

# New Insights into the Mechanism of JNK1 Inhibition by Glutathione Transferase P1-1

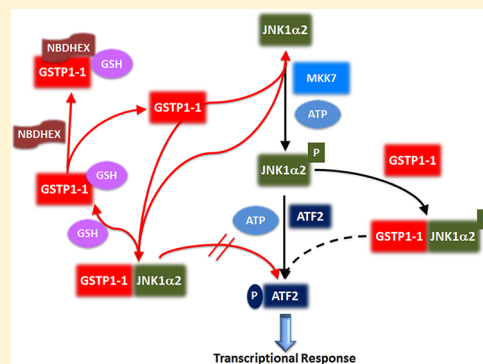
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**ABSTRACT:** The role played by glutathione transferase P1-1 (GSTP1-1) in modulating the c-Jun N-terminal kinase (JNK) pathway has been extensively investigated using JNK isoforms known to exert opposite effects in the cells. We have expressed isoform JNK1 $\alpha$ 2, which has been reported to transmit a pro-apoptotic signal, and we have analyzed both the phosphorylation level and the activity of this kinase in the presence of GSTP1-1. Contrary to what previous studies suggest, we found that GSTP1-1 is able to form a complex with the unphosphorylated and inactive JNK1 $\alpha$ 2 isoform, even in the absence of the substrate. We also analyzed the consequences of this interaction on the activity of both enzymes. The complex strongly reduced the extent of activation of JNK1 $\alpha$ 2 and preserved GSTP1-1 from inactivation. Unexpectedly, glutathione (GSH) exerted a negative effect on the affinity of GSTP1-1 for JNK1 $\alpha$ 2, suggesting that the intracellular levels of this thiol may allow a fine-tuning of the MAPK signaling pathway. Moreover, we found that the adduct formed by GSH and the strong GSTP1-1 inhibitor NBDHEX abolishes the interaction between GSTP1-1 and JNK1 $\alpha$ 2. These data confirm and extend at the molecular level previous evidence obtained in tumor cell lines.



The c-Jun N-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs) are serine/threonine protein kinases encoded by at least three genes: *JNK1* and *JNK2* encode ubiquitously expressed JNK proteins, whereas the *JNK3* protein product is mainly found in the brain, testis, and heart.<sup>1–9</sup> Alternative splicing generates additional complexity, which results in up to 10 different protein products. Specifically, four splice forms arise from both the *JNK1* gene and the *JNK2* gene ( $\alpha 1$ ,  $\alpha 2$ ,  $\beta 1$ , and  $\beta 2$ ), and two arise from the *JNK3* gene ( $\alpha 1$  and  $\alpha 2$ ).<sup>5</sup> The JNK kinases are at the end of the mitogen-activated protein kinase (MAPKs) signaling pathway and, following their activation through concomitant phosphorylation of Thr-183 and Tyr-185 residues, control cellular processes ranging from cell growth to apoptosis. The final effect of activation of JNKs seems to be dependent on the stimulus, cell context, and strength of the signal; whereas transient kinase activation was shown to promote cell survival, prolonged activation mediates tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-dependent apoptosis. Moreover, despite their high levels of sequence similarity, there is evidence that the JNK isoforms play different roles in cell proliferation and apoptosis as a consequence of a different interaction with transcription factor c-Jun.<sup>10</sup> *JNK2* seems to mainly target c-Jun for degradation in unstimulated cells, whereas following stimulation, *JNK1* becomes the major c-Jun interacting kinase; it phosphorylates and stabilizes c-Jun, leading to transcriptional activation.<sup>11</sup> In addition, different studies have shown that only *JNK1* is activated by TNF- $\alpha$  and is required for TNF- $\alpha$ -induced apoptosis in the absence of NF-

$\kappa$ B activation. Conversely, activation of *JNK2* by TNF- $\alpha$  was found to be negligible and appeared to interfere with *JNK1* activation.<sup>11,12</sup> Not only *JNK1* and *JNK2* but also the individual isoforms of *JNK1* might have different functions. A recent study indicates that, in TRAIL-induced apoptosis of colon cancer cell lines, the *JNK1* isoform (*JNK1* $\alpha 1$ ) transmits an anti-apoptotic signal, whereas the longer isoform (*JNK1* $\alpha 2$ ) acts in a pro-apoptotic manner.<sup>13</sup>

Other signaling networks feed into the canonical MAPK cascade. Among the proteins acting as JNK regulators are the glutathione transferases (GSTs), a family of enzymes that play a key role in the cellular detoxification of noxious compounds.<sup>14</sup>

The role of GSTs as inhibitors of JNK activity was revealed approximately a decade ago in the work of Adler and colleagues.<sup>15</sup> They found that the GST isoform GSTP1-1 inhibits JNK-mediated c-Jun phosphorylation through direct association with the c-Jun–JNK complex; however, their data suggested that GSTP1-1 would not bind to either JNK or c-Jun alone. Moreover, they reported that, under physiological conditions, GSTP1-1 inhibits only the activated form of JNK and does not interfere with JNK activation. The GSTP1-1 catalytic activity was not required for JNK inhibition, but some structural changes in GSTP1-1 could be critical for the interaction with the c-Jun–JNK complex.

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These pioneering findings, however, referred specifically to the JNK2 isoform, which is not involved in the apoptotic process, and therefore do not necessarily apply also to JNK1. With this in mind, recently, Collman and colleagues investigated the interaction of different GSTs isoenzymes with both JNK2 and JNK1 isoforms, showing that GSTP1-1 binds preferentially the phosphorylated active form of JNK1, while the interaction with the unphosphorylated, inactive form of JNK1 occurs only in presence of the substrate ATF2. They also reported a direct interaction between GSTP1-1 and ATF2 and explained the ability of GSTP1-1 to inhibit JNK catalytic activity as a consequence of the competition between GSTP1-1 and the active JNK for the substrate ATF2.<sup>16</sup> These findings, however, do not agree with a previous study of Wang and colleagues that provided evidence of a direct physical interaction between GSTP1-1 and the recombinant nonactive JNK1 isoform. As the catalytic kinase domain of JNK1 was not involved in the interaction with GSTP1-1, they hypothesized that JNK inhibition could occur through an allosteric mechanism.<sup>17</sup>

In view of this conflicting evidence, we attempt here to clarify some poorly understood aspects of the interaction between GSTP1-1 and JNK1. We have expressed isoform JNK1 $\alpha$ 2, and through different experimental approaches, we have investigated the occurrence of formation of the heterocomplex with GSTP1-1 and the consequences of this interaction on the activity of both enzymes. Moreover, we analyzed whether the complex may be influenced by the presence of GSH or the adduct between GSH and 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX). This molecule is conjugated to GSH in the active site of GSTP1-1 to form a  $\sigma$ -complex that represents one of the strongest GST inhibitors reported so far.<sup>18</sup> We have demonstrated that NBDHEX induces, in several tumor cell lines, the dissociation of the GSTP1-1–JNK1 heterocomplex and the phospho-activation of JNK1, leading to cell cycle arrest and apoptosis.<sup>19</sup> However, until now there had been no biochemical evidence proving that the formation of a GSH–NBDHEX  $\sigma$ -complex could directly affect the protein–protein interaction between GSTP1-1 and JNK1.

