Loss of Protein Inhibitors of Activated STAT-3 Expression in Glioblastoma Multiforme Tumors: Implications for STAT-3 Activation and Gene Expression

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Abstract

Purpose: STATs activate transcription in response to numerous cytokines, controlling proliferation, gene expression, and apoptosis. Aberrant activation of STAT proteins, particularly STAT-3, is implicated in the pathogenesis of many cancers, including GBM, by promoting cell cycle progression, stimulating angiogenesis, and impairing tumor immune surveillance. Little is known about the endogenous STAT inhibitors, the PIAS proteins, in human malignancies. The objective of this study was to examine the expression of STAT-3 and its negative regulator, PIAS3, in human tissue samples from control and GBM brains.

Experimental Design: Control and GBM human tissues were analyzed by immunoblotting and immunohistochemistry to determine the activation status of STAT-3 and expression of the PIAS3 protein. The functional consequence of PIAS3 inhibition by small interfering RNA or PIAS3 overexpression in GBM cells was determined by examining cell proliferation, STAT-3 transcriptional activity, and STAT-3 target gene expression. This was accomplished using [3H]Tdr incorporation, STAT-3 dominant-negative constructs, reverse transcription-PCR, and immunoblotting.

Results and Conclusions: STAT-3 activation, as assessed by tyrosine and serine phosphorylation, was elevated in GBM tissue compared with control tissue. Interestingly, we observed expression of PIAS3 in control tissue, whereas PIAS3 protein expression in GBM tissue was greatly reduced. Inhibition of PIAS3 resulted in enhanced glioblastoma cellular proliferation. Conversely, PIAS3 overexpression inhibited STAT-3 transcriptional activity, expression of STAT-3-regulated genes, and cell proliferation. We propose that the loss of PIAS3 in GBM contributes to enhanced STAT-3 transcriptional activity and subsequent cell proliferation.

Who grade 4 glioblastoma multiforme (GBM) is the most aggressive malignant astrocytic glioma because of the high degree of cellularity, vascular proliferation, and necrosis. Patients diagnosed with GBMs have a median life expectancy of less than 1 year (1). GBMs are characterized by their propensity to infiltrate throughout the brain, which results in the inability of surgery to cure patients even when surgical resection is possible. In addition, the majority of GBMs are resistant to standard radiotherapy and chemotherapy (1).

Aberrant signaling through receptor tyrosine kinases, including the epidermal growth factor receptor and platelet-derived growth factor receptor, is a hallmark of GBM (2). Constitutive activation of epidermal growth factor receptor and platelet-derived growth factor receptor promotes cell growth and evasion of apoptosis, events that lead to maintenance of a tumor-promoting environment. Deregulated signaling through the mitogen-activated protein kinase, phosphatidylinositol 3-kinase/AKT, protein kinase C, nuclear factor-κB (NF-κB), and Janus-Activated Kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) pathways has also been implicated in glioma development and progression (2, 3).

The JAK family of receptor-associated tyrosine kinases is activated by phosphorylation after ligand binding and activates STAT proteins to induce gene expression (4). The STAT family consists of seven members (STAT-1, STAT-2, STAT-3, STAT-4, STAT-5A, STAT-5B, and STAT-6) and is activated by many stimuli, including the interleukin (IL)-6 cytokine family. Members include IL-6, oncostatin M (OSM), leukemia inhibitory factor, ciliary neurotrophic factor, and IL-11 (5). IL-6 cytokines preferentially activate STAT-3, leading to dimerization, nuclear translocation, and binding to IFN-γ-activated site-like DNA.
Translational Relevance

STAT-3, a cytoplasmic transcription factor that becomes activated in response to a variety of cytokines, chemokines, and growth factors is aberrantly activated in several human cancers, including GBM. STAT-3 is a promising target for GBM therapy because it is a convergence point for several signaling pathways that promote glioma growth and maintenance, and because aberrant STAT-3 activation results from upstream dysregulation, not constitutively active STAT-3 mutations. Pharmacologic inhibitors of STAT-3, including AG490, WP1066, curcubatin I, and gefitinib, have shown promising results in glioblastoma cells in vitro and are in early stages of clinical trials. PIAS3, a negative regulator of STAT-3, is a protein whose function is of great importance in understanding the regulation of STAT-3 signal transduction in vivo. This work provides evidence of PIAS3 dysregulation in GBM and the promotion of STAT-3 transcriptional activity in human GBM. The presence or absence of PIAS3 may determine GBM patients that would be responsive to STAT-3 inhibitors in future clinical trials.

