

Signal Transducer and Activator of Transcription-3: A Molecular Hub for Signaling Pathways in Gliomas

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Abstract

Glioblastoma is the most common and severe primary brain tumor in adults. Its aggressive and infiltrative nature renders the current therapeutics of surgical resection, radiation, and chemotherapy relatively ineffective. Accordingly, recent research has focused on the elucidation of various signal transduction pathways in glioblastoma, particularly aberrant activation. This review focuses on the signal transducer and activator of transcription-3 (STAT-3) signal transduction pathway in the context of this devastating tumor. STAT-3 is aberrantly activated in human glioblastoma tissues, and this activation is implicated in controlling critical cellular events thought to be involved in gliomagenesis, such as cell cycle progression, apoptosis, angiogenesis, and immune evasion. There are no reports of gain-of-function mutations in glioblastoma; rather, the activation of STAT-3 is thought to be a consequence of either dysregulation of upstream kinases or loss of endogenous inhibitors. This review provides detailed insight into the multiple mechanisms of STAT-3 activation in glioblastoma, as well as describing endogenous and chemical inhibitors of this pathway and their clinical significance. In glioblastoma, STAT-3 acts a molecular hub to link extracellular signals to transcriptional control of proliferation, cell cycle progression, and immune evasion. Because STAT-3 plays this central role in glioblastoma signal transduction, it has significant potential as a therapeutic target. (Mol Cancer Res 2008;6(5):675–84)

Introduction

Malignant gliomas, the most common type of primary brain tumors, are highly aggressive, infiltrative, and destructive. The most common and severe form of malignant glioma, the WHO

grade 4 astrocytic glioblastoma (1, 2), affects ~13,000 people each year, with men more often affected than women. The infiltrative and aggressive nature of glioblastoma renders current treatments, such as surgical resection, radiation, and chemotherapy, relatively ineffective (1, 3). Median survival after treatment is 14 months, and despite advances in the basic understanding of cancer biology, this poor prognosis has not improved for several decades (1, 4). For this reason, recent studies have focused on understanding the molecular signaling pathways implicated in glioblastoma progression.

Glioblastoma progression presents molecular biologists with a challenge because components of several different signaling pathways, such as phosphoinositide-3 kinase, AKT, Ras, and mitogen-activated protein kinases (MAPK), and receptor tyrosine kinases, including the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor receptor (VEGFR), all seem to contribute strongly to the growth and promotion of glioblastoma (5, 6). We now know, however, that these diverse signaling pathways converge at specific transcription factors, including signal transducer and activator of transcription-3 (STAT-3). STAT-3 gene targets affect proliferation, growth, and apoptosis, and aberrant activation of STAT-3 has been identified not only in glioblastoma but also in a number of other human cancers, including breast, lung, ovarian, pancreatic, skin, and prostate cancers, and Hodgkin's lymphoma, myeloma, and acute myeloid leukemia (7). This review describes how STAT-3 activation and the resulting downstream effects play a role in glioblastoma progression, and how therapeutic strategies to inhibit STAT-3 signaling provide new opportunities for glioblastoma treatment.

STAT-3 and Its Activation Pathways

The STAT family of cytoplasmic latent transcription factors consists of seven members: STAT-1-4, STAT-5a, STAT-5b, and STAT-6 (8). STAT-3 activation results in expression of genes that control cell proliferation, survival, differentiation, and development. Like all STAT proteins, STAT-3 is activated by tyrosine phosphorylation in response to stimulation by cytokines and growth factors. Specifically, STAT-3 activation is downstream of receptor engagement by members of the interleukin-6 (IL-6) cytokine family, including IL-6, oncostatin M (OSM), and leukemia inhibitory factor, and by growth factors such as platelet-derived growth factor, fibroblast growth factor (FGF), and EGF (9). In addition to initial activation by tyrosine phosphorylation, phosphorylation of STAT-3 on serine residue 727 maximally activates its transcriptional activity (10).

Received 11/29/07; revised 1/3/08; accepted 1/23/08.

Grant support: NIH grants P50 CA-97247 and R01 NS-54158 (E.N. Benveniste).

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doi:10.1158/1541-7786.MCR-07-2180

STAT-3 is tyrosine phosphorylated by three types of kinases: receptor tyrosine kinases such as EGFR, FGF receptor (FGFR), or platelet-derived growth factor receptor, Janus kinase (JAK) family members, which are constitutively bound to the cytoplasmic tails of cytokine receptors, or nonreceptor-associated tyrosine kinases, including Ret, Src, or the Bcl-Abl fusion protein (Fig. 1A). After tyrosine phosphorylation, STAT-3 homodimerizes or heterodimerizes with STAT-1, translocates to the nucleus, and binds to consensus DNA sequences, such as the TTN₄₋₅AA sequence (where N refers to any nucleotide base), within promoters of its target genes (ref. 8; Fig. 1A). STAT proteins then cooperate with other transcription factors to regulate expression of numerous genes, including *bcl-2*, *bcl-xL*, *mcl-1*, *p21^{WAF1/CIP1}*, and *cyclin D1* (11, 12).