## MATERIALS AND METHODS

**Drugs.** GSH, dansyl chloride (DNCS), and 1-chloro-2,4-dinitrobenzene (CDNB) were from Sigma. NBDHEX was synthesized as reported by Ricci and colleagues.<sup>18</sup>

**Construction of Expression Plasmids.** The coding sequence for human JNK1 $\alpha$ 2 was obtained from the Ultimate ORF Clones collection (clone ID IOH53990) by Invitrogen (Life Technologies, Paisley, U.K.). The DNA sequence was amplified by polymerase chain reaction (PCR) using primers 5'-GGCATATGAGCAGAAGCAAGCGTGACAAC-3' and 5'-CCGGATCCTATCTACAGCAGCCCAGAGG-3' (restriction sites are underlined; the start and stop codons are italicized). After digestion with NdeI and BamHI, the amplicon was ligated to the corresponding restriction sites of the pET-28a expression vector (Novagen-Merck, Darmstadt, Germany), yielding pET-JNK1 $\alpha$ 2. This construct was used to transform *Escherichia coli* BL21(DE3) and expressed an N-terminal six-His-tagged form of JNK1 $\alpha$ 2. Because of the poor solubility of the full-length protein (residues 1–427), a truncated form of JNK1 $\alpha$ 2 was used in fluorescence experiments. This protein was obtained by amplifying the JNK1 $\alpha$ 2 coding sequence by PCR with primers 5'-AGCCATGGGCAGAAGCAAGCGTGAC-3' and 5'-CCTCGAGCTCCAAGTCCATAACTTCCTT-3' and cloning

the PCR product in NcoI and XhoI restriction sites of the pET-28a vector. This construct, named pET-JNK1 $\Delta$ , expressed a C-terminally deleted six-His-tagged form of JNK1 $\alpha$ 2 (residues 1–364).

**Expression and Purification of JNK1 $\alpha$ 2 and JNK1 $\Delta$ .** *E. coli* BL21 cells (Invitrogen), transformed with either His-tagged JNK1 $\alpha$ 2 or His-tagged JNK1 $\Delta$ , were grown in Luria broth containing 25  $\mu$ g/mL kanamycin sulfate. The expression of JNK1 $\alpha$ 2 or JNK1 $\Delta$  was induced by the addition of 0.5 mM isopropyl 1-thio- $\beta$ -galactopyranoside when the absorbance at 600 nm was 0.8. Cells were grown for 18 h at 25 °C, harvested by centrifugation, and resuspended in lysis buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 20 mM imidazole, 10% glycerol, 1 mM DTT, and EDTA-free inhibitor of protease]. The cell suspension was sonicated and clarified by centrifugation. The cellular extract was loaded on a 10 mL Ni-NTA column pre-equilibrated with lysis buffer, and then the column was washed with 200 mL of lysis buffer and the protein eluted using a linear gradient consisting of 50 mL of lysis buffer and 50 mL of the same buffer containing 250 mM imidazole as previously described.<sup>20</sup> The JNK content and its purity were analyzed in the fractions eluted by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Imidazole was then removed from the JNK sample by filtration through a Sephadex G25 column (Amersham Biosciences) pre-equilibrated with kinase storage buffer [20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 10% glycerol]. The protein concentration was determined by measuring the absorbance at 280 nm using an extinction coefficient of 48820 M<sup>-1</sup> cm<sup>-1</sup> for both JNK1 $\alpha$ 2 and JNK1 $\Delta$ . Proteins were then stored at –80 °C.

**Expression and Purification of GSTP1-1.** GSTP1-1 was expressed and purified as previously described.<sup>21,22</sup> The protein concentration was determined by measuring the absorbance at 280 nm, using an extinction coefficient of 25460 M<sup>-1</sup> cm<sup>-1</sup> for the GSTP1-1 monomer.

**JNK1 $\alpha$ 2 Kinase Assay.** The JNK1 $\alpha$ 2 activity was evaluated by measuring the incorporation of [ $\gamma$ -<sup>33</sup>P] into ATF2 (JNK activity assay kit, Millipore). Briefly, JNK1 $\alpha$ 2 was phospho-activated in the first step of the assay by incubating the kinase for 15 min at 30 °C in 25  $\mu$ L (final volume) of 50 mM Tris-HCl (pH 7.5) containing 0.1 mM EGTA, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM magnesium acetate, 0.1 mM ATP, and 0.1  $\mu$ M active MKK7 $\beta$ 1 (Millipore). At the end of the incubation, 2.5  $\mu$ L aliquots were withdrawn from the reaction mixture and incubated for 15 min at 30 °C in 25  $\mu$ L (final volume) of 50 mM Tris-HCl (pH 7.5) containing 0.1 mM EGTA, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM magnesium acetate, 0.1 mM [ $\gamma$ -<sup>33</sup>P]ATP (specific activity of ~800 cpm/pmol, Perkin-Elmer), and 3  $\mu$ M ATF2 (Millipore). Aliquots (20  $\mu$ L) were then spotted on P-81 paper filters (Millipore) and washed sequentially with 75 mM phosphoric acid (Sigma Aldrich) and acetone (Sigma Aldrich). Each paper was then transferred to a vial containing 4 mL of scintillation cocktail (Perkin-Elmer) and read by a  $\beta$ -counter (Beckman LS6500) to evaluate the amount of [ $\gamma$ -<sup>33</sup>P] ATF2 formed.

**Effect of GSTP1-1 on JNK1 $\alpha$ 2 Phosphorylation and Activity.** The effect of GSTP1-1 on the phospho-activation of JNK1 $\alpha$ 2 was evaluated by incubating increasing amounts of GSTP1-1 (from 0.5 to 30  $\mu$ M) with JNK1 $\alpha$ 2 (2  $\mu$ M) for 10 min at 20 °C, before the addition of ATP and active MKK7 $\beta$ 1. The kinase assay was also performed in the presence of a saturating GSH concentration (1 mM). At the end of the incubation, aliquots of the reaction mixture were withdrawn

and used to measure the amount of total and phosphorylated JNK1 $\alpha$ 2 by Western blotting. Moreover, 2.5  $\mu$ L was diluted in 25  $\mu$ L (final volume) of the reaction mixture containing ATF2 and [ $\gamma$ -<sup>33</sup>P]ATP as substrates. At the end of the incubation, aliquots of the reaction mixture were withdrawn and used to evaluate the amount of total and phosphorylated ATF2 by Western blotting and to measure the incorporation of [ $\gamma$ -<sup>33</sup>P] into ATF2.

The inhibition curve of JNK1 $\alpha$ 2 was fit to the general eq 1<sup>23</sup>

$$a_r = 100 - \alpha \left[ \frac{[P]_t + [L]_a + IC_{50}}{\sqrt{([P]_t + [L]_a + IC_{50})^2 - 4[P]_t[L]_a}} \right] / (2[P]_t) \quad (1)$$

where  $a_r$  is the residual activity (as a percentage of the activity of free JNK1 $\alpha$ 2),  $[P]_t$  and  $[L]_a$  are the total concentrations of JNK1 $\alpha$ 2 and GSTP1-1, respectively, and  $\alpha$  is the maximal percent inhibition.

