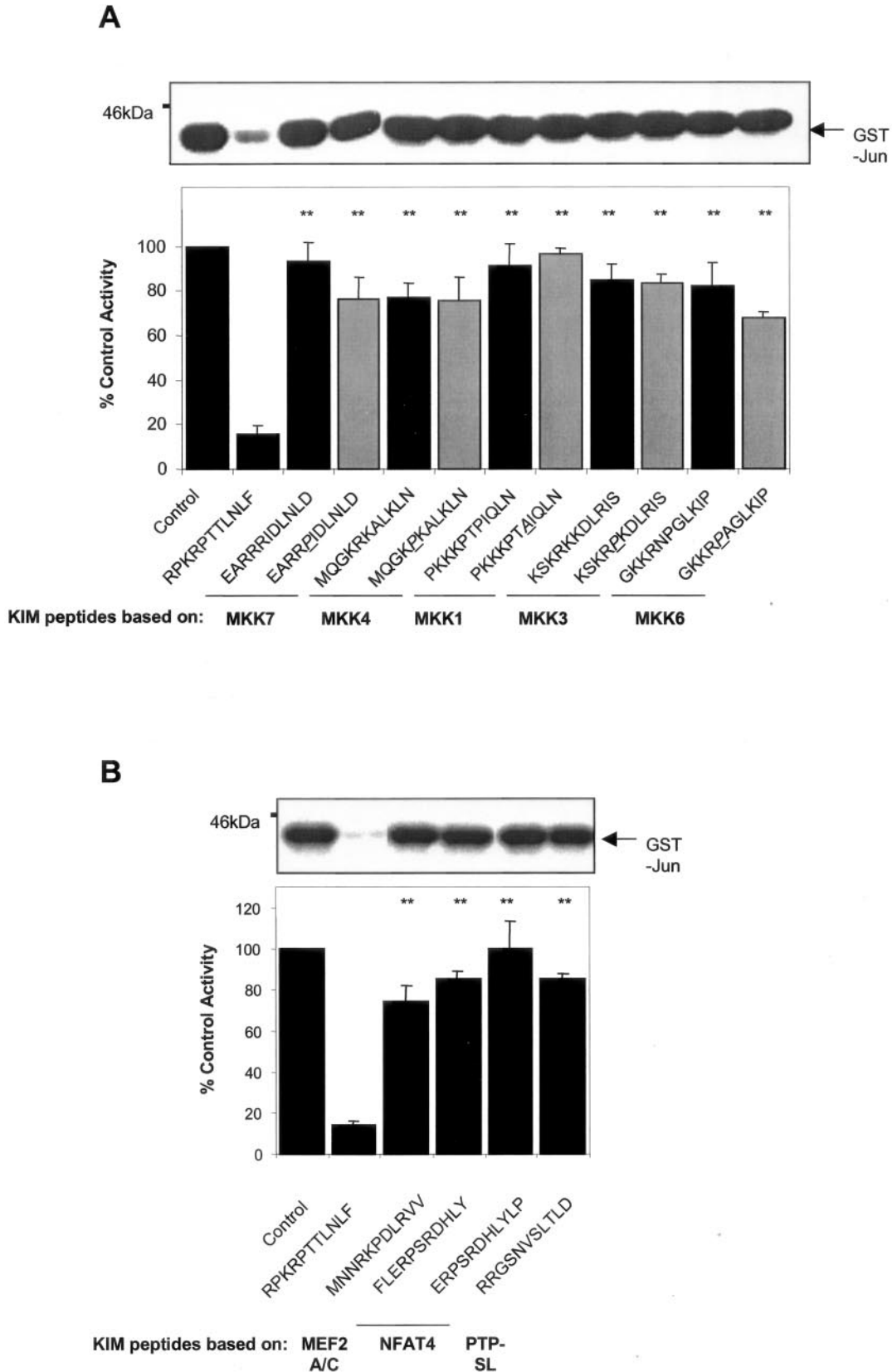


FIG. 4. Assessing JNK inhibition produced by peptides based on the KIMs of MKK proteins and other MAPK-interacting proteins. Activated JNK1 was preincubated for 10 min at 30 °C with either a buffer control, TI-JIP (1700 nM final assay concentration), or equivalent concentrations of KIM-based peptides, before assaying its kinase activity toward GST-c-Jun(1–135). Peptides were based on the KIMs of MKK7, MKK4, MKK1, MKK3, and MKK6 (A), and MEF2A/C, NFAT4, and PTP-SL (B) (see Table I for further information). Where the sequences of the parent proteins were altered, residues that were changed are *underlined* and shown in *italics*, and columns are shown in *gray*. ³²P incorporated into the substrate (as indicated by the *arrow* to the *right* of the autoradiographs (*upper panel*)) was differentially inhibited by the TI-JIP variant peptides. The *lower panel* summarizes data from three independent experiments, normalized to the activity in the uninhibited reaction. *Error bars* represent the S.E. of the means, and *asterisks* indicate that values are significantly different (**, $p \leq 0.01$) from the TI-JIP inhibition.