Materials and Methods

Cells. U251-MG, U87-MG, SNB-19, M059K-MG, U138-MG, and U118-MG human astroglioma cells were cultured as previously described (8). Primary murine astrocytes were >97% positive for GFAP, and microglia were >90% positive for Mac1, as previously described (22). Neuronal cultures were prepared by isolating cerebral hemispheres from P0 mice and removing the meninges, as described (23). GBM cell cultures from four patients were received from the University of Alabama at Birmingham (UAB) Brain Tumor Bank of the Cooperative Tissue Network, in accordance with the UAB Human Tissue Committee policies, Institutional Review Board Exemption #X050415007. GBM primary cells were obtained after 30 days and grown in DMEM/F-12 medium supplemented with 2 mmol/L l-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum, as previously described (24).

Reagents. Recombinant human OSM, IL-6, and soluble IL-6 receptor (sIL-6R) were obtained from R&D Systems. Phorbol 12-myristate 13-acetate was purchased from Calbiochem. Antibodies to STAT-3, PIAS3 (COOH terminus), and p21 were obtained from Santa Cruz Biotechnology, Inc. Anti-PIAS3 (NH2 terminus) antibody was purchased from Abgent. Antibodies to phosphoserine STAT-3, phospho-tyrosine STAT-3, and STAT-3 were from Cell Signaling Technology. Anti-STAT-3, photyrosine STAT-3, and STAT-3 were from Cell Signaling Technology. Anti-STAT-3 antibody was from Zymed and anti-actin antibody was from Sigma. The 3xLySepZlac-TK (STAT-3) luciferase construct was obtained from Addgene. The 1,556-bp SOCS-3 promoter has been previously characterized (25). The matrix metalloproteinase-9 (MMP-9)-Luc luciferase reporter plasmid containing 670 bp of the human MMP-9 promoter was a generous gift from Dr. D. Boyd (M. D. Anderson Cancer Center, Houston, TX). The STAT-3F dominant-negative expression construct has been previously described (26). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were purchased from Clontech. Human PIAS3 cDNA (clone ID: 3528679; accession number: BC001154) was purchased from Open Biosystems and cloned into the pcDNA3 vector. MG-132 was purchased from Calbiochem.

Human tissues. Tissues were received from the UAB Brain Tumor Bank of the Cooperative Tissue Network, in accordance with the UAB Human Tissue Committee policies, Institutional Review Board Exemption #X050415007. The nonneoplastic brain tumor biopsy samples were obtained from patients diagnosed with epilepsy. The tumor biopsies were diagnosed as GBM (WHO grade 4 tumors).

Small interfering RNA transfection. U87-MG cells were untreated or transiently transfected using DharmaFECT 1 reagent (Dharmacon) with either 100 nmol PIAS3 siPOOL Reagent or 100 nmol of negative control small interfering RNA (siRNA; Ambion) according to the manufacturer's instructions to achieve ~85% transfection efficiency. Twenty-four hours after transfection, cells were collected and analyzed for target knockdown by reverse transcription-PCR with primers for human PIAS3 and GAPDH. Cells were collected 24, 48, and 72 h after transfection and analyzed by immunoblotting for PIAS3 and actin.

Immunoblotting. Nonneoplastic brain biopsy (controls) and GBM biopsy samples were lysed, as described previously (8). Total protein (40 μg) was resolved on an 8% SDS-PAGE gel, transferred to
followed by 40 cycles of 95°C cycling conditions were 48°C, quantifying an internal RNA control (S9) and a standard curve. The total RNA from U251-MG cells was extracted using Trizol. PCRs were done in triplicate on an 8% SDS-PAGE gel and analyzed as described above. Densitometry was used to quantify the immunoblotting results, and actin served as an internal control for sample loading. All results were normalized by the respective actin values.

