Regulation of JNK signaling by GSTp

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Studies of low basal Jun N-terminal kinase (JNK) activity in non-stressed cells led us to identify a JNK inhibitor that was purified and identified as glutathione $\beta$-transferase Pi (GSTp) and was characterized as a JNK-associated protein. UV irradiation or H2O2 treatment caused GSTp oligomerization and dissociation of the GSTp–JNK complex, indicating that it is the monomeric form of GSTp that elicits JNK inhibition. Addition of purified GSTp to the Jun–JNK complex caused a dose-dependent inhibition of JNK activity. Conversely, immunodepleting GSTp from protein extracts attenuated JNK inhibition. Furthermore, JNK activity was increased in the presence of specific GSTp inhibitors and a GSTp-derived peptide. Forced expression of GSTp decreased MKK4 and JNK phosphorylation which coincided with decreased JNK activity, increased c-Jun ubiquitination and decreased c-Jun-mediated transcription. Co-transfection of MEKK1 and GSTp restored MKK4 phosphorylation but did not affect GSTp inhibition of JNK activity, suggesting that the effect of GSTp on JNK is independent of the MEKK1–MKK4 module. Mouse embryo fibroblasts from GSTp-null mice exhibited a high basal level of JNK activity that could be reduced by forced expression of GSTp cDNA. In demonstrating the relationships between GSTp expression and its association with JNK, our findings provide new insight into the regulation of stress kinases.

Keywords: GSTp/JNK/signaling/stress kinase

Introduction

Jun N-terminal kinases (JNKs) belong to the multi-member family of stress kinases that are activated transiently in response to UV- or X-irradiation, heat shock, osmotic shock or inflammatory cytokines (Galcheva-Gargova et al., 1994; Kyriakis et al., 1994; Westwick et al., 1994). JNK activation in response to UV irradiation is mediated by upstream signaling components, including cdc42, p21PAK, ASK1, MLK, MEKK1, SEK1/MKK4, MKK7 (Coso et al., 1995; Fanger et al., 1997; Tournier et al., 1997; reviewed by Ip and Davis, 1998) and p21ras (Minden et al., 1994; Adler et al., 1995a, 1996), in concert with nuclear DNA lesions (Adler et al., 1995b). Different forms of stress have been shown to mediate JNK activation via various cellular pathways (Adler et al., 1995c). Activated JNK phosphorylates the transcription factors c-Jun, ATF2, p53 and ELK-1 (Kyriakis et al., 1994; Gupta et al., 1995; Whitmarsh et al., 1995; Adler et al., 1997; Fuchs et al., 1998a). Phosphorylation by JNK has been implicated in stabilization (Fuchs et al., 1997, 1998b) and transcriptional activity (Kyriakis et al., 1994) of its substrates, which directly contribute to the mammalian stress response through changes in the cell cycle, DNA repair or apoptosis (Xia et al., 1995; Fuchs et al., 1998a; Kasibhatla et al., 1998).

Despite the significant advances in identifying the components of stress-activated protein kinases, the mechanisms underlying the regulation of JNK before and immediately after stress are not well understood. In cells maintained under normal growth conditions, the basal activity of JNK is low, although JNK phosphorylation by upstream kinases occurs in response to growth factors (Minden et al., 1994) and hence should also be observed in cells proliferating under normal growth conditions. While some unstressed cell types have been found to contain high levels of JNK activity (Dhar et al., 1996), the reason for this high basal level is unknown. A 2- to 4-fold increase in constitutive JNK activity has been reported and, while this is lower than the activation upon exposure to DNA-damaging agents, it is equivalent to tumor necrosis factor-α (TNF-α) treatment and the degree of activation elicited by multiple exposures to low dose UV-B (Adler et al., 1995d). As a key component in regulating the stability and activity of its substrates (Fuchs et al., 1998c; Kyriakis et al., 1994), changes in JNK’s basal activity could influence key cellular functions, including growth, apoptosis and transformation.

Neither transcript nor protein levels of JNK are affected by stress. JNK activation is accomplished upon its phosphorylation by upstream kinases in response to stress (Coso et al., 1995; Tournier et al., 1997). Dephosphorylation of JNK at a later stage (Cavigelli et al., 1996; Hanada et al., 1998) is thought to constitute the primary mechanism underlying the regulation of JNK activity as a kinase. Nevertheless, several studies have demonstrated the existence of JNK inhibitors in normal growing cells. Thus, stress-mediated JNK activation may be independent of its upstream kinases. Among the cellular components...
involved in regulating JNK substrates is a ‘δ inhibitor’ which blocks transactivation of c-Jun by interacting with the δ domain (Baichwal et al., 1991). This is an integral 27 amino acid sequence (30–57) from the N-terminal region of c-Jun required for JNK binding to c-Jun, enabling Jun phosphorylation (Adler et al., 1992) or ubiquitination (Treier et al., 1994; Fuchs et al., 1996). Other JNK regulatory proteins include p21GTP/Maf, which has been shown to exert greater inhibition of JNK activity in response to stress (Shim et al., 1996).

Our interest in exploring the nature of JNK’s low basal activity originated in the observation that extracts of non-stressed cells efficiently inhibited c-Jun phosphorylation when added to a solid-phase kinase reaction. Our further studies led to the purification, identification and characterization of glutathione S-transferase Pi (GSTp) as a JNK inhibitor, described in this report.

GSTs comprise a multigene family, of which GSTp is the most prevalent and ubiquitous non-hepatic isozyme (Jakoby, 1978). Among cellular functions attributed to GSTs are ligand binding and xenobiotic detoxification (Tew, 1994). Reduced glutathione (GSH) binds to the ‘G’ site of GSTp (and other GST isozymes) and plays an important role in detoxification of reactive oxygen species (ROS) and the maintenance of the cellular redox state (Sato et al., 1989).

Among factors implicated in regulating JNK activity are ROS and altered redox potential (Adler et al., 1995c; Gomez-del-Arco et al., 1996; Cui et al., 1997; Wilhelm et al., 1997). ROS have also been associated with regulation of other signaling cascades, e.g. certain isozymes of protein kinase C (PKC; Konishi et al., 1997) and mitogen-activated protein kinase (MAPK; Guyton et al., 1996). The addition of exogenous oxidants or anti-oxidants has been found to influence the activation of MAPK/JNK (Lo et al., 1996; Wilhelm et al., 1997).

Changes in ROS can also directly influence transcriptional activity, as demonstrated for NF-κB (Wang et al., 1995), Ref-1 (Xanthoudakis et al., 1992) and c-Jun (Gomez-del-Arco et al., 1996). Thus, evidence is accumulating that redox status can play an integral role in kinase-mediated stress response pathways. Our characterization of GSTp as an endogenous regulator of JNK activity provides a novel function for a protein with previously characterized catalytic and ligand-binding properties.

