deviation from topological equilibrium for relaxation predicted by the model agrees with our experimental results and provides an explanation for the puzzling observation that type II topoisomerases are much better at decatenation and unknotting than relaxation (10, 13, 14).

The ability of type II topoisomerases to directionally simplify DNA topology is in accord with their physiological role in DNA replication and chromosome segregation (25). Every turn of the double helix that is replicated introduces a positive supercoil or catenane link into topologically constrained DNA, which must be faithfully and rapidly removed by topoisomerases. However, given the high intracellular DNA concentration (26) and the presence of DNA condensing agents, the topoisomerases might instead be expected to ensnarl chromosomes (27). Our discovery that type II topoisomerases untangle DNA molecules against the thermal drive may help solve this problem.

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# A Cytoplasmic Inhibitor of the JNK Signal Transduction Pathway

Martin Dickens, Jeffrey S. Rogers, Julie Cavanagh, Art Raitano, Zhengui Xia, Jocelyn R. Halpern, Michael E. Greenberg, Charles L. Sawyers, Roger J. Davis\*

The c-Jun amino-terminal kinase (JNK) is a member of the stress-activated group of mitogen-activated protein (MAP) kinases that are implicated in the control of cell growth. A murine cytoplasmic protein that binds specifically to JNK [the JNK interacting protein-1 (JIP-1)] was characterized and cloned. JIP-1 caused cytoplasmic retention of JNK and inhibition of JNK-regulated gene expression. In addition, JIP-1 suppressed the effects of the JNK signaling pathway on cellular proliferation, including transformation by the *Bcr-Abl* oncogenę. This analysis identifies JIP-1 as a specific inhibitor of the JNK signal transduction pathway and establishes protein targeting as a mechanism that regulates signaling by stress-activated MAP kinases.

The JNK signal transduction pathway is activated in response to environmental stress and by the engagement of several classes of cell surface receptors, including cytokine receptors, serpentine receptors, and receptor tyrosine kinases (1). Genetic studies of *Drosophila* have demonstrated that JNK is required for early embryonic development (2). In mammalian cells, JNK has been implicated in the immune response (3), oncogenic transformation (4, 5), and apoptosis (6, 7). These effects of JNK are mediated, at least in part, by increased gene expression. Targets of the JNK

M. Dickens, J. S. Rogers, J. Cavanagh, R. J. Davis, Howard Hughes Medical Institute and Program in Molecular Medicine, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, 373 Plantation Street, Worcester, MA 01605, USA. A. Raitano, J. R. Halpern, C. L. Sawyers, Department of Medicine, University of California School of Medicine, Los Angeles, CA 90095, USA. Z. Xia and M. E. Greenberg, Division of Neuroscience, Department of Neuroscience, Angeles of Department of Depar

\*To whom correspondence should be addressed

signal transduction pathway include the transcription factors c-Jun, activating transcription factor–2 (ATF2), and Elk-1 (1).

Although JNK is located in both the cytoplasm and the nucleus of quiescent cells, activation of JNK is associated with accumulation of JNK in the nucleus (8). Interaction with anchor proteins is one mechanism that may account for the retention of JNK in specific regions of the cell. Anchor proteins participate in the regulation of multiple signal transduction pathways, including the nuclear factor kappa B inhibitor IkB, the A-kinase anchor protein (AKAP) group of proteins that bind type II cyclic adenosine 3',5'monophosphate-dependent protein kinase, and the p190 protein that binds Ca<sup>2+</sup>-calmodulin-dependent protein kinase II (9). These anchor proteins localize their tethered partner to specific subcellular compartments or serve to target enzymes to specific substrates (10). Anchor proteins may also create multienzyme signaling complexes, such as the Ste5p MAP

Department of Neurology, Children's Hospital, and Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA.

kinase scaffold complex and the AKAP79 kinase-phosphatase scaffold complex (11).

We used the yeast two-hybrid method to identify proteins that interact with JNK (12). A group of seven independent clones corresponding to overlapping fragments of one cDNA were identified. This protein, designated as the JNK interacting protein–1 (JIP-1), contains an  $NH_2$ -terminal JNKbinding domain (JBD) and a putative SRC homology 3 (SH3) domain in the COOHterminus. The putative SH3 domain is most closely related to domains in the tyrosine kinase c-Fyn and the p85 subunit of phosphoinositide-3 kinase. JIP-1 is widely expressed in murine tissues (Fig. 1A).