The  $IC_{50}$  value was calculated by assuming the formation of a 1:1 complex between JNK1 $\alpha$ 2 and the GSTP1-1 monomer

Alternatively, GSTP1-1 was added only during the second step of the kinetic assay, to evaluate the effect of GSTP1-1 on the activity of a preactivated JNK1 $\alpha$ 2. The kinetic assay protocol provides that the mixture for the first step (JNK activating step) is diluted 1:10 in the second step (JNK activity step), keeping constant the GSTP1-1:P-JNK ratio. Therefore, we incubated P-JNK1 $\alpha$ 2 (0.2  $\mu$ M) for 10 min at 20 °C with GSTP1-1 concentrations ranging between 0.05 and 3  $\mu$ M. ATF2 and [ $\gamma$ -<sup>33</sup>P]ATP were then added, and the mixture was further incubated for 15 min at 30 °C. The incorporation of  $\gamma$ -<sup>33</sup>P into ATF2 was measured as reported above.

**Western Blot Analysis.** Samples (10  $\mu$ L) were diluted 1:2 in sample buffer [0.5 M Tris-HCl (pH 6.8) containing 10% (v/v) glycerol, 10% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 1% (w/v) bromophenol blue], resolved via 12% SDS-PAGE, and transferred onto an Immobilon-PVDF Transfer Membrane (Millipore). The mouse monoclonal anti-GSTP1-1 (1:3000), rabbit monoclonal anti-phospho JNK (Thr183/Tyr185) (1:3000), mouse monoclonal anti-JNK1 (1:3000), rabbit monoclonal anti-phospho ATF2 (Thr69/Thr71) (1:3000), and rabbit monoclonal anti-ATF2 (1:3000) antibodies (Cell Signaling Technology) and anti-GSH-protein complexes (1:3000) (Virogen) were used as primary antibodies. Anti-rabbit and anti-mouse antibodies (1:30000) (Cell Signaling Technology) were used as secondary antibodies. The membranes were developed with chemiluminescent substrate LITEAbLot Extend (EuroClone). The densitometric analysis was performed using ImageJ64.

**JNK1 $\Delta$  Labeling.** JNK1 $\Delta$  [50  $\mu$ M in 0.1 M potassium phosphate buffer (pH 8.4)] was incubated with 500  $\mu$ M DNSC for 3 h, at 4 °C, in the dark with continuous stirring. After being labeled, the sample was subjected to gel filtration chromatography (Sephadex G25, Amersham Bioscience) to remove unreacted probe and secondary product such as that obtained from the hydrolysis of DNSC. The stoichiometry of the labeling was determined by measuring the protein and probe concentrations in labeled samples. The concentration of labeled JNK1 $\Delta$  was determined by the absorbance at 280 nm of the protein corrected for the contribution of the dye. The following equation was used:

$$[\text{labeled JNK1}\Delta] \text{ (mg/mL)} \\ = (A_{280} - A_{340} \times 0.227) / 1.133$$

where  $A_{280}$  and  $A_{340}$  are the absorbances of JNK1 $\Delta$  at 280 and 340 nm, respectively, 1.133 is the extinction coefficient at 280 nm ( $\text{mL mg}^{-1} \text{cm}^{-1}$ ) for JNK1 $\Delta$ , and 0.227 is the correction factor for the contribution of the fluorophore to the absorbance at 280 nm.<sup>24</sup>

The dye concentration was calculated by the absorbance at 340 nm using an extinction coefficient of  $3370 \text{ M}^{-1} \text{cm}^{-1}$ .<sup>25</sup> The probe:protein mole ratio was determined to be 4:1.

**Fluorometric Analysis.** Fluorometric analysis of dansylated JNK1 $\Delta$  [JNK1 $\Delta$ -dansyl, 0.2  $\mu$ M in 20 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and 10% glycerol] was performed before and after the addition of GSTP1-1 (from 0.1 to 2.5  $\mu$ M). Emission spectra were recorded between 400 and 600 nm (bandwidth of 5 nm), and the excitation wavelength was 340 nm. Fluorescence measurements were performed at 25 °C using a Fluoromax2 (Horiba) fluorometer. The apparent equilibrium dissociation constant ( $K_d$ ) was calculated by assuming the formation of a 1:1 complex between JNK1 $\Delta$ -dansyl and the GSTP1-1 monomer and fitting the experimental data to the general eq 1, modified as follows:

$$\langle \lambda \rangle = \langle \lambda \rangle_0 + (\langle \lambda \rangle_{\text{sat}} - \langle \lambda \rangle_0) \left\{ \frac{[P]_t + [L]_a + K_d}{\sqrt{([P]_t + [L]_a + K_d)^2 - 4[P]_t[L]_a}} \right\} / (2[P]_t) \quad (2)$$

where  $\langle \lambda \rangle_0$ ,  $\langle \lambda \rangle_{\text{sat}}$ , and  $\langle \lambda \rangle$  are the average fluorescence wavelengths measured when dansylated JNK1 $\Delta$  is completely free, complexed, or in the presence of a GSTP1-1 concentration equal to  $[L]_a$ , respectively, and  $[P]_t$  and  $[L]_a$  are the total concentrations of JNK1 $\Delta$ -dansyl and GSTP1-1, respectively.

**Enzyme-Linked Immunosorbent Assay (ELISA).** Two hundred microliters of 18 nM His-tagged JNK1 $\alpha$ 2 in kinase storage buffer [20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 10% glycerol] were added to each well of a 96-well His-Sorb plate (Qiagen) and incubated overnight at 4 °C on a rocking platform. Afterward, wells were washed three times with PBS [50 mM potassium phosphate (pH 7.2) and 150 mM NaCl] and incubated for 30 min with GSTP1-1 (0.2–2  $\mu$ M) in 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA. Alternatively, GSTP1-1 incubation was performed in the presence of either 1 mM GSH or a mixture of 1 mM GSH and 8  $\mu$ M NBDHEX. After the incubation, wells were washed with PBS and incubated with 200  $\mu$ L of the primary anti-GSTP1-1 antibody (1:1000 in 5% nonfat dry milk TBS containing 0.1% Tween) for 2 h, at room temperature. Subsequently, wells were washed with PBS and incubated with the anti-mouse IgG antibody (1:5000 in 5% nonfat dry milk TBS containing 0.1% Tween) for 45 min at room temperature. The wells were then washed with PBS, and 200  $\mu$ L of the 1-Step-Turbo TMB substrate solution (Pierce) was added to each well. Color development was monitored over a period of 45 min, and the reaction was then stopped by the addition of 50  $\mu$ L of 2 M  $\text{H}_2\text{SO}_4$ . The absorbance was measured at 450 nm, and the data were fit to a simple binding isotherm. Differences in the amount of GSTP1-1 bound to JNK1 $\alpha$ 2 were also revealed by Western blot. Wells pretreated with His-tagged JNK1 $\alpha$ 2 were incubated with suitable amounts (1 and 1.5  $\mu$ M) of GSTP1-1, in the absence and presence of

GSH (1 mM), and subsequently washed with PBS and sample buffer [0.5 M Tris-HCl (pH 6.8) containing 10% (v/v) glycerol, 10% (v/w) SDS, 5% (v/w) 2-mercaptoethanol, and 1% (w/v) bromophenol blue] to detach the GSTP1-1 bound to JNK1 $\alpha$ 2. Protein samples were then subjected to Western blot analysis.

