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Phosphorylation of Glutathione *S*-Transferase P1 (GSTP1) by Epidermal Growth Factor Receptor (EGFR) Promotes Formation of the GSTP1-c-Jun N-terminal kinase (JNK) Complex and Suppresses JNK Downstream Signaling and Apoptosis in Brain Tumor Cells*

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Background: GSTP1 is a downstream EGFR phosphorylation target.
Results: EGFR-dependent C-terminal Tyr-198 phosphorylation shifts GSTP1 to the monomeric state, facilitates JNK binding and inhibition, and suppresses apoptosis in brain tumor cells.
Conclusion: Enhanced suppression of JNK signaling by EGFR-phosphorylated GSTP1 provides a survival advantage for tumors.
Significance: The GSTP1-EGFR cross-talk is a mechanism of tumor cell survival and drug resistance.

Under normal physiologic conditions, the glutathione *S*-transferase P1 (GSTP1) protein exists intracellularly as a dimer in reversible equilibrium with its monomeric subunits. In the latter form, GSTP1 binds to the mitogen-activated protein kinase, JNK, and inhibits JNK downstream signaling. In tumor cells, which frequently are characterized by constitutively high GSTP1 expression, GSTP1 undergoes phosphorylation by epidermal growth factor receptor (EGFR) at tyrosine residues 3, 7, and 198. Here we report on the effect of this EGFR-dependent GSTP1 tyrosine phosphorylation on the interaction of GSTP1 with JNK, on the regulation of JNK downstream signaling by GSTP1, and on tumor cell survival. Using *in vitro* and *in vivo* growing human brain tumors, we show that tyrosine phosphorylation shifts the GSTP1 dimer-monomer equilibrium to the monomeric state and facilitates the formation of the GSTP1-JNK complex, in which JNK is functionally inhibited. Targeted mutagenesis and functional analysis demonstrated that the increased GSTP1 binding to JNK results from phosphorylation of the GSTP1 C-terminal Tyr-198 by EGFR and is associated with a >2.5-fold decrease in JNK downstream signaling and a significant suppression of both spontaneous and drug-induced apoptosis in the tumor cells. The findings define a novel mechanism of regulatory control of JNK signaling that is mediated by the EGFR/GSTP1 cross-talk and provides a survival advantage for tumors with activated EGFR and high GSTP1 expression. The results lay the foundation for a novel strategy of dual EGFR/GSTP1 for treating EGFR+ve, GSTP1 expressing GBMs.

c-Jun N-terminal kinases (JNKs) belong to the family of mitogen-activated protein kinases (MAPK). These serine/threonine kinases are activated within the MAPK kinase kinase (MAPKKK)-MAPK kinase (MAPKK)-MAPK signaling cascade in response to intra- and extracellular stresses and stimuli, including oxidative stress, UV, gamma irradiation, heat shock, proinflammatory cytokines, growth factors, and anticancer agents (1, 2). Upon activation, JNKs phosphorylate a number of nuclear substrates, mainly transcription factors, including c-Jun, c-Fos, JunD, and ATF2, involved in the formation and activation of the AP-1 transcription complex (1, 3). The activation and subsequent stabilization of these transcription factors leads to alterations in the expression of a broad spectrum of genes involved in critical cellular processes such as cell cycle progression, proliferation, inflammatory response, apoptosis/survival, and DNA damage response and repair. Activated JNKs also directly phosphorylate a variety of non-nuclear substrates, including cytoskeletal proteins, ubiquitin-protein ligases, scaffold/adaptor proteins, mitochondrial proteins, focal adhesion proteins, and other protein kinases (1). As a result of its varied downstream targets, the JNK signaling pathway is functionally complex with diverse and sometimes opposing cellular outputs. For example, depending on the cellular context, the duration and level of JNK activation, cell type, stimulus type, and level of activity of other signaling pathways, the activation of JNK can either be anti-apoptotic or lead to apoptosis (2, 4, 5). Additionally, the oncogenic transformation and tumorigenicity associated with some activated oncogenes, such as Ras, have been shown to involve activation of the JNK pathway (6–8), consistent with the constitutively high levels of JNK in tumors, such as B-cell lymphoma (9), mantle cell lymphoma (10), acute myeloid

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leukemia (11), non-small cell lung cancer (12), and malignant glioma (13–15). In several of these tumors, the level of GSTP1 increases with their increasing malignant progression, and this could function in part to down-regulate the activity of the high tumor JNK to growth supportive levels and to drive tumor resistance to therapy (16–19).

Abnormalities, notably overexpression, gene amplification, and mutations in the EGFR gene are a hallmark of many human cancers, and increased wild-type EGFR expression and activation and the presence of the ligand-independent, constitutively activated mutant EGFRvIII are frequent in primary glioblastoma (GBM).[2] Activation of EGFR downstream pathways, including the JNK pathway, have been associated with increased tumor growth and both drug and radiation resistance (20, 21). Interestingly, although >85% of GBMs with activated EGFR or EGFRvIII have been reported to have an activated JNK signaling pathway (13, 14, 22), the underlying mechanisms and regulation of the EGFR-associated JNK signaling are still not well understood.

Similar to EGFR, in many human cancers expression of the glutathione *S*-transferase P1, *GSTP1*, gene is abnormally high at both the transcript and protein levels (16, 23–25). In addition to its role in Phase II metabolism, GSTP1 functions as a key regulator of cell signaling in response to intra- and extracellular stimuli, notably, of pathways involving MAPKs (26–30). The signaling regulatory functions of GSTP1 are mediated primarily by interactions of GSTP1 with a variety of protein partners including MAPKs, TRAF2/ASK1, transglutaminase 2, Fanconi anemia group C protein, and 1-cysteine peroxireductase (for review, see Ref. 18). Because the first report of the interaction of GSTP1 with the MAPK/SAPK, JNK (30), several studies have shown GSTP1 to be a potent endogenous inhibitor of JNK and that the inhibition occurs via direct interaction of the C terminus of GSTP1 with the C terminus of JNK to form a GSTP1-JNK complex that prevents JNK from phosphorylating its downstream targets. Dissociation of this GSTP1-JNK1 complex results in restoration of JNK activity (30–32). Thus, GSTP1 functions as an endogenous molecular regulatory switch that allows the cell to regulate JNK signaling in response to its physiological status and/or different stimuli. A consequence of the dual metabolic and signaling regulatory function is that GSTP1 can mediate the response of both normal and tumor cells to agents regardless of whether or not they are direct GSTP1 substrates (17, 29, 33–36).

Despite the role of GSTP1 in the regulation of MAPK/SAPK signaling, to date the cellular context in which this regulatory control functions and how it interacts with other cell signaling pathways are not well understood. We recently reported that the human GSTP1 protein is a downstream target of the EGFR-tyrosine kinase and undergoes EGFR-dependent tyrosine phosphorylation, with tyrosines 7 and 198 being the major EGFR-specific phospho-acceptor residues in the GSTP1 protein (35). Given that activated EGFR and overexpression of GSTP1 are common features of many human malignancies, we sought in this study to gain insight into the impact of the EGFR-GSTP1 cross-talk on the GSTP1-dependent regulatory control of cellular JNK signaling. Using human brain tumor cell lines and xenografts, we examined *in vitro* and *in vivo* the functional effects of the EGFR-dependent GSTP1 tyrosine phosphorylation on GSTP1-JNK physical interaction and on JNK downstream signaling and apoptotic response.

Experimental Procedures

Antibodies and Chemicals—Anti-human GSTP1 mouse monoclonal antibodies were from BD Transduction Laboratories. GST-c-Jun fusion protein, anti-phosphotyrosine (Tyr(P)-100), anti-phospho EGFR (Tyr-1068), anti-phospho-JNK (Thr-183/Tyr-185), anti-phospho-c-Jun (Ser-63), anti-phospho-MKK4 (Thr-257) antibodies were from Cell Signaling Technology (Danvers, MA). JNK1α1/SAPK1c active and inactive full-length recombinant proteins, rabbit anti-JNK/SAPK1 polyclonal antibody, and EGFR active catalytic domain were from Millipore (Billerica, MA). Recombinant full-length human c-Jun was purchased from GloboZymes (Carlsbad, CA). Rabbit anti-JNK1 (C-17) polyclonal, mouse anti-c-Jun (G-4) monoclonal antibody, and horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-V5 monoclonal antibodies, LDS sample loading buffer, and Dynabeads Protein G were from Invitrogen, and human recombinant GSTP1-1 protein was from Calbiochem. All custom-made peptides were from Biosynthesis Inc. (Lewisville, TX). Anti-β-actin antibody, streptavidin-HRP, streptavidin-agarose, recombinant EGF, and all other chemicals and biochemicals were from Sigma unless otherwise stated.