Regulation of STAT-3 Activity

Because STAT-3 affects transcription of genes involved in apoptosis and cell cycle, tight control of STAT-3 activity is imperative to prevent malignant transformation of cells. Following induction of target gene expression, endogenous negative regulators attenuate STAT protein activity on a number of levels. Suppressors of cytokine signaling (SOCS) proteins down-regulate the upstream kinase activity responsible for STAT-3 phosphorylation (13), whereas the protein inhibitors of activated STATs (PIAS) proteins and protein tyrosine phosphatases target STAT proteins directly (Fig. 1B; refs. 14, 15).

STAT-3 Inhibition by SOCS-3

SOCS-3 is a member of the SOCS protein family, which comprises seven SOCS proteins (SOCS-1 through SOCS-7) and cytokine inducible SH2 domain containing protein (16). The cytoplasmic, inducibly expressed SOCS proteins attenuate

STAT activity by inhibiting upstream JAK activation in a classic negative feedback loop (Figs. 1B and 2; ref. 16). Activated STAT-3 induces the expression of SOCS-3 (17, 18), which subsequently inhibits STAT-3 signaling by binding to and attenuating the signal transduction of gp130-related cytokine receptors and their associated JAK kinases (13).

STAT-3 Inhibition by PIAS Proteins and Protein Tyrosine Phosphatases

The highly conserved PIAS family of proteins includes PIAS1, PIAS3, PIASx, and PIASy. Except for PIAS1, each protein has two isoforms, and PIAS3 regulates STAT-3. In contrast to the inducibly expressed SOCS proteins, PIAS proteins are constitutively expressed in the nucleus and mediate transcriptional repression by directly interfering with the binding of STAT proteins and other transcription factors to their target DNA sequences (19). All PIAS proteins contain a zinc ring finger domain, an NH₂-terminal LXXLL motif, a COOH-terminal acidic domain, a serine/threonine-rich domain, and a recently discovered PINIT motif that is involved in nuclear retention (refs. 19, 20; Fig. 3). PIAS proteins inhibit STAT transcriptional activity by interfering with DNA binding via their NH₂-terminal domains (14). PIAS proteins also recruit transcriptional corepressors such as histone deacetylases to target gene promoters to inhibit transcription (21, 22). Furthermore, PIAS proteins influence the activation status of transcription factors by directly modifying the proteins themselves. Some PIAS proteins, including PIAS3, exhibit E3-SUMO (Small Ubiquitin-like Modifier) ligase activity and SUMOylate a variety of transcription factors, including p53, c-Jun, and c-Myb (19, 23, 24). SUMO modification affects their transcriptional ability, either by activation or inhibition (25).