**Effect of JNK1 $\alpha$ 2 on GSTP1-1 Activity.** GST activity was determined spectrophotometrically with a Lambda Bio 40 UV-vis spectrometer (Perkin-Elmer) thermostated at 25 °C. The assay mixture consisted of 1 mL (final volume) of 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM GSH and 1 mM CDNB as substrates. The reaction was followed at 340 nm (where the product of the reaction absorbs,  $\epsilon = 9600 \text{ M}^{-1} \text{ cm}^{-1}$ ); 1 unit of GST activity is defined as the amount of enzyme that catalyzes the formation of 1  $\mu\text{mol}$  of product per minute at 25 °C.<sup>26</sup>

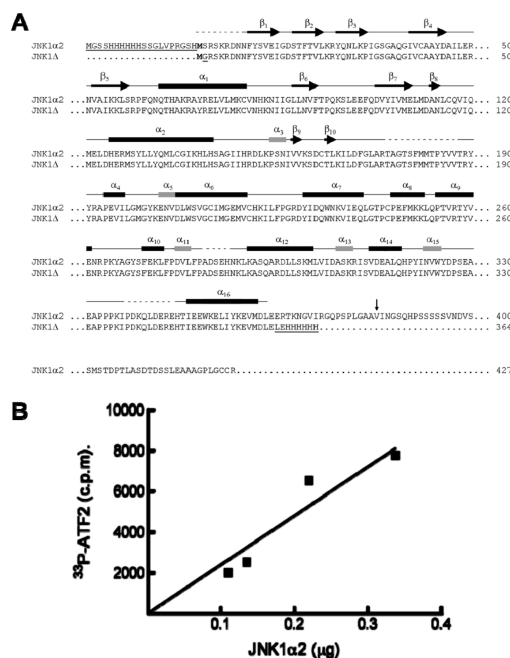
To evaluate the effect of JNK1 $\alpha$ 2 on GSTP1-1 activity, 22 nM GSTP1-1 [in 0.1 M potassium phosphate buffer (pH 6.5) containing 0.1 mM EDTA] was incubated for up to 24 h at 25 °C in the absence and presence of 0.1–2  $\mu\text{M}$  JNK1 $\alpha$ 2. Alternatively, bovine serum albumin (BSA) (Sigma Aldrich) from 0.1 to 2  $\mu\text{M}$  or kinase storage buffer was utilized. Incubations were also repeated in the presence of saturating (1 mM) GSH concentrations. GST activity was assayed at fixed time intervals (10 min, 2 h, and 24 h) after the addition of GSH and CDNB to a suitable volume of the incubation mixture.

## RESULTS

**Expression and Purification of JNK1 $\alpha$ 2 and Its Truncated Form, JNK1 $\Delta$ .** His-tagged JNK1 $\alpha$ 2 and its truncated form, His-tagged JNK1 $\Delta$  (Figure 1A), were purified to homogeneity by using immobilized ion affinity chromatography followed by a gel filtration chromatography step. Afterward, we evaluated the ability of the recombinant JNK1 $\alpha$ 2 isoform to catalyze the phosphorylation of the substrate ATF2. The recombinant kinase was activated by the upstream kinase MKK7 $\beta$ 1, and the activity was revealed by measuring the rate of [ $\gamma$ -<sup>33</sup>P]ATF2 formation. Figure 1B shows the linear relationship between the rate of product formation and the amount of JNK1 $\alpha$ 2 present in the assay, confirming that the recombinant protein was catalytically competent.

**GSTP1-1 Inhibits the Phospho-Activation of JNK.** The kinase assay was used to evaluate the effect of increasing concentrations of GSTP1-1 on the activity of JNK1 $\alpha$ 2. The addition of GSTP1-1 (from 0.5 to 30  $\mu\text{M}$ ) to JNK1 $\alpha$ 2 (2  $\mu\text{M}$ ), in the kinase activation step, caused a significant inhibition of the JNK1 $\alpha$ 2 activity as revealed by the decrease in the level of radiolabeled [ $\gamma$ -<sup>33</sup>P]ATF2 (Figure 2A). Moreover, immunoblotting analysis demonstrated a decrease in the level of both JNK1 $\alpha$ 2 and ATF2 phosphorylation (Figure 2B,C) that well correlates with the decrease in kinase activity. The best fit of the kinetic data to eq 1 yielded an  $\text{IC}_{50}$  value of  $0.13 \pm 0.10 \mu\text{M}$ . This is in accordance with the apparent  $K_d$  of 0.19  $\mu\text{M}$  previously found by Wang et al. for the interaction of GSTP1-1 with the inactive JNK1.<sup>17</sup>

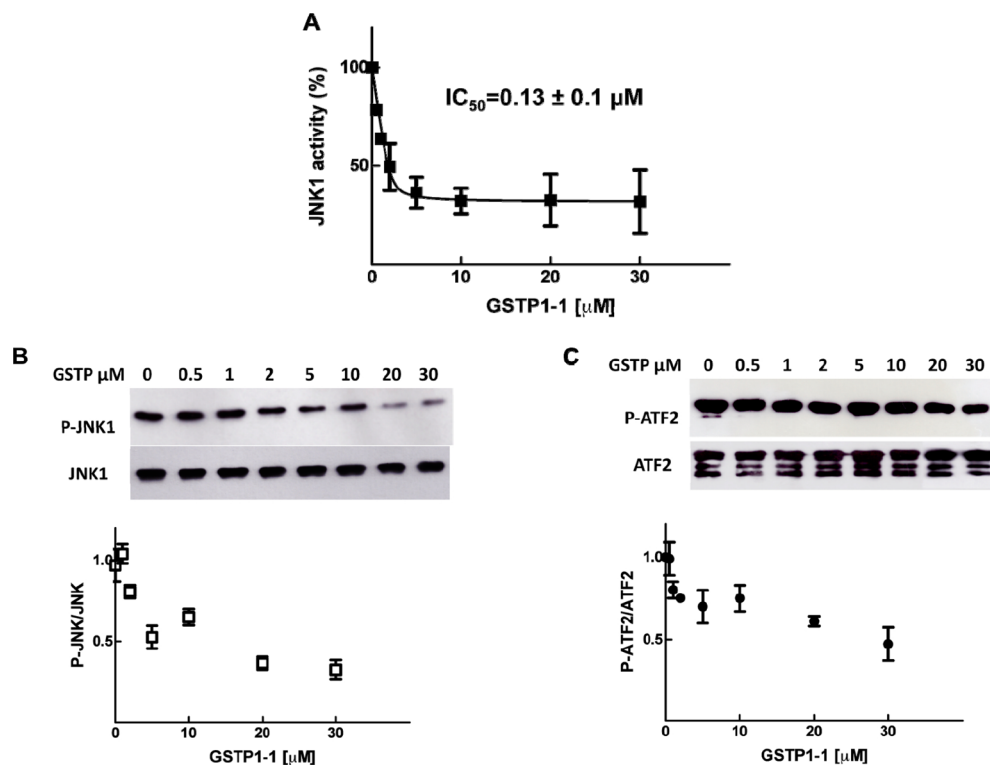
**GSTP1-1 Inhibits the Phosphorylation of ATF2.** We also investigated whether GSTP1-1 may inhibit the activity of a preactivated JNK1 $\alpha$ 2. To do so, we incubated a phospho-activated JNK1 $\alpha$ 2 (0.2  $\mu\text{M}$ ) with different amounts of GSTP1-1 (from 0.05 to 3  $\mu\text{M}$ ) before the addition of [ $\gamma$ -<sup>33</sup>P]ATP and ATF2. Under these conditions, GSTP1-1 caused a decrease in JNK1 $\alpha$ 2 activity with a maximum of inhibition of approximately 35% (Figure 3). A similar result (25–30% inhibition) was



