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Tyrosine Phosphorylation of the Human Glutathione *S*-Transferase P1 by Epidermal Growth Factor Receptor*[S]

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Tatsunori Okamura[‡], Simendra Singh[‡], John Buolamwini[§], Timothy Haystead[¶], Henry Friedman[‡], Darell Bigner[‖], and Francis Ali-Osman[‡1]

From the Departments of [‡]Surgery, [¶]Pharmacology, and [‖]Pathology, Duke Comprehensive Cancer Center, and the Preston Robert Tisch Brain Tumor Center, Duke University, Durham, North Carolina 27710 and the [§]Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Sciences Center, Memphis, Tennessee 38163

Epidermal growth factor receptor (EGFR) gene amplification, mutations, and/or aberrant activation are frequent abnormalities in malignant gliomas and other human cancers and have been associated with an aggressive clinical course and a poor therapeutic outcome. Elevated glutathione *S*-transferase P1 (GSTP1), a major drug-metabolizing and stress response signaling protein, is also associated with drug resistance and poor clinical outcome in gliomas and other cancers. Here, we provide evidence that GSTP1 is a downstream EGFR target and that EGFR binds to and phosphorylates tyrosine residues in the GSTP1 protein *in* vitro and *in vivo*. Mass spectrometry and mutagenesis analyses in a cell-free system and in gliomas cells identified Tyr-7 and Tyr-198 as major EGFR-specific phosphoacceptor residues in the GSTP1 protein. The phosphorylation increased GSTP1 enzymatic activity significantly, and computer-based modeling showed a corresponding increase in electronegativity of the GSTP1 active site. In human glioma and breast cancer cells, epidermal growth factor stimulation rapidly increased GSTP1 tyrosine phosphorylation and decreased cisplatin sensitivity. Lapatinib, a clinically active EGFR inhibitor, significantly reversed the epidermal growth factor-induced cisplatin resistance. These data define phosphorylation and activation of GSTP1 by EGFR as a novel, heretofore unrecognized component of the EGFR signaling network and a novel mechanism of tumor drug resistance, particularly in tumors with elevated GSTP1 and/or activated EGFR.

Epidermal growth factor receptor (EGFR),[2] a 170-kDa receptor-type tyrosine kinase, mediates diverse signaling pathways and cellular processes, including, proliferation, differentiation, motility, and survival (1–4). Ligand binding and activation of EGFR result in receptor dimerization, autophosphorylation, and activation of downstream effector pathways, such as phosphatidylinositol 3-kinase/AKT, Janus kinases/signal transducers and activators of transcription (STAT), and Ras/Raf/mitogen-activated protein kinase (MAPK) (1, 2). The cellular signaling cascades initiated and transduced by EGFR have been implicated in oncogenesis and functional dysregulation of EGFR, and its downstream pathways are frequently observed in malignant gliomas and other human cancers and have been shown to regulate features of the malignant phenotype, such as tumor progression, adhesion, invasion, angiogenesis, and apoptosis (3, 5).

EGFR gene amplification is a hallmark of glioblastoma multiforme (GBM), the most aggressive and most common intrinsic malignant brain tumor (6). In primary (*de novo*) GBM, which accounts for ~95% of all GBM (7, 8), EGFR amplification follows Loss of heterozygosity of chromosome 10q as the most frequently observed genetic alteration (8). In preclinical studies, a strong correlation has been reported between high aberrant EGFR signaling and ligand-dependent GBM cell proliferation (9) and the resistance of GBM to chemotherapy and radiation therapy (10). Consistent with this, clinical studies of GBM have shown EGFR gene amplification to be a significant negative predictor of patient survival (4), and EGFR overexpression has been associated with failure to respond to radiation therapy (11). In addition to its amplification and overexpression, a mutant EGFR, EGFRvIII, characterized by deletion of exons 2–7, is present in almost half of GBMs with amplified *EGFR* (4, 12). EGFRvIII is unique in that the loss of a large portion of the extracellular ligand binding domain leads to its constitutive and ligand-independent activation (13). In GBM, EGFRvIII has been associated with increased tumor growth, cell proliferation, and drug resistance, which, similar to wild-type EGFR, occurs via constitutive activation of downstream EGFR pathways (14, 15).

Glutathione *S*-transferase P1 (GSTP1), a major phase II-metabolizing enzyme, encoded in a polymorphic gene locus (16), catalyzes the *S*-conjugation of endogenous and exogenous electrophiles, including many genotoxins, carcinogens, and anticancer agents, to the nucleophilic thiol group of reduced glutathione, GSH (17). In addition, GSTP1 is a major regulator of cell signaling in response to stress, hypoxia, growth factors, and other stimuli. This results in part from its ability to inhibit downstream mitogen-activated protein kinase signaling, notably that mediated by c-Jun N-terminal kinase (18–20). GSTP1 also regulates important normal cellular functions through interaction with a number of critical cellular proteins, including transglutaminase 2, apoptosis signal-regulating kinase 1, and

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[1] To whom correspondence should be addressed: 421 Medical Science Research Bldg. I, Duke University Medical Center, Durham, NC 27710. Tel.: 919-681-5769; Fax: 919-684-5483; E-mail: francis.aliosman@duke.edu.
[2] The abbreviations used are: EGFR, epidermal growth factor (EGF) receptor; GBM, glioblastoma multiforme; GSTP1, glutathione *S*-transferase P1; GSH, glutathione; EA, ethacrynic acid; MAPK, mitogen-activated protein kinase; IP, immunoprecipitation; MS, mass spectrometry; LC, liquid chromatography; siRNA, small interfering RNA.

Fanconi anemia group C protein (21). Recently, we reported that GSTP1 is a substrate for two Ser/Thr protein kinases, *viz.* cAMP-dependent protein kinase and protein kinase C (22). In many human cancers, including gliomas, leukemias, lymphomas, melanoma, and carcinomas of the breast, ovary, colorectum, lung, liver, etc. (23–25), GSTP1 is frequently overexpressed, and the high expression is associated with a more aggressive tumor biology and poor patient survival.

Given the roles that both EGFR and GSTP1 play in cell signaling and in both normal and neoplastic biology, we investigated using GBM and inflammatory breast cancer cell lines the possibility and consequences of the interaction between the two proteins *in vitro* and in GBM xenografts growing *in vivo*. The nature of the interaction was characterized structurally and functionally by a combination of mass spectrometry and other biochemical analyses, and its effects on the response of the tumor cells to chemotherapy were investigated. Our findings support EGFR-mediated tyrosine phosphorylation of GSTP1 to be a heretofore unrecognized component of the EGFR cellular network and constitutes an important mechanism of cellular protection and drug resistance, particularly in tumors and/or tissues with activated EGFR and/or elevated GSTP1 expression.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—Recombinant human GSTP1-1 protein was purchased from Invitrogen, and recombinant human EGFR active kinase domain and normal mouse IgG was from Upstate Biotechnology Inc., (Lake Placid, NY). [γ-^{32}P]ATP and Protein A-Sepharose were from Amersham Biosciences. Anti-human GSTP1-1 rabbit polyclonal antibody was from Oxford Biomedical Research (Oxford, MI), and anti-human GSTP1 mouse monoclonal antibody was from BIODESIGN International (Saco, ME). Anti-phosphotyrosine (phospho-Tyr-100) and anti-phospho-EGFR (Tyr-1068) monoclonal antibodies were from Cell Signaling Technology (Danvers, MA). Anti-GRB2 and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The EGFR inhibitor, lapatinib, was purchased from LC Laboratories (Woburn, MA) and prepared in DMSO stock solution. All other chemicals and biochemicals were purchased from Sigma-Aldrich unless otherwise stated.

Cell Lines—The human cell lines MGR1 (anaplastic astrocytoma) and MGR3 (GBM) were established in our laboratory from primary specimens (22). The high EGFR expressing human GBM U87MG.wtEGFR was derived by stable transfection of the parental U87MG cells with wild-type EGFR (26). SUM 149 (Asterand PLC, Detroit, MI) is a human inflammatory breast cancer cell line. Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum (FCS) (MGR1, MGR3), Improved MEM Zinc Option with 10% FCS (U87MG, U87MG.wtEGFR), or Ham's F-12 with 5% FCS (SUM 149).