**RNA isolation, quantitative real-time PCR, and reverse transcription-PCR.** Total RNA from human tissues was extracted using a PowerGen 125 with Trizol. The GeneAmp 7700 Sequence Detection System (Applied Biosystems) was used for detection of real-time PCR products amplified from reverse-transcribed total RNA (25 ng). Total RNA from U251-MG cells was extracted using Trizol. PCRs were done in triplicate for each sample on a 96-well plate that included separate wells to control for loading. The enhanced chemiluminescence method was used for protein detection, as previously described (25). For SOCS-3, 100 µg protein was used.

U251-MG cells were treated with either 50 ng/mL OSM or 10 ng/mL IL-6 plus 50 ng/mL sIL-6R for various times. Cells were lysed as previously described (25). Total cell lysate (40 µg) was resolved on an 8% SDS-PAGE gel, transferred to nitrocellulose membrane, and then blocked for 1 h in 5% milk. Membranes were probed as described above.

For proteasome inhibition, U251-MG, SNB-19, and GBM primary cells were incubated with MG-132 (10 µmol/L) for various times and then collected as described above. Total cell lysate (40 µg) was resolved on an 8% SDS-PAGE gel and analyzed as described above. Densitometry was used to quantify the immunoblotting results, and actin served as an internal control for sample loading. All results were normalized by the respective actin values.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded tissues were obtained from the UAB Brain Tumor Bank, in accordance with the UAB Human Tissue Committee policies, Institutional Review Board Exemption #X050415007. Briefly, tissue sections were deparaffinized, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS. Sections were then incubated in PBS to block for 1 h in 5% milk. Membranes were probed as described above.

**Fig. 1.** STAT-3 protein activation is elevated and PIAS3 protein expression is reduced in GBM tissues compared with control brain tissue. **A,** immunoblot of phospho-Tyr705-STAT-3, phospho-Ser727-STAT-3, total STAT-3 (1:1,000), PIAS3 (1:100 dilution; Abgent), SOCS-3 (1:500), and actin (1:1,000 dilution) to control for loading. The enhanced chemiluminescence method was used for protein detection, as previously described (25). For SOCS-3, 100 µg protein was used.

**B** shows PIAS3 mRNA expression in control samples and GBM samples. The PIAS3 mRNA expression is represented as a ratio to the housekeeping gene S9. Columns, mean of three experiments on control (n = 11) and GBM (n = 11) tissue samples; bars, SD. **C,** immunoblot of PIAS3 protein in control and GBM tissues. Blots were reprobed for actin to verify protein loading between samples. Representative of four experiments.

**References.**

Transfection and luciferase assay. U251-MG cells were transiently transfected using the Fugene 6 reagent (Roche Diagnostics) with either 500 ng of 3xLy6epZLuc-TK, which contains three STAT-3 sites (27), 500 ng of the 1,556-bp SOCS-3 promoter (25), or 200 ng of the MMP-9 promoter. Cells were also transfected with either the STAT-3F plasmid or the PIAS3-pcDNA (100-500 ng). Cells were incubated for 16 h in the presence or absence of either OSM or phorbol 12-myristate 13-acetate, and then cell lysates were assayed in triplicate for luciferase activity (28) and normalized to total protein. The luciferase activity from the untreated condition was arbitrarily set at 1 for calculation of fold induction.

[^3H]TdR incorporation. [^3H]TdR incorporation was done as previously described (24). Briefly, U87-MG and U251-MG cells were labeled with 1 μCi of [^3H]TdR for 16 h before terminating the culture. Radioactivity was counted in duplicate samples using a scintillation counter (Beckman).

Fig. 1 Continued. D, human control (a-f; n = 2) and GBM (g-o; n = 3) tissues were analyzed by histochemistry. a, d, g, j, and m, tissue integrity was verified by H&E staining. b, e, h, k, and n, immunohistochemistry using normal rabbit serum (NRS) served as a negative control for staining in control and GBM tissues. c, f, i, l, and o, control and GBM tissues were stained with anti-PIAS3 antibody. Nuclei were counterstained with hematoxylin. Magnifications, ×100 and ×400 (insets).
**Statistical analysis.** Levels of significance for comparison between samples were determined by the Student’s t test distribution. P values of \( \leq 0.05 \) were considered to be statistically significant.