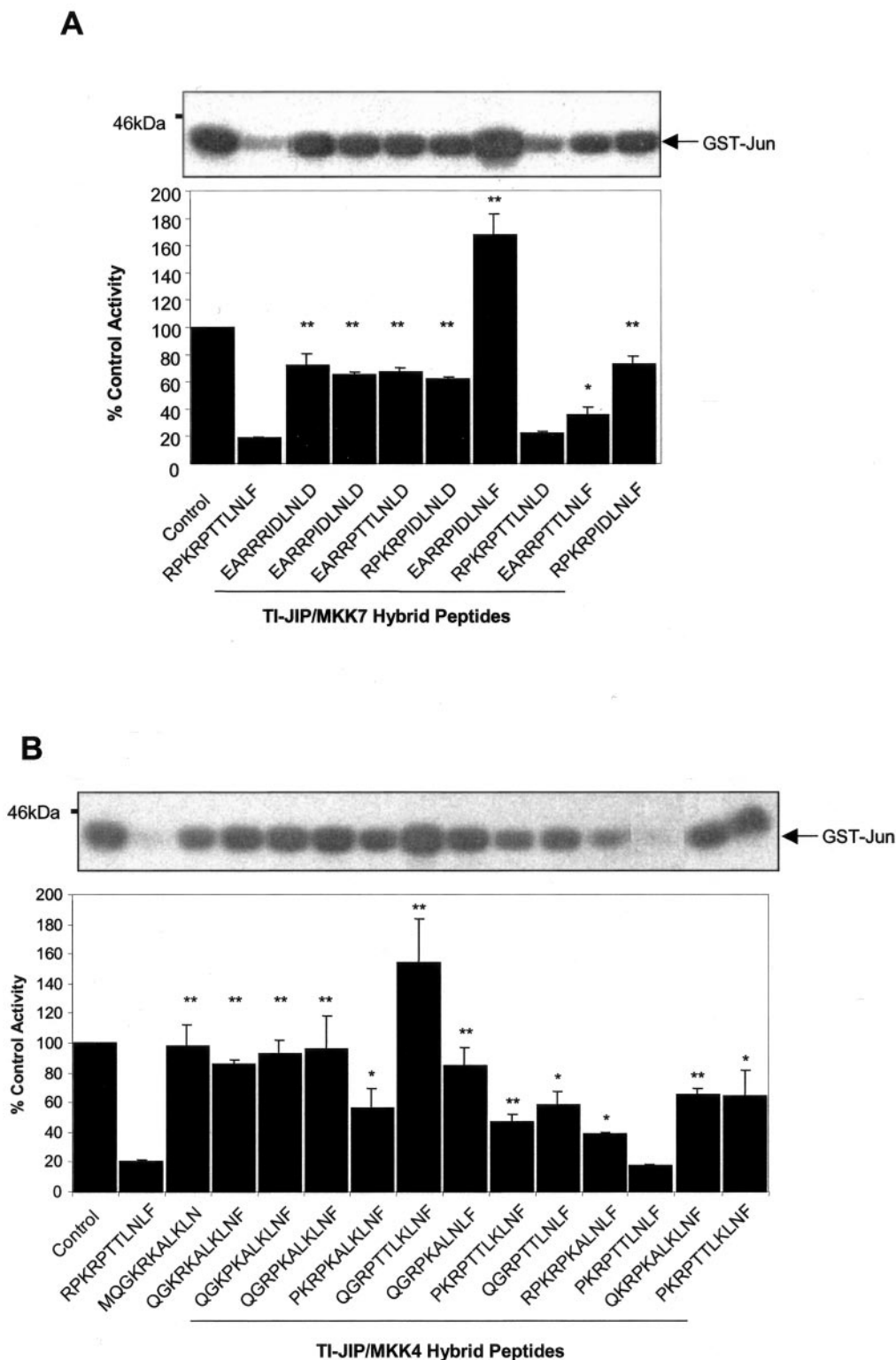


FIG. 5. Assessing JNK inhibition produced by hybrid peptides based on TI-JIP and the KIM of either MKK7 or MKK4. Activated JNK1 was preincubated for 10 min at 30 °C with either a buffer control, TI-JIP (1700 nM final assay concentration), or equivalent concentrations of hybrid TI-JIP/MKK7 peptides (A) or TI-JIP/MKK4 peptides (B) before assaying its kinase activity toward GST-c-Jun-(1-135). ^{32}P incorporated into the substrate (as indicated by the arrow to the right of the autoradiographs (upper panel)) was differentially inhibited by the hybrid peptides. The lower panel summarizes data from three independent experiments, normalized to the activity in the uninhibited reaction. Error bars represent the S.E. of the means, and asterisks indicate that values are significantly different (**, $p \leq 0.01$; *, $p \leq 0.05$) from the TI-JIP inhibition.

inhibition than TI-JIP (Fig. 5B). Although the most N-terminal residue of TI-JIP was removed without significant decreases in JNK inhibition (Fig. 5B), when the C terminus of this peptide was altered to resemble the MKK4 KIM (*i.e.* NLF to KLNLF), the JNK inhibition was significantly reduced (Fig. 5B). The

JNK inhibition was also significantly reduced when the central residues of the TI-JIP peptide were substituted (*i.e.* TT to KA), or when the N terminus was substituted (*i.e.* PK to QG) (Fig. 5B). Therefore, it appeared that all regions of the MKK4 KIM contributed to its significantly weaker JNK inhibition com-