GSTP1 Phosphorylation by EGFR in Cell-free System—To mimic intracellular conditions in which the GSTP1 protein exists in equilibrium with GSH bound to its GSH binding site (22, 27), 1 μg of human recombinant GSTP1 was preincubated with 5 mM GSH for 20 min at 37 °C and then added to a reaction mixture containing EGFR active kinase domain (25 ng) and [γ-^{32}P]ATP in Mn^{2+}, Mg^{2+}-containing kinase buffer. After 1 h of incubation at 30 °C, the reaction was terminated by boiling and resolved by SDS-PAGE followed by Coomassie Blue staining and autoradiography. For stoichiometry of the GSTP1 phosphorylation by EGFR, the phosphorylation reaction was set up containing 1 μg of GSTP1, 0.05 μM EGFR, and a saturating (100 μM) ATP concentration. Over 0–4 h, aliquots were removed and subjected to SDS-PAGE, and the phosphorylated GSTP1 bands were excised and solubilized, and the radioactivity counted by β-scintillation. The incorporated ^{32}P phosphate was computed from the specific activity of the [γ-^{32}P]ATP, expressed per mol of the dimeric GSTP1 protein, and plotted against time.

Analysis of GSTP1 Phosphorylated Amino Acid Residues by Thin Layer Electrophoresis—GSTP1 was ^{32}P-phosphorylated by EGFR, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride membranes. After autoradiography, the phospho-GSTP1 bands were excised, hydrolyzed at 110 °C in 5 M HCl for 1 h, vacuum-dried, and resuspended in a loading buffer of acetic acid:pyridine:water (10:1:189) containing phosphoserine, phosphothreonine, and phosphotyrosine standards, and electrophoresed at pH 3.5 on a cellulose TLC plate. The plate was dried, stained with ninhydrin, and autoradiographed.

Western Blotting for GSTP1 Tyrosine Phosphorylation in Cell-free System—A mixture of GSTP1 preincubated with or without 5 mM GSH was applied to the GSTP1 phosphorylation assay with 50 nM EGFR and 200 μM ATP followed by SDS-PAGE/Western blotting with anti-phosphotyrosine (Tyr(P)) antibody. After stripping, the membrane was reprobed with anti-GSTP1 antibody to control for loading. To inhibit the EGFR activation, a 50 nM EGFR preincubated for 30 min with 0–1 mM lapatinib, an EGFR inhibitor, was used for the GSTP1 phosphorylation assay. Tyrosine-phosphorylated GSTP1 was normalized against total GSTP1 protein.

EGFR-dependent GSTP1 Phosphorylation in Glioma and Breast Cancer Cells—Tumor cells grown in serum-free medium for 24 h were treated with 100 ng/ml EGF for 10 min, rinsed with ice-cold phosphate-buffered saline, and lysed in 50 mM Tris-HCl (pH 7.5) containing 1% Triton X-100, protease and phosphatase inhibitors mixture (Pierce). The lysates were centrifuged at 20,000 rpm for 15 min. Supernatants were subjected to Western blotting with anti-Tyr(P), phospho-EGFR, and GSTP1 antibodies. For a combined immunoprecipitation (IP)-Western blotting, supernatants (1 mg total protein) were incubated (4 °C; overnight) with anti-GSTP1, phospho-EGFR antibodies, or normal mouse IgG (as a negative control). The protein A-Sepharose beads were incubated with immunocomplexes for 1 h and washed 4 times with the lysis buffer, and the immunoprecipitates were subjected to Western blotting. To examine the effect of EGFR inhibition, tumor cells with activated EGFR were treated with 2.5 μM lapatinib for 30 min, as per experimental protocol before lysis and Western blotting.

Enzyme Kinetic Analysis of EGFR-phosphorylated GSTP1—These were performed as we had previously described (22). Briefly, 0.1 unit each of unphosphorylated recombinant GSTP1 and GSTP1 phosphorylated by EGFR as described earlier were used to set up reactions containing 0.05–0.5 mM GSTP1-specific substrate, ethacrynic acid (EA), and 0.25 mM GSH in a 0.1

mM potassium phosphate buffer (pH 6.8). The rate of formation of the reaction product between EA and GSH was monitored at 270 nm. Reaction rates, normalized against that of the nonenzymatic reaction, were computed and used to generate double reciprocal plots from which the enzyme kinetic constants, K_m, V_{max}, K_{cat}, and K_{cat}/K_m, were computed for phosphorylated and unphosphorylated GSTP1 (28), and the results are presented as the mean ± 1 S.D. of triplicate experiments.

In Vivo EGFR Phosphorylation of GSTP1 in GBM Xenografts—Xenografts (approximately, 200 mm^2 in diameter) of U87MG and U87MG.wtEGFR growing subcutaneously in the flanks of 4-week-old male athymic BALB/c *nu*/*nu* mice were excised, minced, and sonicated on ice in the lysis buffer. Supernatants were subjected to the IP (anti-GSTP1)-Western (anti-Tyr(P)) procedure as described earlier.

Phosphorylation Site Analysis in EGFR-phosphorylated GSTP1 by Mass Spectrometry (MS)—Recombinant GSTP1 was EGFR-phosphorylated GSTP1, reduced, and alkylated, and second dimension acrylamide gel electrophoresis was performed. SYPRO Ruby-stained protein spots were robotically excised, reduced with dithiothreitol, alkylated with iodoacetamide, digested with trypsin, and subjected to LC-MS/MS. MS/MS data were analyzed using the MASCOT MS/MS Ions Search, and *de novo* sequence analysis performed with the Scaffold Software (Proteome Software Inc., Portland, OR). Details of the protocols used are available online at Proteome Software Inc. and in supplemental material.

EGFR Phosphorylation of GSTP1 Peptides—Peptides containing each of the 12 tyrosine residues in the GSTP1 protein, namely, Tyr-3, Tyr-7, Tyr-49, Tyr-63, Tyr-79, Tyr-103, Tyr-108, Tyr-111, Tyr-118, Tyr-153, Tyr-179, and Tyr-198 as well as human angiotensin II peptide (DRVYIHPF as a positive control; Calbiochem) and Crosstide (GRPRTSSFAEG as a negative control; AnaSpec Inc., San Jose, CA), were EGFR-phosphorylated using [^{32}P]ATP, spotted on Whatman P81 cellulose phosphate filters, acetone-washed, and air-dried. The radioactivity was quantitated by β-scintillation counting and used to compute the incorporated phosphate in each peptide. To better ascertain the GSTP1 phospho-acceptor residues, Tyr-3/Tyr-7, Tyr-63, Tyr-118, and Tyr-198 in six peptides selected from the LC-MS/MS analysis were mutated to aspartic acid (BioSynthesis, Louisville, TX), and the level of EGFR phosphorylation was determined as described above. Peptide information is available in supplemental Table S1.

Mutagenesis and Phosphorylation of GSTP1 in Glioma Cells—Mutant GSTP1 cDNAs were created by PCR on a template plasmid vector pBK-CMV/GSTP1A (16) using GSTP1-specific primers containing tyrosine to phenylalanine mutations at Tyr-3, -7, and -198. All mutations were verified by DNA sequencing. Cloning was performed using the Gateway technology (Invitrogen) with the pcDNA-DEST40 destination vector to allow C-terminal fusions with a six-histidine tag. Transient transfections were performed with FuGENE HD (Roche Applied Science) according to the manufacturer's instructions. Briefly, 5×10^5 U87MG.wtEGFR cells were plated in 6-well plates and transfected with 2 μg of pcDNA-DEST40 expression vector carrying the wild-type GSTP1, the single tyrosine to phenylalanine mutants Y3F, Y7F, and Y198F, the double mutants, Y3F/Y7F, Y3F/Y198F and Y7F/Y198F, the triple mutant Y3F/Y7F/Y198F, and the empty vector (negative control). After 48 h the cells were treated with 100 ng/ml EGF for 10 min and lysed. The histidine-tagged GSTP1 proteins were immunoprecipitated with TALON cobalt Dynabeads (Invitrogen) according to the manufacturer's instructions followed by SDS-PAGE and Western blotting with anti-Tyr(P) antibody as described earlier. The relative levels of tyrosine phosphorylation of the mutant GSTP1 proteins relative to the wild-type GSTP1 were quantified by densitometry using ImageJ Version 1.34s software. Primer information is available in supplemental Table S2.

Molecular Dynamics Simulations—X-ray crystallographic data were imported from the Brookhaven Protein Data Bank, and using the Insight II modeling program (Accelerys Software, San Diego), the three-dimensional structures of the GSH-bound GSTP1 monomer with and without the hydroxyl group of Tyr-7 phosphorylated were created. The modeled structures were soaked in a cubic box of water molecules and subjected to energy minimization and long-duration molecular dynamics simulation using the NAMD program 2.5 running on a 5 node Scyld Beowulf linux cluster. The coordinate and parameter files for input were generated using the "psfgen" utility in the CHARMM PARAM 22 topology file, whereas the all atom CHARMM PARAM 22 force field was used to describe the potential energy. The results of the analyses of energies and structure frames of the simulated system were extracted using the VMD software and illustrations produced with both the VMD and SYBYL software. Details of the simulation procedure are provided in the supplemental material.