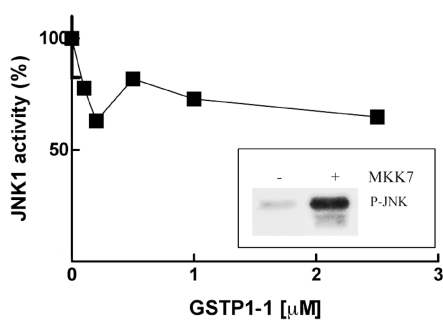
**Figure 1.** JNK1 $\alpha$ 2 activity. (A) Sequence alignment of six-His-tagged forms of JNK1 $\alpha$ 2 and JNK1 $\Delta$ . Sequence numeration starts from the N-terminal methionine of the native form of JNK1 $\alpha$ 2, which is shown in bold. Underlined amino acids are not present in native JNK1 $\alpha$ 2 but were introduced by recombinant DNA manipulation. The vertical arrow indicates the C-terminal site of the JNK1 $\alpha$ 1 isoform. Secondary structure elements are shown above the protein sequences:  $\beta$ -strands as black arrows and  $\alpha$ -helices and  $3_{10}$ -helices as black and gray rods, respectively. Dashed lines indicate the high-mobility regions that were not structurally characterized. Structural information was taken from Protein Data Bank entry 1UKI. (B) Dependence of kinase activity on His-tagged JNK1 $\alpha$ 2 concentration (from 0.1 to 0.4  $\mu\text{g}$ ). Different amounts of His-tagged JNK1 $\alpha$ 2 were activated in the presence of active MKK7 $\beta$ 1 and ATP and then incubated for 15 min at 30 °C with ATF2 and [ $\gamma$ -<sup>33</sup>P]ATP (for details, see Materials and Methods). The incorporation of  $\gamma$ -<sup>33</sup>P into ATF2 was utilized to monitor catalysis.

obtained by Thevenin et al. after incubation of JNK1 with haplotype A of GSTP1-1 (the same utilized in this study).<sup>16</sup>

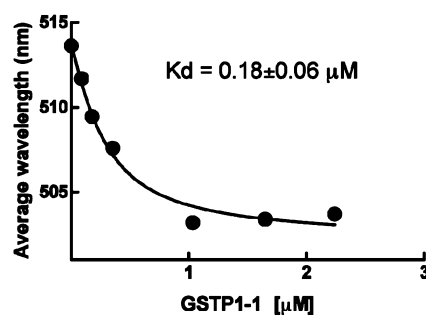
**Direct Evidence of the Binding between GSTP1-1 and the Unphosphorylated Inactive JNK1 $\alpha$ 2.** To directly detect a complex between GSTP1-1 and the unphosphorylated inactive JNK1 $\alpha$ 2, we used two alternative approaches: a fluorescence method and an enzyme-linked immunosorbent assay. In the fluorescence study, we followed changes in the fluorescence emission spectrum of JNK1 $\Delta$ -dansyl upon addition of GSTP1-1. In these experiments, we used the truncated form of JNK1 $\alpha$ 2 to increase the solubility of the labeled protein. After the addition of GSTP1-1 (from 0.05 to 2.5  $\mu\text{M}$ ) to a fixed concentration of JNK1 $\Delta$ -dansyl (0.2  $\mu\text{M}$ ), we observed a concentration-dependent blue shift of the fluorescence maximum, which was indicative of the interaction between the two proteins. Analysis of data using the general eq 2 yielded a  $K_d$  value of  $0.18 \pm 0.06 \mu\text{M}$  (Figure 4). In the second approach, different amounts of GSTP1-1 (from 0.2 to 2  $\mu\text{M}$ ) were added to His-tagged JNK1 $\alpha$ 2 (18 nM) immobilized on a Ni-NTA-coated plate, and the amount of GSTP1-1 bound was revealed by a GSTP1-1 specific antibody. We observed a dose-dependent increase in the magnitude of the antibody signal, indicative of the binding between GSTP1-1 and the immobilized kinase. The best fit of data to a simple binding



**Figure 2.** Effect of GSTP1-1 on the level of phospho-activation of JNK1 $\alpha$ 2. The kinase (2  $\mu$ M) was incubated in the presence of increasing concentrations of GSTP1-1 (from 0.5 to 30  $\mu$ M) in the activation step of the kinase assay. (A) The JNK1 $\alpha$ 2 activity was evaluated in the second step of the assay by measuring the incorporation of  $\gamma$ -<sup>33</sup>P into ATF2 (see Materials and Methods for details), and data were fit to eq 1. (B and C) Representative Western blots showing the levels of (B) P-JNK1 $\alpha$ 2 and JNK1 $\alpha$ 2 and (C) P-ATF2 and ATF2. Densitometric analysis was used to determine the P-JNK1 $\alpha$ 2:JNK1 $\alpha$ 2 ( $\square$ ) and P-ATF2:ATF2 ( $\bullet$ ) ratios; data are reported as a function of GSTP1-1 concentration. Each point represents the mean  $\pm$  SD of at least three different experimental sets.



**Figure 3.** Effect of GSTP1-1 on the activity of P-JNK1 $\alpha$ 2. Active JNK1 $\alpha$ 2 (0.2  $\mu$ M) (preactivated in the absence of GSTP1-1) was incubated in presence of increasing concentrations of GSTP1-1 (from 0.05 to 3  $\mu$ M) before the addition of [ $\gamma$ -<sup>33</sup>P]ATP and ATF2. The extent of ATF2 phosphorylation by JNK1 $\alpha$ 2 was then evaluated as described in Materials and Methods. Each point represents the mean  $\pm$  SD of at least three different experimental sets. The inset shows the phosphorylation level of the preactivated JNK1 $\alpha$ 2 used in the experiment.