Results

A JNK inhibitor is present in cellular extracts of normally growing cells

JNK activities were measured in solid-phase kinase reactions using Jun as a substrate. Proteins prepared from UV-treated 3T3-4A mouse fibroblast cells exhibited marked phosphorylation of c-Jun. The kinetics of JNK activation in these cells increased within 1–5 min, reaching peak levels within 20 min and declining to basal levels within 3–4 h (Adler et al., 1995b). This is a transient activation that is monitored as a multifold increase of kinase activity relative to the control (untreated) cells. Since the assays are performed under conditions that enable selective binding of JNK isozymes (Adler et al., 1995b), we refer to the Jun-bound kinase as JNK and to the overall complex as Jun–JNK.

When cellular extracts prepared from unstressed cells were added to the pre-formed Jun–JNK complex before adding [γ-32P]ATP, c-Jun phosphorylation was inhibited by 80% (Figure 1A, compare lanes 1 and 2). To elucidate further the nature of this inhibition, proteins derived from the non-stressed cells were absorbed by pre-incubation with increasing amounts of Jun–JNK. Subsequently, beads-bound c-Jun–JNK complexes were spun and the supernatant, which lacked Jun–JNK-bound proteins, was tested to determine its ability to alter the degree of JNK-mediated Jun phosphorylation. Increasing the amounts of Jun–JNK complexes used to absorb the putative inhibitor resulted in a dose-dependent decrease of inhibitory activity (Figure 1A, lanes 3–6). To determine whether this inhibitor activity was heat stable, the protein fraction that contained the inhibitor activity was heated to 95°C for 5 min. This treatment abolished the fraction’s ability to block Jun phosphorylation by JNKs (not shown).

UV irradiation abolishes JNK inhibitor activities

The presence of JNK inhibitory activity in non-stressed cells prompted us to determine possible changes to the inhibitory activity after UV irradiation. As shown in Figure 1B, extracts prepared after UV treatment lacked inhibitory activity. The decrease in JNK inhibitor activity depended on the UV dose. Whereas a 10 J/m² dose (a 6 s exposure) caused a 40% reduction (as quantified using a phospho-imager), a 40 J/m² dose (a 25 s exposure) completely abolished the inhibition. In all instances, analysis was performed using the same amount (20 μg) of protein prepared 30 min after administering UV irradiation (Figure 1B). A correlation between JNK activation and the inactivation of its inhibitory activity was also noted in human melanoma cells, which require a 60 J/m² dose for JNK activation. In melanoma cells, 40 J/m² doses caused a 50% inhibition, whereas a 60 J/m² dose abolished JNK inhibitory activities (not shown).

Purification of the JNK inhibitor

To purify the putative inhibitor, extracts of normally growing cells were subjected to the six steps described below. After each step, the presence of the putative inhibitor (tested by measuring inhibition of c-Jun phosphorylation) was monitored in a solid-phase kinase assay using the Jun–JNK (1 μg) complex. Proteins from non-stressed cells were first precipitated in ammonium sulfate (50%). After dialysis against kinase buffer, the non-precipitated proteins were concentrated on membranes with various molecular weight cut-offs. Inhibitor activity was found in the flow-through of both 100 and 30 kDa cut-off filters; it was retained on the 10 kDa membrane, indicating that the inhibitor is between 10 and 30 kDa in molecular weight (Figure 1C). The 10–30 kDa fractions were loaded on a gel filtration column; several fractions within the 20–25 kDa range (based on calibration of the gel filtration column with known molecular weight standards) retained inhibitory activity (Figure 1D). Active fractions were pooled and fractionated on a MonoQ anion exchange column; a 70 mM NaCl eluate was found to contain most of the inhibitor activity (Figure 1E). Active MonoQ fractions were then loaded onto a phenyl-
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Fig. 1. (A) UV-mediated JNK activation is inhibited by proteins obtained from cells maintained under normal growth conditions. Phosphorylation of Jun by JNK (lane 1) is inhibited when the pre-formed complex (Jun-JNK; total 1 μg at 1:1 ratio) is incubated with proteins (10 μg) from untreated cells before adding the \([\gamma-32P]ATP\) (lane 2). Pre-incubation of proteins from non-stressed cells with increasing concentrations of Jun-JNK complex resulted in a dose-dependent decrease of inhibitory activity (lanes 3–6 represent increasing amounts of the pre-formed complex 0.25, 0.5, 1 and 2 μg, respectively). (B) UV irradiation abolishes inhibitor activities. Pre-formed Jun-JNK complex was incubated with whole-cell extract prepared 30 min after sham or UV-C irradiation at the doses indicated prior to addition of \([\gamma-32P]ATP\). (C) Purification of JNK inhibitor on molecular weight cut-off columns. Whole-cell extracts from non-stressed cells were subjected to an ammonium sulfate precipitation and the supernatants were fractionated on the indicated membranes with a cut-off mol. wt of 100, 30 or 10 kDa before being added to pre-formed Jun–JNK complex, followed by addition of \([\gamma-32P]ATP\) (the basal activity in UV-treated cells is shown in lane JNK). Shown is an autoradiograph of the phosphorylated c-Jun. (D) Gel filtration of 3–30 kDa fractions. Fractions <30 kDa were concentrated and loaded (50 μl) onto a Superdex 75 column. From each fraction, 20 μl were added to the pre-formed Jun–JNK complex to measure the ability to inhibit JNK activity (the first lane points to JNK activity with UV-treated extract before this inhibition; numbers represent the respective fractions). (E) Fractionation of JNK inhibitor via an anion exchange column. Fractions 18–24 obtained by Superdex 75 separation were pooled and applied to a MonoQ column. The fractions were assayed for inhibitor activities as shown in the inset. (F) Identifying the inhibitor component on the basis of its binding to Jun–JNK complex. \(\text{his}^+\text{Jun}\) or \(\text{his}^+\text{Jun-JNK}\) complex was incubated with the phenyl-Sepharose eluate (A) or with a purified form of GSTp (B). Beads-bound material was washed and separated on SDS-PAGE. Silver staining of bound proteins is shown in the left panel, whereas the immunoblot of a duplicate gel with antibodies to GSTp is shown on the right. The arrow points to the position of GSTp.
Table I. Purification of JNK inhibitor

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (μg/hisJun-JNK)</th>
</tr>
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<tbody>
<tr>
<td>Total lysate</td>
<td>80</td>
<td>10 160</td>
<td>0.127</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>32</td>
<td>8128</td>
<td>0.254</td>
</tr>
<tr>
<td>Membrane cut-off</td>
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<td>6096</td>
<td>0.91</td>
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<tr>
<td>MonoQ</td>
<td>2.1</td>
<td>5425</td>
<td>2.69</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>1.4</td>
<td>4820</td>
<td>3.46</td>
</tr>
<tr>
<td>hisJun-JNK</td>
<td>0.063</td>
<td>4511</td>
<td>70.70</td>
</tr>
</tbody>
</table>

All purification steps were performed using the hisJun–JNK complex (on nickel beads) as a substrate to measure the degree of JNK activity/inhibition (see Materials and methods for details).

aTotal activity is defined in units: 1 U is the ability to decrease JNK activity by 50%.
bSpecific activity is calculated as inhibitor activity of 1 μg of protein.