Effect of EGFR Modulation on GSTP1 Enzymatic Activity in Tumor Cells—EGFR was activated in exponentially growing tumor cells by a 10-min treatment with 100 ng/ml EGF. Extracts from cells with and without subsequent treatment with 2.5 μM EGFR inhibitor, lapatinib, for 30 min were assayed for specific GSTP1 activity as we described earlier (22).

Effect of EGFR Activation on Glutathione-platinum Metabolite Formation in Tumor Cells—These studies were performed with the GSTP1- and EGF-overexpressing human inflammatory breast cancer cell line, SUM 149. Approximately 5×10^6 cells in exponential growth were pretreated with 100 ng/ml EGF for 10 min in triplicate, after which the medium was replaced with fresh medium containing 100 μM cisplatin. After an additional 2 h at 37 °C, the cells were washed twice, harvested, and homogenized in 500 μl of phosphate-buffered saline. Supernatants after centrifugation at 15,000 × *g* for 20 min were removed, and protein was precipitated by adding trichloroacetic acid to 10% (final concentration) and incubating at 4 °C for 3 h. After final centrifugation, the supernatants (normalized for equal protein content) were used for glutathionylplatinum metabolite quantitation, as we previously described (30). Briefly, aliquots of the supernatant were diluted 1:10 with 10% trichloroacetic acid, and the absorbance was measured by scanning spectrophotometry over a wavelength range of 240–400 nm (Beckman DU-70 spectrophotometer). The absorbance at 265 nm, A_{265} (peak absorbance of the glutathionylplatinum conjugate), of the supernatants with and without EGF pretreatment were normalized against that of control

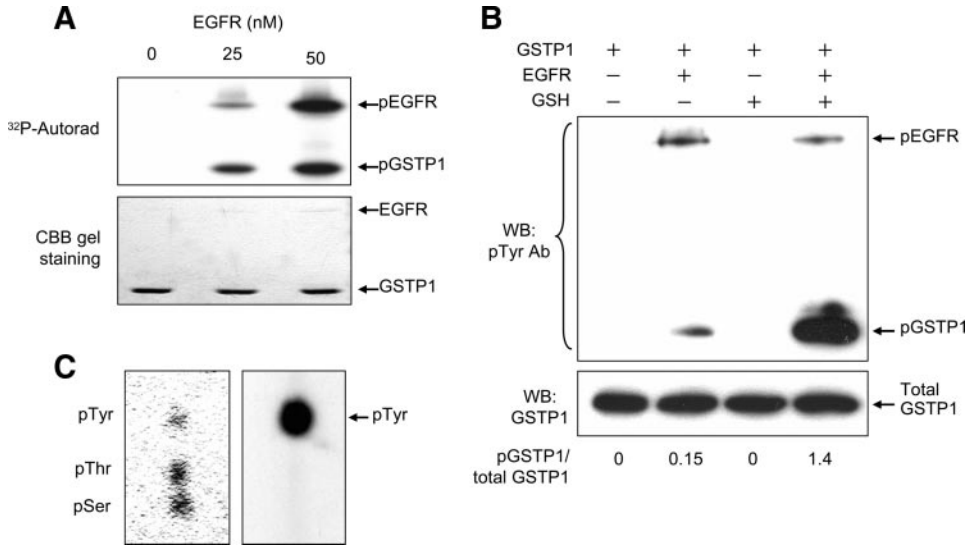


FIGURE 1. **Phosphorylation of GSTP1 by EGFR in a cell-free system.** *A*, autoradiograph showing tyrosine-phosphorylated GSTP1 and autophosphorylated EGFR. *B*, Western blotting (*WB*) demonstrating GSH dependence of EGFR phosphorylation of GSTP1. *Ab*, antibody. *C*, thin layer cellulose electrophoresis showing tyrosine residues to be the only amino acids phosphorylated by EGFR in GSTP1 (*left*, phospho-amino acid standards; *right*, ^{32}P-labeled phospho-GSTP1 hydroxylate. *CBB*, Coomassie Brilliant Blue.

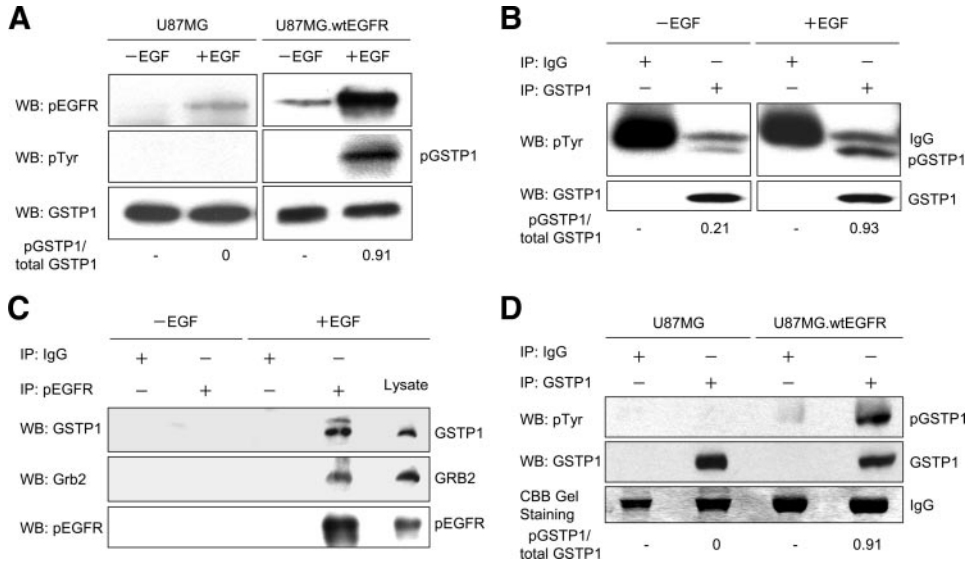


FIGURE 2. ***In vivo*** **EGFR-mediated phosphorylation of GSTP1 in glioma cell cultures and xenografts.** *A*, Western blot (*WB*)showing Tyr phosphorylation of a 23-kDa protein in cultured U87MG.wtEGFR but not in parental U87MG. After stripping and reprobing with anti-GSTP1 antibody, this 23-kDa band was confirmed to be GSTP1. *B*, IP-Western blotting showing an enhancement of a 23-kDa tyrosine-phosphorylated GSTP1 in U87MG.wtEGFR cells after EGF stimulation, whereas no altered expression of a 24-kDa IgG light chain. *C*, phospho-EGFR in complex with GSTP1 and with GRB2 (positive control) in U87MG.wtEGFR cells. *D*, IP-Western blotting showing the presence of phospho-GSTP1 in xenografts of U87MG.wtEGFR but not in parental U87MG growing in nude mice. Coomassie Brilliant Blue (*CBB*) gel staining shows an equal loading (IgG heavy chain).

without cisplatin treatment, and the resulting ΔA_{265} was used as a measure of the level of glutathionylcisplatin in the cells.

Effect of EGFR Activation on Cisplatin Sensitivity of Tumor Cells—Tumor cells with activated EGFR were treated with and without 2.5 μM lapatinib for 30 min followed by 0–50 μM cisplatin for 3 h. The cells were washed, and cell survival was examined after 48 h using the CellTiter-Blue Assay (Promega, Madison, WI) according to the manufacturer's instructions.

Effect of siRNA-mediated GSTP1 Down-regulation on EGFR-induced Cisplatin Resistance in Glioma Cells—MGR3 and SUM 149 cells were plated at 1×10^3–10^4 cells in 100 μl of Dulbecco's modified Eagle's medium containing 10% fetal calf serum in a flat-bottomed microtiter plate. After 24 h at 37 °C, the cells were transfected with siRNA with the sequence (5′-ACCAGAUCUCCUUCGCUGACUACAA-3′) targeting the N-terminal region of GSTP1 mRNA using Lipofectamine^{TM} (Invitrogen) according to the manufacturer's instructions. After 6 h the cultures were refed with fresh medium and incubated at 37 °C for a further 24 h. Untransfected cells and cells transfected with scrambled siRNA served as controls. After 24 h the cells were treated with and without 100 ng/ml EGF for 10 min followed by 0–50 μM cisplatin. Cell survival was determined as described earlier. Cisplatin sensitivity (surviving fraction) after each treatment relative to controls (normalized against scrambled siRNA) was determined, and IC_{50} values were computed. Replicate cells after siRNA treatment were lysed and subjected to Western blotting for GSTP1 expression to monitor the level of GSTP1 knockdown.