We examined the interaction of JIP-1 and JNK1 by coimmunoprecipitation analysis of lysates from COS-1 cells that were transfected with vectors encoding JIP-1 and JNK (Fig. 1B). JIP-1 was detected in JNK1 immunoprecipitates by protein immunoblot analysis. Conversely, JNK1 was detected in JIP-1 immunoprecipitates. Coimmunoprecipitation of transfected JIP-1 with endog-

Fig. 1. Binding of JIP-1 to JNK in vivo and in vitro. (A) The tissue distribution of JIP-1 mRNA. Northern (RNA) blot analysis of polyadenylmRNA isolated ated from different murine tissues. The integrity of the mRNA was examined by hybridization to an actin probe. The mobility of RNA molecular size standards is indicated on the right in kilobases. (B) JNK-JIP-1 complex formation in vivo. COS-1 cells were mock-transfected [control (Con.)] or transfected with Flag-JIP-1 and HA-JNK1. The cells were irradiated without (-) or with (+) UV-C (40 J/m<sup>2</sup>) and incubated for 1 hour. Lysates prepared from the cells were examined by protein immunoblot (IB) analysis with a mixture of antibodies to Flag and HA (bottom panel). Flag immunoprecipitates (IP) were probed with a polyclonal antibody to JNK1

enous JNK1 and JNK2 was also observed (13). Exposure of the cells to ultraviolet (UV) radiation caused no change in the amount of the JNK–JIP-1 complex that was detected. The related MAP kinases extracellular signal-related kinase–2 (ERK2) and p38 did not coimmunoprecipitate with JIP-1 (13).

To test whether JIP-1 interacts directly with JNK, we performed in vitro binding assays. The putative JBD (residues 127 to 281) was expressed as a glutathione-Stransferase (GST) fusion protein. Recombinant JNK prepared by in vitro translation (14) was incubated with GST-JIP-1 that was immobilized on glutathione-agarose. A similar amount of binding to JIP-1 was detected in assays with 10 different human JNK isoforms (14).

JNK binds to the transcription factors c-Jun and ATF2 in an isoform-dependent manner (14, 15). Thus, a larger amount of JNK2 than JNK1 bound to c-Jun and ATF2 (Fig. 1C). In contrast, the amount of JIP-1 that bound to JNK1 and JNK2 was similar (Fig. 1C). Furthermore, the amount of JNK that bound to JIP-1 was greater than the amount of JNK that bound to ATF2 or c-Jun (Fig. 1C). Titration experiments demonstrated that the affinity of JNK2 for JIP-1 was approximately 100-fold greater than the affinity of JNK2 for c-Jun (13). Control experiments demonstrated that JIP-1 did not bind to p38 MAP kinase (13). Together, these data demonstrate that JNK binds JIP-1 with greater affinity than the transcription factors ATF2 and c-Jun.

The JBD of JIP-1 (residues 127 to 281) bound to JNK1 and JNK2 (Fig. 1D). No JNK binding with the central region of the JBD (residues 164 to 240) or with the COOH-terminal region (residues 203 to 281) was detected (Fig. 1D). However, JNK binding activity was observed in experiments that used the NH<sub>2</sub>-terminal region (residues 127 to 202). The effect of progressive deletions within the JBD indicated that JIP-1 residues 144 to 163 are important for the interaction of JIP-1 with JNK (Fig. 1D). We prepared a series of synthetic peptides corresponding to this region. Competition studies demonstrated that a peptide representing the wild-type JIP-1 sequence caused a dose-dependent inhibition of JNK bind-



(middle panel), and HA immunoprecipitates were probed with an antibody to Flag (top panel). (C) Deletion analysis of JIP-1. The GST fusion proteins (Coomassie-stained gel) and the results of the binding assay (immunoblot) are shown. The binding of JNK1 and JNK2 to GST and GST fusion proteins corresponding to residues 127 to 202, 203 to 281, 164 to 240, and 127 to 281 of JIP-1; residues 1 to 79 of c-Jun; and residues 1 to 109 of ATF2 is also shown. The mobility of molecular size standards is indicated on the left in kilodaltons. (D) Binding of JNK1 and JNK2 to GST and GST fusion proteins corresponding to JIP-1 with small deletions between residues 127 and 202. The mobility of molecular size standards is indicated on the left in kilodaltons.



