Glutathione S-transferase P1-1 (GSTP1-1) inhibits c-Jun NH$_2$ terminal kinase (JNK1) signaling through interaction with the carboxyl terminus

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Running Title: GSTP1-1 interacts with the carboxyl-terminal of JNK

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c-Jun NH2 terminal kinase (JNK) mediated cell signaling pathways are regulated endogenously in part by protein-protein interactions with glutathione S-transferase P1-1 (GSTP1-1) (1). Using purified recombinant proteins, combined with fluorescence resonance energy transfer (FRET) technology, we have found that the carboxyl terminus of JNK is critical to the interaction with GSTP1-1. The apparent Kd for full-length JNK was 188 nM and for a C-terminal fragment (residues 200-424) 217 nM. An N-terminal fragment (residues 1-206) did not bind to GSTP1-1. Increased expression of the c-terminal JNK fragment in a tetracycline-inducible transfected NIH3T3 cell line produced a concentration-dependent increase in the kinase activity of JNK under normal, unstressed growth conditions indicating a dominant:negative effect. This suggests that the fragment can compete with endogenous full-length functional JNK resulting in dissociation of the GSTP1-1:JNK interaction and concomitant JNK enzyme activation. By using an antibody to HA-tagged C-JNK, a concentration dependent co-immunoprecipitation of GSTP1-1 was achieved. These data provide evidence for direct interactions between the C-terminal of JNK and GSTP1-1 and a rationale for considering GSTP1-1 as a critical ligand binding protein with a role in regulating kinase pathways.
INTRODUCTION

C-Jun NH₂-Terminal kinase (JNK)¹, or stress activated kinase (SAPK) is a member of the mitogen activated stress kinase family (MAPK), which also includes extracellular signal regulated kinase (ERK) 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 (4), and the Sap-1α transcription factor (5), and JNK may influence p53 (6,7) and NF-κB pathways (8,9). C-Jun, a component of the transcription factor AP-1 complex is the most characterized downstream target of JNK, where regulation is achieved by phosphorylation at ser-63 or ser-73 (3). Extracellular signals such as growth factors, transforming oncoproteins and UV irradiation stimulate phosphorylation of c-Jun at ser-63/73 and activate c-Jun dependent transcription. The binding of JNK to the N-terminal region of c-Jun permits substrate phosphorylation. The JNK pathway has also been shown to be important in the control of cell survival and death pathways and interference with the JNK pathway suppresses the induction of apoptosis by a variety of agents (10).

The glutathione transferases (GST) are a multigene family of isozymes that catalyze the nucleophilic attack of the sulfur atom of glutathione on electrophilic groups of substrate molecules. While catalytic efficiencies for many of the substrates are low, their biological relevance as enzymes has frequently been linked to pleiotropic substrate specificity. Among this family of isozymes, GSTP1-1 is generally the most prevalent in mammalian cells. The link
between GST overexpression, especially with respect to GSTP1-1, and anticancer drug resistance has been extensively studied (11). Because of the defined role of GST in drug metabolism, elevated expression of GSTP1-1 in solid tumors or in drug-resistant cells has frequently been associated with detoxification reactions even in instances where there is no evidence that the selecting drug is a substrate for GSTP1-1. More recently, however, the link between the redox active components of GSTP1-1 and stress activated kinases such as JNK has been redefined as a non-catalytic, ligand-binding activity which mediates both stress response and apoptotic responses (1,10). In a parallel series of studies, either GSTµ or thioredoxin have also been identified as a ligand-binding partner for apoptosis signaling kinase (ASK1) (12,13), extending the role that “redox proteins” may have in kinase regulation. In order to elucidate further the mechanism by which GSTP1-1 mediates JNK signal transduction, we have used biophysical methods in reconstituted protein systems to provide direct evidence for protein:protein interactions and to measure binding affinities. Our results demonstrate that GSTP1-1 has significant affinity for the C-terminus of JNK and confirm the ligand-binding regulatory role for this ubiquitously expressed protein.
EXPERIMENTAL PROCEDURES

Protein Purification-- Recombinant JNK1 protein was expressed in *Escherichia coli* (*E. coli*). The polymerase chain reaction (PCR) product was constructed with a leader his-tag, digested with NdeI and BamHI, purified, and ligated into NdeI, 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 Mᵣ 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), pH11.0 with 0.3% N-lauroylsarcosine, supplemented with 1 mM DTT and slowly diluted to 20 mM Tris-HCl, pH 8.5 before subjecting to Ni²⁺ column chromatography. Renaturation was accomplished by dialyzing for 12 h at 4°C with a change of buffer with DTT 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 DTT for the same length of time was enacted.