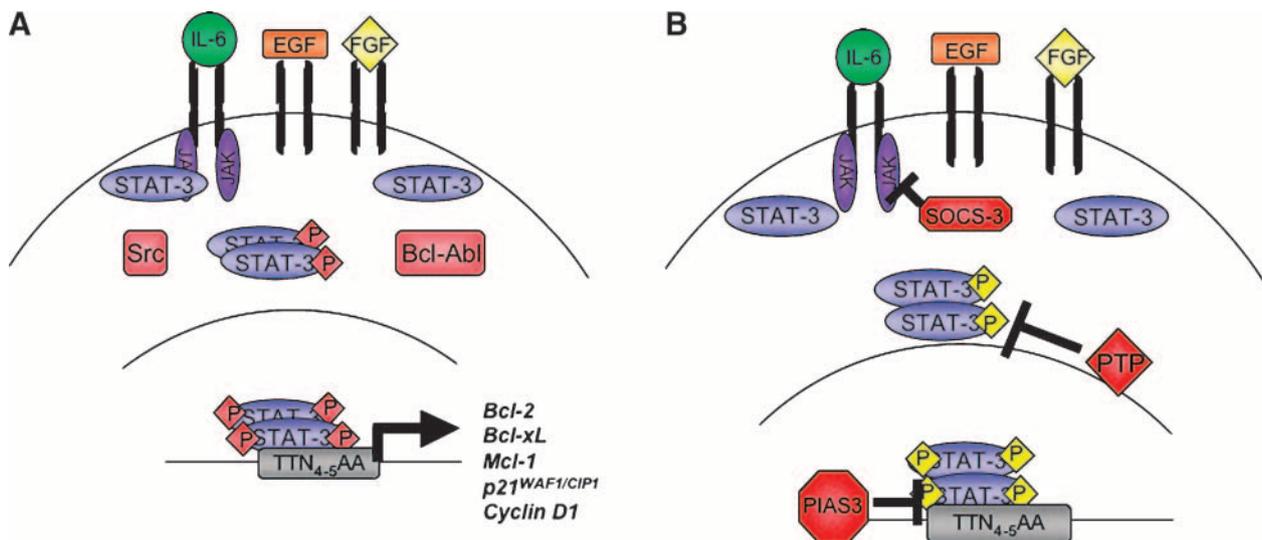


FIGURE 1. The STAT-3 signaling pathway. **A.** Cytokines such as IL-6, or growth factors including EGF and FGF, initiate STAT-3 signal transduction when they bind to their receptors and activate intracellular kinases. JAK proteins or receptor tyrosine kinases recruit inactive STAT-3 monomers and phosphorylate them on tyrosine 705. STAT-3 is also activated directly through interaction with the oncogenic kinases Src and Bcl-Abl. Tyrosine phosphorylated STAT-3 dimerizes and translocates to the nucleus, where STAT-3 transcriptional regulation is further modulated by serine phosphorylation. Active STAT-3 dimers bind to consensus sequences in the promoters of genes that regulate cell growth and antiapoptotic behavior. **B.** Multiple STAT-3 endogenous inhibitors attenuate STAT-3 signaling. SOCS-3 inhibits JAK activation and subsequent signal transduction in the cytoplasm, whereas PIAS3 inhibits STAT-3 DNA binding in the nucleus. Protein tyrosine phosphatases such as SHP-1 and SHP-2 dephosphorylate active STAT-3 complexes.

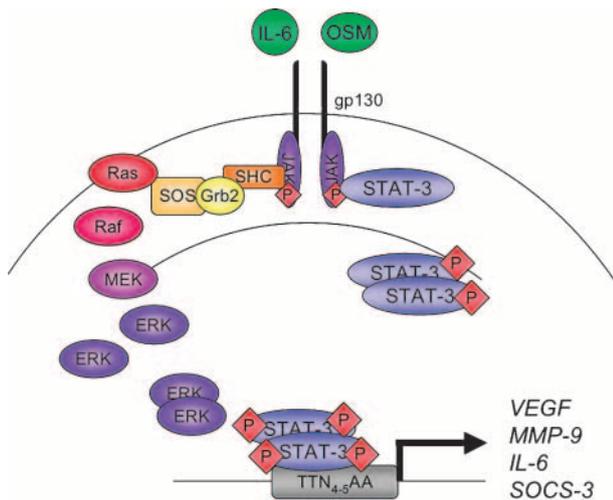


FIGURE 2. IL-6 cytokine signaling pathways. Members of the IL-6 cytokine family bind to a common receptor subunit, gp130, which either homodimerizes or heterodimerizes upon ligand binding. JAK proteins are autophosphorylated and transphosphorylate and phosphorylate one of four tyrosine residues on the intracellular portion of the gp130 subunit. JAK protein activation results in downstream signal transduction through both the MAPK and STAT-3 pathways. Receptor interactions with the adaptor proteins SHC and growth factor receptor binding protein 2 (*Grb2*) activate the MAPK/extracellular signal-regulated kinase (*ERK*) kinase (*MEK*) pathway. Alternatively, STAT-3 monomers are recruited to the receptor, become tyrosine phosphorylated, and dimerize through their SH2 domains. STAT-3 dimers translocate to the nucleus and bind to consensus sequences in the promoters of genes, including *VEGF*, *MMP-9*, *IL-6*, and *SOCS-3*.

Protein tyrosine phosphatases act in both the cytoplasm and nucleus to inhibit target protein phosphorylation events. STAT-3 signaling is dampened by protein tyrosine phosphatases, such as the SH2-domain containing tyrosine phosphatase family (SHP-1 and SHP-2), which down-regulate STAT-3 activation directly by dephosphorylating active STAT-3 complexes (15).

The Role of STAT-3 in Oncogenesis and Immune Evasion

In mice, the STAT-3^{-/-} phenotype is embryonic lethal at E6.5-7 (26), demonstrating that STAT-3 is essential to normal cellular functions. Conditional STAT-3 knockout mice and other deletion models have revealed a role for STAT-3 in wound healing, T-cell development, mammary gland development, cell cycle progression, apoptosis, and proliferation (26-28). A number of these basic cellular events are involved in tumor development; consequently, STAT-3 has been implicated in oncogenesis by promoting abnormal cell cycle progression, angiogenesis, apoptosis, tissue invasion, and immune evasion (29). Many tumor-derived cell lines require STAT proteins, particularly STAT-3, to maintain a transformed phenotype (7), and STAT-3 is constitutively activated in 50% to 90% of diverse human cancers (7, 30). In experimental settings, introduction of a constitutively active STAT-3 mutant, STAT-3C, was sufficient to transform cells, and these cells could form tumors in nude mice (31). Furthermore, a dominant-negative mutant of STAT-3 blocked transformation by v-src (32). These observations suggest that many types of oncogenesis may be dependent on STAT-3 activity.

In addition to promoting oncogenesis, constitutively active STAT-3 also perpetuates tumor growth by undermining tumor recognition pathways in the immune system (33). In several experimental systems, STAT-3 promoted tumor immune evasion on multiple fronts by inhibiting pro-inflammatory cytokine signaling, blocking the antitumor activity of immune cells themselves, and promoting tolerogenesis by amplifying regulatory T (T_{Reg}) cells. Blocking STAT-3 activity in tumor cells, with either dominant-negative or antisense STAT-3, resulted in elevated expression of pro-inflammatory mediators, including IFN- β , tumor necrosis factor- α , IL-6, and the chemokines RANTES and IP-10. Conversely, activating STAT-3 inhibited expression of both IL-6 and RANTES in normal fibroblasts (34).

