Glutathione S-transferase P1-1 (GSTP1-1) Inhibits c-Jun N-terminal Kinase (JNK1) Signaling through Interaction with the C Terminus*

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c-Jun N-terminal kinase (JNK),1 or stress activated kinase, is a member of the mitogen activated stress kinase family (MAPK), which also includes extracellular signal regulated kinase and p38-MAPK. JNK activation was initially identified as a cellular response to environmental stresses, proinflammatory cytokines, and interleukins (2). In addition, the JNK pathway participates in activating transcription factor 2 (3), ELK-1 as a cellular response to environmental stresses, proinflammatory protein-protein interactions and to measure binding affinities. Experimental Procedures—Recombinant JNK1 protein was expressed in Escherichia coli. The polymerase chain reaction product was constructed with a leader His-tag, digested with Ndel and BamHI, purified, and ligated into Ndel-digested, BamHI-digested, and phosphatase-treated pET-15b to create JNK-pET-15b. The JNK1 protein was expressed in E. coli and purified using a standard Ni2+ column for His-tagged proteins. The purified protein was homogeneous as judged by the single polypeptide band of the predicted Mx on silver-stained SDS-polyacrylamide gels (data not shown).

Both the C-terminal and N-terminal truncated proteins expressed in E. coli were found mainly in the insoluble fraction of the bacterial extracts. Thus, inclusion bodies were solubilized in 50 mM CAPS (3-(cyclohexylamino)-1-propanesulfonic acid), pH 11.0, with 0.3% N-lauremaldehyde and 4 M guanidine·HCl. The solubilized inclusion bodies were denatured, histidine affinity purified, and digested with thrombin to generate His6-c-Jun-N-term. As determined by gel filtration, the His6-c-Jun-N-term fragment bound to the GSTP1–1 recombinant bacterial fusion protein. The His6-c-Jun-N-term fragment was negatively charged and could be loaded onto the His affinity column. On-column digestion with thrombin resulted in the cleavage of the His6-c-Jun-N-term fragment. The gel filtration studies established the presence of the His6-c-Jun-N-term fragment. The His6-c-Jun-N-term fragment eluted at the void volume, indicating that the His6-c-Jun-N-term fragment is similar to the His6-c-Jun-N-term fragment. The His6-c-Jun-N-term fragment was positively charged and could be loaded onto the His affinity column. On-column digestion with thrombin resulted in the cleavage of the His6-c-Jun-N-term fragment. The gel filtration studies established the presence of the His6-c-Jun-N-term fragment. The His6-c-Jun-N-term fragment eluted at the void volume, indicating that the His6-c-Jun-N-term fragment is similar to the His6-c-Jun-N-term fragment.

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1 The abbreviations used are: JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; GST, glutathione S-transferases; ASK, apoptosis-signaling kinase; WCE, whole cell extract; FRET, fluorescence resonance energy transfer; HA, hemagglutinin; ECL, enhanced chemiluminescence.
roylsarcosine supplemented with 1 mM dithiothreitol and slowly diluted to 20 mM Tris-HCl, pH 8.5, before subjection to Ni2+- column chromatography. Renaturation was accomplished by dialyzing for 12 h at 4 °C with a change of buffer with dithiothreitol and continued dialysis for an additional 6 h. To promote refolding, a further period of dialysis in the above buffer (20 mM Tris, pH 8.5) without dithiothreitol for the same length of time was enacted.

The GSTP1–1 construct was made by direct digestion of GSTP1–1 from Pichia pastoris with HindIII and XhoI followed by cloning into pET21 vector previously treated with HindIII and XhoI. This was expressed in E. coli, and protein was purified using GSH affinity chromatography. Circular dichroic spectra of the C-terminal and N-terminal JNK1 in 20 mM PO4,3- were obtained using an Aviv 62A DS spectrometer at the Fox Chase Cancer Center core facility. The corresponding structural elements were calculated using an algorithm for the estimation of the percentages of protein secondary structure from UV circular dichroism spectra using a Kohonen neural network with a two-dimensional output layer using K2D software (14, 15). The program uses a self-organizing neural network to extract the secondary structure features present in the data from a set of circular dichroism spectra ranging from 200–241 nm. The result provides an estimate of percentage helix, b-sheet, and random structure for the protein. K2D also estimates the probable error in the estimation based on the training procedure results.