Tumor Cell Lines and in Vivo GBM Xenografts—The MGR3 (GBM), MGR1 (anaplastic astrocytoma), and UW228 (medulloblastoma) cell lines were all established by one of the co-authors, Francis Ali-Osman, from primary patient specimens (37). UW228 is naturally GSTP1−ve because the *GSTP1* gene is *de novo* transcriptionally silent, a result of hypermethylation of its promoter. We created a GSTP1-overexpressing cell line, UW228*1C, from the parental UW228, via stable transfection with the human *GSTP1*C* allelic variant.[3] The high EGFR expressing human GBM U87MG.wtEGFR was derived by stable transfection of the parental U87MG cells with wild-type EGFR (38). All cell lines were maintained in DMEM with 10% FCS except for U87MG.wtEGFR, which was maintained in Improved MEM Zinc Option with 10% FCS in a humidified atmosphere containing 5.0% CO_2 at 37 °C. The GBM xenografts, GBM6 and GBM10, were derived from patient GBM samples in the laboratory of Dr. David James, University of California, San Francisco, as previously described (39) and maintained in our laboratory as 6B and 10T, respectively, by serial *in vivo* passage (40). For the *in vivo* studies, briefly, the freshly obtained tumor (xenograft) specimens were minced, passed through a modified tissue press, and sieved through two layers of mesh. The resulting tissue homogenate was passed through a 19-gauge needle, and 500 μl was injected subcutaneously into

[2] The abbreviations used are: GBM, glioblastoma; GSTP1, glutathione *S*-transferase P1; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PLA, proximity ligation assay; EGFR, epidermal growth factor receptor; TAT, trans-activator of transcription.

[3] F. Ali-Osman, unpublished data.

the right flank of Balb/C nu/nu mice. The mice were monitored daily for tumor growth, and when the tumors had attained 300–500 mm^3, the animals were euthanized, and the tumors were removed and used in the analyses.

Protein Extraction and Western Blot Analyses—Tumor xenografts or exponentially growing tumor cell cultures were rinsed with ice-cold PBS and lysed in buffer containing 40 mM HEPES-KOH pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, and Halt protease and phosphatase inhibitor mixture (Thermo Fisher Scientific Inc., Rockford, IL). After brief sonication and subsequent high speed centrifugation, the particle-free tumor and/or cell supernatants were collected and assayed for protein content (Bio-Rad). For experiments requiring EGFR activation, tumor cells were grown in serum-free media overnight, and EGF was added to 100 ng/ml. After 20 min at 37 °C, cell extracts were prepared as described above. All protein gel electrophoreses were performed using NuPAGE® Novex® Bis-Tris Gel Systems (Invitrogen). Briefly, samples prepared in LDS sample loading buffer containing reducing agent were boiled for 10 min and electrophoresed on a 10% Bis-Tris gel in MOPS buffer. The gels were electrophoretically transferred to Immobilon P membrane (Millipore) and stained with Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories). After blocking in 1× TBS-T containing 5% BSA, the blots were treated overnight with the appropriately diluted primary antibody followed by horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized with the ECL system (Thermo Fisher Scientific), after which the membranes were stripped and reprobed with β-actin or other required antibody.

JNK Pathway Activation in Tumor Xenografts and Cells—The level of activation of the JNK pathway in tumor xenografts and *in vitro* growing cells was determined as the level of JNK-mediated phosphorylation of its downstream target, c-Jun, at Ser-63 after immunoprecipitation with an anti-JNK1 antibody. Briefly, 700 μg of tumor or cell extract protein was incubated with 2 μg of rabbit anti-JNK1 (C-17) polyclonal antibody overnight at 4 °C. Dynabeads Protein G was added to the sample and incubated for 1 h at 4 °C. The JNK1-bound beads were washed 4 times with cell lysis buffer and once with kinase buffer (10 mM $MgCl_2$, 2 mM DTT, 0.1 mM Na_3VO_4, 5 mM β-glycerol phosphate, 25 mM Tris, pH 7.5) and incubated in kinase buffer supplemented with 0.2 μg of c-Jun fusion protein and 200 μM ATP for 30 min at 30 °C. The phosphorylation was terminated with LDS sample loading buffer, and the mixture was subjected to Western blotting with anti-phospho-cJun (Ser-63) antibody. Protein loading was monitored by stripping and reprobing the membrane with anti-c-Jun antibody. Immunoreactive bands were quantified by densitometry, and phospho-c-Jun levels were normalized to total c-Jun.

GSTP1 Tyrosine Phosphorylation by EGFR in a Cell-free System—The EGFR-mediated GSTP1 phosphorylation assay was performed as we previously described (35). Briefly, a 30-μl reaction mixture in a kinase buffer (10 mM $MgCl_2$ + 10 mM $MnCl_2$ in 20 mM HEPES, pH7.4) containing 0.4 μM human recombinant GSTP1, 0.05 μM human recombinant EGFR active kinase domain, and 200 μM ATP was incubated for 1 h at 30 °C, and the reaction was terminated by adding loading buffer without any reducing agents. To mimic intracellular GSH concentrations, unless otherwise stated, GSTP1 was preincubated with 5 mM GSH for 20 min at 37 °C before use in the phosphorylation assays (41, 42). The samples were subjected to SDS-PAGE under non-reducing condition in which samples did not contain reducing reagents without heating followed by Western blot analysis as described earlier.

Cell-free JNK Kinase Assay—To 0.5 μM human recombinant GSTP1 preincubated with 5 mM GSH for 20 min at 37 °C was added human recombinant full-length c-Jun to 0.15 μM, activated human recombinant full-length JNK1α1 to 0.10 μM and human EGFR active catalytic domain to 0.05 μM, and the mixture was incubated in a 200 μM ATP-supplemented kinase buffer for 30 min at 30 °C. To terminate the reaction, the mixture was boiled for 2 min and subjected to SDS-PAGE and Western blotting with anti-phospho-c-Jun (Ser-63), anti-c-Jun, Tyr(P)-100, anti-GSTP1, anti-phospho-JNK, and anti-phospho-EGFR antibodies.

Mutagenesis of Human GSTP1—The human *GSTP1* (pcDNA-DEST40/WT) and its mutant counterpart in which tyrosine 198 was mutated to phenylalanine (pcDNA-DEST40/Y198F) were generated essentially as we described previously (35). Tyrosine 198 was replaced with aspartic acid by PCR-based mutagenesis on the template plasmid vector pcDNA-DEST40/WT using the following primer sets: forward, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCCGCCCTACACCGTG-3′; reverse 1: 5′-GTTGCCATTGATGGGGAGGTTCACGTCCTCAGGGGAGGCC-3′; reverse 2, 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGTTTCCCGTTGCCATTGATGGG-3′ (the underlined sequences are for Gateway cloning). The resulting PCR product was cloned into the V5-tagged pcDNA-DEST40 destination vector (pcDNA-DEST40/Y198D) using the Gateway technology (Invitrogen), and the sequence of the product was verified by DNA sequencing. Stable transfections were performed with FuGENE HD (Roche Applied Science) into UW228 and MGR1 cell lines as described previously (35). After G418 selection, colonies were isolated, and GSTP1-V5 expression was assessed by Western blot analysis with ani-V5 antibody. Control UW228 and MGR1 cell lines were generated by transfection with the expression vector pcDNA-DEST40. All cell lines were cultured in DMEM with 10% FCS.

Detection of GSTP1-JNK Complex Formation—GSTP1-JNK interaction was examined in both a cell-free system and in tumor cells. In the former, 0.1 μM EGFR-phosphorylated and 0.5 μM unphosphorylated GSTP1 preincubated with 5 mM GSH were incubated in 200 μM ATP-supplemented kinase buffer for 30 min at 30 °C. Human recombinant full-length inactive JNK1α1 was added at 0.5 μM to a total volume 400 μl and incubated for an additional 30 min at 30 °C. The GSTP1-JNK1 complex was immunoprecipitated with Dynabeads Protein G pre-coupled with a rabbit anti-JNK1 polyclonal antibody (or normal rabbit serum control) for 10 min at room temperature, washed, and resolved by SDS-PAGE followed by Western blotting with anti-GSTP1, anti-JNK1, and anti-Tyr(P)-100 antibodies.

To examine intracellular GSTP1-JNK complex formation, parental UW228 were engineered to overexpress V5-tagged GSTP1, grown overnight in serum-free medium, and treated

with 100 ng/ml of EGF for 20 min. Cell extracts were prepared as described earlier, and 1 mg of supernatant protein was immunoprecipitated with rabbit anti-JNK antibody or normal rabbit IgG followed by Western blotting with an anti-V5 and anti-JNK1 antibodies. For the mutant GSTP1 studies, cell extracts were prepared as described earlier from V5-tagged wild-type GSTP1 (control) and V5-tagged mutant Y198F GSTP1 expressing UW228 cells that had been grown overnight in serum-free medium and treated with or without 100 ng/ml EGF for 20 min. The wild-type (GSTP1-WT) and mutant (GSTP1-Y198F) proteins were pulled down from 1 mg of cell extract protein using Dynabeads Protein G pre-coupled with a mouse anti-V5 monoclonal antibody. The pulled-down proteins were subjected to the *in vitro* binding assay with 0.2 μg of recombinant full-length inactive JNK1α1 protein in 5 mM GSH-supplemented kinase buffer for 15 min at 30 °C. After washing, the beads coupled with the GSTP-JNK complex were eluted with LDS sample buffer and analyzed by Western blot analysis. The GSTP1-bound JNK1 protein was computed as the total amount of JNK1 protein expressed in the cells to the relative amount of V5-GSTP1.