In addition to blocking pro-inflammatory cytokine signals, STAT-3 activation blunts the immunogenic response of immune cells themselves, further tipping the balance to a tolerogenic response. Animal and patient studies support this idea; in tumor-bearing mice, inhibiting STAT-3 increased the antitumor activity of T cells, natural killer cells, and neutrophils (35). Bone marrow-derived dendritic cells, which are thought to promote immune tolerance, are immature and dysfunctional in both humans and animals with cancer (36, 37), but inhibition of STAT-3 enhanced dendritic cell maturation in tumor-bearing mice. Additionally, mice that lack STAT-3 in hematopoietic cells had elevated levels of MHC class II, CD80, and CD86, compared with mice with intact STAT-3 expression (35). Taken together, these results show that STAT-3 is involved in maintaining the immature dendritic cell phenotype and promoting tumor immune tolerance.

More specifically, in gliomas, activation of STAT-3 has been implicated in inhibiting the T-cell response. Glioma-infiltrating CD8⁺ T cells were characterized as neither activated nor proliferating (CD8⁺CD25⁻), a hallmark of the immunosuppressive tumor environment (38). One route to reversing immune tolerance is by targeting CD8⁺CD25⁻ T cells themselves. When STAT-3 activity in monocytes from glioma patients was inhibited, the expression of costimulatory molecules such as CD80 and CD86 was up-regulated, an event that presumably enhanced T-cell activation and is a critical step in reversing immune evasion (39). These data show that inhibiting STAT-3 affects the activation of glioma-infiltrating CD8⁺ T cells, which serve as one of many potential targets in overcoming immune suppression.

STAT-3 activity was shown to be elevated in tumor-associated T_{Reg} cells that maintain tumor immune evasion

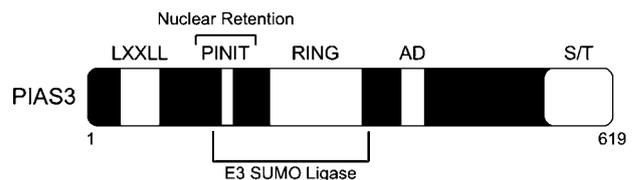


FIGURE 3. Structural domains of the PIAS3 protein. PIAS3 is a 619-amino-acid protein that shares 40% homology to the other PIAS proteins. PIAS3 contains an NH₂-terminal LXXLL motif, a PINIT motif, which is involved in nuclear retention, a zinc ring finger domain (RING), a COOH-terminal acidic domain (AD), and a serine/threonine (S/T) rich domain. The PINIT motif and the RING finger domain are involved in the E3 SUMO ligase activity exhibited by PIAS3.

(35, 40–42). In these T_{Reg} cells, elevated activation of STAT-3 increased proliferation and promoted expression of forkhead box P3, transforming growth factor- β , and interleukin-10, all of which inhibit $CD8^+$ T-cell differentiation and dendritic cell maturation (33, 40, 41, 43). T_{Reg} cells have been shown in the blood and within the tumor microenvironment of glioblastoma patients and are thought to contribute to the lack of effective immune responsiveness against glioblastomas (38, 44). The STAT-3 activation status of the T_{Reg} cells was not examined in these studies. Interestingly, T_{Reg} cells have been reported to be deficient in SOCS-3 expression, whereas SOCS-3 overexpression in these cells decreased their proliferation and suppressive function (45). The observed activation of STAT-3 in tumor-associated T_{Reg} cells may be attributable to the associated absence of the endogenous inhibitor SOCS-3. Taken together, these data show that STAT-3 activation promoted not only oncogenesis but also immune evasion, specifically by inhibiting expression of pro-inflammatory mediators, suppressing both dendritic cell maturation and $CD8^+$ T-cell activation, and promoting proliferation of T_{Reg} cells. These results show how two separate and equally important steps in oncogenesis, transformation and immune evasion, intersect at the point of STAT-3 activation.

STAT-3 Activation in Gliomas

It is clear that STAT-3 activation is critically involved in tumorigenesis, but *in vitro*, glioblastoma cells have shown varying levels of constitutive STAT-3 activation. Using electrophoretic mobility shift assays, immunofluorescence, and immunoblotting, we and others have shown that numerous glioblastoma cell lines express very little activated STAT-3 under basal, unstimulated conditions (46, 47).¹ However, using similar techniques, others have reported that some glioblastoma cells exhibited constitutive STAT-3 activation, as well as constitutively high levels of antiapoptotic proteins such as Bcl-xL, Bcl-2, Mcl-1, and c-Myc (48, 49). Several studies examining human glioblastoma tissues observed constitutive activation of STAT-3, as assessed by tyrosine phosphorylation (48, 50, 51).¹ Additionally, we detected elevated levels of serine-phosphorylated STAT-3 in human glioblastoma tissues,¹ demonstrating for the first time the presence of the maximally activated form of STAT-3 in glioblastoma. Immunohistochemistry studies showed that tyrosine phosphorylated STAT-3 localized to tumor endothelial cells (50), suggesting a role for STAT-3 in angiogenesis. Also, tyrosine phosphorylated STAT-3 has been localized to tumor cells (51). Taken together, these observations implicate a role for STAT-3 in glioblastoma pathology.