Protein Labeling and Reconstitution—GSTP1–1 was labeled at a 1:1 molar ratio (probe:protein) with the fluorescence energy transfer donor 7-methoxycoumarin succinyl ester using previously described procedures (16). The JNK1 partner proteins (full-length and partial) were similarly labeled with the non-fluorescence energy transfer acceptor, 4(dimethylamino) phenylazophenol-4-sulfonil chloride succinyl ester (Molecular Probes Inc., Eugene, OR). The protein:probe labeling ratios were determined by absorption spectroscopy (17) using the calculated extinction coefficients for the protein and the coefficients provided by the probe manufacturer.

Cells and Protein Preparation—NIH3T3 mouse fibroblast cells that stably express the pSV40-Hyg plasmid (CLONTECH, Palo Alto, CA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (Life Technologies Inc.). Cells were grown at 37 °C with 5% CO2. The pTet-C-JNK was assembled by subcloning the cDNA of the C-terminal 224 amino acids of wild type JNK1 into the tet-regulated promoter of the pUHD-10- vector. Cell clones that stably express both constructs were selected in 600 µg/ml genetin in the presence of hygromycin (100 µg/ml) 24 h after N,N,N-trimethyl-1–23-bis (1-oxo-9-octadecenyl)oxy- (Z,Z)-1-propamino-aminomethyl methyl sulfonyl fluoride transfection with the pTet-C-JNK-UHD-10- construct. Cells expressing C-terminal JNK were maintained in Dulbecco's modified Eagle's medium containing fetal bovine serum (10%), hygromycin (100 µg/ml), and genetin (400 µg/ml). To maintain suppression of C-terminal JNK expression, tetracycline was added to the medium every 3 days (to a final concentration of 1 µg/ml). Analysis of JNK1 activity was achieved by measurement of phosphorylation of c-Jun as a substrate (1). Hydrolysis of ATP is thought to be associated with c-Jun-dependent transcriptional activity. Antisense oligomers can inhibit c-Jun transcriptional activity. Circular dichroism of these purified proteins was used to monitor the refolding process. Fig. 1 shows the circular dichroic spectra of the C-terminal and N-terminal JNK1 solubilized in 20 mM phosphate buffer (pH 7.2). Data were collected at room temperature. Spectra were fit to secondary structural elements that are listed under “Experimental Procedures” and analyzed to yield the secondary structures listed in Table I.

Antibodies, Immunoprecipitation, and Immunoblotting—Antibodies to C-JNK and phospho-c-jun were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and New England BioLabs (Beverly, MA). Immunoblot analysis was performed using 200 µg of whole cell extract (WCE) separated on SDS-polyacrylamide gel electrophoresis followed by electrotransfer to a polyvinylidene difluoride membrane in 20 mM CHAPS (3-(3-cholamidopropyl) dimethylammonio-1-propane-sulfonate) buffer, pH 11, for 30 min membrane blocking (5% non-fat milk) and incubating with the respective antibodies. Reactions were visualized using chemiluminescence (ECL) reagents. Immunoblot analysis was carried out following the protocol from the manual provided by Roche Diagnostics Corporation using 1 µg of WCE and 2 µg of the respective antibodies for 16 h at 4 °C. Protein G beads (Roche Molecular Biochemicals) were added (15 µl) for 4 h at 4 °C. Immunoprecipitated material was subjected to immunoblot analysis.

Protein Kinase Assays—Protein kinase assays were carried out using JNK kinase assay kits from New England BioLabs according to the manufacturer’s instructions. The kinase reaction was carried out in the presence of excess unlabeled ATP. c-Jun phosphorylation was selectively measured using a phospho-c-Jun antibody. This antibody specifically recognizes JNK-induced phosphorylation of c-Jun at Ser-63, an important site for c-Jun-dependent transcriptional activity.

Fluorescence Resonance Energy Transfer Experiments—Associations between GSTP1–1 and JNK were measured by fluorescence resonance energy transfer (FRET) as described previously (16). Protein concentrations were measured using UV absorption (A280) and colorimetric assays. Extinction coefficients for each protein were assessed as described previously (18). FRET was performed on a PC1 (photo counting) spectrophuorimeter (ISS, Inc., Champagne, IL) using a 3-mm cuvette with a magnetic stirrer. Coumarin-labeled proteins were excited at 350 nm and scanned from 380–480 nm. To determine the binding affinities for protein-protein interactions, the energy transfer data were analyzed using Sigma Plot.

Data Analysis—All immunoblot and SDS-polyacrylamide gel electrophoresis gels were scanned using a UMAX Powerbook III flatbed with Adobe Photoshop software. Scanned images were quantified by NIH image program. Prism software was used for data analysis.