Cell-penetrating GSTP1-TAT Peptides—To further clarify the role of the GSTP1 C terminus in the interaction of EGFR-phosphorylated GSTP1 with JNK, we used wild-type and mutant Y198F GSTP1 C-domain peptides conjugated to the cell-penetrating TAT sequence (43, 44) to optimize their intracellular delivery. The TAT peptide used GRKKRRQRRRP (GenScript, Piscataway, NJ), which was derived from HIV-TAT protein transduction domain (amino acids 47–57) via two proline residues as a spacer. Exponentially growing parental UW228 cells were treated with 10 μM wild-type TAT-Tyr-198 GSTP1 peptide, the mutant TAT-Y198F GSTP1 peptide, or control TAT peptide in serum-free medium. After 15 h, the UW228-WT cells were treated with 100 ng/ml EGF for 20 min, and cell extracts prepared and used to assay JNK activity, as described earlier.

In Situ Proximity Ligation Assay (PLA) for Localization of GSTP1-JNK Complex in Cells—The intracellular localization of the GSTP1-JNK complex and the impact of EGFR-mediated GSTP1 phosphorylation on it was performed by a proximity ligation assay, PLA (45), using the Duolink™ *in situ* PLA (Olink Bioscience, Uppsala, Sweden) performed as recommended by the manufacturer with minor modifications. Briefly, 10^5 cells were grown overnight on a glass coverslip in a 6-well plate. After fixation in 4% formaldehyde/PBS for 20 min, the cells were permeabilized with 0.1% Triton X-100, PBS for 30 min and blocked in Duolink™ Blocking stock. Primary antibodies, rabbit anti-GSTP1 polyclonal and mouse anti-MAPK8 monoclonal antibodies (Abnova Taiwan Corp., Taipei, Taiwan), were to 1:1200 (v/v) and 1:50 (v/v), respectively. After primary antibody staining at 4 °C overnight, the diluted PLA probes, anti-mouse MINUS and anti-rabbit PLUS, were incubated for 1 h at 37 °C. Subsequent hybridizations, ligations, amplifications, and final wash steps were performed according to the Abnova modified protocol. Detection was performed using DuoLink™ Detection kit 613 that includes 613 fluorophore-labeled oligonucleotides and Hoechst 33432 nuclear dye. Coverslips were air-dried, mounted, and analyzed using an Zeiss Axioskop 2 fluorescence microscope with oil immersion lenses at ×100 magnifications. Band-pass filter setting for Hoechst33342 and red signals were 420 – 480 nm and 560 – 615 nm, respectively. PLA signal dots were counted using Blobfinder image analysis software (The Centre for Image Analysis, Uppsala University, Uppsala, Sweden).

Caspase-3/7 Activation Assay—Apoptotic induction was determined after a 24-h treatment of the cells with 25 μM cisplatin and/or 300 nM SP600125 as we previously described. This was determined as the level of activation of caspases 3/7 in the cells using the SensoLyte® Homogeneous Rh110 Caspase 3/7 assay kit (AnaSpec, San Jose, CA). The level of caspases-3/7 activation was measured as the enzymatic cleavage of the profluorescent substrate $(Z\text{-DEVD})_2$-Rh110 (Z-, benzyloxycarbonyl), and the release of the fluorescent rhodamine 110-cleaved group. Briefly, cells were incubated in a 96-well plate in the presence or absence of drug for 24 h. The $(Z\text{-DEVD})_2$-Rh110-containing caspase-3/7 reagent (AnaSpec) was added to each well, and after 3 h at room temperature the fluorescence was measured at excitation and emission wavelengths of 485 nm and 528 nm, respectively. The level of caspase 3/7 activation was expressed as relative fluorescence units to untreated or untransfected controls.

siRNA-mediated GSTP1 Down-regulation—Small interfering RNA targeting human *GSTP1* (HSS104546) and control siRNA (Stealth RNAi Negative Universal Control Duplexes LO, 46-2002) were obtained from Invitrogen. Tumor cells grown in antibiotic-free medium were transfected with Lipofectamine RNAiMAX (Invitrogen) containing GSTP1 or control siRNA at a final concentration of 15 nM. After a 72-h incubation the cells were treated and processed as per experimental protocol. Aliquots were monitored for the level of GSTP1 gene transcripts and protein.

Statistical Analyses—Differences between cell lines and between different treatments were assessed for statistical significance using Student's *t* test ($p < 0.05$). Each experiment was repeated at least three times, and the means ± 1 S.D. or S.E. were used in all statistical analyses.

Results

GSTP1-dependent Inhibition of JNK Is Significantly Enhanced in Tumor Cells after EGFR Ligand-dependent Activation—The effect of EGFR-dependent tyrosine phosphorylation of GSTP1 on the ability of GSTP1 to regulate JNK signaling was first examined using the parental GSTP1−ve UW228 and the UW228*1C cell lines, the latter engineered to overexpress GSTP1. The status of the JNK signaling pathway was monitored in the cells after treatment with the EGFR ligand, EGF. The results are summarized in Fig. 1, *A* and *B*. In both cell lines EGF treatment (EGFR activation) induced a rapid and significant phosphorylation of the JNK downstream target, c-Jun; however, the level of phospho-cJun was 2.1-fold higher in the parental UW228 than in the GSTP1+ve UW228*1C despite the presence of equal levels of cJun (Fig. 1*A*). Similarly, although in both cell lines the Thr-183/Tyr-185 double phosphorylation of JNK was induced after EGFR activation, the level in the parental GSTP1−ve cells was twice that in the GSTP1+ve cells. The histogram in Fig. 1*B* quantifies the