**Results**

The **STAT-3 protein is constitutively activated and PIAS3 protein expression is diminished in human GBM tissue.** There are conflicting reports about the activation status of STAT-3 in GBM. Although tyrosine-phosphorylated STAT-3 was detected in GBM samples by several investigators (10–13), a tissue microarray study failed to confirm these findings (29). STAT-3 activation is reflected by phosphorylation of both tyrosine and serine residues; thus, we examined both variables in this study. Tissue samples were analyzed by immunoblotting, and higher levels of tyrosine- and serine-phosphorylated STAT-3 protein were detected in GBM tissue compared with controls (Fig. 1A). This is the first demonstration of serine-phosphorylated STAT-3 in GBM tissues. These results indicate that GBM samples contain elevated levels of activated STAT-3.

PIAS3 functions as a negative regulator of STAT-3 signaling by interfering with its ability to bind DNA (16). Using tissue samples from both GBM and control brains, PIAS3 mRNA and protein levels were examined by real-time reverse transcription-PCR and immunoblotting, respectively. Similar levels of PIAS3 mRNA expression were observed in control and GBM tissues (Fig. 1B). Due to the instability in housekeeping gene expression in human tumor tissues, the levels of PIAS3 were compared with both S9 (Fig. 1B) and GAPDH (data not shown), and comparable results were obtained. The 68-kDa PIAS3 protein was expressed in all control tissues tested; however, little to no expression of the PIAS3 protein was detected in GBM tissues (Fig. 1C). In 35 GBM samples tested, PIAS3 protein expression was detected in only 4 samples, translating to 89% of GBM samples with loss of PIAS3 protein expression, whereas 100% of control samples \( (n = 33) \) expressed PIAS3 (data not shown). Because of the striking difference in PIAS3 protein levels between control and GBM groups, a second antibody recognizing a different epitope on the PIAS3 protein was used to confirm the results (data not shown). To determine if any correlates could be established between the absence/presence of PIAS3 in GBM samples and STAT-3 activation, we compared these two variables. Of the 14 GBM samples analyzed in Fig. 1A, we were able to obtain PIAS3 status on 11 samples. Two of the 11 GBM samples expressed PIAS3 (samples 14 and 18), and those samples also had low levels of

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**Fig. 2.** PIAS3 is constitutively expressed in glioblastoma cell lines and primary central nervous system cells. **A,** U251-MG cells were incubated in the presence or absence of IL-6 (10 ng/mL) plus sIL-6R (50 ng/mL) for up to 24 h, and then cell lysates were assessed by immunoblotting for phospho-Tyr\(^{705}\)STAT-3, STAT-3, and PIAS3 protein expression. Immunoblots were reprobed for actin. Representative of three experiments. **B,** constitutive PIAS3 protein expression in four glioblastoma cell lines, SNB-19, M059K-MG, U138-MG, and U118-MG, is shown by immunoblotting. Cells were maintained in 10% fetal bovine serum (FBS) or serum starved (no fetal bovine serum) before collection. Blots were stripped and reprobed for actin to control for protein loading. **C,** four primary human GBM cultures were analyzed for phospho-Tyr\(^{705}\)STAT-3, phospho-Ser\(^{727}\)STAT-3, STAT-3, and PIAS3 protein expression by immunoblotting. Resected human GBM tissues were cultured, as described in Materials and Methods, and lysates were collected for analysis. Anti-actin antibody was used to control for protein loading. **D,** SNB-19, U251-MG, and primary human GBM cells were incubated in the presence or absence of MG-132 (10 \( \mu \)mol/L) for up to 6 h, and then cell lysates were assessed by immunoblotting for PIAS3 expression. Immunoblots were reprobed for actin. Data are representative of three independent experiments. Fold induction is calculated based on densitometry measurements, normalized to actin, and compared with the untreated sample.
Loss of PIAS3 in GBMs

Fig. 3. OSM activates STAT-3 in human glioma cells. A, U87-MG cells were incubated in the presence or absence of OSM (50 ng/mL) for up to 6 h and cell lysates were assessed by immunoblotting with phospho-Tyr$^{705}$ STAT-3 antibody. Blots were reprobed for total STAT-3 and actin. Representative of four experiments. B, U251-MG cells were transiently transfected with the 3×Ly6epZLuc-TK (STAT-3) luciferase construct (500 ng), either alone or with pcDNA3 and/or STAT-3F (0-500 ng), and then recovered overnight before treatment with medium or OSM for 16 h. Lysates were analyzed in triplicate for luciferase activity and normalized to protein levels. Columns, mean of one of three independent experiments; bars, SD. *, $P < 0.002$, compared with medium only condition; **, $P ≤ 0.005$, compared with OSM plus pcDNA3 (500 ng) condition.