RESULTS

Purification of JNK Proteins—Full-length JNK1 was soluble and purified directly by Ni2+ chromatography. Both truncated forms were insoluble in aqueous buffer and were initially solubilized in detergent followed by slow dialysis against renaturing buffers. The renatured proteins were then similarly purified by Ni2+ chromatography. The purity of these proteins was confirmed by single band staining on SDS-polyacrylamide gel electrophoresis (data not shown).

Fig. 1. Circular dichroic spectra of (A) C-terminal JNK1 and (B) N-terminal JNK1 solubilized in 20 mM phosphate buffer (pH 7.2). Data were collected at room temperature. Spectra were fit to secondary structural elements that are listed under “Experimental Procedures” and analyzed to yield the secondary structures listed in Table I.
GSTP1–1 was labeled with the succinyl ester of 7-methoxycoumarin. Full-length and truncated JNK1 was labeled with the non-fluorescence energy transfer acceptor, 4-(dimethylamino)phenylazophenyl-4-sulfonyl chloride succinyl ester. Saturation binding experiments were used to measure the specific binding at equilibrium at various concentrations of the fluorescent acceptor protein to determine acceptor affinity. Fig. 2 shows that for the full-length JNK1 a loss in 7-methoxycoumarin fluorescence was observed as the protein interacted with GSTP1–1. A similar result was found for the C-terminal fragment (Fig. 2B), while neither the N-terminal fragment nor buffer conditions produced any change in fluorescence. The calculated $K_d$ for GSTP1–1 association with full-length JNK1 was $188 \pm 38 \text{nM}$ and for the C-terminal fragment, $217 \pm 72 \text{nM}$. These data provide quantitative evidence for the direct protein–protein interaction between GSTP1–1 and JNK1.

**Immunoprecipitation Analysis of GSTP1–1–C-JNK Association**—These results were extended to a cellular system by using a tet-inducible construct of HA-tagged C-JNK. To measure the in vivo association of C-JNK and GSTP1–1, immunoprecipitation of WCE from cells expressing HA-tagged C-JNK was performed. Induction of C-JNK expression following removal of tetracycline resulted in an enhanced level of GSTP1–1 co-immunoprecipitation when an HA-tag antibody was used (Fig. 3).

The precise reason for the high levels of expression in tumor cells has not been explained adequately. Recently, we have carried out a series of studies to show that GSTP1–1 can act as a ligand-binding protein and an endogenous switch for the control of the catalytic activity of JNK1 (1, 10). This has led to other studies implicating that GSTP1–1 can act as a ligand-binding protein and an endogenous switch for the control of the catalytic activity of JNK1 (1, 10). This has led to other studies implicating that GSTP1–1 can act as a ligand-binding protein and an endogenous switch for the control of the catalytic activity of JNK1 (1, 10). This has led to other studies implicating that GSTP1–1 can act as a ligand-binding protein and an endogenous switch for the control of the catalytic activity of JNK1 (1, 10). This has led to other studies implicating that GSTP1–1 can act as a ligand-binding protein and an endogenous switch for the control of the catalytic activity of JNK1 (1, 10). This has led to other studies implicating that GSTP1–1 can act as a ligand-binding protein and an endogenous switch for the control of the catalytic activity of JNK1 (1, 10). This has led to other studies implicating that GSTP1–1 can act as a ligand-binding protein and an endogenous switch for the control of the catalytic activity of JNK1 (1, 10). This has led to other studies implicating that GSTP1–1 can act as a ligand-binding protein and an endogenous switch for the control of the catalytic activity of JNK1 (1, 10). This has led to other studies implicating that GSTP1–1 can act as a ligand-binding protein and an endogenous switch for the control of the catalytic activity of JNK1 (1, 10). This has led to other studies implicating that GSTP1–1 can act as a ligand-binding protein and an endogenous switch for the control of the catalytic activity of JNK1 (1, 10). This has led to other studies implicating that GSTP1–1 can act as a ligand-binding protein and an endogenous switch for the control of the catalytic activity of JNK1 (1, 10). This has led to other studies implicating that GSTP1–1 can act as a ligand-binding protein and an endogenous switch for the control of the catalytic activity of JNK1 (1, 10). This has led to other studies implicating that GSTP1–1 can act as a ligand-binding protein and an endogenous switch for the control of the catalytic activity of JNK1 (1, 10).
pathways in stress response and cell survival (21). It now seems reasonable to conclude that GSTP1–1 can elicit protection against apoptosis induced by reactive oxygen species by controlling the balance of kinase activity elicited by JNK1 versus other cellular kinases such as extracellular signal regulated kinase, IκB kinase, and p38 (1, 10). Our earlier experiments showing interactions between GSTP1–1 and JNK1 relied primarily on immunoprecipitation technology. In this report, we combine a number of technical approaches to provide evidence for direct protein-protein interaction between GSTP1–1 and JNK1. These data also establish quantitative binding kinetics for these two proteins with $K_D$ values in the nanomolar range. The specificity of the C terminus of JNK1 was confirmed by a number of controls that included the N-terminal and other unlabeled proteins, all of which failed to bind. The affinity of the truncated C-JNK fragment for GSTP1–1 was similar to the full-length protein.