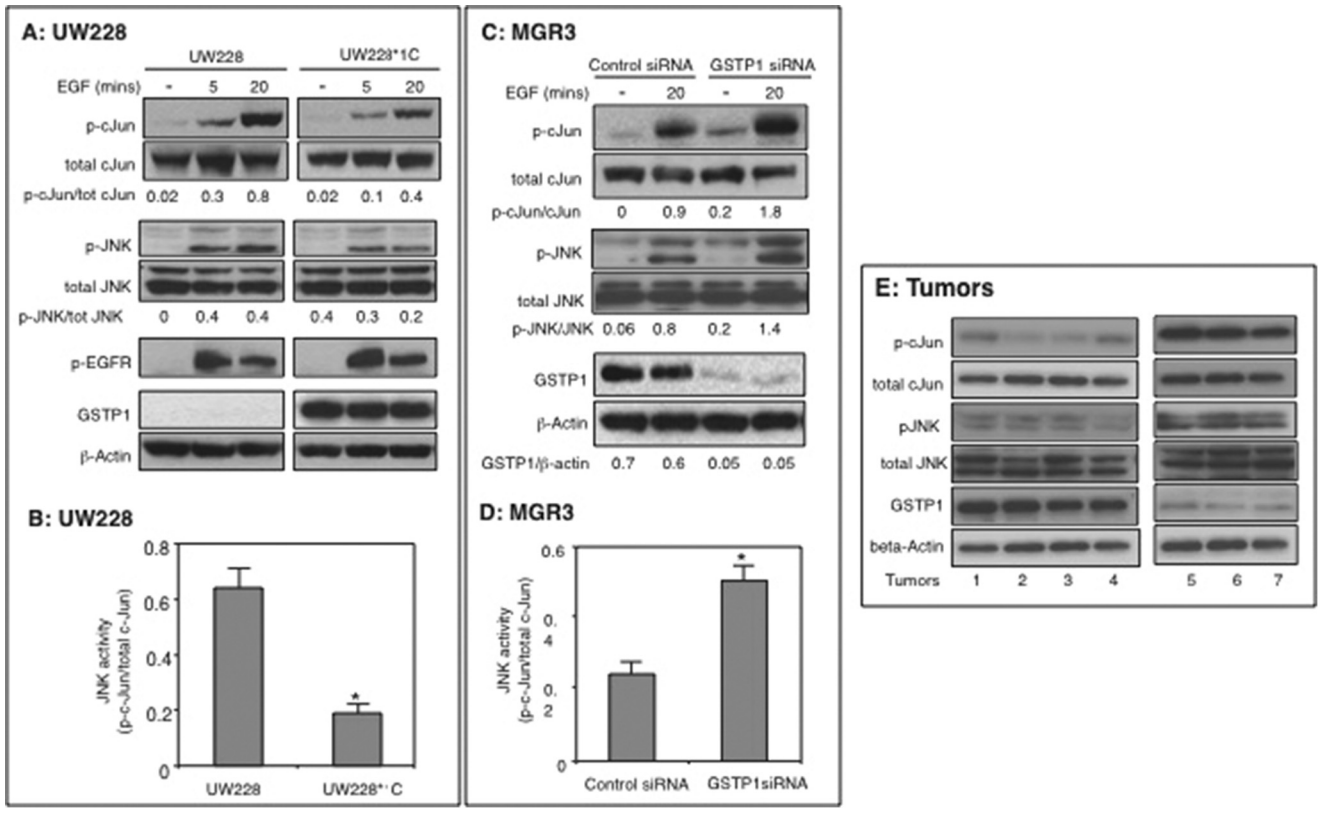

FIGURE 1. **EGFR activation enhanced GSTP1-dependent suppression of JNK activation in human brain tumor cells.** *A*, Western blots showing EGF-dependent induction of phosphorylation of JNK (Thr-183/Tyr-185) and its downstream target, c-Jun (Ser-63), in UW228 cells. The EGF-induced c-Jun phosphorylation in parental GSTP1−ve UW228 cells was suppressed in GSTP1+ve UW228*1C cells. *B*, histogram showing a 3-fold lower EGF treatment-induced suppression of JNK activity in UW228*1C cells compared with control UW228 cells. *C*, Western blots showing increased c-Jun phosphorylation (JNK pathway activation) in MGR3 cells after siRNA-mediated GSTP1 knockdown. Note the increase in c-Jun phosphorylation after EGF treatment of the cells. *D*, JNK activity in MGR3 cells was increased >2-fold (*, $p < 0.01$) after GSTP1-knockdown. *E*, relationship between GSTP1 expression and JNK pathway activity (phospho-JNK and phospho-cJun levels) in primary human glioblastomas. Note the significantly suppressed JNK pathway activity in the four high GSTP+ve tumors compared with the three low GSTP1-expressing tumors.

EGF treatment-induced changes in JNK activity, determined as the ratio of phosphorylated to total c-Jun in the two UW228 cell lines. After EGF treatment, JNK activity was decreased by 64% in UW228*1C cells compared with control UW228 cells. The results of EGF activation on JNK activity and downstream signaling in the naturally GSTP1-overexpressing human GBM cell line, MGR3, with and without siRNA-mediated GSTP1 suppression are summarized in Fig. 1*C*. There was a significant, 2.0-fold increase in the level of phospho-cJun in the EGF-treated, GSTP1 siRNA-transfected cells relative to the control without GSTP1 suppression. Total c-*jun* levels were the same in all the cells. Fig. 1*D* summarizes the results quantitatively and shows that GSTP1 knockdown increased EGF-induced JNK activity by >2-fold ($p < 0.01$) over controls. Fig. 1*E* shows the relationship between GSTP1 and JNK signaling in seven human primary GBMs specimens that we grew *in vivo* as xenografts. The four tumors (Fig. 1*E*, *left panel*) with constitutively high GSTP1 had significantly lower levels of both activated JNK and phospho-cJun despite the presence of relatively high levels of JNK and cJun. In contrast, in the three tumors (Fig. 1*E*, *right panel*) with low levels of GSTP1, phospho-JNK and phospho-cJun levels were significantly elevated relative to total JNK and total cJun, respectively. These results demonstrate that GSTP1 suppresses activation of JNK and its downstream target, cJun, and as shown with the UW228 and MGR3 cell lines, EGFR activation enhances this GSTP1-dependent suppression of JNK signaling.

Tyrosine Phosphorylation of GSTP1 Shifts the GSTP1 Dimer-Monomer Equilibrium toward the Monomeric State and Enhances Intracellular JNK Inhibition—Previous studies (30–32) have suggested that monomeric GSTP1 binds to JNK with a higher affinity and regulates JNK signaling more effectively than dimeric GSTP1. We, therefore, examined the effect of EGFR-mediated GSTP1 tyrosine phosphorylation on the equilibrium between the monomeric and dimeric/oligomeric forms of GSTP1 in both a cell-free system and in tumor cells. In the cell-free system, after phosphorylation of GSTP1 by EGFR, SDS-PAGE under non-reducing conditions was performed followed by Western blotting with anti-GSTP1, anti-phosphotyrosine, and anti-EGFR antibodies. The results (Fig. 2*A*) show that in the presence of 5 mM GSH (to mimic intracellular GSH levels), unphosphorylated GSTP1 was present as the 23-kDa monomer, the 46-kDa dimer, and other multimeric forms (*second lane*). In contrast, in the presence of EGFR, GSTP1 existed predominantly as the 23-kDa monomeric form (Fig. 2*B*, *fourth lane*).

In the EGFR overexpressing GBM cell line, U87MG.wtEGFR, the results (Fig. 2*C*) of Western blotting for GSTP1 after non-reducing SDS-PAGE showed the dimeric form of GSTP1 to predominate over the monomeric form in untreated cells. In

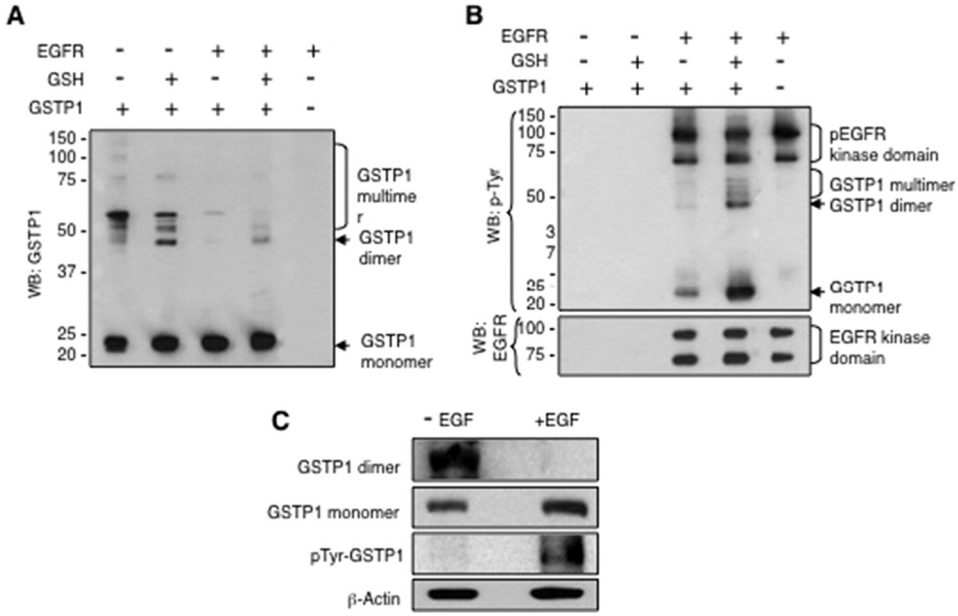

FIGURE 2. **EGFR-mediated GSTP1 phosphorylation shifts the GSTP1 dimer-monomer equilibrium toward the monomeric form.** *A* and *B* are from SDS-PAGE conducted under non-reducing conditions followed by Western blotting (*WB*) with anti-GSTP1. After its phosphorylation by EGFR in a cell-free system in the presence of physiological concentration of GSH, GSTP1 dimerization/oligomerization was significantly diminished (2.2-fold) relative to the 23-kDa GSTP1 monomer. In *C*, EGF treatment of EGFR-overexpressing U87MG.wtEGFR cells increased GSTP1 phosphorylation and with it the level of monomeric GSTP1. Note the almost undetectable levels of GSTP1 dimers in cells treated with EGF.

contrast, after EGF treatment, dimeric GSTP1 was undetectable, and only the monomer, present exclusively as tyrosine-phosphorylated GSTP1, was observed. These results are consistent with recent reports in which kinetic time-resolved and steady-state anisotropy showing that GSTP1 exists predominantly in the more stable dimeric form (46–48). Our results demonstrate an increased propensity of tyrosine-phosphorylated GSTP1 to dissociate from the dimeric state into monomers.