activated STAT-3 (see Fig. 1A). Samples 9, 10, 11, 17, 19, 20, 21, and 22 lacked PIAS3 expression and had high levels of activated STAT-3, except for sample 21 (see Fig. 1A). These results suggest that a lack of PIAS3 in GBMs may contribute to constitutive STAT-3 activation.

Tissues were examined using immunohistochemistry, and sections were analyzed by a neuropathologist blinded to the antibodies used. Strong nuclear staining for PIAS3 was observed in two control brain specimens (Fig. 1D, c and f, arrowheads). Positive staining appeared mainly in neurons, with weaker staining in astrocytes and endothelial cells. Human GBM ($n = 3$) tissues had little to no positive reactivity for PIAS3 (Fig. 1D, i, l, and o). These data indicate that loss of PIAS3 protein expression in GBM samples is frequent.

The PIAS3 protein is constitutively expressed in vitro. Because the expression levels of PIAS3 were substantially different between control and GBM samples, we examined the expression of PIAS3 in human glioblastoma cells. Constitutive expression of PIAS3 was observed in U251-MG cells (Fig. 2A). IL-6/sIL-6R and OSM treatment induced STAT-3 tyrosine phosphorylation but had little effect on PIAS3 expression (Fig. 2A; data not shown). Comparable results were obtained with U87-MG cells (data not shown). Whole-cell extracts from four additional glioblastoma lines, SNB-19, M059K-MG, U138-MG, and U118-MG, showed constitutive, although varying, levels of PIAS3 expression, which were unaffected by the presence of serum (Fig. 2B). Primary human GBM cultures expressed PIAS3 and had nondetectable to low constitutive STAT-3 activation (Fig. 2C), which is in contrast to the GBM tissues. These data suggest that regulation of the PIAS3 protein differs between GBM tissues and the in vitro systems tested, including cultures established from resected GBM tissues.

To further examine the in vitro expression pattern of PIAS3, primary murine astrocytes, murine microglia, and rat cortical neurons were examined (Supplementary Fig. S1). In all central nervous system cell types, expression of PIAS3 was detected. Collectively, these data indicate that in vitro, human glioblastoma cell lines, as well as primary glial and neuronal cultures, show constitutive PIAS3 expression.

The discrepancy of PIAS3 protein expression patterns between primary GBM tissues and cell lines suggested that the absence of PIAS3 in vivo might be due to degradation. We tested this hypothesis using the SNB-19 and U251-MG cell lines as well as primary human GBM cultures. Treatment with MG-132, a classic inhibitor of the proteasome, resulted in an accumulation of the PIAS3 protein (Fig. 2D), which increased over time in U251-MG cells. These results showed that expression of PIAS3 in vitro is enhanced by inhibition of the proteasome, suggesting that ubiquitin-mediated degradation may promote the loss of PIAS3 in vivo.

STAT-3 is activated by OSM in U251-MG cells. Having illustrated the activation of STAT-3 and loss of PIAS3 in vitro, the activation status of this signaling pathway was analyzed in vivo. In untreated U251-MG cells, STAT-3 had little to no basal tyrosine phosphorylation. Treatment with OSM induced STAT-3 tyrosine phosphorylation at 30 min, which persisted to 6 h (Fig. 3A). Comparable results were observed using IL-6/sIL-6R (Fig. 2A). Other GBM cell lines, including U87-MG, CH235-MG, and D54-MG, were tested for STAT-3 activation, and similar results were observed (data not shown). A measure of the transcriptional activity of STAT-3 is its ability to activate a STAT-3–driven promoter. On treatment with OSM, U251-MG cells showed a 35-fold increase in STAT-3 promoter activity (Fig. 3B), which was inhibited by a dominant-negative STAT-3 construct, STAT-3F (Fig. 3B). Collectively, these data showed that basal levels of STAT-3 activation in glioblastoma cells were minimal. Importantly, activation was observed on OSM or IL-6/sIL-6R treatment, which resembled the pattern observed in human GBM tissues.