Upstream Mediators of Aberrant STAT-3 Activation

Missense mutations of STAT-3 have been reported in patients with hyper-IgE syndrome (52, 53). In human cancers, however, aberrant STAT-3 activity stems from dysregulation of upstream tyrosine kinases or loss of negative feedback

mechanisms. For example, stably transforming cells with the oncogenic Src tyrosine kinase was sufficient to induce constitutive activation of STAT-3 (54) and downstream C-reactive protein and c-fos promoter activities (55). To understand how STAT-3 is improperly activated in glioblastoma and other cancers, research has focused on unraveling the upstream mechanisms by which STAT-3 is activated, and understanding how regulation of these mechanisms fail in the context of glioblastoma. Below, we discuss how, in the context of glioblastoma, members of the IL-6 cytokine family or growth factors initiate the STAT-3 activation pathway by interacting with their receptors.

STAT-3 Activation by IL-6 Cytokines

The IL-6 cytokine family consists of a number of structurally related proteins, such as IL-6, OSM, ciliary neurotrophic factor, and leukemia inhibitory factor. These pleiotropic cytokines mediate signal transduction through the MAPK or the JAK–STAT-3 pathways, and initiate these signaling pathways by binding to their receptors. This binding step leads to the homodimerization of gp130 or heterodimerization of gp130 with other gp130-related receptor subunits, such as the OSM receptor (9, 27). JAK proteins then phosphorylate the gp130 receptor subunit on one of four specific tyrosine residues, which recruits and activates the STAT-3 protein (ref. 9; Fig. 2). IL-6 expression is regulated by multiple stimuli, examples of which include hypoxia, pro-inflammatory mediators, and IL-6 cytokines themselves, including both IL-6 and OSM. These many factors are up-regulated in various diseases of the central nervous system, including glioblastoma (56).

Substantial data gathered from cell lines, mouse models, and patient samples support a role for IL-6 proteins in glioblastoma-associated STAT-3 activation. Several groups observed constitutive expression of IL-6 and OSM in human glioblastoma cells, both *in vivo* and *in vitro* (57–59). The elevated expression of IL-6 cytokines is thought to be responsible, at least in part, for both constitutive and induced activation of STAT-3 in glioblastoma. In some glioblastoma cell lines, autocrine IL-6 expression resulted in constitutive activation of STAT-3, and neutralizing antibodies to IL-6 reduced STAT-3 activation, inhibited cell proliferation, and induced apoptosis (48). In a number of other human brain tumor cell lines, stimulation with IL-6, leukemia inhibitory factor, ciliary neurotrophic factor, or OSM resulted in induced STAT-3 activation (46), and treatment of human astrogloma cells with OSM increased the STAT-3-dependent expression and activation of matrix metalloproteinase-9 and VEGF (59, 60).

In a glioblastoma mouse model, development of gliomas required the presence of IL-6; mice that lacked IL-6 failed to develop tumors. To further explore this IL-6 dependence, heterozygous GFAP–v-src transgenic mice were used to compare astrocytic tumor incidence between animals that express IL-6 ($v\text{-src}^{+/-}/IL\text{-6}^{+/+}$) and those that lack IL-6 ($v\text{-src}^{+/-}/IL\text{-6}^{-/-}$). Although 21% of the $v\text{-src}^{+/-}/IL\text{-6}^{+/+}$ mice (12 of 56) developed tumors of varying grades, only 2.8% of the $v\text{-src}^{+/-}/IL\text{-6}^{-/-}$ mice (1 of 35) developed tumors (61). These results suggest that loss of IL-6 in predisposed mouse models suppressed glioma formation.

¹E.C. Brantley, et al. Loss of PIAS3 in glioblastoma multiforme tumors: implications for STAT-3 activation and gene expression, submitted for publication.

Human glioblastoma tissues also amplify expression of the *IL-6* gene (57, 62). Immunohistochemistry studies on patient samples revealed that IL-6 was localized to glioblastoma tumor cells, and IL-6 activity was detected in the cerebrospinal fluid and tumor cysts of glioblastoma patients (57). Additionally, expression of prosurvival effectors downstream of STAT-3, such as matrix metalloproteinase-9 and VEGF, was elevated in human glioblastoma tissues and implicated in tumor angiogenesis (63). It is clear from these studies that IL-6 promotes glioma development *in vivo*, at least in part through the action of STAT-3.

STAT-3 Activation by EGFR

The transmembrane receptor tyrosine kinase EGFR is amplified in ~50% of glioblastomas. In ~50% of these cases, the glioblastomas express a mutant EGFR that lacks a portion of the extracellular ligand-binding domain (EGFRvIII). In studies comparing EGFRvIII with wild-type EGFR, the mutant was persistently autophosphorylated at low levels (64) and could not be down-regulated. Autophosphorylation of EGFRvIII resulted in inefficient EGF signal attenuation and persistent activation of downstream kinase pathways, including those involving STAT-3, Ras/MAPK, and AKT (5). In fact, the expression of tyrosine phosphorylated STAT-3 correlated significantly with the expression of EGFRvIII in human glioblastoma tissues (51).