GSTP1 Tyrosine Phosphorylation by EGFR Promotes Intracellular Formation of the GSTP1-JNK1 Complex and Enhances Inhibition of JNK and Its Downstream Signaling—The physiological and functional consequences of the GSTP1 monomer-dimer transition has been a subject of debate (30–32, 46–48), with some data indicating that the monomer is preferred for binding to JNK, whereas other reports support the dimer as the preferred form for JNK binding. Our data (Fig. 2) show that GSTP1 tyrosine phosphorylation results in a shift in the GSTP1 monomer-dimer equilibrium to favor the monomeric state. We, thus, examined the effect of this on the ability of GSTP1 to bind to JNK and to inhibit JNK downstream signaling both in a cell-free system and in tumor cells. In the former, using recombinant cJun, activated JNK1, GSTP1, and EGFR proteins, we showed that suppression of phosphorylation of Ser-63 in the JNK downstream target, c-Jun, by EGFR-phosphorylated GSTP1 was approximately 2.0-fold more than that by unphosphorylated GSTP1 ($p < 0.01$; Fig. 3, *A* and *B*) despite the presence of equal levels of phospho-JNK in both cases. We then examined in a cell-free system the effect of the phosphorylation on the binding of GSTP1 to JNK1 using recombinant JNK1 incubated with EGFR (kinase domain)-pre-phosphorylated GSTP1 and ATP followed by immunoprecipitation with anti-JNK1 antibody and Western blotting with anti-GSTP1 antibody. The results (Fig. 3*C*) show that the binding of tyrosine-

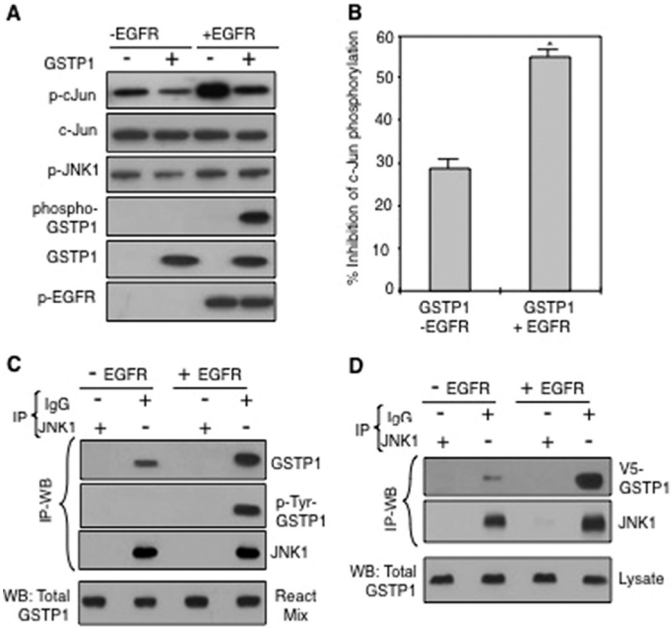

FIGURE 3. **Tyrosine phosphorylation of GSTP1 by EGFR enhanced GSTP1-dependent suppression of GSTP1-JNK complex formation and JNK activity.** *A–C*, cell-free system. *D*, UW228 cells. *A*, Western blotting showing suppression of c-Jun Ser-63 phosphorylation by GSTP1 incubated with EGFR in a cell-free system. *B*, quantitation of effect of EGFR-dependent phosphorylation on GSTP1 inhibition of JNK activity (c-Jun phosphorylation) in cell-free system. Phospho-cJun levels were normalized to those of total c-Jun and expressed as % inhibition by GSTP1 (mean ± S.E.; *, $p < 0.05$). *C*, immunoprecipitation (*IP*)/Western blotting showing enhanced GSTP1 binding to JNK1 after tyrosine phosphorylation of GSTP1 by EGFR in a cell-free system. Western blotting (*WB*) was used to monitor levels of GSTP1 levels in the reaction mixtures (*lowest panel*). *D*, immunoprecipitation/Western blotting showing enhancement of GSTP1-JNK1 complex formation after EGFR activation in GSTP1+ve UW228 cells relative to UW228 cells. Western blotting of total GSTP1 in cell lysates is shown in the *lowest panel*.

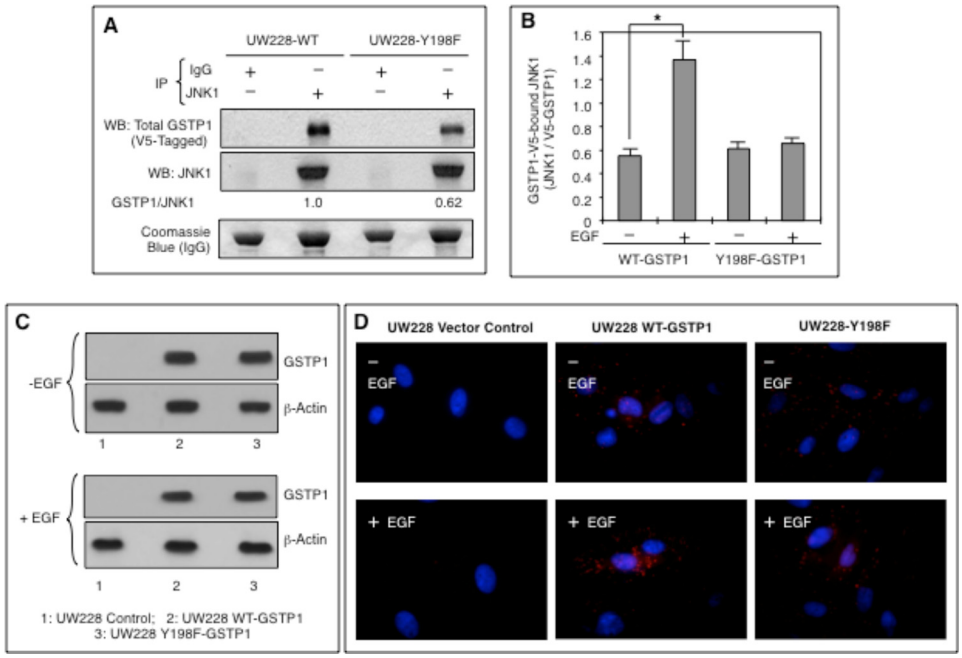

FIGURE 4. **GSTP1 carboxyl-terminal tyrosine 198 is critical for formation of JNK-GSTP1 complex in tumor cells.** *A*, immunoprecipitation (*IP*)/Western blotting (WB) blotting of cell extracts showing EGF treatment-induced GSTP1-JNK complex formation in UW228 V5-GSTP1 (wild-type GSTP1) but not mutant V5-Y198F GSTP1-expressing UW228 cells. Coomassie Blue Brilliant-stained IgG heavy chain bands were used to monitor loading of lanes. *B*, quantitation of EGFR activation-induced GSTP1-JNK1 complex formation in wild-type GSTP1-transfected UW228 cells relative to Y198F GSTP1 cells (*, $p < 0.01$). *C*, Western blot for GSTP1 in lysates of UW228 cells transfected with vector control, wild-type GSTP1, and mutant Y198F GSTP1. Cells were used in the *in situ* PLA. *D*, visualization of the subcellular localization of GSTP1-JNK complex by PLA of control and wild-type- and mutant Y198F-expressing UW228 cells after EGFR activation. Note the significantly increased cytoplasmic/extranuclear GSTP1-JNK1 complex formation (*red dots*) after EGF treatment of wild-type GSTP1-expressing UW228 cells compared with cells expressing the mutant Y198F GSTP1. Hoechst 33342 blue counter-fluorescence of DNA was used to identify cell nuclei.

phosphorylated GSTP1 to JNK1 (*fourth lane*) was 3.1-fold higher than that of unphosphorylated GSTP1 (*second lane*). After the cell-free studies we examined the effect of EGFR-dependent GSTP1 phosphorylation on GSTP1-JNK1 binding in tumor cells by co-immunoprecipitating JNK1 from lysates of V5-GSTP1 overexpressing UW228 cells followed by Western blotting for GSTP1. The results (Fig. 3*D*) showed the GSTP1-JNK1 complex, barely detectable in untreated cells, to increase dramatically upon EGF treatment. Together, these results demonstrate that tyrosine phosphorylation of GSTP1 by EGFR promotes formation of the GSTP1-JNK1 complex and inhibits JNK activation. The observation that tyrosine phosphorylation of GSTP1 shifts the GSTP1 monomer-dimer balance to the monomeric form and increases the formation of the JNK-GSTP1 complex argues for a higher JNK binding affinity of the GSTP1 monomer over the dimer.

EGFR-dependent Phosphorylation of C-terminal Tyrosine 198 Promotes Binding of GSTP1 to JNK1—The C-terminal domain of GSTP1, which includes residues 194–201, has been suggested to be involved in the interaction of GSTP1 with JNK (31). Because this putative JNK-interacting domain contains tyrosine residue 198, which we had previously shown to be phosphorylated by EGFR (35), we examined the effect of its phosphorylation on both GSTP1-JNK1 binding and JNK downstream signaling. For this, *in vitro* binding of GSTP1 to recombinant JNK1 protein was assayed with V5-tagged GSTP1 proteins pulled down from extracts of UW228 cells with and without EGF treatment. The cells had been stably transfected with a V5-tagged wild-type GSTP1 or Y198F GSTP1 in which Tyr-198 had been mutated to phenylalanine and, thus, rendered unphosphorylatable. The results (Fig. 4*A*) show that in EGF-treated cells wild-type GSTP1 binding to JNK1 was significantly higher than in the EGF-naive cells. In contrast, in cells expressing the unphosphorylatable GSTP1 Y198F mutant protein, EGF treatment had no effect on GSTP1-JNK1 binding. Co-immunoprecipitation with anti-JNK1 antibody (Fig. 4*B*) showed a 39% lower level of GSTP1 binding to JNK1 in cells expressing the GSTP1 Y198F mutant than in those expressing wild-type GSTP1, indicating a significant effect of GSTP1 C-terminal Tyr-198 phosphorylation on GSTP1-JNK1 complex formation in cells.