pared with TI-JIP. The results obtained with both the MKK7 and MKK4 KIM hybrid peptides further emphasized the notion that although all residues of TI-JIP were not demonstrated to be independently critical for potent inhibition of JNK activity, only a limited tolerance for change is permitted and they collectively contribute to the potent JNK inhibition afforded by the TI-JIP peptide.

Assessing JNK Inhibition Produced by Peptides Based on KIMs from the Sab Protein—The Sab protein was identified as a novel JNK-interacting protein by yeast two-hybrid screening (24). Using deletion analysis and site-directed mutagenesis, the most N-terminal KIM in Sab was shown to be essential for JNK binding (24). This KIM sequence (KIM 1: RPGSLDL) contained all critical JNK-inhibitory residues identified in the TI-JIP sequence (underlined), whereas the C-terminal KIM 2 sequence (KIM 2: RMKQLSL) lacked a Pro at position 5. We predicted that a peptide based on the KIM 1 sequence would be an effective JNK inhibitor, whereas a similar peptide based on the KIM 2 sequence would be a relatively poor JNK inhibitor. Using JNK activity assays, we found that the KIM 2 peptide did not cause any significant reduction in JNK activity (Fig. 6A). However, the KIM 1 peptide reduced JNK activity by only 14%, which was significantly less than the 82% reduction by TI-JIP (Fig. 6A). This result indicated that the presence of critical inhibitory residues in a peptide was not sufficient for JNK inhibition.