D54-MG glioma cells expressing EGFRvIII showed higher motility than their wild-type counterparts (65), which suggests that expression of EGFRvIII enhanced glioblastoma cell invasion. EGFRvIII expression also promotes tumorigenesis *in vivo*; when implanted s.c. or intracerebrally into nude mice, EGFRvIII-expressing U87-MG cells produced larger and faster-growing tumors than those formed by wild-type EGFR-expressing cells (66). Current evidence suggests that the mechanism behind the increased tumor size and growth rate in EGFRvIII-expressing cells lies in the activation of downstream effectors such as STAT-3 and AKT. Recent work in lung cancer cells showed that STAT-3 cooperates with AKT in EGFR signal transduction, and that the cooperative activation of these signaling intermediates contributed to changes in expression of the cell cycle intermediates cyclin D1 and p27 (67). In support of this observation, tumors formed by a variety of glioblastoma cells expressing EGFRvIII had larger volumes than tumors formed by wild-type EGFR-expressing cells, due to higher levels of proliferation as measured by Ki67 immunoreactivity (68). To determine the mechanism behind the increased proliferation, the EGFRvIII-expressing cells were examined for expression of cell cycle-related proteins. Compared with wild-type controls, EGFRvIII-expressing glioblastoma cells had lower levels of the cyclin-dependent kinase inhibitor, p27, higher CDK2-cyclin A activity, and constitutively high levels of hyperphosphorylated RB proteins. Importantly, this pro-growth phenotype was dependent on the phosphoinositide-3 kinase/AKT pathway, because inhibition of AKT resulted in decreased tumor volume and increased p27 expression (68). Taken together, these data illustrate that expression of EGFRvIII in glioblastoma cells enhances gliomagenesis by promoting cooperative activation of multiple downstream effectors such as STAT-3 and AKT.

STAT-3 Activation by FGFR

Signaling initiated by FGF through the FGFR is another mechanism by which a growth factor promotes the transcriptional activation of STAT-3 (69). Glioma cell growth was dependent on a functional FGFR pathway in C6 glioma cells; introduction of a dominant-negative FGFR attenuated FGF signaling, which resulted in reduced anchorage-dependent growth rates in these cells (70). When cells expressing the mutant FGFR were xenografted into immunodeficient mice or transplanted into rat brain, onset of tumor development was delayed and tumor volume was reduced compared with wild-type FGFR controls. Tumors formed from FGFR dominant-negative expressing cells exhibited lower levels of VEGF compared with tumors from wild-type counterparts (70), which helps to explain why the dominant-negative FGFR-expressing tumors displayed slower growth rates. Interestingly, STAT-3 is a downstream transcriptional target of FGFR (69), and activation of STAT-3 results in expression of VEGF (59). These observations suggest that STAT-3 was the critical link between FGFR signaling and VEGF-mediated tumor growth in C6 glioma cells. In summary, although multiple cytokines and growth factors may independently contribute to glioblastoma pathology, STAT-3 seems to be a critical “molecular hub” that links these pathways together.

Mechanisms of STAT-3 Inhibition in Gliomas

STAT-3 is a promising target for glioblastoma therapy, not only because it is a convergence point for several signaling pathways that promote glioma growth and maintenance but also because aberrant STAT-3 activation results from upstream dysregulation, not constitutively active STAT-3 mutations. STAT-3 should therefore retain its ability to respond to direct

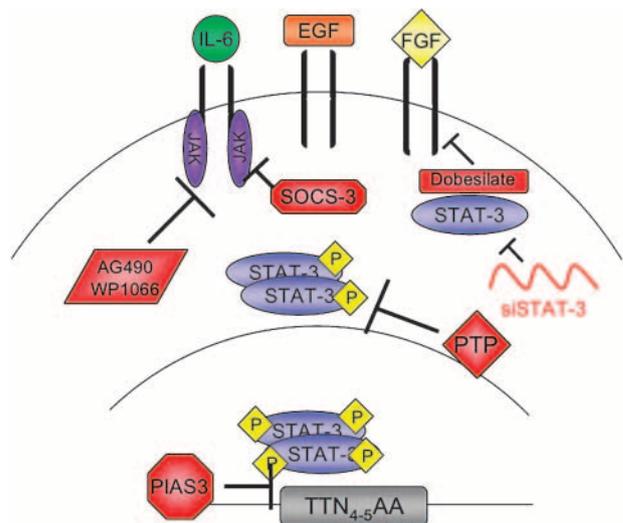


FIGURE 4. Inhibition of STAT-3 signal transduction. A variety of endogenous and pharmacologic inhibitors can attenuate STAT-3 signaling. SOCS-3, PIAS3, and various protein tyrosine phosphatases (PTP) inhibit STAT-3 activity endogenously. STAT-3-specific siRNA degrades STAT-3 mRNA. Pharmacologic inhibition of JAK activity by AG490 and WP1066 dampens the signals that result in STAT-3 activation. Attenuation of FGF signaling by dabesilate also inhibits STAT-3-mediated gene expression by attenuating kinase signals upstream of STAT-3 activation.

regulatory stimuli or external inhibition, independent of upstream signaling. Several current therapeutics target STAT-3 activity in cancers other than glioblastoma (Fig. 4). Recent work has approached STAT-3 inhibition from two fronts: (a) through RNA interference or chemical inhibitors, and (b) through modulation of endogenous regulators such as PIAS3 and SOCS-3.