siRNA-mediated inhibition of PIAS3 increases proliferation. To mimic the observed in vivo loss of PIAS3, siRNA was used to inhibit PIAS3 expression in U87-MG cells. These cells were selected as they were the most amenable to siRNA knockdown. Transient transfection using PIAS3 siRNA showed specific inhibition of PIAS3 mRNA at 24 h (Fig. 4A). PIAS3 protein levels decreased over time in culture (Fig. 4B, lanes 1, 3, and 5); however, PIAS3 knockdown was detected at 48 and 72 h (lanes 4 and 6). STAT-3 activation in the PIAS3 knockdown cells was examined, and minimal to no enhancement of basal tyrosine-phosphorylated STAT-3 was observed (data not shown), indicating that in cells with reduced PIAS3 expression, levels of phosphorylated STAT-3 remain relatively unchanged. Although activation of STAT-3 is measured by tyrosine and serine phosphorylation, one functional indicator of STAT-3 transcriptional activation is cell proliferation (30). We examined this variable using the $[^{3}H]$TdR incorporation assay. Knockdown of PIAS3 resulted in a significant increase in cell


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PIAS3 overexpression inhibits STAT-3 and SOCS-3 expression is elevated in human GBM tissues. Unlike the constitutively expressed PIAS proteins, SOCS proteins are cytoplasmic, inducibly expressed proteins that act in a negative feedback loop to inhibit JAK activation. SOCS-3 is not only an endogenous inhibitor of STAT-3 (32) but also a STAT-3 transcriptional target (28). Recent investigations have revealed a role for SOCS-3 in cancer cells (33, 34). Ectopic PIAS3 expression repressed OSM activation of the SOCS-3 promoter in a dose-dependent manner (Fig. 6A). In addition, levels of OSM-induced SOCS-3 mRNA were significantly inhibited on PIAS3 overexpression (Fig. 6B). These results indicated that overexpression of PIAS3 can reduce STAT-3 transcriptional activity and inhibit the expression of the STAT-3 target gene SOCS-3.

Given the observation that activated STAT-3 levels were elevated, and PIAS3 levels were diminished in GBMs, we examined the expression of SOCS-3 in vivo. Enhanced levels of the SOCS-3 protein were observed in GBM tissues compared with controls (Fig. 6C), with variability in expression levels. SOCS-3 protein expression was observed in 16 of 18 GBM tissues, whereas only 1 of 8 control samples expressed SOCS-3 (data not shown). These data showed that SOCS-3, a STAT-3 transcriptional target gene, was elevated at the protein level in GBM tissues.

PIAS3 inhibits transcriptional activation of the NF-κB–regulated gene MMP-9. PIAS3 is classically defined as an inhibitor of the JAK-STAT pathway, but its expression has recently been shown to modulate several signal transduction pathways, many of which are implicated in glioma cell biology, such as the NF-κB and transforming growth factor-β pathways (16). To test the effect of PIAS3 on the NF-κB pathway, we examined transcriptional activation of the MMP-9 promoter, as we have previously shown that MMP-9 gene expression is dependent on the NF-κB signaling pathway (35). We observed that ectopic expression of PIAS3 in U251-MG glioma cells inhibited phorbol 12-myristate 13-acetate–induced activation of the MMP-9 promoter in a dose-dependent manner (Supplementary Fig. S2). These results confirm that PIAS3 expression modulates the transcriptional activation of NF-κB target genes and describe this effect on the MMP-9 gene for the first time in human glioma cells.

Discussion

The role of PIAS3 in human disease states is largely unclear. Although PIAS3 expression has been shown to correlate with malignancy (17, 36), other reports describe deficient PIAS3 expression in various human cancers (18). PIAS3 function has been examined in vitro using transformed cell lines. Infection of prostate cancer cells with adenovirus encoding PIAS3 increased apoptosis (19). Furthermore, PIAS3 overexpression prevented further explore the effects of PIAS3 on the cell cycle, the expression of p21, the cyclin-dependent kinase inhibitor, was evaluated. Expression of the p21 protein was enhanced by ~2-fold on PIAS3 ectopic expression (Fig. 5D). Collectively, these data indicated that PIAS3 overexpression attenuated STAT-3 transcriptional activity, inhibited the expression of survivin and Bcl-xl, and reduced the proliferation of glioma cells. The inhibition of cell proliferation may be due to enhanced expression of p21, which regulates the cell cycle at the transition from G1 to S phase.