To further investigate why the Sab KIM 1 peptide was a weaker JNK inhibitor than TI-JIP, we tested a series of hybrid peptides constructed from the TI-JIP sequence and the Sab KIM 1 sequence. Initially, we tested TI-JIP peptides with either Thr-6 or Thr-7 individually substituted with the corresponding residues from Sab KIM 1. Although the conservative Thr-7 → Ser substitution was well tolerated, the Thr-6 → Gly substitution significantly reduced the JNK activity produced by TI-JIP (Fig. 6B). In addition, the substitution of either the N terminus or C terminus of TI-JIP for the corresponding sequence present in Sab KIM 1 resulted in peptides that were significantly weaker JNK inhibitors than TI-JIP (Fig. 6B). Therefore, like the MKK4 KIM sequence, all regions of the Sab KIM1 sequence resulted in its reduced ability to inhibit JNK activity. We decided to investigate in the following section whether this reduced inhibition resulted from a weaker interaction with JNK.

Using Yeast Two-hybrid Analysis to Investigate the Interaction between KIM-based Peptides and JNK—Using yeast two-hybrid analysis, we were able to assess the interaction between KIM-based peptides and JNK, used as “bait” and “prey,” respectively. Fig. 6C illustrates the results of an overlay assay performed on yeast that were replica plated onto UHW⁻ synthetic complete medium containing either 2% glucose, which repressed bait and prey expression, or in the presence of 0.05% galactose, which induced bait and prey expression. In the presence of glucose (Fig. 6C, left panel), all yeast remained colorless due to repression of bait and prey protein expression. However, in the presence of galactose (Fig. 6C, right panel), yeast expressing JNK and TI-JIP became colored, indicative of an interaction between the proteins. In contrast, yeast expressing JNK and either TI-JIP(P5A) or TI-JIP(L8A) remained colorless, indicating that these proteins did not interact. Because these TI-JIP variants are unable to inhibit JNK activity (20), these results from yeast two-hybrid analysis suggest that this is caused by impaired docking of the peptides to JNK.

We also used this system to investigate whether the lack of JNK inhibition produced by the Sab KIM 1 peptide resulted from a less avid interaction with JNK. Here we used two reporters, the *LEU2* reporter (growth in the absence of

leucine), and the *lacZ* reporter (conversion of the colorless X-gal substrate into a colored product). Overlay assays demonstrated that yeast expressing JNK and TI-JIP grew on medium lacking leucine, and became colored (Fig. 6D, panel i, upper), whereas yeast expressing JNK and the Sab KIM 1 peptide failed to grow or become colored (Fig. 6D, panel i, lower). This suggested a lack of interaction between the Sab KIM 1 peptide and JNK.

We performed a quantitative assay to assess *lacZ* reporter activity in yeast expressing JNK and either TI-JIP, Sab KIM 1, or hybrid peptides constructed from both of these sequences. We found that yeast expressing JNK and any of the hybrid peptides exhibited significantly weaker reporter activity than yeast expressing JNK and TI-JIP (Fig. 6D, panel ii). In addition, the reporter activity was further reduced as the sequence of the bait peptide deviated from TI-JIP to more closely resemble Sab KIM 1. Therefore, the relatively weak JNK inhibition produced by the Sab KIM 1 peptide (Fig. 5A) is partially explained by the relatively weak interaction between Sab KIM 1 and JNK.