RESULTS

GSTP1 Is Phosphorylated by EGFR in a Cell-free System—Figs. 1, *A–C*, summarizes the results of the cell-free analysis of the phosphorylation of GSTP1 by EGFR, performed with recombinant GSTP1 and EGFR proteins. The ^{32}P-labeling results (Fig. 1*A*) show that, after its pre-equilibration with GSH, GSTP1 undergoes dose-dependent phosphorylation by EGFR. The Western blots with an anti-phosphotyrosine (Tyr(P))-specific antibody (Fig. 1*B*) show that, while for GSTP1, tyrosine phosphorylation required GSH and was significantly reduced in its absence, the presence of GSH resulted in a slight reduction in the level of EGFR autophosphorylation, consistent with previous reports that EGFR is a redox-regulated protein and that its intracellular activation is suppressed by reducing agents (31). Equal loading of GSTP1 in the lanes is shown by the Western blots for GSTP1 (*lowest panel* in Fig. 1*B*). To determine which amino acids in the GSTP1 protein undergo phosphorylation by EGFR, recombinant GSTP1 was ^{32}P-phosphorylated by EGFR, acid-hydrolyzed, and subjected to thin layer electrophoresis and

autoradiography. The results (Fig. 1*C*) show tyrosine to be the only amino acid residue phosphorylated by EGFR in the GSTP1 protein. No phosphoserines or phosphothreonines were detected. In the cell-free system and at saturating ATP concentrations EGFR phosphorylated the dimeric GSTP1 protein with a stoichiometry of 0.0436 ± 0.0003 mol of phosphate/mol of GSTP1.

GSTP1 Undergoes Tyrosine Phosphorylation after EGFR Activation in Human Glioma Cells—Intracellular EGFR-dependent GSTP1 phosphorylation was examined using the isogenic pair of human GBM cell lines, U87MG and U87MG.wtEGFR, with low and high constitutive wild-type EGFR expression, respectively. Protein extracts prepared from tumor cells that had been treated with and without 100 ng/ml EGF for 10 min were subjected to Western blotting with anti-Tyr(P) antibody. The results, Fig. 2*A*, show no detectable tyrosine-phosphorylated proteins of around 23 kDa in either EGF-treated and untreated U87MG cells. Similarly, phospho-EGFR was undetectable in control untreated U87MG cells and increased only modestly after EGF treatment. In contrast, in untreated U87MG.wtEGFR cells moderate levels of phospho-EGFR were observed even without EGF treatment, and after EGF treatment these levels increased by more than 10-fold. Western blotting with Tyr(P) antibody showed the level of 23-kDa tyrosine-phosphorylated protein, absent in the non-EGF treated cells, to be significantly increased after EGF treatment. After stripping and reprobing with anti-GSTP1 antibody, this 23-kDa band was confirmed to be GSTP1.

The results of GSTP1 tyrosine phosphorylation in GBM cells examined by immunoprecipitation with an anti-GSTP1 antibody followed by Western blotting for phosphotyrosine (Fig. 2*B*) were similar to those of the direct Western blotting and showed that, in U87MG.wtEGFR, levels of tyrosine-phosphorylated GSTP1 were low in non-EGF-treated cells but increased significantly after EGF treatment. Western blotting with anti-GSTP1 confirmed the amount of GSTP1 immunoprecipitated to be equal in all the lanes.

To examine binding of GSTP1 to activated EGFR, a prerequisite for EGFR-dependent GSTP1 phosphorylation, extracts of U87MG.wtEGFR with and without EGF treatment, were immunoprecipitated with anti-phospho-EGFR antibody followed by immunoblotting for GSTP1. The results, Fig. 2*C* show that GSTP1 forms a complex with phospho-EGFR only in EGFR-activated cells. As a positive control, we also showed that GRB2, an adaptor protein that binds to EGFR-phosphorylated at Tyr-1068 (32), also formed a complex with phospho-EGFR in EGF-treated U87MG.wtEGFR cells.

GSTP1 Undergoes EGFR-dependent Tyrosine Phosphorylation in GBM Xenografts in Vivo—Subcutaneously growing xenografts of U87MG and U87MG.wtEGFR in nude mice were surgically removed and homogenized in phosphate-buffered saline. Proteins in the particle-free supernatants from the tissue lysates were immunoprecipitated with anti-GSTP1 antibody and Western blotted for phospho-Tyr. The results (Fig. 2*D*) show high levels of tyrosine-phosphorylated GSTP1 in the EGFR-overexpressing U87MG.wtEGFR and none in the parental U87MG. Western blotting with the anti-GSTP1 antibody showed that the amount of immunoprecipitated GSTP1 from both xenografts was equal, and Coomassie Brilliant Blue staining also showed equal levels of mouse IgG in the immunoprecipitates.

GSTP1 Phosphorylation by EGFR Increases GSTP1 Catalytic Activity—We investigated the effects of EGFR phosphorylation on the ability of GSTP1 to catalyze the conjugation of GSH to the GSTP1-specific substrate, EA. The results, summarized in Table 1, show that although the turnover number, K_{cat}, of the GSTP1 protein was only modestly altered by phosphorylation, the affinity of EA for phosphorylated GSTP1 increased significantly, as evidenced by a 3.8-fold decrease in the K_m relative to the unphosphorylated protein. The increased EA affinity correlated with a 2.9-fold increase in catalytic efficiency, K_{cat}/K_m, of GSTP1, from 685.2 to 1967.5 $mM^{-1}\cdot min^{-1}$, after its phosphorylation by EGFR.

Computer-assisted Analyses of Putative Phosphorylation Sites in Human GSTP1—Using the NetPhos 2.0 Server program (33), which produces neural network predictions for serine, threonine, and tyrosine phosphorylation sites in eukaryotic proteins, we identified Tyr-3, Tyr-118, and Tyr-198 as potential phospho-acceptors in GSTP1, with scores of 0.946, 0.983, and 0.989, respectively. With the Scansite 2.0 program (34), which searches for short sequence motifs within proteins likely to be

TABLE 1

Effect of EGFR phosphorylation on GSTP1 catalytic activity measured with the GSTP1-specific substrate, EA, as we had described earlier (24)

Reaction rates were normalized against the rate of the non-enzymatic reaction and used to compute the enzyme kinetic constants.

	K_m	V_{max}	K_{cat}	K_{cat}/K_m
	mM	$mM.min^{-1}$	s^{-1}	$mM^{-1}\cdot min^{-1}$
Unphosphorylated GSTP1	0.088 ± 0.006	975.5 ± 26.7	59.8 ± 1.6	685.2 ± 33.5
EGFR-phosphorylated GSTP1	0.023 ± 0.003	719.1 ± 50.9	44.1 ± 3.1	1967.5 ± 305.3