PIAS3 overexpression inhibits SOCS-3, and SOCS-3 expression is overexpression inhibits transcriptional activation of the NF-κB–regulated gene MMP-9. PIAS3 is classically defined as an inhibitor of the JAK-STAT pathway, but its expression has recently been shown to modulate several signal transduction pathways, many of which are implicated in glioma cell biology, such as the NF-κB and transforming growth factor-β pathways (16). To test the effect of PIAS3 on the NF-κB pathway, we examined transcriptional activation of the MMP-9 promoter, as we have previously shown that MMP-9 gene expression is dependent on the NF-κB signaling pathway (35). We observed that ectopic expression of PIAS3 in U251-MG glioma cells inhibited phorbol 12-myristate 13-acetate–induced activation of the MMP-9 promoter in a dose-dependent manner (Supplementary Fig. S2). These results confirm that PIAS3 expression modulates the transcriptional activation of NF-κB target genes and describe this effect on the MMP-9 gene for the first time in human glioma cells.

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growth of prostate tumor xenografts in nude mice (19). Overexpression of PIAS3 in lung cancer cells led to inhibition of cell growth, increased susceptibility to chemotherapeutic agents, and suppression of AKT activation (20). Additionally, PIAS3 overexpression in COS-7 cells suppressed gene induction by v-src–activated STAT-3 (37).

This study examined PIAS3 expression in human GBM tissues. The striking absence of PIAS3 protein in GBM tissues compared with control tissues was observed by both immunoblotting and immunohistochemistry (Fig. 1C and D) and confirmed by two PIAS3 antibodies targeting different PIAS3 epitopes. Our results contrast those of Wang and Banerjee (17) who detected PIAS3 in GBM specimens. However, they examined a very small number of GBM tissues and used a different PIAS3 antibody, which may account for the differences observed. There were no differences between control and GBM tissue expression of PIAS3 mRNA (Fig. 1B); thus, we postulated that the differences seen at the protein level were most likely due to posttranslational changes to PIAS3. In the GBM tissue microenvironment, it is possible that PIAS3 was quickly degraded by the ubiquitin-proteasome system, releasing constraints on STAT-3 signal transduction. MG-132 treatment of primary GBM cells and glioma cell lines induced an appreciable accumulation of the PIAS3 protein in vitro (Fig. 2D), supporting the notion that PIAS3 is rapidly degraded in GBM tissues. PIAS3 protein stability was shown to be regulated by nitric oxide, which promoted its association with an E3 ubiquitin ligase and subsequent degradation (38). Nitric oxide metabolism is closely associated with glioma progression, and the enzyme responsible for nitric oxide production has been detected in human glioma tissues (39). The loss of PIAS3 protein, either by rapid degradation or another mechanism, may allow activated STAT-3 to sustain transcription of genes, which control proliferation and the cell cycle. Proteins including p53 and p27^kip-1 are destabilized and degraded by ubiquitination in a variety of cancers (40). The loss of PIAS3 protein provides a mechanism to promote aberrant STAT-3 signal transduction in GBM tissues, which is a downstream target of mutated epidermal growth factor receptor, vascular endothelial growth factor receptor, and the IL-6 cytokine family.
The constitutive expression of PIAS3 provides a unique role for this protein to act as a “buffer” to inhibit sustained STAT-3 transcriptional activity, and in the absence of PIAS3, in conjunction with aberrant growth factor signal transduction, activation of STAT-3 and enhanced STAT-3 transcriptional activity is observed in GBMs.

PIAS3 expression was inhibited in astrogliaoma cells using siRNA to mimic the in vivo loss observed in GBM tissue. PIAS3 knockdown resulted in a significant increase in proliferation of astrogliaoma cells (Fig. 4C) but did not affect the overall levels of phosphorylated STAT-3 (data not shown). These data support the previous findings of Herrmann et al. (42), which show that PIAS3 expression does not modulate cellular levels of phosphorylated STAT-3 but does influence its ability to activate transcription. Accordingly, overexpression of PIAS3 resulted in significant inhibition of STAT-3 transcriptional activity and down-regulation of survivin and Bcl-xL (Fig. 5A and B). Attenuation of cell proliferation (Fig. 5C) and enhanced p21 protein expression (Fig. 5D) was observed in cells that ectopically expressed PIAS3. These data support the hypothesis that PIAS3 influences the expression of genes that control cell proliferation and survival. Thus, the PIAS3 protein exhibits several antitumorigenic functional properties important for restraining GBM cell growth and gene expression.