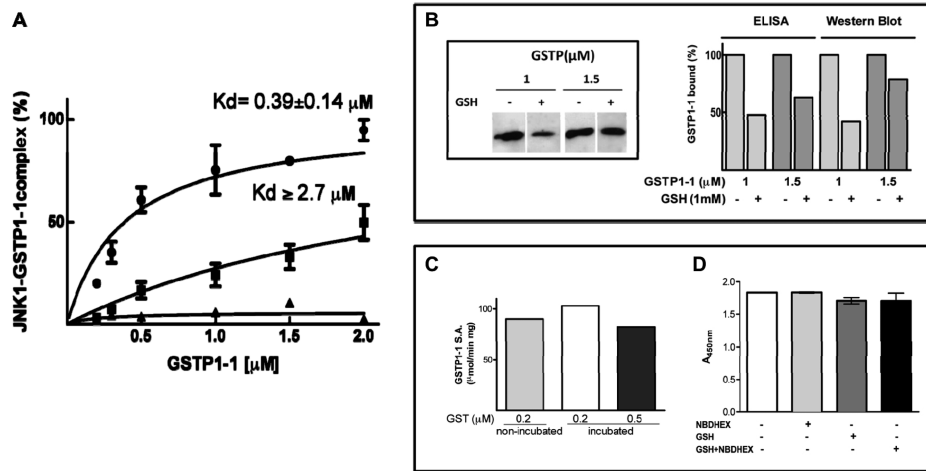


**Figure 4.** Fluorometric analysis of dansylated JNK1 $\Delta$ . The truncated form of JNK1 $\alpha$ 2 (0.2  $\mu$ M) was labeled with DNSC, and the emission spectra were recorded before and after the addition of increasing GSTP1-1 concentrations (from 0.1 to 2.5  $\mu$ M). Each point is the average wavelength of the JNK1 $\Delta$ -dansyl spectrum (between 350 and 600 nm) recorded after the addition of a given amount of GSTP1-1. The curve represents the best fit of data to eq 2.

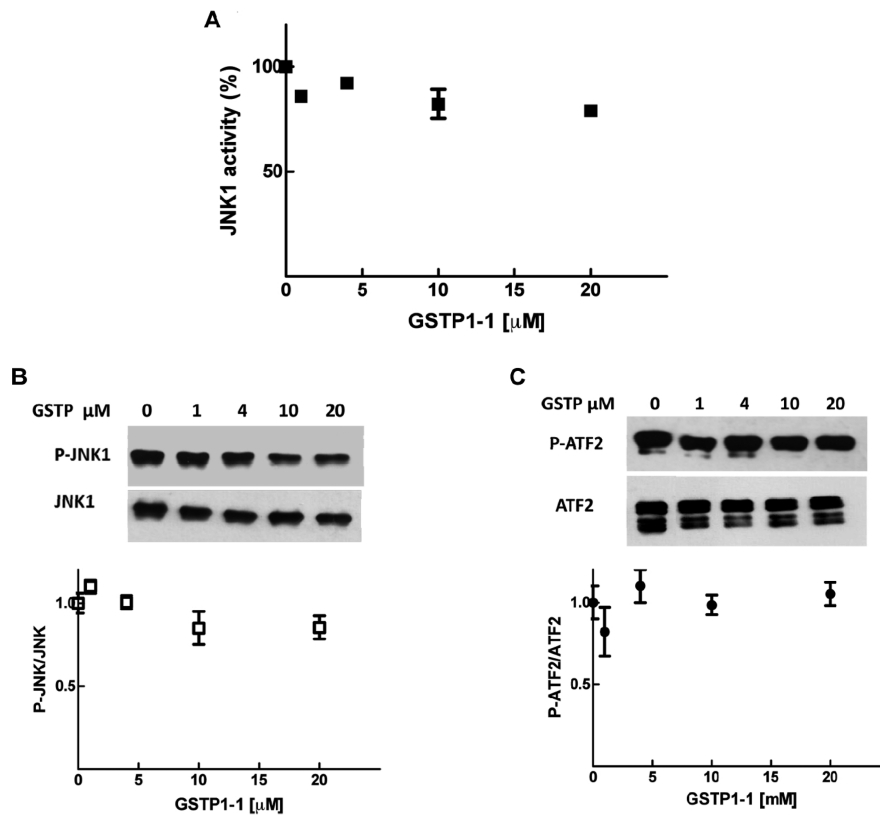
isotherm yielded an apparent dissociation constant of approximately  $0.39 \pm 0.14 \mu\text{M}$  (Figure 5A), in agreement with the IC<sub>50</sub> and K<sub>d</sub> values obtained from kinetic and fluorometric measurements, respectively (Figures 2 and 4). These findings clearly prove the formation of a complex between GSTP1-1 and the inactive JNK1 $\alpha$ 2, ruling out the possibility that the inhibitory effects of GSTP1-1, observed in the kinase assay, may be due to the inhibition of the upstream kinase MKK7 $\beta$ 1. Furthermore, because similar dissociation

constants were observed using JNK1 $\Delta$  or JNK1 $\alpha$ 2, we conclude that the long C-terminal tail that distinguishes JNK1 $\alpha$ 2 from the shorter splicing variant JNK1 $\alpha$ 1 is not implicated in GSTP1-1 recognition.

**GSH Affects the Ability of GSTP1-1 To Inhibit the Phospho-Activation of JNK1 $\alpha$ 2.** Next we investigated the effect of GSH (1 mM) on the interaction by adding the thiol to either the first or the second step of the kinase assay. Under both conditions, GSH protected JNK1 $\alpha$ 2 against inactivation by GSTP1-1. In the attempt to explain these unexpected findings, we focused our attention on a component of the