TABLE 2

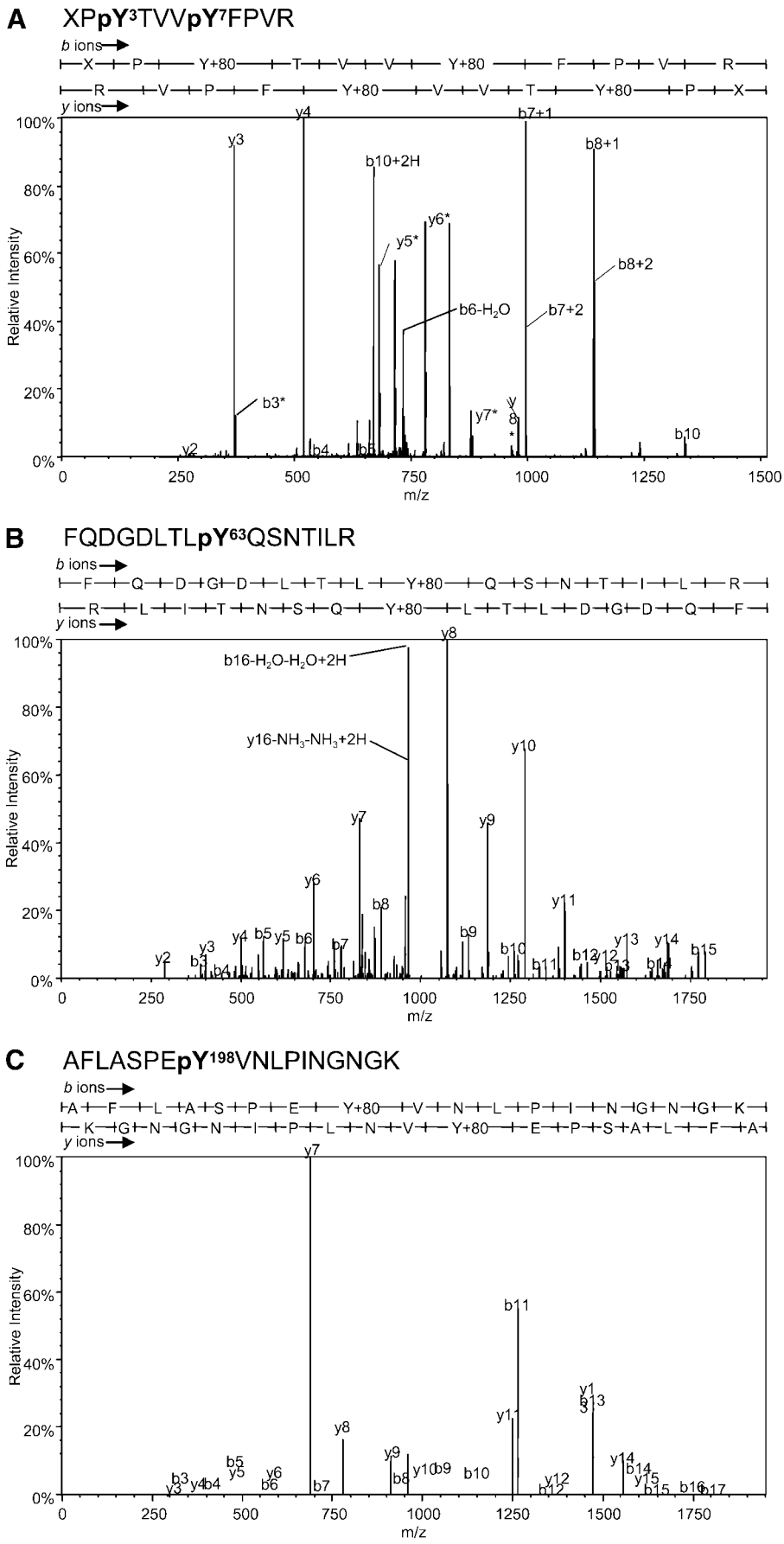
Nano-LC-MS/MS characterization of amino acid residues phosphorylated by EGFR in the GSTP1 protein

The putative phospho-acceptor tyrosine residues are bold and underlined. a.m.u., atomic mass units.

Peptide sequence	Mass	Charge	Modifications	Amino acids	Phosphorylated residues
	a.m.u.				
XP**Y**TVV**Y**FPVR	1512.16	2	Phospho (+80)	1–11	Tyr-3, Tyr-7
FQDGDLTL**Y**QSNTILR	1963.15	2	Phospho (+80)	191–208	Tyr-198
AFLASPE**Y**VNLPINGNGK	2023.99	2	Phospho (+80)	191–208	Tyr-198
AFLASPE**Y**VNLPINGNGK	1984.10	2	Phospho (+80)	191–208	Tyr-198
AFLASPE**Y**VNLPINGNGK	2023.99	3	Phospho (+80)	191–208	Tyr-198
AFLASPE**Y**VNLPINGNGKQ	2112.13	2	Phospho (+80)	191–209	Tyr-198
AFLASPE**Y**VNLPINGNGKQ	2112.36	3	Phospho (+80)	191–209	Tyr-198
LKAFLASPE**Y**VNLPINGNGK	2225.52	2	Phospho (+80)	189–209	Tyr-198
AFLASPE**Y**VNLPINGNGK	1983.03	2	Phospho (+80)	191–208	Tyr-198

phosphorylated by specific protein kinases, however, only Tyr-198 was identified as a residue phosphorylated by EGFR with high stringency (0.3734; 0.191%). Finally, using the PhosphoMotif Finder (35), which contains known published kinase/phosphatase substrate and binding motifs, we identified Tyr-118 and Tyr-198 to be in regions homologous with a reported EGFR phosphorylation motif, *X*(E/D)pY(I/L/V), where *X* is any amino acid (36). Taken together, these results predict Tyr-3, Tyr-118, and Tyr-198 as residues in the GSTP1 protein with a potential of being phosphorylated by EGFR. This information was used to design experiments to determine the phospho-acceptor residues in the GSTP1 protein.

Nano-LC-MS/MS Identifies Tyrosine Residues Phosphorylated by EGFR in the GSTP1 Protein—Five SYPRO Ruby-stained spots (molecular mass 23 kDa) from two-dimensional gel electrophoresis of EGFR-phosphorylated GSTP1 were in-gel tryptic-digested and subjected to nano-LC-MS/MS. The results showed nine phosphopeptides containing phosphotyrosines, detected by the neutral loss of 80 Da (HPO_3) upon collision-induced dissociation. Computed theoretical values of *b* and *y* ions for the phosphopeptides were compared with the fragment ions observed in the mass spectra to confirm the sequence assignment. The results are summarized in supplemental Table S1 and in Table 2. Designated *b* and *y* ions of *m*/*z* 1512.16 atomic mass units corresponded to a region ^{1}*X*Pp**Y**TVV**pY**-FPVR^{11}, residues 55–70, in the GSTP1 N terminus, containing Tyr-3 and Tyr-7 (Fig. 3*A*). Similarly, the fragmentation mass spectrum of *m*/*z* 1963.29 atomic mass units (Fig. 3*B*) was consistent with FQDGDLTL**pY**QSNTIL^{69} (residues 55–70). The sequence, AFLASPE**pY**VNLPINGNGK^{208} (residues 191–208) with *m*/*z* of 1984.15 atomic mass units (Fig. 3*C*) and all the other phosphopeptides identified contained the same phosphotyrosine, Tyr-198, in the GSTP1 C terminus.