The GSTP1-1 construct was made by direct digestion of GSTP1-1 from PCR2.1 (+) with Hind III and XhoI followed by cloning into pET21 vector previously treated with Hind III and
Xho1. 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 PO$_4^{3-}$ 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 2-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 nm to 241 nm. The result provides an estimate of percentage helix, β-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-fluorescent energy transfer acceptor, 4 (dimethylamino) phenylazophenyl-4-sulfonyl 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., Rockville, MD). Cells were grown at 37°C with 5% CO₂. The pTet-C-JNK was assembled by subcloning the cDNA of the carboxyl-terminal 224 amino acids of wild type JNK1 into the tet-regulated promoter of the pUHD-10-3 vector. Cell clones that stably express both constructs were selected in 600 µg/ml geneticin in the presence of hygromycin (100 µg/ml, 24 h after N,N, N-trimethyl 1-2-3-bis (1-oxo-9-octadecenyl)oxy-(Z,Z)-1-propanaminium methyl sulfate (DOTAP) transfection with the pTet-C-JNK-UHD-10-3 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 geneticin (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).

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-PAGE followed by electrotransfer to a PVDF membrane in 20 mM CHAPS (3-[(3-cholamidopropyl) dimethylammonio-1-propane-sulfonate) pH11 buffer for 30 min, membrane blocking (5% non-fat milk) and incubating with the respective antibodies. Reactions were visualized using chemiluminescence (ECL) reagents. Immunoprecipitations were carried out following the protocol from the manual provided by
Roche Diagnostics Corporation using 1 mg of whole cell extracts (WCE) and 2 µg of the respective antibodies, for 16 h at 4°C. Protein G beads (Roche Diagnostics Corporation, Indianapolis, IN) 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 manufacturer’s instructions (Beverly, MA). 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 measures 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 fluorescent 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 an ISIS spectrofluorimeter (Champagne, IL) using a 3 mm cuvette with a magnetic stirrer. Coumarin-labeled proteins were excited at 350 nm and scanned from 380 nm to 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-PAGE 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-PAGE (data not shown).

Circular dichroism of these purified proteins was used to monitor the refolding process. Figure 1 shows the proportional secondary structure of the truncated fragments derived from the circular dichroism spectra. These data were analyzed further using a Kohonen neural network with 2-dimensional output layer using K2D software (14,15). JNK1 shows a >95% identity to JNK3, a protein which has been crystallized (19). The C-terminal of JNK3 is comprised primarily of α-helices, while the N-terminal is predominantly β-sheet. Such structures are in agreement with the data shown in Table 1, confirming that accurate folding in solution has occurred.

FRET Analysis of GSTP1-1 Association With JNK-- In vitro association kinetics between the purified JNK proteins and GSTP1-1 was analyzed using fluorescence resonance energy transfer (FRET) and is shown in Fig. 2. GSTP1-1 was labeled with the succinyl ester of 7-methoxycoumarin. Full-length and truncated JNK1 was labeled with the non-fluorescent 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 in order to determine acceptor affinity. Figure 2A 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 Kd for GSTP1-1 association with full-length JNK1 was 188 ± 38 nM and for the C-terminal fragment, 217 ± 72 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).

These data are consistent with the conclusion that c-JNK and GSTP1-1 interact in a cellular milieu. Similarly, the intracellular expression of the C-terminal truncated form of JNK1 leads to an increase in JNK1 kinase activity, as a consequence of competing for the GSTP1-1 binding site and liberating catalytically active JNK1 (see Fig. 5).

**C-JNK Blocks the Interaction Between GSTP1-1 and Full-length JNK and Increases JNK Enzymatic Activity**-- For confirmation that the C-terminal portion of JNK1 is critical to the
JNK1:GSTP1-1 interaction, a tetracycline-inducible expression system was used to examine whether the increased expression of the C-terminal truncated JNK1 could have a dominant:negative effect upon GSTP1-1 inhibition of JNK1 activity. Figure 4 shows the time dependent increase in expression of the C-terminal truncated JNK1 fragment, following removal of tetracycline. Induction of the C-terminal fragment was detected by 1 h and persisted for at least 48 h. In these same cells, full-length JNK1 expression was constitutive and unaltered throughout this period (Fig. 4). JNK1 activity was measured by the phosphorylation of the downstream c-Jun substrate (Fig. 5). The time dependent increase in phosphorylated c-Jun was coincident with the enhanced expression of the truncated C-terminal fragment. If tetracycline was not removed from the media, there was no enhanced production of phosphorylated c-Jun product (Fig. 5). Overall, these results support the conclusion that the C-JNK fragment through competitive binding with GSTP1-1 can act in a dominant:negative fashion and increase the catalytic activity of JNK1.
DISCUSSION