Sepharose column in the presence of 0.6 M ammonium sulfate. Using decreased concentrations of ammonium sulfate, the inhibitor activity was found in 0.2 M eluate fractions (not shown). The active fractions from the phenyl-Sepharose column were incubated in a hisJun–JNK column. Analysis of the hisJun-JNK-bound material revealed a single band on silver-stained gels (Figure 1F). Microsequencing analysis of the corresponding band obtained after a large-scale purification (Table I) revealed a 12 amino acid peptide (Pro-Pro-Tyr-Thr-Val-Val-Tyr-Phe-Pro-Val-Arg-Gly) that exhibited 100% homology to the human form of GSTp. To confirm this protein’s identity, a purified form of GSTp was separated parallel to the hisJun–JNK-bound material and subjected to Western blot analysis using polyclonal antibodies to GST. Both migration and immunoreactivity of purified GSTp were identical to those of the hisJun–JNK-bound material (Figure 1F).

**GST inhibition of JNK activity does not alter phosphorylation of Jun or JNK**

To determine whether GSTp affected the degree of JNK or Jun phosphorylation, increasing concentrations of GSTp (purified form) were added to pre-formed Jun–JNK complex which contained the phosphorylated form of JNK obtained from UV-treated cells. GSTp decreased JNK phosphorylation of c-Jun in a dose-dependent manner (within a range of 0.05–1 μg) (Figure 2A), but it did not decrease the number of phosphate groups on JNK, as revealed by immunoblots with phospho-JNK antibodies (Figure 2B). Dual activity protein phosphatase (Ishibashi et al., 1992) was used as a positive control in these reactions (Figure 2C).

To determine whether GSTp affects the number of phospho groups on c-Jun, cellular extracts from normally growing cells were incubated with pre-formed Jun–JNK complex for the indicated periods of time before or after the phosphorylation step with [γ-32P]ATP. The extent of c-Jun phosphorylation was not altered when cell extracts were added after the phosphorylation reaction (Figure 2D). This observation suggested that GSTp did not reduce the number of phospho groups on c-Jun.

**Effect of different GST isoforms on JNK activity**

Incubation of whole-cell extract prepared from non-stressed mouse fibroblasts with the hisJun–JNK complex identified GSTp as the associated protein. A marked decrease in this association was found in proteins prepared after UV irradiation (Figure 3A). In addition to GSTp, isozymes of the GSTα and GSTμ families were also capable of associating with the Jun–JNK complex in vitro (Figure 3A). GSTp exhibited greater JNK inhibitory activity than did GSTμ, which was more potent than GSTα (Figure 3B). Bacterially expressed GST (GST-2T) also mediated JNK inhibition (Figure 3B). This excludes the
peptide efficiently alter JNK inhibition. Another GSH peptidomimetic related to TER-117, also GSTp inhibition of JNK activity. Similarly, TER-117, an inactive prodrug of TER-117 (Flatgaard et al., 1993), was also without effect in vitro.

Molecular dynamic calculations on the energy-minimized X-ray crystal structure of GST were performed to identify the most flexible regions of the protein, i.e. those that were computed to have the highest degree of fluctuations. These regions are most likely to undergo significant conformational changes during catalysis or ligand binding. Two such flexible domains, corresponding respectively to amino acids 36–50 and 194–201, which can participate in the GST–Jun–JNK association and inhibition, were tested. Peptides corresponding to each of these domains were synthesized, and their ability to alter GSTp inhibitor activity was determined in vitro. As shown, the GSTp-derived peptide from amino acids 194–201 (at the C-terminal domain designated P1) abrogated GSTp inhibition of JNK activity (Figure 3F). Conversely, neither P2 (aa 36–50; Figure 3F) nor three unrelated, non-GST-based peptides of varying lengths (8–24 amino acids; not shown) were capable of affecting GSTp inhibition of JNK. Treatment of cells with TER-199 (the prodrg form of TER-117 which is converted to active form in vivo and has been shown to elicit effective inhibition of GST activity in vivo; Flatgaard et al., 1993) led to a 2-fold increase in basal JNK activity in non-stressed cells. UV irradiation also increased (~2.5-fold) JNK activation in TER-199-treated cells (Figure 3G). That TER-199 also affected JNK activities after UV irradiation suggests that the dose of UV irradiation was insufficient to mediate complete inactivation of GST as a JNK inhibitor; thus, the presence of a specific GSTp inhibitor led to an additive effect on overall JNK activities in these cells. The effect of these GSH peptidomimetic agents is likely to be mediated either by altered conformation of the GSTp molecule or by a competition for the binding site, as non-active (bacterially expressed) GSTp was incubated with substrates for other protein kinases. Incubation with PKA, PKC, casein kinase II (CKII) or MAPK revealed 6, 3, 36 and 9% inhibition, respectively. Under the same conditions, GST elicited 86% inhibition of JNK (Figure 3D). In all cases, the activities of the various kinases tested were normalized (c.p.m./µg protein and an equal ratio between the respective substrate and GSTp) to ensure an equal degree of substrate phosphorylation.

GSTp immunodepletion attenuates JNK inhibition

We further elucidated the ability of GSTp to inhibit JNK activity by modulating its levels or activity, or both, in vitro and in vivo. The in vitro kinase assay in which proteins from non-stressed cells were added to the preformed Jun–JNK complex revealed a dose-dependent increase in the degree of JNK inhibition (of up to 80%). Immunodepletion of GST from whole-cell extract decreased the degree of JNK inhibition from 80 to 45% (Figure 3E, lane GST-1). Repeated immunodepletion by GST antibodies further decreased the degree of JNK inhibition to 20% (Figure 3E, lane GST-2). Control immunodepletion reactions with normal rabbit serum (NRS) and protein A/G beads did not affect the degree of JNK inhibition (Figure 3E, lanes NRS-1 and NRS-2, respectively).