Intracellular Ligation of GSTP1-JNK Complex by in Situ PLA—The results of the *in situ* PLA visualization of the intracellular localization of the GSTP1-JNK1 protein complex are summarized in Fig. 4, *C* and *D*. The Western blots of the cell lysates (Fig. 4*C*) show that GSTP1 levels in UW228 cells transfected with wild-type and mutant GSTP1 were relatively equal, and as expected, control UW228 cells transfected with vector alone were negative for GSTP1. The fluorescence micrographs in Fig. 4*D* show that, in wild-type GSTP1 expressing cells, the GSTP1-JNK1 complex (*red spots*) increased significantly upon EGF treatment relative to untreated cells without EGF treatment. In contrast, there was no difference in the levels of the GSTP1-JNK1 complex in cells with the mutant Y198F GSTP1 without and without EGF treatment. Interestingly, in all the cells the GSTP1-JNK1 complex was exclusively cytoplasmic even after EGFR activation. This and our earlier results (Fig. 3) demonstrating that GSTP1 binds to both phosphorylated and unphosphorylated JNK indicate that in both forms the JNK-GSTP1 complex does not translocate to the cell nucleus.

TABLE 1

Effect of EGFR ligand activation on JNK activity in UW228 cells carrying wild-type and mutant GSTP1 C-terminal peptides

Exponentially growing UW228 cells were treated overnight with 10 μM concentrations of wild-type (Tyr-198) and mutant (Y198F) GSTP1 effector domain peptides conjugated to the cell-penetrating HIV TAT sequence followed by 100 ng/ml EGF for 20 min. Cell extracts were prepared, and JNK activity was determined as described under "Experimental Procedures." Underlines indicate sequences 194–201.

Cell-permeable GSTP1-Tyr-198 peptide	JNK activity (phospho-cJun/total cJun)		
	−EGF	+EGF	-Fold increase
No treatment	0.28 ± 0.02	0.32 ± 0.04	1.14
GRKKRRQRRR-PP (control TAT)	0.27 ± 0.01	0.35 ± 0.03	1.29
TAT-Tyr-198 GRKKRRQRRR-PP-ASPEYVNL201	0.31 ± 0.04	0.91 ± 0.07	2.24
TAT-Y198F GRKKRRQRRR-PP-ASPEFVNL	0.30 ± 0.03	0.39 ± 0.02	1.3

Cell-penetrating GSTP1-TAT Peptides—The results of these studies are summarized in Table 1. As shown, relative to control untreated cells, treatment of UW228 cells with the cell-permeable TAT-GSTP1-Y198 peptides increased intracellular JNK activity in UW228 cells by 2.24-fold relative to controls. In contrast, the change in JNK activity was the same as in untreated controls for cells treated with the mutant TAT-GSTP1-Y198F or unconjugated TAT peptides (control). These results are similar to those observed with the full-length wild-type and mutant GSTP1 proteins in which EGF treatment had no effect on binding of the GSTP1 Y198F mutant protein to JNK but significantly increased JNK binding of wild-type GSTP1 with the intact C-terminal Tyr-198. Together these data indicate that the GSTP1 C terminus containing tyrosine 198 is critical for the EGFR-mediated suppression of JNK signaling by GSTP1.

C-terminal Phosphorylation of GSTP1 Suppresses JNK Activity and Apoptotic Induction in Tumor Cells—GSTP1 has been implicated in the apoptotic process via both JNK-mediated and JNK-independent pathways (27, 49), consistent with the multifunctionality of the GSTP1 protein. In this study we examined the significance of the GSTP1 C-terminal tyrosine 198 phosphorylation on both cellular JNK downstream signaling and drug-induced apoptosis. For this, the GSTP1−ve UW228 and the low GSTP1-expressing MGR1 were engineered to stably express high levels of wild-type GSTP1, the previously described unphosphorylatable mutant GSTP1 Y198F, or the phosphomimic mutant GSTP1 Y198D in which tyrosine 198 was mutated to aspartic acid. The resulting cell lines, UW228-GSTP1-WT, UW228-GSTP1-Y198F, MGR1-GSTP1-WT, MGR1-GSTP1-Y198F, and MGR1-GSTP1-Y198D were treated with EGF to activate EGFR, and the level of JNK signaling was examined. The results, summarized in Fig. 5*A*, show that, relative to controls, EGFR ligand activation resulted in a significant decrease in JNK downstream signaling (phosphorylation of JNK and c-Jun) in cells with wild-type GSTP1 (UW228 and MGR1) and Y198D GSTP1 (MGR1). In contrast, in both UW228 and MGR1, JNK signaling remained unchanged in cells expressing Y198F GSTP1 compared with controls. To rule out any effects of an upstream kinase on these differences in JNK signaling, we examined phospho-MKK4, the immediate intracellular upstream kinase of JNK, and showed no GSTP1-dependent difference in its level between the cell lines. The results of the JNK activity assays were consistent with those of the phosphorylation assays and are summarized in Fig. 5, *C* and *D*, for UW228 and MGR1, respectively. In both cell lines, expression of wild-type GSTP1 or Y198D-GSTP1 was associated with decreases in JNK activity compared with vector control of 69% (*, $p < 0.01$) for UW228-GSTP1-WT, 58% (*, $p < 0.01$) for MGR1-GSTP1-WT, and 49% (**, $p < 0.05$) for MGR1-GSTP1-Y198D. In contrast, JNK activity increased 2.0-fold (**, $p < 0.05$) and 2.5-fold (*, $p < 0.01$), respectively, in UW228 and MGR1 cells expressing the mutant Y198F-GSTP1 relative to the wild-type GSTP1-expressing cells.

The impact of the GSTP1 Tyr-198 phosphorylation on apoptotic induction was investigated using the GSTP1 wild type and GSTP1 mutant transfectant cells described above, following treatment with the apoptosis-inducing agent, cisplatin. The results (Fig. 5, *E* and *F*) show a significantly higher level of apoptosis (caspase 3/7 activation) induced by cisplatin in cells of both UW228- and MGR1-expressing mutant GSTP1-Y198F by 1.9-fold (*, $p < 0.05$) and 3.2-fold (*, $p < 0.01$), respectively, than in cells expressing WT-GSTP1 or the GSTP1-Y198D. Treatment with 300 nM JNK-specific inhibitor, SP600125, decreased apoptotic induction in both UW228 and MGR1 cell lines by 2.8-fold and 3.0-fold, respectively (*, $p < 0.01$) (Fig. 5*G*). The ability of WT-GSTP1 and the phospho-mimic Y198D mutant GSTP1 to protect cells against drug-induced apoptosis together with the decreased apoptotic response associated with pharmacological inhibition of JNK supports a significant role of JNK and its inhibition by EGFR-mediated tyrosine-phosphorylated GSTP1 in cellular apoptotic response.

EGFR-dependent GSTP1 Phosphorylation Shifts GSTP1 to the Monomeric State and Increases Apoptosis in Tumors in Vivo—These studies were performed using *in vivo* nude mouse xenografts of two human GBMs, 10T and 6B, that express low and high levels of EGFR, respectively. To determine the state of the GSTP1 monomer-dimer equilibrium, we analyzed the levels of GSTP1 dimers and monomers in extracts of exponentially growing 10T and 6B GBMs by non-reducing SDS-PAGE/Western blotting. The results (Fig. 6) show that in the low EGFR-expressing 10T xenografts, dimeric GSTP1 was more dominant than the monomer. In contrast, in the 6B xenografts with high levels of EGFR, the GSTP1 dimer-monomer equilibrium shifted to the monomeric form (Fig. 6, *A* and *B*). Immunoprecipitation with anti-GSTP1 followed by SDS-PAGE and Western blotting with anti-phosphotyrosine antibody confirmed GSTP1 in the 6B GBM to be more highly tyrosine-phosphorylated by 3.2-fold than that in the 10T (Fig. 6*D*). The results of the analysis of JNK signaling in the two *in vivo* tumors are summarized in Fig. 6*C* and show a higher level of phospho-c-Jun in 10T than in 6B. We then examined the level of spontaneous apoptosis in 6B- and 10T-growing xenografts relative to the level of GSTP1 phosphorylation in the tumors. The results (Fig. 6*E*) show a significant, ~3-fold, decrease in the level of apopto-