DISCUSSION

Although JIP-1-based peptides have demonstrated efficacy as JNK inhibitors, both *in vitro* (20) and *in vivo* (21–23), their mechanism of action has remained undefined. The sequence similarities between TI-JIP and the KIM sequences in JNK substrates such as c-Jun and ATF-2 suggests that these inhibitors may act by competing with the substrate for docking to JNK. However, peptides based on the KIMs of c-Jun and ATF-2 are relatively poor inhibitors of JNK when compared with TI-JIP (20). It might therefore be predicted that these inhibitors allosterically modulate JNK activity and inhibit by non-competitive or uncompetitive mechanisms. For example, the insulin receptor interacting domain of the adapter protein Grb14 behaves as an uncompetitive inhibitor with respect to the insulin receptor substrate (31). Here, we have demonstrated that the TI-JIP peptide competes with the c-Jun protein substrate for binding JNK. This is supported by recent findings that JIP-1 and c-Jun interact with similar regions of JNK2 (32).

Competitive peptide inhibitors of kinases can be divided into two groups. First, naturally occurring peptide inhibitors include the PKI (5–22) peptide derived from the heat-stable inhibitor protein that inhibits the activity of protein kinase A (33–35), and regulatory domain peptides that inhibit the activity of protein kinase C (36). The second group includes peptide inhibitors modified from known substrates. These include non-phosphorylatable substrate-based peptides that inhibit protein kinase C (37), calmodulin-dependent protein kinase II (38), MAPK-activated protein kinase-2 (39), Akt/protein kinase B (40), p60 c-Src protein-tyrosine kinase (41) and ZAP-70 tyrosine kinase (42). Inhibitors from both of these groups act as pseudosubstrates for the relevant kinases. However, this is not true for all competitive peptide inhibitors of kinases, and a third class of inhibitors based on protein-protein interaction partners can be defined. For example, a peptide inhibitor derived from the consensus sequence of the cyclin/cyclin-dependent kinase-2 (cdk2) binding motif, PVKRRRLFG, that serves as the docking site for cyclin-dependent kinase-2/cyclin complexes, blocks the phosphorylation of substrates by cyclin A/cdk2 or cyclin E/cdk2 (43). Similarly, cell-permeable peptides based on MKK1 inhibited activation of ERK MAPK both *in vitro* and *in vivo* and also inhibited ERK-mediated activation of the transcriptional activity of Elk-1 (44). It would appear that the JIP-based peptides function by a similar mechanism to this third group of inhibitors, being based on the docking motif for JNK/JIP-1 complexes and not acting as pseudosubstrates.