Glutathione S-transferases are a multigene family of isozymes which have primarily been linked functionally with phase II metabolism or xenobiotics. In particular, GSTP1-1 is frequently overexpressed in solid tumors and in cells which have acquired resistance to anticancer drugs even when the selecting drug is not a GST substrate (11). The precise reason for the high levels of expression in tumor cells has not been adequately explained. 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 GSTP1-1 in control of apoptosis (20). Maintenance of cellular kinases which influence cell growth and apoptosis must be finely tuned. In particular, external stimuli such as reactive oxygen species may influence signaling kinase activities influencing 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 ERK, 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 Kd values in the nM 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
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 which 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 which 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 α-helix, rich in negatively charged residues, particularly glutamic and aspartic acid (Fig. 6). These residues are either in the flexible loop structure (D377, D381, E382, E384) or the α-helix (E388, E389, E392, E397, D400, E402, E403) 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. Figure 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 101) (23) suggesting that JNK may interact in vivo with homodimeric GSTP1-1. In addition, since 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 inhibitor 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 (Ruscoe et al, unpublished observations²). It is plausible that pharmacological manipulation with such agents control of the co-ordinated regulation of stress kinases could achieve significant in the management of cancer.
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REFERENCES


FOOTNOTES

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1 The abbreviations used are: JNK, C-jun NH2 terminal kinase; FRET, fluorescent energy transfer; PCR, polymerase chain reaction; MC, 7-methoxycoumarin; DAB, 4 (dimethylamino) phenylazophenyl-4-sulfonyl chloride; WCE, whole cell extract; ECL, chemiluminescence; CAPS, 3-(cyclohexylamino)-1-propane sulfonic acid; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate.

FIGURE LEGENDS

Fig. 1. Circular dichroic spectra of (A) C-terminal JNK1 and (B) N-terminal JNK1 solubilized in 20 mM phosphate buffer (pH7.2). Data were collected at room temperature. Spectra were fit to secondary structural elements that are listed in Experimental Procedures and analyzed to yield the secondary structures listed in Table 1.

Fig. 2. Binding GSTP1-1 to full-length or truncated JNK1. The FRET assay was carried out as described under Experimental Procedures at room temperature. GSTP1-1 labeled with 7-methoxy coumarin (20nM) titrated with either N or C-terminal JNK and full-length JNK labeled with dabsyl. The reaction buffer contained 20 mM Hepes, 150 mM Nacl. (A) The normalized decrease in the fluorescence intensity, obtained by dividing the zero-point fluorescence intensity with background subtraction of 2 nM methoxycoumarin labeled GSTP1-1 titrated with full-length JNK1 (l); N-terminal-JNK1 (s) or buffer only (n). (B) The association of GSTP1-1 with C-terminal JNK1 under the same experimental procedures as Fig. 4A; C-terminal JNK (l).

Fig. 3. Co-immunoprecipitation of GSTP1-1 with C-JNK. HA antibody was added to WCE. Protein G beads were added to the mixture to pull down HA-tagged C-JNK followed by immunoblotting with GSTP1-1 polyclonal antibodies. Endogenous GSTP1-1 expression levels stayed the same throughout the 48 h induction after removal of tetracycline. The histogram quantified (mean ± SD of three experiments) that the GST association with C-JNK increased as more C-JNK was expressed.
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-10-3 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 Kd 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 ± SD 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 (approximately 200 ug of protein) were electrophoresed on 12% polyacrylamide gels. Immunoblot detection was performed using the ECL system with rabbit monoclonal antibodies to phosphorylated c-Jun for the detection of JNK kinase catalytic activity. Absorption was quantified and plotted in arbitrary units in the presence (s) or absence (n) 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 red) are possible sites interacting with the basic residues of GSTP1-1 identified by Monaco et al. (22).
**TABLE 1.** K2D software analysis of circular dichroism results

<table>
<thead>
<tr>
<th></th>
<th>C-JNK</th>
<th>N-JNK</th>
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<td>Random Coils</td>
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</tr>
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</table>
Figure 1

A. 

B. 

Wavelength (nm)

Molar Ellipticity

Wavelength (nm)
Figure 3

GSTP1-1 association with C-JNK

HA-C-JNK

GSTP1-1

0 1 2 4 8 12 24 48 hours

HA-C-JNK immunoppt

Endogenous GSTP1-1
Figure 5

Graph showing JNK1 kinase activity over time with error bars. The graph compares -Tet (■) and +Tet (▲) conditions.

- Tet (■)
- +Tet (▲)

Time in hours: 0, 1, 2, 4, 8, 12, 24, 48
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