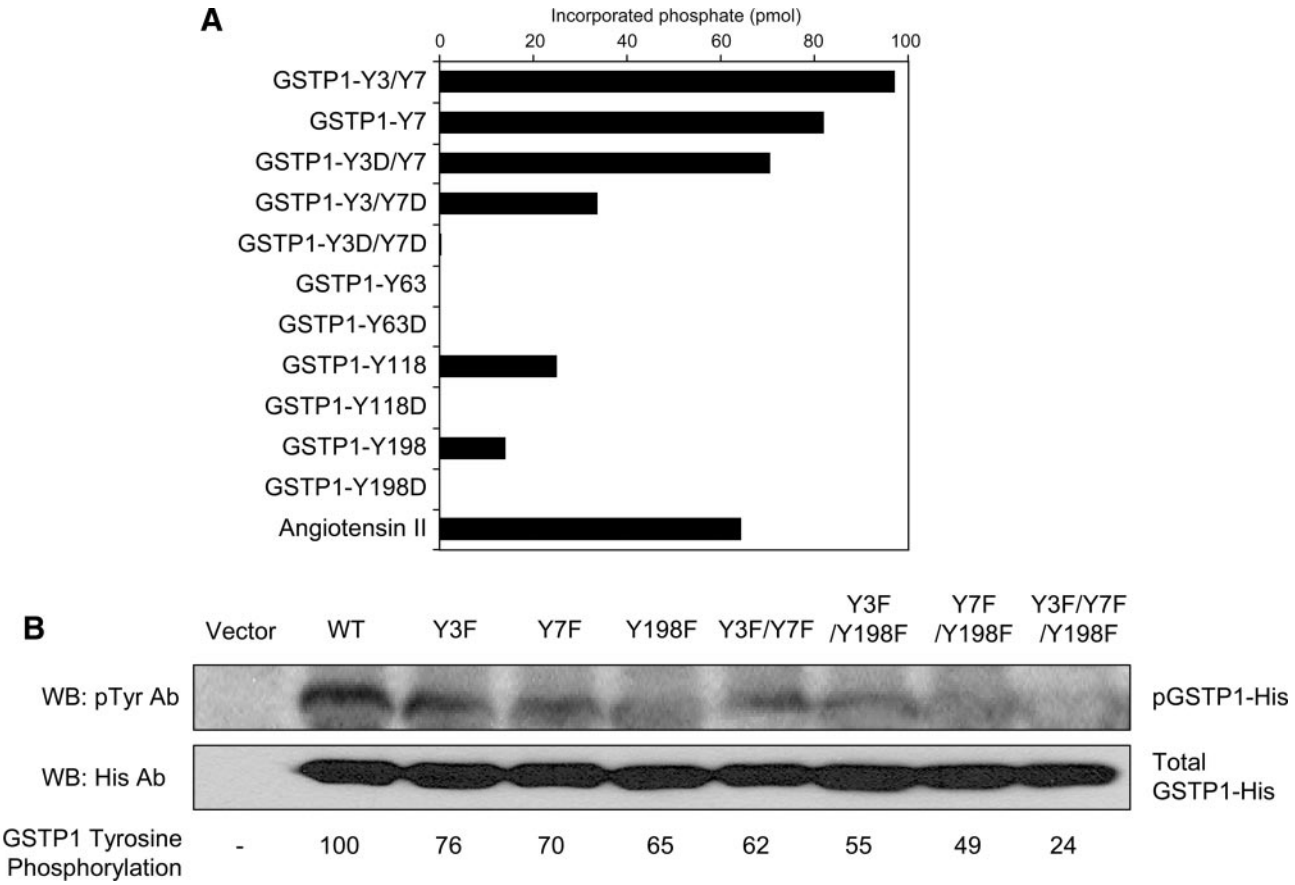


FIGURE 4. *A*, EGFR-mediated ^{32}P phosphorylation of GSTP1 peptides containing putative phospho-acceptor tyrosines. Each of the single mutant peptides (Y3D and Y7D) was EGFR-dependently phosphorylated, whereas the double mutant peptide (Y3D/Y7D) was not. Angiotensin II peptide (DRVYIHPF) was used as a positive control. *B*, analysis of EGFR-mediated tyrosine phosphorylation of GSTP1 mutants in cultured glioma cells. Exponentially growing U87MG.wtEGFR cells were transiently transfected, with the indicated expression vectors containing single, double, and triple tyrosines 3, 7, and 198 mutated to phenylalanine. After 48 h the cells were exposed to EGF for 10 min, cell lysates were prepared, and the His-tagged GSTP1 mutant proteins were pulled down with TALON cobalt beads and Western-blotted (*WB*) with anti-Tyr(P) and anti-His antibodies (*Ab*). The tyrosine phosphorylation levels in the GSTP1 mutants were determined relative to that of wild-type (*WT*) GSTP1.

Together, the LC-MS/MS revealed four tyrosine residues, Tyr-3, Tyr-7, Tyr-198, and, to a lesser extent, Tyr-63, to be phosphorylated by EGFR in the GSTP1 protein.

Mutational Peptide Analysis Confirms Putative N-terminal GSTP1 Phospho-acceptor Tyrosine Residues—The MS/MS fragmentation and peptide analyses showed Tyr-3 and Tyr-7 to be phosphorylated by EGFR (Fig. 4*A* and Table 2) but could not differentiate between the two residues because of their close proximity. To resolve this, we created two peptides with each tyrosine mutated to aspartate (Y3D and Y7D) and a third with both tyrosines mutated (Y3D/Y7D) and examined their ability to be phosphorylated by EGFR. The results (Fig. 4*A*) showed each of the single mutant peptides to be EGFR-dependently phosphorylated, whereas the double mutant Y3D/Y7D peptide was not, indicating that both Tyr-3 and Tyr-7 are EGFR phospho-acceptors. Taken together, these results and those of the MS/MS analyses confirmed Tyr-3, Tyr-7, and Tyr-198 to be primary residues phosphorylated by EGFR in the GSTP1 protein. Tyr-63 was excluded as a major phospho-acceptor because of the relatively weak signal from the mass spectral analysis and the lack of EGFR phosphorylation of its peptide. Similarly, we eliminated Tyr-118 because the MS analysis showed no phosphopeptides containing it.

Tyrosine Mutations Decrease GSTP1 Phosphorylation in Glioma Cells—Fig. 4*B* summarizes the results of the mutagenesis examining the effects of mutating tyrosine residues to phenylalanine on GSTP1 phosphorylation in glioma cells. There was a significant reduction in the level of tyrosine phosphorylation of all the mutant GSTP1 proteins in gliomas cells after activation of the EGFR pathway. In the EGFR-overexpressing U87MG.wtEGFR glioma cells treated with EGF, the phosphorylation levels of the GSTP1 single mutants, Y3F, Y7F, and Y198F were decreased by 24, 30, and 35%, respectively, relative to that of the wild-type GSTP1. In cells transfected with the

FIGURE 3. **Isolation and identification of GSTP1 phosphorylated peptides by nano-LC-MS/MS.** Shown are fragmentation mass spectra of the three phosphopeptides from human recombinant GSTP1 phosphorylated by EGFR tyrosine kinase in cell free systems. Only the positively identified *b* and *y* ions are labeled. The sequence analysis was performed using the Scaffold Software distributed by Proteome Software followed by manual validation. The peptide sequence corresponding to the *b* and *y* ions labeled in the spectra is shown *above the spectra*. The fragment ions produced due to the neutral loss of HPO_3 (mass = 80) are marked with *asterisks*. *A*, ^{1}XPpYTVVpYFPVR^{11} (mass = 1512.16 atomic mass units, +2H). *B*, ^{55}FQDGDLTLpYQSNTILR^{70} (mass = 1963.29 atomic mass units, +2H). *C*, ^{191}AFLASPEpYVNLPINGNGK^{208} (mass = 1984.15 atomic mass units, +2H).

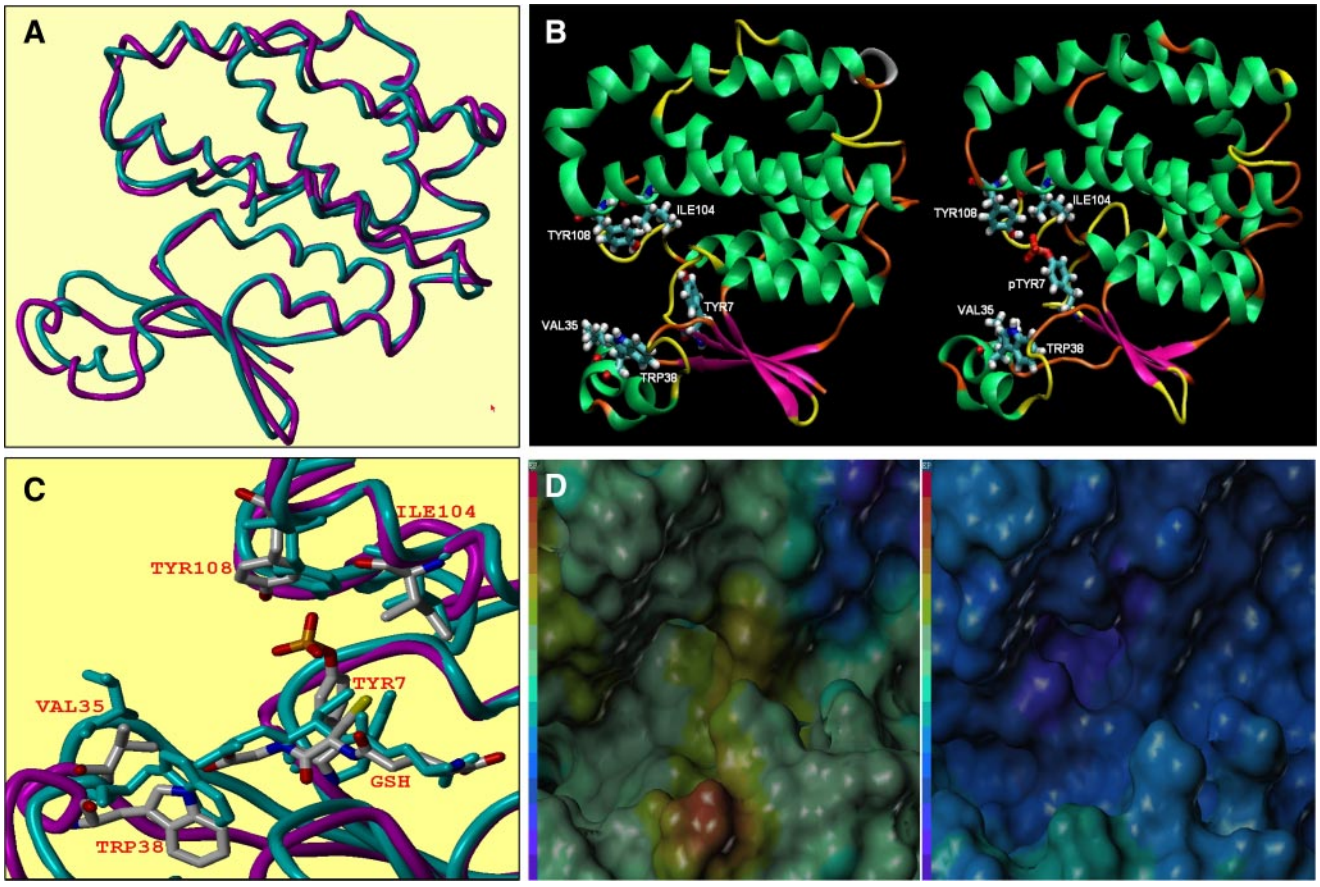


FIGURE 5. **Computer modeling of the effect of tyrosine phosphorylation on GSTP1 protein structure.** *A*, ribbon representation of superimposed structures of unphosphorylated GSTP1 (*cyan*) and Tyr-7-phosphorylated GSTP1 (*magenta*). *B*, tube representation of structures of unphosphorylated GSTP1 (*left*) and Tyr-7-phosphorylated GSTP1 (*right*) showing significant conformational changes in the active site region. *C*, superimposition of key GSTP1 active site residues showing shifts after phosphorylation of Tyr-7. Residues are colored by atom type (*gray*, C; *red*, O; *blue*, N; *yellow*, S; *orange*, P). Corresponding residues in the native protein are colored *cyan*. Hydrogens are omitted for clarity. *D*, electrostatic potential surface maps of the active site regions of unphosphorylated (*left panel*) and Tyr-7-phosphorylated GSTP1 (*right panel*). *Red* is the most positive electrostatic potential, whereas *purple* is the most negative.

three double mutants, the reduction in GSTP1 phosphorylation was greatest for the Y7F/Y198F. The highest reduction in GSTP1 phosphorylation (76% of controls) was obtained with the triple mutant Y3F/Y7F/Y198F. These results support Tyr-3, Tyr-7, and Tyr-198 as major GSTP1 phospho-acceptor residues for EGFR.