We have demonstrated that TI-JIP, based on the JIP-1 KIM,

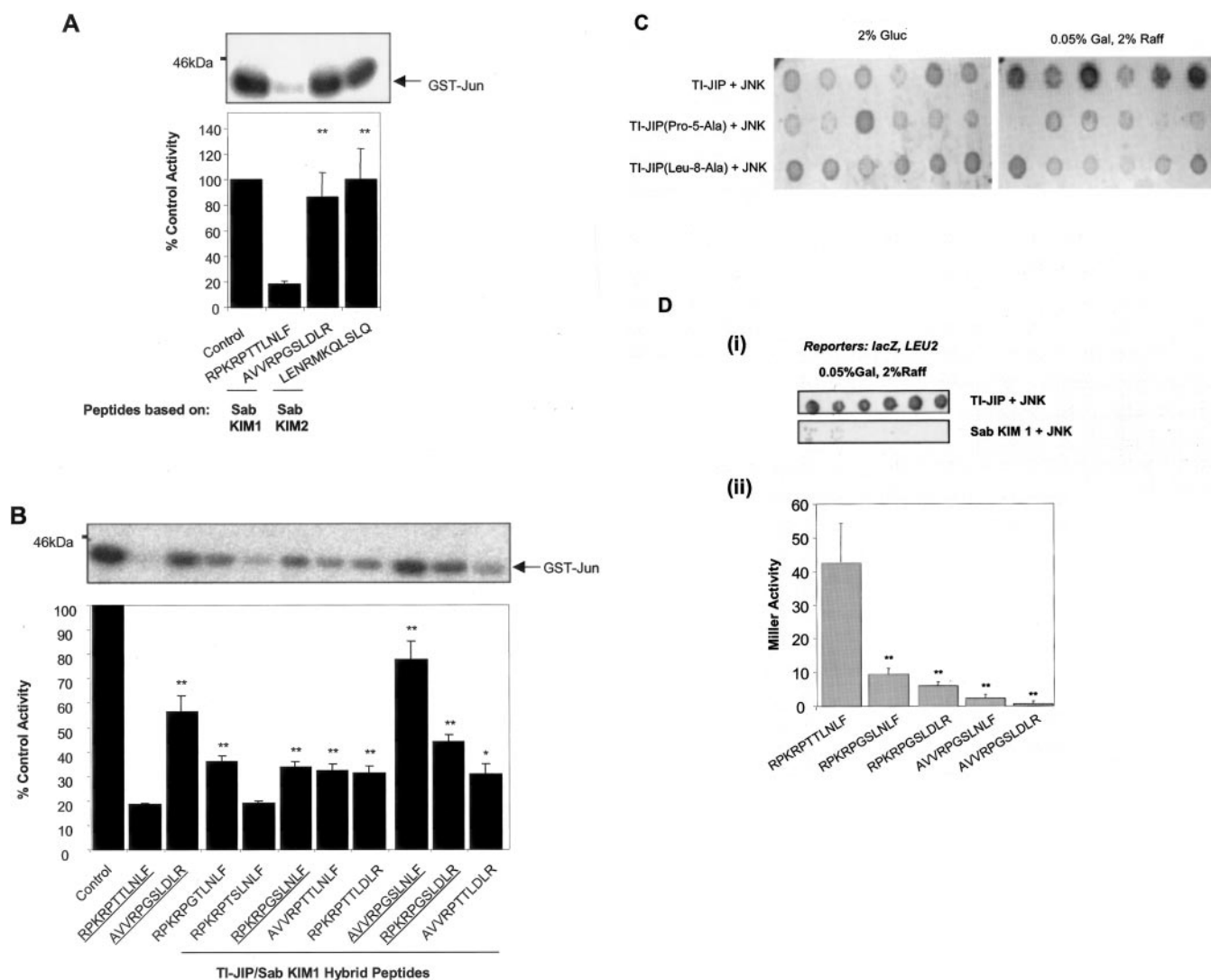


FIG. 6. Investigating the inhibition of JNK by Sab KIM 1- and Sab KIM 2-based peptides, and comparing the JNK-TI-JIP interaction with the JNK-Sab KIM 1 interaction. *A*, activated JNK1 was preincubated for 10 min at 30 °C with either a buffer control, TI-JIP (1700 nM final assay concentration), or equivalent concentrations of Sab KIM 1- and Sab KIM 2-based peptides, before assaying its kinase activity toward GST-c-Jun-(1–135). ³²P incorporated into the substrate (as indicated by the arrow to the right of the autoradiograph (upper panel)) was differentially inhibited by the peptides. The lower panel summarizes data from three independent experiments, normalized to the activity in the uninhibited reaction. Error bars represent the S.E. of the means, and asterisks indicate that values are significantly different (**, $p \leq 0.01$) from the TI-JIP inhibition. *B*, activated JNK1 was preincubated for 10 min at 30 °C with either a buffer control, TI-JIP (1700 nM final assay concentration), or equivalent concentrations of peptides representing hybrids of the TI-JIP and Sab KIM 1 sequences, before assaying its kinase activity toward GST-c-Jun-(1–135). ³²P incorporated into the substrate (as indicated by the arrow to the right of the autoradiograph (upper panel)) was differentially inhibited by the hybrid peptides. The lower panel summarizes data from three independent experiments, normalized to the activity in the uninhibited reaction. Error bars represent the S.E. of the means, and asterisks indicate that values are significantly different (**, $p \leq 0.01$; *, $p \leq 0.05$) from the TI-JIP inhibition. Sequences that are underlined were tested for their interaction with JNK using yeast two-hybrid assays as described in *D* (panel ii). *C*, diploid yeast were replica plated onto synthetic complete media lacking uracil, histidine and tryptophan (UHW⁻ medium) containing either 2% glucose or 0.05% galactose/2% raffinose. Following growth for 48 h, yeast were analyzed for β -galactosidase reporter activity using the overlay assay technique (see “Experimental Procedures”). A colored product was obtained for yeast expressing JNK and TI-JIP, but not for yeast expressing JNK and either of the TI-JIP variants. *D*, panel i, diploid yeast were replica plated onto synthetic complete media lacking uracil, histidine, tryptophan, and leucine (UHWL⁻ medium) containing either 2% glucose or 0.05% galactose/2% raffinose. Following growth for 48 h, yeast were analyzed for β -galactosidase reporter activity using the overlay assay technique (see “Experimental Procedures”). Yeast expressing JNK and TI-JIP grew in the absence of leucine and became colored, whereas yeast expressing JNK and Sab K1 were unable to grow in the absence of leucine or become