Specific GSTp inhibitors and a GSTp-derived peptide efficiently alter JNK inhibition

As an independent approach to inhibit GSTp, we used specific inhibitors which were shown to inactivate GSTp enzymatic activity in vitro and in vivo (Flatgaard et al., 1993). Adding TER-117, a specific in vitro GSTp inhibitor, to a Jun–JNK complex at the same time as GSTp prevented GSTp inhibition of JNK activity. Similarly, TER-293, another GSH peptidomimetic related to TER-117, also blocked GSTp inhibition of JNK activity (Figure 3F). TER-317, structurally similar to TER-117, yet void of GSTp inhibitory properties, failed to affect GSTp inhibition of JNK. Similarly, TER-199, an inactive prodrug of TER-117 (Flatgaard et al., 1993), was also without effect in vitro.

Changes in ROS affect GST oligomerization and its association with, and inhibition of, JNK

We next assessed whether modulation of the cellular redox potential would affect JNK inhibition by GSTp. We monitored the GST–JNK complex in vivo by means of immunoprecipitations followed by immunoblot analysis. Exposure of mouse fibroblast cells to either UV or H2O2 reduced the amount of the JNK–GSTp complex and increased JNK activity, whereas pre-treating cells with the free radical scavengers N-acetylcysteine (NAC) or the ethyl-ester of glutathione (eeGSH) prevented JNK dissociation from GSTp and maintained GSTp inhibitory activity (Figure 4A I–III). These observations indicate that GST–JNK–Jun association is inversely correlated with JNK activity. To elucidate further changes to GST that take place upon altered redox conditions, we measured the possible formation of GST–GST dimers/multimers through disulfide bonds on neighboring cysteines. Monitoring the migration of GSTp on non-reducing SDS–PAGE revealed decreased monomer/dimer forms of GSTp in UV- or
H$_2$O$_2$-treated cells (Figure 4A IV). Instead, a high molecular weight band corresponding to a multimer form of GSTp was detected (Figure 4A IV, upper arrow in panel a). Pre-treatment with NAC or eeGSH maintained the lower molecular weight forms of GSTp (Figure 4A IV, the single arrow in panel a points to the dimer and panel b shows the monomer). H$_2$O$_2$ treatment is known to cause the formation of intra- or intersubunit disulfide bonds between cysteines at amino acids 47 and 101 of GST (Shen et al., 1993), resulting in a multimerization
of GST subunits which no longer accommodates JNK molecules.

**Monomer form of GSTp mediates JNK inhibition**

The observation of changes in GSTp from monomer/dimer to multimer forms upon exposure to increased ROS prompted us to determine which of the two prevalent GSTp forms, monomer or dimer, exerts inhibitory activity on JNK. A gel filtration column was used to separate the monomer from the dimer form of GSTp (Figure 4B). A purified form of either monomer or dimer was added to a solid-phase kinase reaction in which pre-formed hisJun/full-length_JNK complex served as a substrate. As shown, only the monomeric form of GSTp was capable of mediating efficient inhibition of JNK phosphorylation of c-Jun (Figure 4B).

**GSTp expression is inversely correlated with JNK activities**

In a complementary approach to study GSTp effects on JNK activity, increasing amounts of GSTp cDNA were transiently transfected into mouse fibroblasts. As shown in Figure 5A, GSTp transfection resulted in a dose-dependent decrease in JNK activation by UV. While transfection of 0.5 μg of GSTp caused a 20% decrease, 2 μg led to a 40% and 10 μg to a 50% decrease in JNK activation by UV irradiation. These observations suggest that it is possible to alter the degree of JNK activation by GSTp transfection, albeit within the relatively narrow range of 0.5–2 μg.

**Effect of GSTp on phosphorylation of JNK kinase MKK4/JNK/SEK1**

To elucidate further the mechanism underlying GSTp inhibition of JNK activity, we monitored possible changes at the level of MKK4 phosphorylation. In vitro phosphorylation of JNK by MKK4 was not inhibited by GSTp (not shown). In non-stressed 3T3 fibroblasts, MKK4 phosphorylation of c-Jun (Figure 5B).

**GSTp expression results in higher ubiquitination of c-Jun**

Since JNK efficiently targets the ubiquitination of its non-phosphorylated associated proteins c-Jun, ATF2 and p53, we determined the possible effects of GSTp on ubiquitination of JNK substrates in this reaction. Under non-stress growth conditions, c-Jun exhibits a short half-life, which is prolonged upon phosphorylation by JNK (Fuchs et al., 1991), as indicated. Following extensive washes, complex-bound and non-bound (absorbed on Jun–JNK; sup) material was analyzed on immunoblots with polyclonal antibodies that recognize multiple forms of GST (Ramagamaltha and Tew, 1991). Arrows point to the identified forms of GSTp. (B) Effect of different GST isoforms on JNK activity. Pre-formed hisJun–JNK was incubated with the indicated concentrations (per μg of the relevant Jun substrate) to GSTp-c-Jun or GST-Jun–JNK (2 μg/reaction) or subjected to one or two sequential immunodepletions of GSTp (GST-1 or GST-2, respectively) using antibodies to GSTp before addition of [γ-32P]ATP. The insets show representative autoradiographs of the respective reactions (reflects the degree of phosphorylation without inhibitor added). Quantification of three independent experiments is shown in the graph. (D) Specificity of GSTp as a JNK inhibitor. The ability of the purified form of GSTp to inhibit phosphorylation by Src, CKII, MAPK or PKA was tested. Bead-bound substrates were incubated with the respective kinases in the presence of GSTp before [γ-32P]ATP was added to initiate the kinase reaction. After phosphorylation, the beads were washed and phosphorylation was quantified. The values shown represent average results of three different reactions. (E) Immunodepletion of GSTp increases JNK activity. hisJun–JNK complex (containing JNK purified from UV-treated cells) was incubated with whole-cell extract either from non-stressed cells (10 μg) or subjected to one or two sequential immunodepletions of GSTp (GST-1 or GST-2, respectively) using antibodies to GSTp before addition of [γ-32P]ATP. The control reactions with whole-cell extracts treated under the same conditions with NRS and protein A/G beads are also shown. The inhibitory activity depicted was calculated based on the ratio between JNK phosphorylation activities in the absence and in the presence of GSTp. The insets show representative autoradiographs of the respective reactions (reflects the degree of phosphorylation without inhibitor added). Quantification of three independent experiments is shown in the graph. (F) GSTp inhibits JNK activities in vitro. hisJun–JNK or GST–Jun–JNK was incubated with selective GST inhibitors in the presence of the purified form of GSTp, prior to the addition of [γ-32P]ATP. The degree of JNK phosphorylation in the presence of each of the inhibitors is shown. Peptides tested in parallel represent two flexible domains from GSTp, corresponding to the N- (P2) and the C-terminal regions (P1). (G) GSTp inhibits JNK activities in vivo. Mouse fibroblasts were treated with TER-199, a specific inhibitor of GSTp, for 2 h followed by either sham or UV treatment (50 J/m2). Whole-cell extract proteins were prepared after 45 min and assayed for JNK activity by means of his-Jun phosphorylation. The inset shows an autoradiograph of his-Jun phosphorylation (without; + in the presence of TER-199), which was quantified as shown in the graph.
Free radical scavengers prevent UV- or H2O2-mediated GSTp-JNK complex dissociation and maintain low basal JNK activity. Immunoprecipitations using antibodies to JNK (clone 333; PharMingen) were performed on proteins prepared before (−) and after UV irradiation (60 J/m2) or H2O2 treatment (10 μM added in phosphate-buffered saline (PBS) which was replaced after 5 min with medium for another 30 min) of mouse fibroblasts. Cells were pre-treated with NAC (10 mM) or eeGSH (1 mM added to the medium) indicated in the figure under non-reducing (without β-mercaptoethanol in sample buffer) versus reducing SDS–PAGE. The blot shown in (a) was subjected to a 1 min exposure, whereas the blot in (b) was exposed for 1 h. Migration of purified GSTp is shown on the right lane marked GSTp. The positions of the dimer (lower arrow), trimer (double arrow) and large complex (possible tetramer or higher) seen under non-reducing SDS–PAGE conditions (upper arrow) are indicated. Molecular weight markers are indicated on the left panel. (B) The monomer form of GST mediates JNK inhibition. GSTp was immunoprecipitated using antibodies to GSTp from cells treated as indicated in the figure under non-reducing (without β-mercaptoethanol in sample buffer) versus reducing SDS–PAGE. The blot shown in (a) was subjected to a 1 min exposure, whereas the blot in (b) was exposed for 1 h. Migration of purified GSTp is shown on the right lane marked GSTp. The positions of the dimer (lower arrow), trimer (double arrow) and large complex (possible tetramer or higher) seen under non-reducing SDS–PAGE conditions (upper arrow) are indicated. Molecular weight markers are indicated on the left panel.