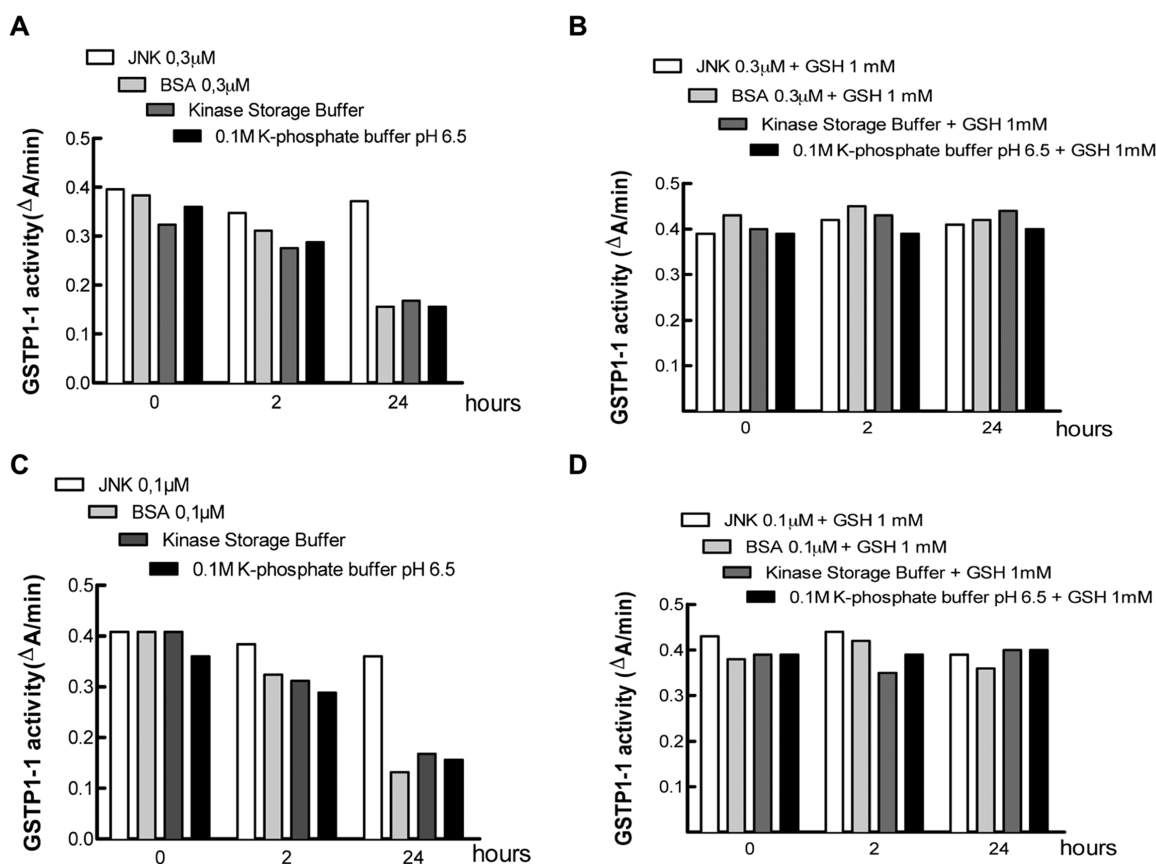
**Figure 5.** Binding of GSTP1-1 to an immobilized His-tagged JNK1 $\alpha$ 2. (A) Immobilized His-tagged JNK1 $\alpha$ 2 (18 nM) was incubated with increasing GSTP1-1 concentrations (from 0.2 to 2  $\mu$ M) (●). Alternatively, incubation of GSTP1-1 was performed in the presence of either 1 mM GSH (■) or a mixture of 1 mM GSH and 8  $\mu$ M NBDHEX (▲). The amount of GSTP1-1 bound to His-tagged JNK1 $\alpha$ 2 was revealed by using a GSTP1-1 specific antibody (ELISA). (B) Immobilized His-tagged JNK1 $\alpha$ 2 (18 nM) was incubated with GSTP1-1 (1 and 1.5  $\mu$ M) in the absence and presence of 1 mM GSH. The GSTP1-1 bound to His-tagged JNK1 $\alpha$ 2 was revealed by an ELISA or detached and subjected to Western blot analysis. The amount of GSTP1-1 bound in the absence of GSH was set to 100%. (C) Effect of a 30 min incubation with His-tagged JNK1 $\alpha$ 2 (18 nM) and GSH (1 mM) on the specific activity (S.A.) of GSTP1-1. (D) Effect of NBDHEX and/or GSH on the absorbance measured at 450 nm indicative of the reaction between the immobilized His-tagged JNK1 $\alpha$ 2 and its specific antibody.



**Figure 6.** Effect of GSH on the GSTP1-1-induced inhibition of JNK1 $\alpha$ 2. JNK1 $\alpha$ 2 (2  $\mu$ M) was incubated for 10 min at 20 °C with increasing concentrations of GSTP1-1 (from 0.5 to 20  $\mu$ M) and 1 mM GSH before the addition of active MKK7 $\beta$ 1 and ATP (JNK1 activating step). (A) The JNK1 $\alpha$ 2 activity was evaluated in the second step of the assay by measuring the incorporation of  $\gamma$ -<sup>33</sup>P into ATF2 (see Materials and Methods for details). (B and C) Representative Western blots showing the levels of (B) P-JNK1 $\alpha$ 2 and JNK1 $\alpha$ 2 and (C) P-ATF2 and ATF2. P-JNK1 $\alpha$ 2:JNK1 $\alpha$ 2 (□) and P-ATF2:ATF2 (●) ratios were determined by densitometric analysis of Western blots and are reported as a function of GSTP1-1 concentration. Each point represents the mean  $\pm$  SD of at least three different experimental sets.

kinase assay buffer, the phosphatase inhibitor sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>) that is known to form a vanadil adduct with GSH.<sup>27</sup> Actually, we found that Na<sub>3</sub>VO<sub>4</sub>, in the

presence of GSH, behaves as a reversible inhibitor of GSTP1-1 with an IC<sub>50</sub> value of approximately 25  $\mu$ M (data not shown). To preserve the activity of GSTP1-1, we decreased to 10  $\mu$ M



**Figure 7.** Effect of JNK1 $\alpha$ 2 on GSTP1-1 stability. Effect of (A) 0.3  $\mu$ M or (B) 0.1  $\mu$ M JNK1 $\alpha$ 2 on the activity of GSTP1-1 (22 nM) incubated at 25  $^{\circ}$ C and pH 6.5. Alternatively, JNK1 $\alpha$ 2 was replaced with (A) 0.3  $\mu$ M or (C) 0.1  $\mu$ M BSA or by a corresponding volume of the kinase storage buffer containing 10% glycerol. (B and D) Incubations were repeated in the presence of saturating (1 mM) GSH concentrations.

the concentration of  $\text{Na}_3\text{VO}_4$  in the kinase assay. On the basis of the  $\text{IC}_{50}$  value of  $\text{Na}_3\text{VO}_4$ , we would expect a >50% inhibition of JNK1 $\alpha$ 2 phospho-activation by high GSTP1-1 concentrations, but we observed a negligible reduction in JNK1 $\alpha$ 2 activity in the presence of GSH (Figure 6A) and a comparable effect on the phosphorylation levels of JNK1 $\alpha$ 2 and ATF2 (Figure 6B,C). Interestingly, Tew and colleagues have reported that GSTP1-1 is subjected to auto-S-glutathionylation and that modification of Cys47 and/or Cys101 interferes with the JNK–GSTP interaction.<sup>28,29</sup> Therefore, as a control experiment, we verified whether GSTP1-1 might be modified during the kinase assay performed in the presence of GSH. After the first step of the kinase assay (JNK activating step), samples were analyzed by Western blotting using anti-GSTP1-1 and anti-GSH-protein antibodies. We did not observe high-molecular weight bands or GSTP1-1 glutathionylation (data not shown). Therefore, we can exclude a mechanism of binding interference based on the GSH-mediated covalent modification of GSTP1-1.

**Effect of GSH and the GSH–NBDHEX Complex on the Interaction between GSTP1-1 and JNK1 $\alpha$ 2.** To assess whether GSH may really interfere with the formation of the GSTP1-1–JNK1 $\alpha$ 2 complex, we also analyzed the binding of GSTP1-1 to the immobilized JNK1 $\alpha$ 2 in the presence of GSH (1 mM). The thiol strongly reduced the affinity of GSTP1-1 for the kinase ( $K_d \geq 2.7 \mu\text{M}$ ) (Figure 5A), confirming the kinetic evidence. As control experiments, we evaluated by Western blot analysis the amount of GSTP1-1 bound to JNK1 $\alpha$ 2 in the absence and presence of GSH; the results are in good

agreement with the ELISA data shown in Figure 5B. Moreover, the activity of GSTP1-1 incubated with His-tagged JNK1 $\alpha$ 2 (18 nM) and GSH (1 mM) was comparable to that of the untreated enzyme (Figure 5C), ruling out the possibility that the decreased affinity for JNK1 $\alpha$ 2 may be a consequence of GSTP1-1 inactivation.