Fig. 2. Small NH<sub>2</sub>-terminal region of JIP-1 is sufficient for interaction with JNK. (A) Cell lysates containing Flag-epitope-tagged JNK1 were incubated with GST and GST-JIP-1 (residues 127 to 281) bound to glutathione-Sepharose, and bound proteins were detected by protein immunoblot analysis with an antibody to Flag. The effect of increasing concentrations (0, 4, 8, 16, 32, and 64 µg/ml) of synthetic peptide corresponding to JIP-1 residues 148 to 174 or to a peptide with a scrambled sequence (control) was examined. (B) Binding of JNK1 to GST and GST-JIP-1 (residues 127 to 281) was examined in the absence and presence of synthetic peptides (64 µg/ml). The effect of the wild-type peptide (JIP-1 residues 148 to 174), peptides with point mutations (substitution with Gly) [Thr<sup>159</sup>  $\rightarrow$  Gly<sup>159</sup> (T159G)] (18), and a peptide with a scrambled primary sequence (control) was examined. The mutant JIP(159-162G) was constructed by replacement of JIP-1 residues 159 to 162 with Gly.

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ing to JIP-1 (Fig. 2A). In contrast, a peptide with a scrambled sequence caused no change in JNK binding. This region of JIP-1 contains three amino acids (Lys<sup>155</sup>, Thr<sup>159</sup>, and Leu<sup>160</sup>) that are conserved in the JBDs of ATF2 and c-Jun. A hydrophobic amino acid (Leu) at residue 162 is also found in c-Jun (Leu) and ATF2 (Phe). Individual replacement of each of these residues with Gly caused a reduction in the peptide competition of JNK binding to JIP-1 (Fig. 2B). Together, these data are consistent with a role for Lys<sup>155</sup>, Thr<sup>159</sup>, Leu<sup>160</sup>, and Leu<sup>162</sup> in the function of the JBD of JIP-1.

The similarity between the JBDs of JIP-1, c-Jun, and ATF2 suggests that JIP-1 may compete with these transcription factors for interaction with JNK. We therefore examined the effect of JIP-1 on JNK activity. Phosphorylation of JIP-1 by JNK was detected. JIP-1 also inhibited phosphorylation of c-Jun by JNK (Fig. 3A). In contrast, JIP-1

A

activity

kinase

С

activity

luciferase

Relative

1.00

0.75

0.50

Belative 0

1.2

0.9

0.6

0.3

0.0

Fig. 3. Inhibition of JNK-reaulated signal transduction in cells expressing JIP-1. (A) Selective inhibition of JNK activity by JIP-1. CHO cells were serum-starved for 1 hour and treated with or without mouse interleukin-1 (IL-1) (10 ng/ml) (JNK and p38) or 100 nM phorbol 12myristate 13-acetate (ERK). Protein kinase activity was measured in immune complex kinase assavs by means of 3 µg of substrate. ERK, JNK, and p38 MAP kinase were assayed with c-Jun, ATF2, and myelin basic protein as substrates, respectively. The effect of the addition of 3 µg of GST or GST-JIP-1 (residues 127 to 202) was examined. (B) Effect of JBD (JIP-1 residues 127 to 281) on reporter gene

expression mediated by the GAL4 DNA binding domain (GAL4) and GAL4 fusions with the c-Myc, E1a, Sp1, and VP16 activation domains (19). (C) Effect of JBD on reporter gene expression mediated by GAL4-c-Jun [wild type (WT)], GAL4-c-Jun (S63A, S73A) [mutation (Mut)], GAL4-ATF2 (WT), GAL4-ATF2 (T69A, T71A) (Mut), GAL4-Elk-1 (WT), and GAL4-Elk-1 (S383A) (Mut) (18). (D) Effect of JIP-1 (WT), a deletion mutant lacking the SH3 domain ( $\Delta$ SH3) (residues 491 to 540), and the JBD on reporter gene expression mediated by GAL4-ATF2 and GAL4-ATF2 (T69A, T71A) (18). (E) Indirect immunofluorescence analysis of the subcellular distribution of transfected JNK1 and JIP-1. The cells were exposed to UV-C (40 J/m<sup>2</sup>) for 1 hour before fixation. Flag-JIP-1 was detected with the M2 antibody and a Texas Redconjugated secondary antibody (red). HA-JNK1 was detected with a polyclonal antibody to HA