Although SOCS-3 is a negative regulator of the STAT-3 signaling pathway, it is also a STAT-3 transcriptional target. Overexpression of the PIAS3 protein caused a significant reduction in SOCS-3 promoter activity and SOCS-3 mRNA expression (Fig. 6A and B), showing SOCS-3 transcriptional repression when PIAS3 is overexpressed. Because SOCS-3 promoter activity and mRNA expression were negatively affected by PIAS3 overexpression, we examined its expression in human tissues and discovered SOCS-3 aberrant expression in GBM tissues (Fig. 6C). SOCS-3 is a member of the SOCS protein family, which is composed of CIS and SOCS-1 to SOCS-7 (32). SOCS-3 is a cytokine-inducible endogenous inhibitor of STAT-3 that works in a classic negative feedback loop (32), but it has recently been described to have a complicated role in cancer cell signaling. A tumor-suppressing function of SOCS-3 was suggested by reports describing hypermethylation of SOCS-3, and subsequent loss of expression, in a variety of cancers (43). This defective expression of SOCS-3, along with loss of feedback inhibition of STAT-3 activation, may contribute to tumor progression. However, other reports revealed that aberrant SOCS-3 expression may actually promote tumorigenesis. Elevated SOCS-3 expression was reported in human breast cancer and melanoma tissues as well as primary lymphoma cells (44, 45). Further, growth and proliferation was markedly induced in cancer cells that overexpressed SOCS-3 (33). SOCS-3 overexpression was attributed to constitutive STAT-3 activation in various cancers (46). Interestingly, human GBM tissues were recently reported to constitutively express SOCS-3, which correlated with enhanced cell growth/survival and radioresistance (34). Because SOCS-3 is a transcriptional target of STAT-3, the parallel, elevated expression of both activated STAT-3 and SOCS-3 proteins in GBM is plausible and may contribute to the antiapoptotic and radioresistant phenotype of GBMs.
In many cancers, including GBMs, mechanisms to regulate STAT-3 activity have failed, thus enabling activated STAT-3 to function as a tumor promoter (30). Interestingly, recent data showed that STAT-3 served conflicting roles, acting either as a tumor suppressor or as a promoter depending on the genetic profile of the tumor (47), furthering the customized, clinical use of STAT-3 inhibitors as potential therapeutics. Abrogation of STAT-3 activity by a variety of inhibitors, such as AG490 and WP1066, inhibited GBM cell growth, promoted apoptosis, and suppressed expression of antiapoptotic genes (3, 10, 48).

The loss of PIAS3 expression observed in GBMs may be a contributing factor to the STAT-3 transcriptional activation observed in this cancer. More recently, PIAS3 was shown to regulate other signaling pathways, including the NF-κB, phosphatidylinositol 3-kinase/AKT, and transforming growth factor-β pathways (16, 20). PIAS3 interacted with NF-κB p65 and repressed its transcriptional activity, thereby functioning as a negative regulator of NF-κB (49). Supporting this finding, we showed that transcriptional activation of the NF-κB–regulated gene MMP-9 is inhibited by PIAS3 overexpression in glioma cells (Supplementary Fig. S2), linking PIAS3 expression to inhibition of NF-κB signaling for the first time in glioma cell biology. Furthermore, others have shown that PIAS3 interacted with AKT in vitro, suppressing its phosphorylation and activation (20). PIAS proteins were also implicated in either positively or negatively regulating SMAD transcriptional activity, which mediate transforming growth factor-β biological activities (16). The aberrant activation of the NF-κB, phosphatidylinositol 3-kinase, and transforming growth factor-β pathways has been correlated with poor prognosis in patients with GBMs (12, 29, 50). Thus, the loss of PIAS3 expression in GBMs has implications for multiple signaling pathways contributing to gliomagenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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