We established embryo fibroblast cells from GSTP1/P2(−/−) mice [MEFGSTP1/P2(−/−)] in order to assess JNK activity in a GSTp-free environment. These cells do not express GSTp as monitored by either RT–PCR (data not shown) or Western blot analysis (Figure 6A). Stress in the form of UV irradiation, sorbitol or anisomycin markedly increased JNK activity in both MEFwt and MEFGSTP1/P2(−/−) cells; of these treatments, UV elicited the greater degree of JNK activation (Figure 6B). Interestingly, a higher basal level of JNK activity was found in the MEFGSTP1/P2(−/−) than in the MEFGSTwt (Figure 6B and C). This high activity could be diminished in a dose-dependent manner upon transfection of GSTp cDNA (Figure 6C). Lesser amounts of GSTp cDNA were required to mediate 80–100% inhibition of UV-mediated JNK activation in the MEGSTP1/P2(−/−) cells, as compared with the MEGSTwt cells (not shown). In both types of MEFs, transfection of GSTp caused 80–100% inhibition of UV-mediated JNK activation, whereas in mouse fibroblast 3T3 cells, GSTp inhibition reached only 50%. Such differences may be attributed to the overall amount and form (monomer and dimer) of GSTp and/or the levels of other radical scavenger enzymes expressed in each of the cell lines.

To elucidate further the possible mechanism by which GSTp elicits its inhibition of JNK, we monitored levels of JNK phosphorylation before and after stress using JNK phospho-antibodies. In MEGSTP1/P2(−/−) cells, a higher basal level of JNK phosphorylation was found in the MEFGSTP1/P2(−/−) than in the MEFGSTwt (Figure 6B and C). MEFs derived from GSTp null mice [MEFGSTP1/P2(−/−)] as well as from GSTp wild-type mice were co-transfected with GSTp and Jun promoter (5×jun2 target sequence)-driven luciferase constructs. MEFGSTP1/P2(−/−) cells exhibited high basal levels of transcriptional activity mediated by the Jun2 promoter sequence (Figure 6E, control transfection with empty construct, pcDNA3). This activity...
reflected the high basal JNK activity found in these cells (Figure 6B and C). Forced expression of GSTp efficiently reduced high basal Jun-driven transcriptional activity in the MEF<sup>GSTp/P2(−/−)</sup> cells (Figure 6E), implicating the capacity of GST to reduce JNK phosphorylation and activity (Figures 6C and D). As the amino acid residues which are important for GST–GST dimerization and enzymatic activity have been identified previously (Shen <i>et al.</i>, 1993), we mutated GSTp at the respective sites and evaluated their ability to elicit JNK inhibition. When tested in MEF<sup>GSTp/P2(−/−)</sup> cells, GSTp whose cysteines were mutated at amino acids 47 and 101 (which are required for GSTp dimerization) and GST whose tyrosine was mutated at amino acid 7 (which abrogates catalytic proton transfer activity) were as potent as wild-type GSTp in inhibiting high basal JNK activity (not shown) and Jun-mediated transactivation (Figure 6E). The lower basal levels of Jun-mediated transactivation found in MEF<sup>wt</sup> were also reduced by each of these constructs.

The capacity of GSTp to reduce transcriptional activity mediated by the Jun promoter could be due to reduced phosphorylation of Jun and/or ATF2 by JNK, increased ubiquitination and degradation of c-Jun, or both. These observations further support the hypothesis that GSTp inhibition of JNK does not require GST enzymatic activity and is mediated by its monomeric form. Forced expression of peptide derived from the GSTp C-terminal domain

Fig. 5. (A) Transfection of GSTp into mouse fibroblasts reduces JNK activities. The figure shows the extent of JNK activation by UV in cells transfected with GSTp cDNA as compared with mock-transfected controls (100%). GSTp cDNA was co-transfected with the β-gal (0.5 μg) construct into 3T3 cells (via lipofection; Gibco-BRL). Control empty vector was added to reach a constant amount of transfected DNA (15 μg). At 48 h post-transfection, cells were subjected to UV treatment (50 J/m²) and whole-cell extract proteins were prepared 45 min later. The inset depicts an immunoblot performed on the same protein extracts, revealing the respective increase in expression of GSTp (reflects mock-transfected, lanes 2–4 represent 0.5, 2 and 10 μg of GSTp cDNA, respectively). Whole-cell extracts (normalized to transfection efficiency based on β-galactosidase values) were used for a kinase reaction using the hist-C-Jun substrate (2 μg).

(B) (I) Forced expression of GSTp reduces MKK4 phosphorylation. Left panel: mouse 3T3 fibroblasts analyzed for MKK4 phosphorylation using MKK4 phospho-antibodies. Forced expression of GSTp was achieved via lipofection of GSTp cDNA at the indicated concentrations (micrograms). TER-199 (at the micromolar concentration indicated in the figure) was added 22 h after transfection (2 h before protein preparations). The lower panel depicts the overall expression level of MKK4 (using non-phospho-antibodies). Right panel: level of MKK4 phosphorylation upon GSTp expression (0.5 μg) or UV irradiation (30 J/m²). PC represents positive control of phosphorylated MKK4.