colored. Panel ii, diploid yeast transformed with pJG4–5-JNK and either pGILDA-TI-JIP, pGILDA-Sab KIM 1, or equivalent constructs encoding TI-JIP/Sab KIM 1 hybrid peptides were grown in UHW⁻ medium containing either 2% glucose (control) or 0.05% galactose/2% raffinose for 48 h. Equal volumes of yeast were pelleted, and then analyzed for reporter activity using liquid β -galactosidase assays (see “Experimental Procedures”). The relative reporter activities present in the different diploids are shown in Miller units. Error bars represent the S.E. of the means. The asterisks indicate that the β -galactosidase activity in the samples with Sab KIM 1 or TI-JIP/Sab KIM 1 hybrids as the bait peptides were significantly different ($p \leq 0.01$) from the sample with TI-JIP as the bait peptide.

also inhibits JNK with greater potency when compared with other KIM-based peptides. Although we have defined KIM-based peptides as relatively poor inhibitors of JNK activity, other groups have used similar peptides as JNK inhibitors. This discrepancy most likely arises from differences in the concentrations of inhibitors used. For example, a 16-mer pep-

tide based on the docking site in MKK4 inhibited JNK2-mediated phosphorylation of c-Jun and ATF-2 (29), but the lowest concentration of peptide tested was 10 μ M. We found that at low micromolar concentrations, an 11-mer peptide based on the KIM of MKK4 was a significantly weaker JNK inhibitor when compared with TI-JIP. In addition, MKK4 and JIP-1 KIM-

based peptides when used at 100 μM final concentration were reported as effective inhibitors of MKK2 phosphorylation of ERK2 (29), suggesting that selectivity for JNK *versus* ERK may be lost at these higher concentrations *in vitro*.

A similar disagreement arises with the use of peptides based on the KIM of the JNK substrate c-Jun. We previously reported that an 11-mer peptide based on the KIM of c-Jun was a significantly weaker JNK inhibitor than TI-JIP (20). However, a cell-permeable version in which a 29-mer peptide from the c-Jun KIM was synthesized together with the TAT protein transduction domain inhibited the induction of glutamate cysteine ligase in response to 4-hydroxynonenal treatment of HBE1 cells (45), and AP-1 activation through endogenous H₂O₂ generation by alveolar macrophages (46). In an *ex vivo* assay, this inhibitor reduced phosphorylation of c-Jun by activated JNK in a dose-dependent manner, where inhibition was observed with 5 μM (but not 1.25 μM) peptide. This is in contrast to TI-JIP, which significantly inhibited phosphorylation of c-Jun by activated JNK in an *ex vivo* assay at a concentration of 0.56 μM , and reduced c-Jun phosphorylation by up to 90% at a concentration of 1.7 μM (20). Therefore, although the c-Jun-based peptides inhibit the phosphorylation of c-Jun by JNK, these appear less potent than TI-JIP.