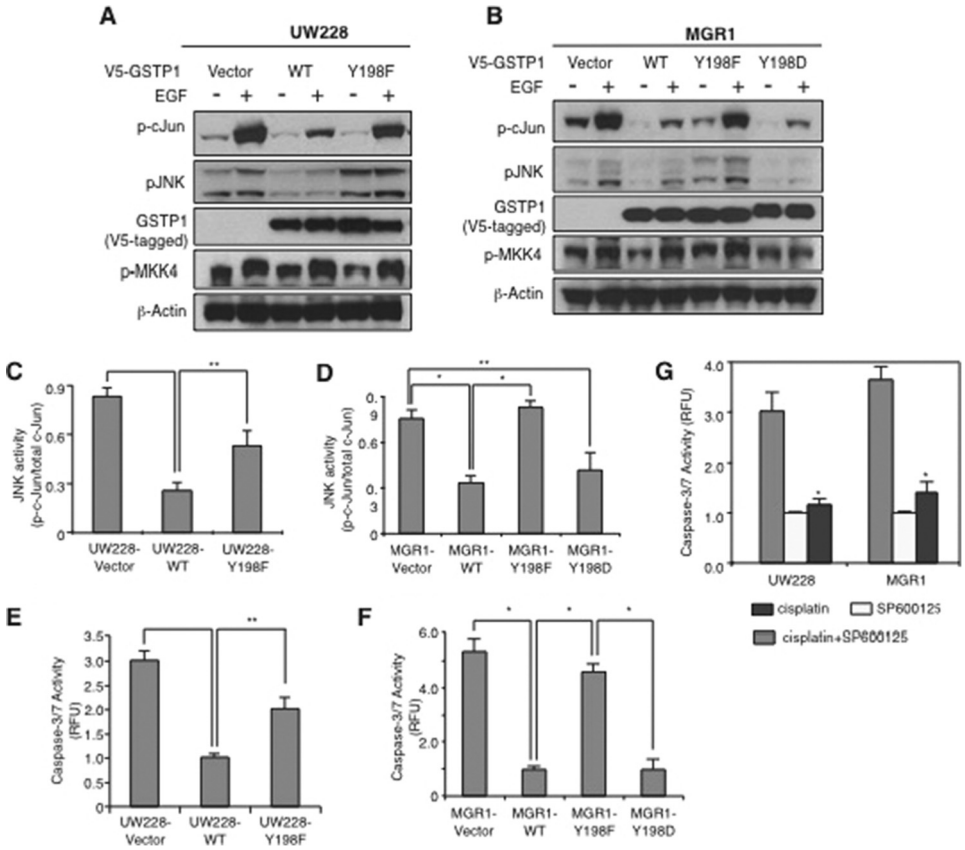

FIGURE 5. **GSTP1 phosphorylation is required for inhibition of JNK activity and JNK downstream signaling in tumor cells.** Using wild-type GSTP1 and mutant phosphomimetic (Y198D) and non-phosphomimetic (Y198F) GSTP1, the inhibition of JNK activity and JNK downstream signaling is shown to be attenuated in both UW228 (*A* and *C*) and MGR1 (*B* and *D*) cells expressing mutant Y198F GSTP1 relative to cells expressing wild-type GSTP1 (*WT*) or mutant Y198D GSTP1 (*A* and *B*). The changes in JNK signaling were independent of the phosphorylation status of MKK4, an upstream kinase of JNK (*C* and *D*). Caspase-3/7 activation was suppressed after 24 h of treatment with 25 μM cisplatin in both in the WT and Y198D transfectant UW228 (*E*) and MGR1 (*F*) cells compared with cells with vector control; caspase-3/7 activity in Y198F GSTP1 transfectants was higher than that in the WT GSTP1 transfectants, UW228-WT (**, $p < 0.05$) and MGR1-WT (*, $p < 0.01$), respectively. *G*, JNK inhibition suppresses caspase 3/7 activation induced by cisplatin in brain tumor cell lines. Combination treatment with the JNK inhibitor and cisplatin reduced the levels of caspase-3/7 activation significantly in both UW228 and MGR1 cell lines (*, $p > 0.01$).

sis in 6B than in 10T xenografts, consistent with the higher level of tyrosine-phosphorylated GSTP1 and higher level of JNK suppression in the former. These *in vivo* observations together with those *in vitro* provide support for a mechanism of protection against apoptosis that involves the increased ability of EGFR-phosphorylated GSTP1 to inhibit the JNK signaling pathway.

Discussion

The JNK signaling pathway is involved in several critical cellular processes, including those of life and death of the cell. The findings in this study, using *in vitro* and *in vivo* human brain tumor models, describe a novel EGFR-dependent GSTP1-mediated mechanism of regulation of JNK signaling, in which the GSTP1-mediated inhibition of JNK and its downstream signaling (30) is significantly enhanced by EGFR-dependent tyrosine phosphorylation of GSTP1. We further characterized the involvement of the GSTP1 C-terminal region in this process primarily because it harbors the putative JNK interacting domain of GSTP1 (31, 32) and contains Tyr-198, a primary phospho-acceptor residue phosphorylated by EGFR in GSTP1 (35). The major finding is that the tyrosine-phosphorylated GSTP1 shifts the GSTP1 dimer-monomer equilibrium to the monomeric state, thereby acquiring an enhanced ability to bind to and suppress the JNK downstream signaling and, ultimately, the cellular apoptotic process.

In the normal unstressed cellular state, GSTP1, a dimeric protein, exists in equilibrium with its monomeric subunits (46–47). Structural factors that contribute to GSTP1 dimer formation include the hydrophobic lock and key interaction between subunit interfaces and the electrostatic attractions associated with the interactions at the dimer interface (50). Under cellular stress, such as that from oxidative metabolism, however, GSTP1 dissociates from the GSTP1-JNK1 complex, leading subsequently to the activation of JNK (30). This intracellular switch from the monomeric to the dimeric form of GSTP1 and the associated altered JNK signaling provides a mechanism of cellular response to and protection from oxidative and other potentially deleterious stress (50, 51).

Throughout this study we observed that whereas increased JNK phosphorylation was always associated with increased cJun phosphorylation, and vice versa, the relationship between the levels of phospho-JNK and phospho-cJun, although always positive, was not necessarily linear and varied from tumor to tumor. This observation suggests that other factors can impact the level of cJun phosphorylation and/or the JNK signaling pathway in cells. Among such factors are the levels of proteins,

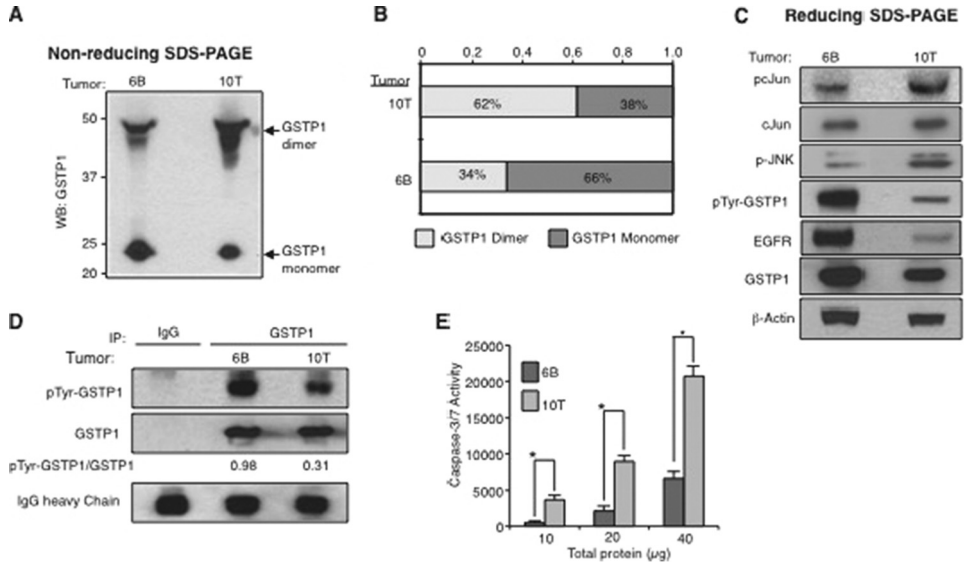

FIGURE 6. **EGFR tyrosine phosphorylation of GSTP1 shifts GSTP1 monomer-dimer equilibrium and enhances JNK signaling in glioblastomas growing *in vivo* in nude mice.** *A*, non-reducing SDS-PAGE and Western blotting of tumor extracts of low (*10T*) and high (*6B*) EGFR-expressing human GBMs show a shift in GSTP1 dimeric to the monomeric form in 6B and to the dimer in 10T tumors. *B*, relative quantitative expression of GSTP1 monomers and dimers in 6B (high EGFR) and 10T low EGFR-expressing glioblastomas. *C*, reducing SDS-PAGE followed by Western blotting showed higher levels of EGFR in 6B xenograft than in 10T. Although high in both 6B and 10T, the level of total GSTP1 in 6B was 1.4-fold higher than that in 10T, and phosphorylation of c-Jun was suppressed in 6B compared with in 10T. *D*, immunoprecipitation with anti-GSTP1 antibody followed by Western blotting analysis with anti-phosphotyrosine showed 3.2-fold higher levels of tyrosine phosphorylation of GSTP1 in 6B than in 10T. *E*, the level of spontaneous apoptosis (caspase-3/7 activity) increased by ~3-fold in 10T than in the 6B (*, $p < 0.05$).

such as JNK inhibitory proteins, dual-specificity phosphatases, which dephosphorylate JNK and other MAPKs, as well as cellular protein/phosphoprotein processing, including ubiquitination, SUMOylation, etc. (51–54).