(III) Forced expression of GSTp does not affect ERK1 phosphorylation. Proteins prepared 24 h after transfection (2 h before protein preparations). The lower panel depicts the overall expression level of MKK4 (using non-phospho-antibodies). Right panel: level of MKK4 phosphorylation upon GSTp expression (0.5 μg) or UV irradiation (30 J/m²). PC represents positive control of phosphorylated MKK4.

C. Ubiquitination of c-Jun increases upon GSTp expression. Mouse 3T3 cells were co-transfected with his-Jun, GSTp and Ub-HA as indicated in the figure. his-Jun was purified on Ni beads as described (Terier <i>et al.</i>, 1994) and the degree of ubiquitinated Jun was assessed by immunoblot analysis using antibodies to hemagglutinin (HA). The area reflecting the polyubiquitination is marked on the right panel. The expression of c-Jun is shown on the lower panel.
(which blocked GSTp inhibition in vitro) increased basal JNK kinase and c-Jun transcriptional activities, respectively (Z. Yin, V. Adler and Z. Ronai, unpublished observations). By monitoring c-Jun transcriptional activities, these experiments point to the biological significance of GSTp as an inhibitor of JNK.

Discussion
In the present study, we identify and characterize GSTp as a JNK inhibitor. Our data point to an additional cellular mechanism that is involved in the regulation of JNK activity before and after stress. In non-stressed cells, the basal levels of JNK phosphorylation are low, in spite of constitutive stimulation by growth factors and endogenously formed ROS. Maintaining a low basal JNK activity is believed to affect the half-life of JNK substrates, including c-Jun, ATF2 and p53 (Fuchs et al., 1996, 1998c; Musti et al., 1997), and to play a key role in maintenance of controlled cell growth. Indeed, forced expression of GSTp in 3T3 fibroblasts increased the degree of Jun ubiquitination and decreased Jun-mediated transactivation.

GSTp inhibition of JNK is found primarily in normally growing non-stressed cells. Stress, as shown in the case of UV irradiation, decreases this inhibition in a dose-dependent manner. Important to our understanding of GSTp’s ability to elicit association with JNK and inhibition of this kinase is the finding that the inhibitory activities are confined to the monomeric form of GSTp. When first identified, the Jun–JNK-associated protein had a mol. wt of 23 kDa, the characteristic size for the monomeric form of GSTp. Immunoprecipitation reactions always identified the monomeric form of GSTp as the Jun–JNK-associated protein. Similarly, the ability of the monomer (but not the dimer) to elicit JNK inhibitory activity in vitro supports the role of the monomer GSTp in JNK inhibition in vivo. UV irradiation reduces GSTp–JNK association, probably as a result of the formation of GST–GST dimers and multimers; because of disulfide bond-induced steric constraints, dimers/multimers cannot accommodate the Jun–JNK complex. ROS scavengers, such as NAC or GSH, inhibit the formation of GSTp multimers, prevent GSTp dissociation from Jun–JNK and maintain the low basal activity of JNK as a kinase. The switch from a monomer to a dimer/multimer form is likely to provide the underlying mechanism for GSTp’s ability to sense and transmit changes in redox potential as a regulator of JNK signaling. Thus, based on its cellular conformation, GSTp dictates the association and inhibition of JNK. Importantly, while our studies demonstrate the effect of ROS elicited by UV and H2O2 on GSTp dimerization, certain type of stress...
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Fig. 7. Model of GST inhibition of JNK signaling. Based on our findings, the following model is proposed: under non-stressed conditions, GSTp can be free or part of a complex with Jun–JNK. Upon stress, in which ROS are formed, GSTp forms dimers and larger aggregates which cannot accommodate Jun–JNK, thus enabling JNK phosphorylation of c-Jun, which as a result is a stable and active transcription factor.

(i.e. certain cytokines) are not expected to cause the same degree of ROS, and yet are strong inducers of JNK. The latter suggests that dissociation of GSTp from JNK may also occur due to changes other than disulfide bond-based dimerization. The effect of post-translational modifications of GSTp on its association with JNK is being investigated.

The finding that GSTp transfection reduces basal and UV-inducible levels of JNK phosphorylation in vivo provides further insight into the mechanism by which GSTp elicits inhibition of JNK signaling. It is possible to rescue GSTp-mediated inhibition of JNK phosphorylation by transfecting a constitutively active form of MEKK1. Independently, under physiological conditions, GSTp inhibition of JNK takes place in spite of activated MKK4, possibly through direct inhibition of JNK-mediated Jun phosphorylation, supporting our original in vitro observation where GSTp inhibited c-Jun phosphorylation by an activated form of JNK. The ability of GSTp to block JNK activity in spite of MKK4 and JNK phosphorylation by MEKK1 suggests that the nature of the GST–JNK association disables JNK activity as a kinase. That endogenous GSTp does not affect the MKK4 phosphorylation level in non-stressed cells provides further evidence for selective inhibition of the Jun–JNK module by physiological levels of GSTp. That high expression of GSTp also reduces MKK4 phosphorylation could be attributed to possible inclusion of MKK4 with the GST–JNK module. Our data suggest that the interplay between upstream MKK4 and GSTp may determine the effect of GSTp on MKK4. Interplay between MEKK1–MKK4 and MKK4–JNK modules (Xia et al., 1998) and their existence in complex with scaffold protein, which determines the specificity of these signaling cascades (Whitmarsh et al., 1998), may explain the nature of changes elicited with different expression levels of GSTp. The specificity of GSTp-mediated JNK inhibition was demonstrated via comparison of different protein kinases in a solid-phase reaction, as well as in vivo, as forced expression of GSTp did not affect ERK1/2 phosphorylation. Our data also imply that MEKK1 is not a target for GSTp inhibition; other MKK4 upstream kinase(s), which include ASK1, TAKs, MLKs and GCKs, are currently being examined. Of interest is the observation that UV doses (10 J/m²) which decrease GST inhibitory activities are insufficient for JNK activation, which requires doses >20 J/m². This suggests that conversion of the monomer to the dimer may precede JNK ability to elicit kinase activity MKK4/7, and is in agreement with the finding that GSTp efficiently reduces JNK phosphorylation of c-Jun in vivo, even when its upstream kinase MKK4 is active.