caused no change in the phosphorylation of substrates by the related MAP kinases p38 and ERK2 (Fig. 3A). We also examined the effect of the JBD (residues 127 to 281) on the activation of transcription by the JNK signal transduction pathway in cells that were stimulated by fetal bovine serum. The JBD did not inhibit the reporter gene expression mediated by the activation domains of the transcription factors c-Myc and Sp1 or the viral proteins VP16 and E1a (Fig. 3B), but it did inhibit the transcriptional activity of c-Jun and ATF2 (Fig. 3C). The partial inhibition of Elk-1 transcriptional activity (Fig. 3C) probably reflects the role of ERK, JNK, and p38 MAP kinases in Elk-1 regulation (8, 16). Inhibition JNK-regulated gene expression was of caused by JIP-1, JBD, and a form of JIP-1 in which the SH3 domain was deleted (Fig. 3D). These data indicate that JIP-1 suppresses signaling by JNK.

JIP-1 was detected in the cytoplasm of control (13) and UV-irradiated cells (Fig. 3E). In contrast, JNK was detected in both the cytoplasmic and the nuclear compartments of cells (Fig. 3E). To investigate how cytoplasmic JIP-1 can inhibit the nuclear function of JNK, we examined the distribution of JIP-1 and JNK in transfected cells. Expression of JIP-1 reduced the amount of nuclear JNK in UV-irradiated cells (Fig. 3E). In contrast, JIP-1 caused no change in the subcellular distribution of p38 MAP kinase (13), which was also located in both nuclear and cytoplasmic compartments of cultured cells (17). Thus, overexpression of JIP-1 causes cytoplasmic retention of JNK (Fig. 3E).

The ability of JIP-1 to inhibit substrate phosphorylation and gene regulation by JNK (Fig. 3) suggests that JIP-1 may inhibit

Δ



and fluorescein-isothiocyanate-labeled secondary antibody (green). Three-dimensional images were collected by digital imaging microscopy (17). Single optical sections of the images are presented.



Fig. 4. JIP-1 inhibits pre-B cell transformation by Bcr-Abl. (A) JNK activation by v-Abl and Bcr-Abl is suppressed by JIP-1. JNK activity was measured in an immune-complex kinase assay of 293 cell lysates by means of a polyclonal JNK antibody and the substrate GST-Jun. The effect of transfection of the cells with plasmid vectors that express v-Abl, Bcr-Abl, and the JBD of JIP-1 (residues 127 to 281) is presented. (B) Primary mouse marrow cells were infected with the bi-cistronic retroviruses illustrated (20). The mean density (×10<sup>4</sup>) (±SE) of nonadherent pre-B cells on day 10 is presented. The data shown are derived from three independent experiments plated in triplicate. The empty box in the first line indicates that the vector does not contain an insert in this position. (C) Photomicrographs of representative plates from (B) are shown.

the biological effects of the JNK signaling pathway. We investigated the effect of JIP-1 on the promotion of apoptosis by JNK after the withdrawal of nerve growth factor (NGF) from PC12 cells (6). Expression of the JBD of JIP-1 (residues 127 to 281) reduced the NGF withdrawal-induced apoptosis by more than 90% (13). These data demonstrate that JIP-1 suppresses the biological actions of the JNK signal transduction pathway.

We also examined the effects of JIP-1 on oncogenic transformation. The Bcr-Abl leukemia oncogene causes activation of JNK, but not ERK (5). Thus, transformation by Bcr-Abl may be mediated, in part, by the JNK signaling pathway. Expression of Bcr-Abl or the viral oncogene v-Abl caused constitutive activation of JNK (approximately fivefold), which was blocked by coexpression of JBD (Fig. 4A). We prepared bi-cistronic retroviruses expressing Bcr-Abl alone or together with JBD in the sense and antisense orientations (Fig. 4B). Immunoblot analysis of Bcr-Abl and JBD confirmed that the retroviral constructs expressed the appropriate proteins. The recombinant retroviruses were used to infect primary marrow cells, and the transformation of precursor B cells (pre-B cells) was monitored in culture. Bcr-Abl caused transformation of pre-B cells (Fig. 4C). JBD inhibited transformation when it was expressed in the sense but not in the anti-sense orientation. These data implicate the JNK pathway in pre-B cell transformation by Bcr-Abl. Because Bcr-Abl has a role in human disease, both JNK and JIP-1 are candidate targets for the design of therapeutic strategies for the treatment of chronic myeloid leukemia.