It must be considered that peptides based on the KIMs of JNK interactors such as MKK7, MKK4, and Sab may have been relatively poor JNK inhibitors because they adopted random conformations when used in an isolated form rather than in the context of their native proteins. However, a larger protein based on Sab residues 219–425 that included the KIM 1 sequence also did not function as a JNK inhibitor,² which supports the results obtained with the shorter Sab KIM 1 peptide (Fig. 6). Furthermore, although the isolated TI-JIP peptide functions as a potent JNK inhibitor, hybrid peptides based on the sequences of TI-JIP and the KIMs of MKK7, MKK4, and Sab were relatively poor inhibitors of JNK, even when these predominantly resembled the TI-JIP sequence (Figs. 5 and 6B). Therefore, although a discrete set of TI-JIP residues are independently critical for potent JNK inhibition, other non-critical residues contribute to the JNK inhibition and simultaneous substitutions of multiple residues are poorly tolerated, even when separately localized to either the N terminus, central region, or C terminus of the peptide. Furthermore, some conservative substitutions such as Thr-7 \rightarrow Ser were well tolerated (Fig. 6B), whereas an Arg-4 \rightarrow Lys substitution significantly decreased JNK inhibition,² as did a TI-JIP variant peptide with rearrangement of Lys-3 and Arg-4.² Finally, the JNK inhibition afforded by TI-JIP was not significantly reduced following an Asn-9 \rightarrow Gly substitution,² but was reduced by either a Thr-6 \rightarrow Gly (Fig. 6B) or a Thr-7 \rightarrow Gly² substitution. Taken together, it appears that the KIM sequence in the JIP-1 scaffold protein has the unique ability to inhibit JNK activity with high potency. The exact biological significance of the JNK inhibition afforded by this scaffold protein remains to be clarified.

The region of JIP-1 involved in binding JNK was identified in 1997 (47). It was possible that the region of JNK binding JIP-1 corresponded to a similar domain on p38 α MAPK, which bound peptides based on the KIMs of both the nuclear substrate, MEF2A, and the activator, MKK3b (30). Structural analysis has indicated that this docking groove was similar between the p38 α , ERK2 and JNK3 MAPKs and it was suggested that proteins with KIMs would bind to this common region (30). However, the crystal structure of JNK1 in complex with the TI-JIP inhibitor peptide was very recently reported (48). Inter-

estingly, all residues identified as independently critical for the potent inhibition of JNK by TI-JIP in our previous study (20) were implicated in the selective binding of TI-JIP to JNK1. Specifically, the van der Waals contacts by the three residues (Pro-5, Leu-8, and Leu-10) of TI-JIP and the hydrogen bonding between Glu-329 of JNK1 and Arg-4 or TI-JIP were critical for the selective binding (48). In addition, binding of the peptide induced a hinge motion between the N-terminal and C-terminal domains of JNK1 and distorted the ATP-binding cleft, reducing the affinity of JNK1 for ATP (48). Therefore, although we demonstrated that TI-JIP was directly competitive with phosphoacceptor substrate for binding to JNK, its potency for inhibiting JNK also results from other conformational changes that occur in JNK1 upon binding to TI-JIP.

In conclusion, it appears that TI-JIP, representing the KIM from the JIP-1 scaffold protein, functions as a unique and potent inhibitor of JNK activity. It competes with protein substrate for binding to JNK, in contrast with SP600125, which competes with ATP and is currently the only other commercially available direct inhibitor of JNK activity. JIP-1-based peptides represent a new class of direct JNK inhibitors that have relatively high specificity for inhibiting JNK (22) and are based on the docking domain involved in the JNK-JIP-1 interaction. It is likely that other peptide inhibitors based on docking domains will become increasingly popular as the interaction interfaces between protein complexes are characterized.

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