Previously, we showed that the three primary phospho-acceptor tyrosine residues, namely, Tyr-3, Tyr-7, and Tyr-198, phosphorylated by EGFR, contribute to the three-dimensional structure of the GSTP1 the subunit interface and active site (33, 35). Our present data demonstrate that EGFR phosphorylation of one of these residues, namely, the C-terminal Tyr-198, affects the dimer-monomer equilibrium of GSTP1 and contributes to reversal of the shift of GSTP1 from the dimeric to the monomeric form after phosphorylation of GSTP1 by EGFR not only in a cell-free system but, more importantly, in cells growing under physiological unstressed conditions *in vitro* and in tumors growing *in vivo*. The monomeric phosphotyrosine-GSTP1 acquires an enhanced ability to bind to and to inhibit JNK. Interestingly, it has been previously reported that under oxidative and nitrosative stress, both *in vitro* and *in vivo*, post-translational *S*-glutathionylation of Cys-47 and Cys-101 in GSTP1 breaks the ligand binding interaction of GSTP1 with JNK, resulting in GSTP1 oligomerization (55).

The studies in the present report were conducted exclusively with the human GSTP1. This is important because to date studies of JNK-GSTP1 interactions have, for the most part, been conducted with the mouse GSTp protein. These studies have shown that residues 194–201 (SSPEHVN in the C terminus of GSTp is an effector domain in the binding of GSTp to, and subsequent inhibition of JNK (31) in part because it contains two positively charged residues, arginine and histidine (56, 57), which allows it to interact with the negatively charged binding face of JNK1. In contrast, in human GSTP1 (33, 58) the C-terminal 194–201 domain containing residues ASPEYVNL, unlike that of the mouse GSTp (56, 57), is not positively charged, suggesting that non-ionic interactions will predominate in its interaction with JNK and, as such, structurally the human GSTP1-JNK1 complex may differ somewhat from the mouse GSTp-JNK complex. In a previous study (33) we showed that the phospho-acceptor C-terminal Tyr-198 in the human GSTP1 is absent from the mouse GSTp.

Based on our results with the mutant Y198D in which phosphotyrosine is replaced by the negatively charged aspartate, we speculate that the increased binding of EGFR phosphorylated GSTP1 to JNK observed in this study is the result at least in part from the increased electronegativity of the human GSTP1 C-terminal region upon phosphorylation of Tyr-198 by EGFR. This is supported by our observation that although tyrosine-phosphorylated GSTP1 binds both unphosphorylated JNK as well as Thr-183/Tyr-185 phosphorylated/active JNK1, the binding of the former is less than that of the latter. Furthermore, the wild-type GSTP1-dependent suppression of JNK is recovered in cells transfected with the Y198D mutant GSTP1, with Tyr-198 mutated to aspartic acid. The Y198D GSTP1 structurally mimics tyrosine-phosphorylated GSTP1, and the charged aspartate restores the interactions of residue amino acid residue 198 in the C-terminal domain with other residues in the GSTP1 protein.

In this context, another important finding in this study is that JNK downstream signaling is suppressed upon activation of EGFR in cells expressing wild-type GSTP1 but not in cells with GSTP1 in which Tyr-198 was mutated to phenylalanine and, thus, cannot be phosphorylated by EGFR. Our results also indicate, as would be expected, that the effect of EGFR-dependent tyrosine phosphorylation of GSTP1 on JNK signaling is dependent on wild-type GSTP1 expression in the cells. Thus, after transfection with GSTP1-Y198F, JNK activation was signifi-

cantly lower in UW228 than in MGR1 cells, consistent with the absence of GSTP1 in the former and its presence in the latter.

An interesting finding in these studies is the relatively modest effect of GSTP1 on JNK activity in UW228 cells. We believe that this is related to the fact that the primary tumor from which the UW228 cell line was derived was naturally GSTP1−ve. The dependence of UW228 on GSTP1 to drive its biology is thus mitigated, in contrast to cells of tumors that naturally express high levels of GSTP1. The results with the MGR3 glioblastoma cell line (Fig. 1*C*) and the new results with the naturally high and low GSTP1 expressing tumors (Fig. 1*E*) support this notion and indicate unequivocally the strong direct relationship between GSTP1 expression and suppression of JNK signaling. The results of the studies using the cell-penetrating TAT-Tyr-198 peptide, in which residues 194–201 of the human GSTP1 were conjugated to the cell-permeable TAT sequence (47, 48) to enhance intracellular delivery, showed this peptide to enhance EGF-induced JNK activation in GSTP1-overexpressing cells, whereas TAT-Y198F mutant and control TAT peptides had no effect on the JNK activity. This suggests a competition of the wild-type and Y198F mutant GSTP1 C terminus for binding to JNK. Together, these data demonstrate that the EGFR-mediated phosphorylation of tyrosine 198 in the C-terminal JNK binding domain of GSTP1 is critical for the interaction and regulation of JNK by GSTP1 and that its phosphorylation by EGFR enhances this inhibitory regulation of JNK.

The apoptosis-inducing agent, cisplatin, widely used to treat a variety of human cancers, induces a persistent hyperactivation of JNK, which has been shown to contribute to the induction of cell death in cisplatin-treated cells (59–62). Paradoxically, at modest levels, JNK activation has been associated with cellular protection against cisplatin cytotoxicity via transcriptional activation of genes, including those involved in DNA repair (62, 63). These opposing effects of JNK signaling are cell-type and physiological state-dependent, and cell fate is frequently determined by the level and duration of the JNK activation. In the present study we showed that a small molecule specific JNK inhibitor, SP600125, significantly protected the cells of two types of brain tumors, namely, medulloblastoma and glioblastoma, from cisplatin-induced cytotoxicity and was associated with increased resistance to apoptosis. The observation that, in contrast to cells with unphosphorylatable GSTP1-Y198F, cells with wild-type GSTP1 or the phosphomimic Y198D GSTP1 were more drug-resistant indicates that the phosphorylation state of Tyr-198 of GSTP1 contributes to cell survival and apoptotic response in part due to a suppression of JNK signaling.

Taken together the data in this study allow us to propose a model of JNK regulation in which, in response to EGF-ligand activation, EGFR rapidly phosphorylates tyrosine residues both in the N and C termini of GSTP1. As we showed in a previous study (35), the N-terminal tyrosine phosphorylation, especially of tyrosine 7, causes structural modification of GSTP1, resulting in increased electronegativity of the GSH binding active site and enhanced catalytic activity of GSTP1 (35). Tyrosine phosphorylation of the GSTP1 C terminus, on the other hand, as shown in the present study, shifts the GSTP1 dimer-monomer equilibrium to the monomeric form, thus promoting the formation of the GSTP1-JNK1 complex, resulting in an enhanced inhibition of JNK downstream signaling. We propose that this novel GSTP1-dependent negative regulation of JNK signaling by EGFR together with the previously described "EGFR-MAP-KKK-MKK-JNK"-positive JNK regulatory pathway (2) will be major determinants of the JNK signaling status of cells. Our findings in this study thus unravel a novel and important component of the regulation of JNK signaling via the EGFR-GSTP1-JNK1 axis, contribute to a better understanding of the cellular functions of these important proteins, and provide the basis for novel strategies for GBM therapy and for overcoming tumor resistance to EGFR-targeted therapy.

Author Contributions—F. A.-O. conceived the study, designed and supervised the experiments, wrote the elements of the manuscript, and revised the completed manuscript for submission. T. O. conducted the majority of the experiments, analyzed the data, and prepared the draft manuscript. G. A. assisted T. O. in the conduct of the experiments, particularly the Western blots of JNK downstream signaling, and performed the additional experiments to respond to the critique of the original submission. S. T. K. established the human glioblastoma xenografts for studying the effect of EGFR phosphorylation of GSTP1 *in vivo*. H. F. and D. D. B. participated in data analyses, critiqued results, and contributed substantially to the interpretation of the findings. All authors reviewed the results and approved the final version of the manuscript.

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