The results of this study demonstrate that one role of JIP-1 may be to suppress signal transduction by the JNK pathway. For example, JIP-1 may compete with substrates that bind JNK. Alternatively, JIP-1 may have a more direct role in targeting JNK to specific regions of the cell or to specific substrates. Indeed, overexpression of JIP-1 caused retention of JNK in the cytoplasm. Therefore, in addition to other possible physiological functions, JIP-1 may act as a cytoplasmic anchor for JNK.

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- Single-letter abbreviations for the amino acid residues are as follows: A, Ala; G, Gly; K, Lys; L, Leu; S, Ser; and T, Thr.
- 19. Luciferase reporter assays in CHO cells were performed 48 hours after transfection with the use of  $\beta$ -galactosidase ( $\beta$ -Gal) to measure transfection efficiency (14). The cells were activated by treatment with fetal calf serum (10%). The data are presented as the relative luciferase activity [mean  $\pm$  SEM of the activity ratio of luciferase/ $\beta$ -Gal (n = 3)].
- 20. We performed bone marrow transformation assays using recombinant retroviruses [C. L. Sawyers, J. McLaughlin, O. N. Witte, *J. Exp. Med.* **181**, 307 (1995)] that were packaged with 293T cells. We constructed the bi-cistronic retroviruses expressing the JBD and *Bcr-Abl* by subcloning p185*Bcr-Abl* in the Cla I site and the JBD in the Eco RI site of pSRαTK.
- 21. We thank J. Cooper and S. Hollenberg for two-hybrid reagents; D. Schmidt and F. Fay for assistance with immunofluorescence microscopy; M. Wysk, R. Tizard, and J. Demarinis for Maxam-Gilbert sequencing; I.-H. Wu for technical assistance; T. Barrett for automated DNA sequencing; and K. Gemme for secretarial assistance. Supported by NIH grants CA43855 (M.E.G.) and CA65861 (R.J.D.) and by a grant from the James S. McDonnell Foundation (C.L.S.). R.J.D. is an investigator of the Howard Hughes Medical Institute. C.L.S. is a scholar of the Leukemia Society of America.

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## Analysis of a Chemical Plant Defense Mechanism in Grasses

Monika Frey, Paul Chomet, Erich Glawischnig, Cornelia Stettner, Sebastian Grün, Albert Winklmair, Wolfgang Eisenreich, Adelbert Bacher, Robert B. Meeley, Steven P. Briggs, Kevin Simcox, Alfons Gierl\*

In the Gramineae, the cyclic hydroxamic acids 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) form part of the defense against insects and microbial pathogens. Five genes, *Bx1* through *Bx5*, are required for DIBOA biosynthesis in maize. The functions of these five genes, clustered on chromosome 4, were demonstrated in vitro. *Bx1* encodes a tryptophan synthase  $\alpha$  homolog that catalyzes the formation of indole for the production of secondary metabolites rather than tryptophan, thereby defining the branch point from primary to secondary metabolism. *Bx2* through *Bx5* encode cytochrome P450–dependent monooxygenases that catalyze four consecutive hydroxylations and one ring expansion to form the highly oxidized DIBOA.

A substantial number of secondary metabolites in plants are dedicated to pathogen defense. These include the cyclic hydroxamic acids, which are found almost exclusively in Gramineae. For example, DIM-BOA and its precursor DIBOA are present in maize. DIMBOA confers resistance to first-brood European corn borer (Ostrinia nubilalis), northern corn leaf blight (Helminthosporium turcicum), maize plant louse (Rhophalosiphum maydis), and stalk rot (Diplodia maydis), as well as to the herbicide atrazine (1). DIBOA is the main hydroxamic acid in rye, whereas DIMBOA is the predominant form in wheat and maize (1). The DIMBOA and tryptophan biosynthetic