That specific inhibitors of GSTp efficiently decrease GSTp inhibition of JNK both in vitro (TER-117, TER-287) and in vivo (TER-199) suggests that their binding to GSTp is within the domain which is also required for association with Jun–JNK or, alternatively, that they alter the conformation of GSTp, affecting its association with JNK. Our results suggest that GSTp does not require transferase activity to mediate inhibition of JNK, in as much as bacterially produced GST, as well as GSTp mutated on Tyr7, which is essential for its catalytic activity, efficiently inhibited JNK enzyme and Jun transcriptional activities, respectively.

Important confirmation of the finding that GSTp is a regulator of JNK kinase activity comes from the use of cells from GSTP1/P2(−/−)-null mice (Henderson et al., 1998). GSTP1/P2(−/−)-derived MEFs revealed a noticeably higher basal level of JNK activity, which was also reflected in JNK's phosphorylation and Jun-driven transcriptional activities. Transfection of GSTp into MEFGSTP(−/−) cells caused a decrease in high basal JNK phosphorylation, kinase activity and Jun-mediated transactivation, further supporting the role of GSTp as an inhibitor of JNK signaling.

It is well documented that GSTp levels vary between different cell types (Tew, 1994). This, together with
variable cellular compartmentalization and the expression of other detoxification enzymes, will contribute to variability of GSTp inhibition of JNK activity. Indeed, comparison of 3T3 fibroblasts and MEFs revealed that while GSTp transfection causes 100% inhibition in MEFs, it is limited to 50% in the 3T3 fibroblasts.

Overexpression of GSTp has been associated with transformation to malignancy (Sato, 1989) and acquired resistance to electrophilic anticancer drugs (Nakagawa et al., 1990; Tew, 1994). The finding that GSTp is a modulator of JNK inhibitor and the relationship between expression of this protein and JNK inhibition suggests that cancer cells prone to overexpress GSTp may exhibit high intrinsic JNK inhibitory activity. In this model, tumor cells overexpressing GSTp may escape apoptosis, which has been implicated as one of the end-points of JNK activity (Xia et al., 1995; Kasibhatla et al., 1998).

The emerging model suggests that through its association with the Jun–JNK complex under non-stressed conditions, GSTp inhibits JNK phosphorylation and activity (Figure 7). Changes in the level of ROS elicited by UV irradiation or H2O2 treatments decrease the amount of monomeric GSTp, resulting in a reduction of the GST–JNK complex, thereby enabling JNK phosphorylation (Figure 7). The fact that transcriptionally active p53 and c-Jun directly affect transcription of radical scavenging enzymes including GST (Pollyak et al., 1997; Komarova et al., 1998) points to the possible existence of an autoregulatory loop for GSTp regulation of JNK. Under such autoregulation, stress-mediated GSTp dimerization/multimerization could enable JNK activation, yielding transcriptionally active p53/Jun and, in turn, newly synthesized GSTp that re-forms a complex with JNK to limit the degree and duration of JNK kinase activity. Our studies support this model, which provides a possible underlying mechanism for the regulation of stress kinases by altered redox potential (Adler et al., 1996; Gomez del Arco, 1996; Kuo et al., 1996; Wilhelm et al., 1997; reviewed by Finkel, 1998). Thus, as a guardian of JNK activities in normally growing cells, GSTp may serve as a sensor of intracellular changes in redox potential that are elicited by various forms of stress.

Materials and methods

Cells and protein preparation

The mouse fibroblast cell line 3T3-4A (Adler et al., 1995a), kindly provided by Dr Claudio Basilico, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum and antibiotics (Gibco-BRL). Cells were grown at 37°C with 5% CO2. MEFs provided by Dr Claudio Basilico, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum and antibiotics (Gibco-BRL). Cells were grown at 37°C with 5% CO2. MEFs provided by Dr Claudio Basilico, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum and antibiotics (Gibco-BRL). Cells were grown at 37°C with 5% CO2. MEFs provided by Dr Claudio Basilico, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum and antibiotics (Gibco-BRL). Cells were grown at 37°C with 5% CO2. MEFs provided by Dr Claudio Basilico, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum and antibiotics (Gibco-BRL). Cells were grown at 37°C with 5% CO2. MEFs provided by Dr Claudio Basilico, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum and antibiotics (Gibco-BRL). Cells were grown at 37°C with 5% CO2. MEFs provided by Dr Claudio Basilico, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum and antibiotics (Gibco-BRL). Cells were grown at 37°C with 5% CO2. MEFs provided by Dr Claudio Basilico, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum and antibiotics (Gibco-BRL). Cells were grown at 37°C with 5% CO2. MEFs provided by Dr Claudio Basilico, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum and antibiotics (Gibco-BRL). Cells were grown at 37°C with 5% CO2. MEFs provided by Dr Claudio Basilico, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum and antibiotics (Gibco-BRL). Cells were grown at 37°C with 5% CO2. MEFs provided by Dr Claudio Basilico, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum and antibiotics (Gibco-BRL). Cells were grown at 37°C with 5% CO2.

Antibodies, immunoprecipitations and immunoblotting

Antibodies to c-Jun, PKA and ERK1/2 were purchased from Santa Cruz. Antibodies to JNK were obtained from PharMingen. Antibodies to CKII were purchased from Upstate Biotechnology and antibodies to phospho-JNK were purchased from Promega. Antibodies to GSTs were as described previously (Ramgamathan and Tew, 1991; Henderson et al., 1998). Antibodies to the phosphorylated form of MKK4, ERK1/2 were obtained from New England Biolabs.

Immunoprecipitations were carried out using 1 mg of protein extracts and 500 ng of the antibodies, for 16 h at 4°C. Protein A/G beads (Gibco-BRL) were added (15 µl) for 30 min at room temperature before washes were carried out in PBS supplemented with Tween-100 (0.5%). Immunoblot analysis was performed using 50 µg of whole-cell extract that had been separated on SDS–PAGE (10%) followed by electrophoretic transfer to PVDF membrane. Ponceau staining was carried out to confirm equal loading, followed by blocking (5% non-fat milk) and reaction with the appropriate antibodies (diluted 1:3000) for 16 h at 4°C. Reactions were visualized using enhanced chemiluminesence (ECL) reagents (Amersham). Analysis of GSTp migration was performed as indicated (Shen et al., 1993), using material that had been immunoprecipitated (0.5 µg of GST antibodies and 1 mg of proteins) from cells. Where indicated, separation under non-reducing conditions was performed in SDS–PAGE without including the reducing agent β-mercaptoethanol in sample buffer.

Peptides and inhibitor

Selective inhibitors of GST, including TER-117, TER-199, TER-291 and TER-317, were synthesized, purified to >95% purity, and kindly provided by Telik Inc. (San Francisco, CA). Peptides corresponding to flexible domains on GST [amino acids 194–201, sequence SSPEHVNPR (P1), and amino acids 36–50, sequence TIDTWMQGLLKPTCL (P2)] were synthesized (Peptide Technologies Corp., Gaithersburg, MD) and purified by HPLC to >98.5%.