We then analyzed the effect of the strong GST inhibitor NBDHEX on the GSTP1-1–JNK1 $\alpha$ 2 interaction. This compound is known to form a stable adduct with GSH ( $\sigma$ -complex) that occupies both the G and H binding sites of GSTP1-1.<sup>30</sup> In the presence of saturating concentrations of NBDHEX (8  $\mu\text{M}$ ) and GSH (1 mM), the interaction of GSTP1-1 with JNK1 $\alpha$ 2 was completely abolished (Figure 5A), confirming the evidence previously obtained in tumor cell lines.<sup>19</sup> We evaluated, as a control, the effect of NBDHEX and/or GSH on the signal intensity measured after the reaction of immobilized JNK1 $\alpha$ 2 with its specific antibody. Figure 5D shows that the presence of NBDHEX and/or GSH does not interfere with the amount of JNK1 $\alpha$ 2 detected, excluding the possibility of a nonspecific decrease in the magnitude of the signal.

**JNK1 $\alpha$ 2 Stabilizes GSTP1-1 Activity.** Then we investigated the effect of JNK1 $\alpha$ 2 on GSTP1-1 activity and the role played by GSH. GSTP1-1 was incubated for 24 h at 25  $^{\circ}$ C and pH 6.5 in the presence of different amounts of JNK1 $\alpha$ 2 (from 0.1 to 2  $\mu\text{M}$ ). Alternatively, GSTP1-1 was incubated with BSA (from 0.1 to 2  $\mu\text{M}$ ), known to behave as a chaperone protein,<sup>31</sup> or with a corresponding volume of the kinase storage buffer containing 10% glycerol (Figure 7). Neither the kinase storage

buffer nor low BSA concentrations ( $<1 \mu\text{M}$ ) were able to stabilize GSTP1-1 activity over a long period of time (24 h). Surprisingly, a JNK1 $\alpha$ 2 concentration of only  $0.1 \mu\text{M}$  was sufficient to keep perfectly active GSTP1-1 for up to 24 h (Figure 7C). The stabilizing effect of JNK1 $\alpha$ 2 became superfluous in the presence of GSH (1 mM) that protected GSTP1-1 against time-dependent inactivation (Figure 7B,D).

## DISCUSSION

Although it is well-known that GSTP1-1 plays a role in modulating the activity of the kinase JNK, many aspects of this interaction remain to be defined. While the studies performed by Adler et al. shed the first light on this nonenzymatic activity of GSTP1-1,<sup>15</sup> they focused on only the JNK2 isoform, which is mainly reported to be involved in transmitting a cell proliferation signal, and not in apoptosis. Furthermore, they found that GSTP1-1 binds the active form of JNK2 only when this forms a complex with its substrate c-Jun. Later results of Thevenin et al.,<sup>16</sup> instead, pointed out that JNK1 was indeed able to bind GSTP1-1 in its unphosphorylated, inactive form, but only in the presence of its substrate ATF2. These studies, however, are partly contradicted by Wang et al.,<sup>17</sup> who reported a direct and efficient protein–protein interaction between GSTP1-1 and the recombinant nonactive JNK1 isoform. However, there was no indication that the GSTP1-1–JNK1 complex might affect the phospho-activation of JNK1.

Our kinetic and binding experiments establish that GSTP1-1 binds the inactive JNK1 $\alpha$ 2 isoform even in the absence of ATF2 and that this interaction significantly weakens the ability of MKK7 $\beta$ 1 to phospho-activate JNK1 $\alpha$ 2 (Figure 2). One of the most controversial aspects of the previous studies is the idea that only the monomeric form of GSTP1-1 might be able to efficiently inhibit JNK.<sup>15</sup> However, recent evidence suggests that the GST dimer is extremely stable with a dissociation constant of  $\ll 1 \text{ nM}$ ,<sup>32</sup> and therefore, GSTP1-1 exists mainly in the dimeric state under our experimental conditions. Thus, when fitting our experimental data, we assumed that each subunit of a GSTP1-1 dimer may interact with JNK1 $\alpha$ 2, even though we cannot strictly rule out the possibility of a 1:1 complex between one JNK1 $\alpha$ 2 molecule and the GSTP1-1 dimer. In addition, we confirmed that GSTP1-1 binding interferes only in part with the catalytic activity of the active kinase, causing only 35% of inhibition as previously reported.<sup>16</sup> Therefore, these data suggest that the main inhibitory activity of GSTP1-1 with respect to JNK1 $\alpha$ 2 resides in preventing its phospho-activation by the upstream kinase. Thevenin et al. have previously reported that the glutathione analogue S-methylglutathione causes a partial decrease in the ability of GSTP1-1 to compete with JNK for the binding of the substrate ATF2.<sup>16</sup> By further investigation, we also proved that GSH affects the ability of GSTP1-1 to bind JNK1 $\alpha$ 2, leading to an increase in the dissociation constant of the JNK1–GSTP1-1 complex from  $0.39$  to  $\sim 2.7 \mu\text{M}$ . However, additional cell-based analysis is required to confirm the physiological relevance of our findings.

We also showed that when the active site of GSTP1-1 is occupied by the  $\sigma$ -complex between GSH and NBDHEX, the enzyme becomes fully unable to inhibit JNK1 $\alpha$ 2 (Figure 5A). This is in perfect agreement with previous observations obtained by our group for several tumor cell lines where NBDHEX treatment resulted in a strong increase in the level of JNK1 phosphorylation and the activation of apoptosis.<sup>19,33–35</sup> Similar results were obtained by Adler and colleagues with the

GSH-peptidomimetic TLK199, which is activated by the intracellular esterases in a selective GSTP1-1 inhibitor.<sup>36</sup>

Finally, we analyzed the effect of formation of the GSTP1-1–JNK1 $\alpha$ 2 complex on the activity of GSTP1-1. It is well-known that the maintenance of catalytic function requires a structured and shaped active site; in fact, a prolonged incubation of GSTP1-1 in the absence of GSH results in the inactivation of the enzyme, while GSH increases the rigidity and structural stability of the active site of GSTP1-1.<sup>37,38</sup> Here, we found that JNK1 $\alpha$ 2 has an effect similar to that of GSH, preventing GSTP1-1 inactivation over a long period of time, in accordance with the idea that JNK1 $\alpha$ 2 may increase the structural rigidity of some protein portions of GSTP1-1. Therefore, we may hypothesize that the active GSTP1-1 may exist in a dynamic equilibrium between a form bound to GSH and a form bound to JNK1 $\alpha$ 2. This latter interaction may preserve GSTP1-1 from inactivation when there is a temporary reduction in GSH concentration.

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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS

JNK, cJun N-terminal kinases; MAPKs, mitogen-activated protein kinases; GST, glutathione transferase; NBDHEX, 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol; GSH, reduced glutathione; CDNB, 1-chloro-2,4-dinitrobenzene;  $\text{Na}_3\text{VO}_4$ , sodium orthovanadate; SD, standard deviation.

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