Direct STAT-3 Inhibition

Although not yet applied to glioblastoma, several compounds attenuate STAT-3 signaling by directly targeting the STAT-3 protein. Researchers showed that platinum compounds interfere with STAT-3 activation and abrogate signaling mediated by constitutively active STAT-3, presumably by direct action. This block of STAT-3 activity was accompanied by inhibition of cell growth and induction of apoptosis in breast, lung, and prostate cancer cell lines (71). Decoy oligonucleotides, or G-quartets, are competitive inhibitory structures comprised of guanine-rich oligonucleotides. By competitively binding activated STAT-3, G-quartets inhibited STAT-3 binding to endogenous gene promoters and therefore attenuated STAT-3–induced gene expression (72, 73). Another chemical inhibitor of STAT-3, S31-201 (NSC 74859), mediated its antitumor activity by inhibiting expression of pro-survival genes and attenuating the growth of human breast tumors *in vivo* (74). S31-201 was found to inhibit STAT-3 homodimer formation, STAT-3 DNA binding, and the resulting transcriptional activity, suggesting that the antitumor activity of this compound was mediated in part by a direct block of STAT-3 signal transduction (74). Using glioblastoma cell lines, direct inhibition of STAT-3 activity using RNA interference triggered apoptosis and inhibited survival (75). RNA interference–mediated down-regulation of STAT-3 in A172 and U251-MG glioma cell lowered levels of pro-survival proteins such as Survivin and Bcl-xL and increased levels of apoptosis, as measured by cleaved caspase 3 levels and Annexin V staining (75). This link between STAT-3 activity and glioblastoma cell survival warrants testing the clinical effectiveness of existing STAT-3 inhibitors on glioblastoma progression.

Inhibition of Upstream Kinases

Because aberrant STAT-3 activation is usually the result of the overactivity of upstream kinases, several pharmacologic interventions that act by inhibiting growth factor receptors or other upstream kinases have been very effective at abrogating STAT-3 activity. Inhibition of EGFR with gefitinib (Iressa, AstraZeneca) resulted in inhibition of STAT-3 tyrosine phosphorylation (76). Because the activation of STAT-3 depends on its direct phosphorylation by tyrosine kinases (27), several pharmacologic JAK [AG490, WP1066, and JSI-124 (cucurbitacin I)] and Src (PD180970) inhibitors showed promising STAT-3 inhibition *in vitro*, and are in various early stages of experimental testing (48, 49, 77). The JAK inhibitor AG490 blocked constitutively active STAT-3 in the astrogloma cell line U251-MG (48), which decreased cell survival and increased cleavage of the apoptotic marker poly(ADP)ribose polymerase. AG490-treated cells also displayed a dose-dependent inhibition in expression of pro-survival

proteins, including Bcl-xL, Bcl-2, and Mcl-1 (48). These proteins are all gene targets of STAT-3, suggesting that the apoptotic response was mediated by STAT-3 inhibition.

Although AG490 is very effective *in vitro*, it did not consistently offer the same antitumor results *in vivo*; therefore, the structure of AG490 was modified to produce the more potent and active WP1066 compound (49). Preliminary *in vivo* studies showed that WP1066 successfully crossed the blood-brain barrier, a key feature in drug design for glioma patients (39), and that it significantly inhibited the growth of glioma xenografts compared with untreated controls (49). Importantly, the effects of WP1066 are tumor specific. This compound specifically inhibited glioblastoma cell viability without affecting the viability of normal human astrocytes (49).

The mechanism behind WP1066-mediated glioblastoma cell growth attenuation seems to be rooted in its potent inhibition of STAT-3. In initial studies, WP1066 blocked tyrosine phosphorylation of STAT-3 by JAK proteins, which resulted in a failure of STAT-3 to translocate to the nucleus and mediate its transcriptional effects. WP1066 treatment of U87-MG and U373-MG cells decreased STAT-3–mediated expression of Bcl-xL, Mcl-1, and c-Myc, which resulted in increased apoptosis and reduced glioblastoma cell viability (49). WP1066 also reversed immune tolerance in glioblastoma patients, presumably by blocking STAT-3–mediated immune suppression and tolerogenesis. WP1066-stimulated proliferation of T cells from glioblastoma patients and enhanced immunogenic responses in glioma-infiltrating microglia, macrophages, and peripheral blood monocytes (39). WP1066 also promoted immunogenic responses by up-regulating the costimulatory molecules CD80 and CD86 and the T-cell effector cytokines IL-2, IL-4, IL-12, and IL-15 (39). Because pharmacologic inhibitors of JAK proteins attenuate STAT-3–mediated glioblastoma cell growth, glioma cell viability, and immune evasion in glioblastoma, the JAK proteins have emerged as attractive targets for subsequent STAT-3 down-regulation.