UV irradiation and H2O2 treatment

Cells in the logarithmic growth phase were exposed to UV-C (60 J/m2, which requires a 15 s exposure in PBS with the lids off), followed by addition of medium and incubation for 45 min. When indicated, NAC (10 mM) was added to the cells 1 h prior to UV irradiation. For H2O2 treatment, medium from the culture dish was mixed with freshly diluted H2O2 (10 µM) and immediately applied to the fibroblasts (105 cells). When indicated, H2O2 was added in the presence of 1 mM eGSH (Sigma).

Protein kinase assays

Protein kinase assays were carried out using a fusion protein, GST-Jun (amino acids 5–89; Adler et al., 1992) or his-Jun (full-length; Treier et al., 1994) as a substrate. JNK2 was purified from UV-treated 3T3-4A cells as previously described (Adler et al., 1995a). The purity of bacterially produced c-Jun and of 3T3-4A-derived JNK was confirmed by silver-stained SDS–PAGE. Solid-phase JNK assays were carried out as previously described (Adler et al., 1995a, 1996). Briefly, the GST–Jun or hisJun (0.5 µg/assay) were bound to glutathione–Sepharose or nickel beads, before incubation with the purified form of JNK (0.5 µg/assay) in the presence of kinase buffer [20 mM HEPES, pH 7.6, 1 mM EGTA, 1 mM dithiofretiol (DTT), 2 mM MgCl2, 2 mM MnCl2, 5 mM NaF, 1 mM NaVO3, 50 mM NaCl] at room temperature for 15 min. The beads were pelleted and washed extensively with PBST [150 mM NaCl, 16 mM sodium phosphate, pH 7.5, 1% Triton X-100, 2 mM EDTA, 0.1% β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM benzamidine], before they were incubated with [γ-32P]ATP (50 c.p.m./mol; Amersham) in the presence of kinase buffer. Following extensive washing, the phosphorylated Jun was boiled in SDS sample buffer and the eluted proteins were run on a 15% SDS–polyacrylamide gel. The gel was dried, and phosphorylation of the Jun substrate was determined by autoradiography, followed by quantification with a phosphoimager (Bio-Rad).

To assay for the presence of the JNK inhibitor, we used Jun–JNK as a pre-formed complex. GST–Jun or hisJun (0.5 µg) was first pre-incubated with the purified form of active JNK (0.5 µg), thus forming a Jun–JNK complex which subsequently was incubated with protein extracts (30 min) before addition of [γ-32P]ATP (10 min) at room temperature. Important for the formation of these pre-formed complexes is the ratio between Jun and JNK molecules (1:1 molar ratio) and the saturation of the nickel or glutathione beads with EDTA or glutathione to prevent non-specific binding of cellular proteins. In all cases, the buffers contained a cocktail of protease and phosphatase inhibitors (Adler et al., 1995c, 1997).

Other kinase assays were performed with peptides that are known to serve as specific substrates for Src tyrosine kinases, MAPKs and for PKA, all of which were purchased from Santa Cruz Biotech. Co. (Santa
Cruz, CA). Histone H1 (Sigma) was used as a substrate for CKII. In all cases, the substrates were covalently bound to beads using the iimmunolink kit (Pierce) according to the manufacturer’s recommendations. In these assays, 20 μl of bead-coupled peptides (1 μg) were incubated with the source of kinase in the presence or absence of the inhibitor fraction, followed by extensive washing and quantification.

**Purification of JNK inhibitor**

Protein extracts prepared from 3T3-4A mouse fibroblast cells (4 mg/ml) were precipitated with ammonium sulfate (50%). The precipitated material was dialyzed against kinase buffer and subjected to fractionation on small concentrators with cut-off membranes of 100 and 30 kDa (Millipore, Bedford, MA). Material that passed through the 100 kDa membrane was placed on a 30 kDa column and the filtrate (<30 kDa) was concentrated further to a volume of 50 μl using a 10 kDa membrane (Amicon). Concentrated material that was 10–30 kDa in size was separated on a Superdex 75 column (SMART system; Pharmacia) pre-calibrated with respect to the position of the expected molecular weight using a combination of protein standards. Using kinase buffer and a flow rate of 40 μl/min, fractions (200 μl) were collected and tested for inhibitor activity. In these assays, 20 μl fractions were added to pre-formed Jun-JNK complex before addition of [γ-32P]ATP in the presence of kinase buffer and a cocktail of protease and phosphatase inhibitors. Active fractions were pooled and applied to a MonoQ column with a gradient of 20–500 mM NaCl in kinase buffer at a flow rate of 75 μl/min. MonoQ fractions were tested for inhibitory activity, and pooled fractions were pooled and loaded on a phenyl-Sepharose column in a buffer consisting of 20 mM K2HPO4 pH 7.5, 10% glycerol and 0.6 M ammonium sulfate. Using a gradient elution scheme, the inhibitory component was found in the 0.2 M ammonium sulfate fraction. To adjust for kinase buffer and to concentrate the fraction that contained inhibitory activity, the phenyl-Sepharose eluate was concentrated on a 3 kDa column and applied to a pre-formed Jun-JNK complex. This fraction inhibited JNK activity (Table 1). Material that was bound to the Jun-JNK complex revealed a single protein on silver-stained SDS–PAGE.

**Microsequencing**

A large-scale preparation of the JNK inhibitor (Table 1) was subjected to multiple separation steps as described above, followed by SDS–PAGE and blotting onto a PVDF membrane. The purity of the single protein identified by Ponceau staining on the PVDF membrane was confirmed in parallel by silver staining of the same band. The band identified on the PVDF membrane was excised and subjected to N-terminal analysis on an ABI protein sequencer Model 402 equipped with a Model 140C phenylthiohydantoin microanalyzer. The sequence Pro-Pro-Tyr-Thr-Val-Val-Tyr-Phe-Pro-Val-Arg-Gly, which was obtained at the α-C domain.

**Regulation of JNK signaling by GSTp**

GSTp was purified from human placenta. GSTα and GSTμ were purified from human liver as described (Ciaccio et al., 1991), followed by Superdex 75 gel filtration (SMART). Purity was confirmed by silver-stained SDS–PAGE.

**Transcription and ubiquitination assays**

Transcriptional analysis of Jun was carried out using the 5’ Jun2-driven luciferase construct as previously reported (van Dam et al., 1998). In all cases, values were normalized with respect to transfection efficiency. In vivo ubiquitination assays were carried out by transfection of Jun and HA-tagged ubiquitin into the 3T3 cells as indicated elsewhere (Treier et al., 1994).

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**References**