Molecular Dynamic Modeling of Tyr-phosphorylated GSTP1—These studies were designed to provide insight into the effect of phosphorylation on the structural dynamics of the GSTP1 protein. Tyr-7 was targeted as the surrogate phosphoacceptor because of its critical role in GSTP1 active site function. After Tyr-7 phosphorylation, the root mean square deviations of the backbone α carbons with time along the dynamics trajectory showed a highly stable structure (data not shown). Superimposition of the unphosphorylated GSTP1 structure on the final 8.2-ns dynamics structure of the Tyr-7-phosphorylated GSTP1 (Fig. 5, *A* and *B*) showed significant conformational changes in the α-super-helical region housing the active site residues, Val-35 and Trp-38. Notably, there was a shift in Tyr-7, resulting from hydrogen bonding of the phosphate group with Tyr-108 (Fig. 5*C*). Such hydrogen bonding could aid deprotonation of OH and/or SH groups and facilitate substrate interactions with the active site. Fig. 5*C* shows a juxtapositioning of the phosphate on Tyr-7 between Tyr-108 and the SH of glutathione (GSH). A major effect of the Tyr-7 phosphorylation (Fig. 5*D*) was a dramatic increase in electronegativity of the GSTP1 active site region.

EGFR Modulation Is Associated with Altered GSTP1 Phosphorylation and Enzymatic Activity, Cisplatin Metabolism, and Drug Resistance in Tumor Cells—The results of these studies are summarized in Figs. 6, *A–D*. In the cell-free system (Fig. 6*A*), the addition of lapatinib (0–10 μM) showed a dose-dependent inhibition of the ability of EGFR to phosphorylate GSTP1, and at 10 μM lapatinib, GSTP phosphorylation was reduced to 8.4% that of the control without lapatinib. Table 3 and Figs. 6, *B* and *C*, show the effects of EGFR activation/inhibition on intracellular GSTP1 tyrosine phosphorylation, GSTP1 activity, and cisplatin-glutathione conjugate formation in cells of SUM 149. After EGFR activation, intracellular GSTP1 specific activity in SUM 149 cells increased 3-fold (Table 3), whereas phospho-GSTP1 levels increased by 2.25-fold (Table 3, Fig. 6*B*), and glutathionylplatinum metabolite levels increased by 2.3-fold (Table 3, Fig. 6*C*). Upon exposure to 2.5 μM lapatinib for 30 min, GSTP1 activity in the EGF-treated cells was reduced to 36.4% that of the peak level, whereas the level of phospho-GSTP1 was reduced to that of control untreated cells (Fig. 6*B*). The effect of EGFR activation and inhibition on cisplatin sensitivity of the inflamma-

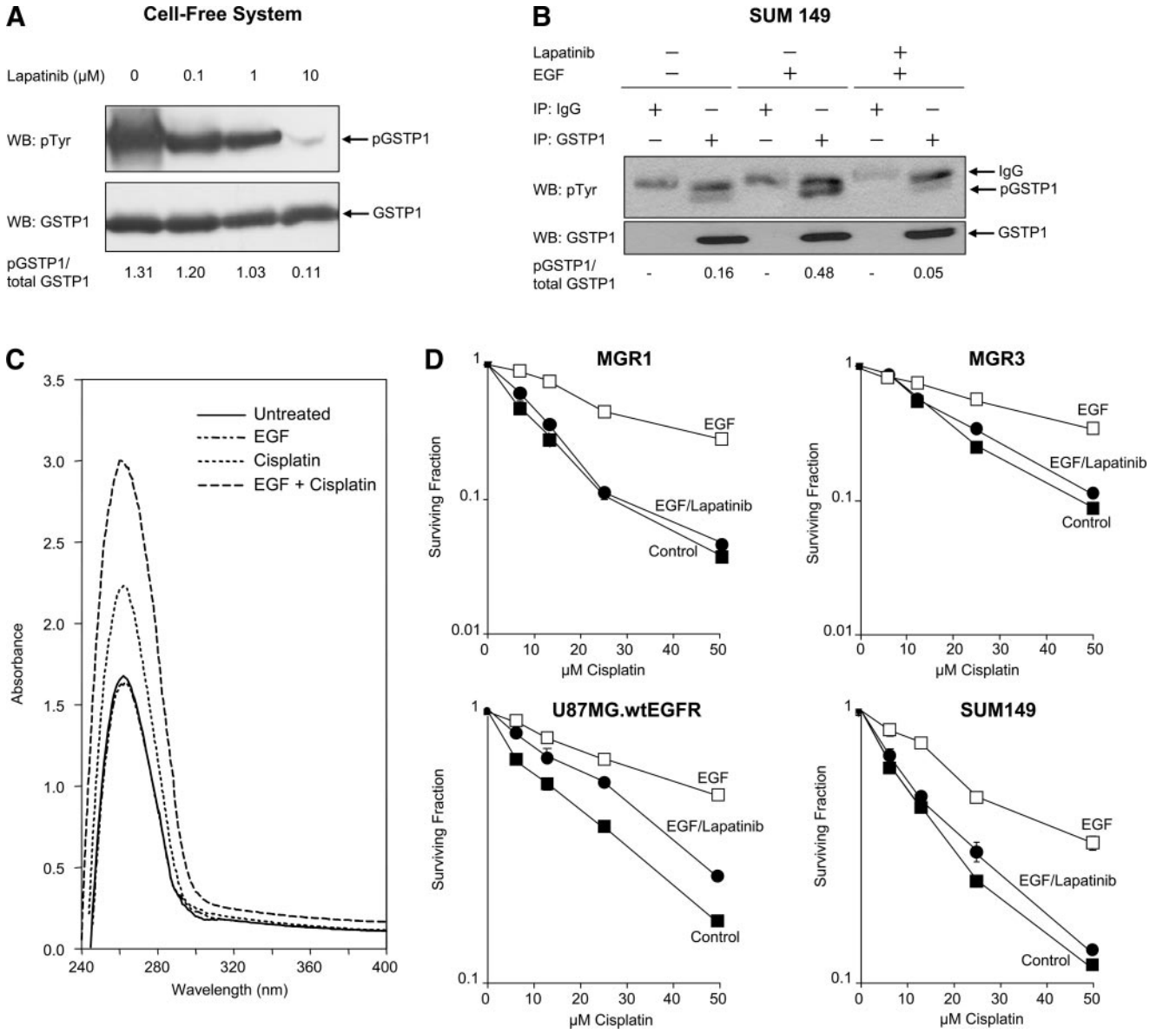


FIGURE 6. **Effect of EGFR modulation on GSTP1 phosphorylation, glutathione-cisplatin conjugate formation, and tumor cell survival.** *A*, Western blotting (*WB*) showing dose-dependent inhibition of GSTP1 phosphorylation by lapatinib in a cell-free system. The tyrosine-phosphorylated GSTP1 bands were normalized against those of total GSTP1 protein. *B*, IP-Western blotting of SUM 149 inflammatory breast cancer cells with and without EGF or lapatinib treatment showing EGF-induced GSTP1 tyrosine phosphorylation and lapatinib-mediated inhibition of the EGF-induced GSTP1 phosphorylation. The tyrosine-phosphorylated GSTP1 is present only in immunoprecipitates of the EGF-treated cells, whereas the IgG light chain is present in all immunoprecipitates. *C*, analysis of glutathione-cisplatin conjugate *in vivo* (cultured tumor cells). SUM 149 cells were treated with EGF to activate EGFR, after which they were treated with 100 μM cisplatin. After 2 h the cells were harvested and used to quantitate the level of glutathionylplatinum. *D*, survival curves of control, EGF-, and EGF and lapatinib-treated cells following by exposure to cisplatin.