JNK1 shares a high level of amino acid identity with JNK3 (Fig. 6). By extrapolation, we were able to establish that the folding pattern of the C-terminal truncated JNK1 was similar to the structure of JNK3 established by crystallography data (19). Both the in vitro and cellular data confirmed that the truncated C-terminal JNK1 contained amino acid residues that can be implicated in the binding to GSTP1–1. A recent study (22) has used computation of average structures to reveal that residues 194–201 of GSTP1–1 may be involved in the interaction with JNK. This sequence (SSPEHVNR) contains residues that are positively charged or contain polar groups in their side chains. Sequence analysis of the C terminus of JNK1 shows both a loop region and an $\alpha$-helix rich in negatively charged residues, particularly glutamic and aspartic acid (Fig. 6). These residues are either in the flexible loop structure (Asp-377, Asp-381, Glu-382, Glu-384) or the $\alpha$-helix (Glu-398, Glu-389, Glu-392, Asp-400, Glu-402, Glu-403) of the protein and can form a negatively charged binding face capable of interacting with GSTP1–1. This, in turn, is on the surface of JNK1 and therefore in a location conducive to protein-protein interaction with GSTP1–1. Fig. 6 shows the sequence alignments for the C-terminal components of JNK family members (19). The potential interaction site implicated in the molecular dynamic analysis (22) is distal to the GST subunit dimerization domain (involving Cys-47 and Cys-101) (23) suggesting that JNK may interact in vivo with homodimeric GSTP1–1. In addition, because the catalytic kinase domain is localized in the N-terminal of JNK1, our results suggest that the capacity of GSTP1–1 to suppress JNK enzyme activity will be through an allosteric inhibition mechanism.

This information may be facile in the design of potential small molecule inhibitors of the ligand-binding properties of these two proteins. Targeting this area on JNK1 may impact kinase expression and, by extrapolation, influence apoptosis (10). We have previously shown that a peptidomimetic inhibi-

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**Fig. 4.** A), induction of protein expression of C-terminal JNK in NIH3T3 cells by removal of tetracycline. HA-tagged C-JNK (residues 200–424) was used to establish NIH3T3 cells that are tet-regulatable. NIH3T3 cells transfected with ptet-C-JNK-UHD-10200–424 were harvested at different time points for protein isolation after tetracycline removal. WCE were analyzed on 12% polyacrylamide gels. Immunoblot analysis revealed the expression of a 30-kDa protein detected with polyclonal antibodies to C-JNK1. B, expression of full-length JNK1 under the same conditions. The full-length JNK protein was used to monitor equal loading for each lane. Immunoblots were scanned and quantified by NIH image software. The final results were plotted as histograms and are mean ± S.D. of three experiments.

**Fig. 5.** JNK kinase activity in the presence of truncated C-terminal JNK expression. Cells were harvested at the indicated time points (hours) after removal of tetracycline. C-Jun fusion protein beads were added to the WCE. Equal amounts of total cell lysate (~200 μg of protein) were electrophoresed on 12% polyacrylamide gels. Immunoblot detection was performed using the ECL system with rabbit monoclonal antibodies to phosphorylated c-Jun (top panel) for the detection of JNK kinase catalytic activity or c-Jun (bottom panel). Absorption was quantitated and plotted in arbitrary units in the presence (A) or absence (B) of tetracycline.

**Fig. 6.** Multiple sequence alignment of JNK proteins using the program MAC showing conserved sequence homology among JNK proteins. A cluster of acidic residues (marked in light gray) are possible sites interacting with the basic residues of GSTP1–1 identified by Monaco et al. (22).
tor of GSTP1–1 (24) causes a dissociation of the GSTP1–1-JNK1 complex resulting in increased JNK catalytic activity (1). This drug has significant effects on proliferative pathways in bone marrow cells. It is plausible that pharmacological manipulation with such agents could achieve control of the coordinated regulation of stress kinases with a significant impact in the therapy of cancer.

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