TABLE 3

Effect of EGFR activation on the GSTP1 activity, GSTP1 phosphorylation and glutathione-platinum metabolite formation in SUM 149 cells

	GSTP1 activity	Phospho-GSTP1	GS-Pt conjugate
	nmol·min/μg protein	*Relative to total GSTP1*	ΔA_{265}
−EGF	33.8 ± 3.56	8.25 ± 1.71	0.59 ± 0.03
+EGF	101.4 ± 9.30	18.6 ± 3.30	1.35 ± 0.02

tory breast cancer cell line, SUM 149, and the three malignant glioma cell lines, MGR1, MGR3, and U87MG.wtEGFR, are summarized in Fig. 6*D*. In all four cell lines, EGFR activation by EGF resulted in a significant increase in cisplatin resistance. In each case a 30-min exposure of the cells to 2.5 μM lapatinib after EGFR activation caused a significant reversal of the acquired cisplatin resistance.

GSTP1-targeted siRNA Reverses Activated EGFR-induced Cisplatin Resistance in Glioma Cells—The results of the GSTP1-targeted siRNA studies, summarized in Table 4, show that GSTP1 down-regulation alone caused a 2.5- and a 2.4-fold sensitization of MGR3 and SUM 149 cells, respectively, to cisplatin. Treatment with EGF, on the other hand, induced a 1.8-fold (MGR3) and a 2.35-fold (SUM 149) increase in cisplatin resistance. The combination of siRNA and EGF treatment resulted in a 1.9- and 1.8-fold reversal of the EGF-induced resistance in MGR3 and SUM 149 cells, respectively. The Western blot of the cells transfected with GSTP1 siRNA showed a

TABLE 4

Effect of GSTP1 knockdown on cisplatin sensitivity in MGR3 and SUM 149 cells

Cells were transfected with GSTP1-targeted siRNA and after 24 h were treated with or without EGF for 10 min followed by cisplatin. Cell survival was determined after 48 h. The control values were corrected against scrambled siRNA controls.

	IC_{50}; 48-h post-cisplatin exposure			
	Control (−EGF/−siRNA)	+EGF	+siRNA	+siRNA/+EGF
		μM		
MGR3	18.91 ± 2.19	34.83 ± 2.43	6.50 ± 0.12	6.73 ± 0.23
SUM 149	10.46 ± 0.91	24.60 ± 1.3	3.29 ± 0.07	4.76 ± 0.70

93% reduction of GSTP1 relative to untransfected controls (results not shown). There was no effect of the scrambled siRNA on GSTP expression.

DISCUSSION

Activation of the receptor-tyrosine kinase, EGFR, resulting from binding of ligands such as EGF or transforming growth factor-α or its constitutive activation, as in EGFRvIII, initiates important cell signaling cascades, including the phosphatidylinositol 3-kinase/AKT/mTOR, Janus kinase/STAT (signal transducers and activators of transcription), and Ras/Raf/MAPK pathways (1, 2). In this study we provide the first evidence that the human GSTP1 protein is a direct downstream EGFR target and an important player in the EGFR signaling network. Our data show that activated EGFR binds to and phosphorylates GSTP1 in a cell-free system and in human tumor cells growing *in vitro* and *in vivo*, and that the phosphorylation results in a structural and functional alteration of the GSTP1 protein. Phosphoamino acid analysis of the hydrolyzed EGFR-phosphorylated GSTP1 confirmed that only tyrosine residues were phosphorylated by EGFR in the GSTP1 protein. The combination of peptide phosphorylation and mutational analysis with LC-MS/MS showed conclusively that Tyr-3, Tyr-7, and Tyr-198 were among the principal amino acid residues phosphorylated by EGFR in the GSTP1 protein. The results of the analysis of GSTP1 phosphorylation in cultured glioma cells that had been transfected with full-length GSTP1 cDNAs carrying Tyr → Phe single, double, and triple mutations in Tyr-3, Tyr-7, and Tyr-198 showed that after EGF stimulation, cells carrying each of the mutant GSTP1 cDNAs showed a significant decrease in phosphorylation in the *in vivo* cellular setting. These results not only confirm the observation in the cell-free systems that all three tyrosines in the GSTP1 proteins are phospho-acceptors, but the fact that the highest decrease in GSTP1 phosphorylation was observed with the Y198F followed by Y7F containing mutants suggests that these residues may be the most significant phospho-acceptors in the cellular context.

An interesting finding in this study was that the phosphorylation of GSTP1 by EGFR was enhanced by GSH, similar to our previous observation with the Ser/Thr kinases, cAMP-dependent protein kinase, and cAMP-dependent protein kinase (22). The basis for the requirement of GSH for efficient phosphorylation of GSTP1 by protein kinases is unclear. We postulate, however, that conformational changes induced by GSH binding to the G-site of the GSTP1 protein may position the phospho-acceptor residues more favorably for phosphorylation. This notion is supported by x-ray crystallographic studies showing that after GSH binding, the G-site of the irregular α-helix 2 (residues 37–46) in the GSTP1 protein assumes a less flexible form and a structural motion occurs that increases the distance between helix 2 and helix 4 (37, 38). The requirement of GSH for EGFR phosphorylation of GSTP1 is also consistent with prior reports that EGFR is a redox-regulated protein (31). The enhancing effect of GSH on GSTP1 phosphorylation by EGFR suggests that in tumors, such as GBM or breast cancer, with high intracellular GSH content, GSTP1 will be in a hyperphosphorylated and catalytically more active state. Furthermore, in tumors with *EGFR* amplification and/or overexpression, GSTP1 will be in a phosphorylated and more active state, thus leading to increased drug conjugation/inactivation and, subsequently, drug resistance. In normal cells, the antioxidant state characterized by elevated GSH will favor a functionally enhanced GSTP1 and a correspondingly increased ability to protect cells from damage by free radicals, alkylating carcinogens, and other genotoxins.

The enzyme kinetic studies showed that EGFR phosphorylation increased the catalytic efficiency of GSTP1, as measured by the utilization ratio, K_{cat}/K_m, for the GSTP1-specific substrate, EA, by almost 3-fold. This increase in catalytic efficiency is the direct result of a significant reduction in the K_m of EA. Given that K_m is an estimate of how well a substrate binds to an enzyme (28), the decrease in K_m indicates an increased affinity of EA for the H-site of the phosphorylated GSTP1.

The results of the computer modeling of the three-dimensional structure of the GSTP1 active site in which the critical active site residue, Tyr-7 (39, 40), was phosphorylated showed a significant increase in electronegativity of the environment around this tyrosine residue. The increase in electronegativity suggests that electrophilic substrates will have an increased affinity for the phosphorylated active site and may explain, in part, the increased affinity (lower K_m) of the GSTP1 substrate, EA, for the EGFR-phosphorylated GSTP1 protein and the associated increase in its enzymatic activity.

The results of the *in vivo* studies using both xenografts and cultured cells of EGFR- and GSTP1-overexpressing glioma and inflammatory breast cancer cell lines place the findings of the cell-free (*in vitro*) studies in context and confirm their relevance *in vivo*. The finding that EGF-mediated EGFR activation results in increased GSTP1 activity, increased GSTP1 tyrosine phosphorylation, and increased levels of cisplatin-glutathione conjugate and that this was associated with increased resistance of tumor cells to cisplatin, a drug metabolized by GSTP1 (29, 30), are particularly relevant. The EGF-induced cisplatin resistance was reversible upon treatment of the cells with lapatinib, a clinically active EGFR inhibitor or upon siRNA-mediated depletion of GSTP1. These observations suggest that in cancer patients the cross-talk between EGFR and GSTP1 has the potential to mediate tumor drug resistance and, possibly, other functions of GSTP1 and EGFR that could contribute to a more aggressive tumor growth and treatment failure.

Although the full implications of the phosphorylation of GSTP1 by EGFR remain to be elucidated, our findings establish it as a novel, heretofore unrecognized component of the downstream pathway of the EGFR receptor tyrosine kinase and, thus, expand our understanding of the biological roles of these two

important proteins. In particular, it will be important to establish whether the stability, ability to metabolize other anticancer drugs, and known carcinogens and/or to interact with other known important GSTP binding, such as N-terminal kinase, apoptosis signal-regulating kinase 1, transglutaminase 2, or Fanconi anemia group C protein, of GSTP1 are altered upon phosphorylation by EGFR. Taken together, the findings in this study suggest that in cancer therapy the dual targeting of EGFR and GSTP1 could potentially be more effective than the current strategy of targeting either protein individually.

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