Dihydroxy-2,5-benzenesulfonate (dobesilate) is thought to inhibit activation of STAT-3 by attenuating the upstream FGF signaling pathway. Dobesilate is currently used to treat diabetic retinopathy and chronic venous insufficiency, because it has been shown to block FGF-driven neovascularization (78, 79). Treatment of C6 glioma cells with dobesilate *in vitro* triggered apoptosis and growth arrest (80). Further studies in glioma cells showed that dobesilate significantly inhibited constitutive expression of tyrosine phosphorylated STAT-3 (81), activation of the MAPK extracellular signal-regulated protein kinases 1/2 (82), and expression of the pro-survival proteins Bcl-xL and cyclin D1 (81). These results support the idea that dobesilate increased apoptosis and decreased cell growth and survival in part by blocking STAT-3 activation.

The observed effects of AG490, WP1066, and dobesilate collectively show that pharmacologic inhibitors of individual kinases that operate upstream of STAT-3 have significant potential as glioblastoma chemotherapeutic agents. Furthermore, inhibition of these various upstream kinases in combination with direct STAT-3 inhibitors may also be effective in glioblastoma therapy. Recent work has shown that inhibition of a single tyrosine kinase pathway provides little benefit in

reducing glioma cell survival and growth; however, a combinatorial strategy to inhibit multiple upstream tyrosine kinases, such as EGFR and platelet-derived growth factor receptor, as well as phosphoinositide-3 kinase, proved to significantly reduce intracellular signaling, survival, and anchorage-independent growth of glioma cells (83). This combinatorial strategy provided novel evidence that inhibition of aberrant signaling pathways by multiple mechanisms may be effective for treatment of glioblastoma. In addition, it supports the notion that STAT-3 inhibition by direct, indirect, or a combinatorial approach using existing pharmacologic inhibitors has promise as a clinical target in glioblastoma.

Endogenous Negative Regulation by PIAS3

In addition to targeting STAT-3 for pharmacologic inhibition, recent studies have focused on enhancing endogenous cellular mechanisms for modulating STAT-3 activity, namely, the inhibitory pathways involving PIAS3, SOCS-3, and protein tyrosine phosphatases (15).

Because of its ability to inhibit STAT-3 transcriptional activation at multiple levels, PIAS3 has recently become a focus of cancer biologists. In support of this, we showed that inhibiting constitutive PIAS3 expression with siRNA significantly increased proliferation of glioblastoma cells.¹ In contrast, PIAS3 overexpression in these cells attenuated proliferation and inhibited OSM-mediated STAT-3 promoter activity, suggesting that ectopic expression of PIAS3 could inhibit STAT-3 transcriptional activity in glioblastoma.¹ Importantly, we recently showed that expression of the PIAS3 protein was largely absent in human glioblastoma tissues, despite unchanged PIAS3 mRNA levels,¹ suggesting that the PIAS3 protein was rapidly degraded in glioblastoma tissues. It was also reported that PIAS3 expression was diminished in human ALK⁺ T-cell lymphoma samples, which was associated with STAT-3 dysregulation (84). Taken together, these observations indicate that down-regulation of PIAS3 may cause or exacerbate gliomagenesis, and that reintroduction of PIAS3 could potentially inhibit glioblastoma progression.

Although PIAS3 was first described as a negative regulator of STAT-3 transcriptional activity, it is now known to also modulate nuclear factor- κ B, phosphoinositide-3 kinase, and transforming growth factor- β signaling pathways, all of which are associated with poor prognosis in glioblastoma patients (51, 85-90). *In vitro*, PIAS3 down-regulated the nuclear factor- κ B pathway by interacting with p65 and repressing its transcriptional activity (91). PIAS3 also modulated the phosphoinositide-3 kinase pathway by interacting with AKT and suppressing its phosphorylation and activation (92). Studies in various cell lines showed that PIAS3 overexpression affected AKT signaling in both prostate and lung cancer cells, with subsequent effects on cell growth and apoptosis (92, 93). PIAS proteins also regulated the transcriptional activity of SMAD proteins, the downstream targets of transforming growth factor- β signaling (25). These results strongly suggest that enhancing PIAS3 expression may inhibit multiple signaling pathways, such as STAT-3, nuclear factor- κ B, phosphoinositide-3 kinase, and transforming growth factor- β , that are overactive in human glioblastoma tissues.

Involvement of SOCS-3 in Gliomas

Because SOCS-3 is an endogenous inhibitor of STAT-3 signaling (16), as well as a STAT-3 transcriptional target (13, 17), the evidence correlating SOCS-3 expression and STAT-3 activity is conflicting and somewhat confusing. SOCS-3 works in a negative-feedback loop to suppress STAT-3 signaling; therefore, it is reasonable to suggest that loss of SOCS-3 may contribute to STAT-3 activation and tumor progression. Indeed, reports describing SOCS-3 hypermethylation and subsequent loss of expression in a variety of cancers support the idea that SOCS-3 may have a tumor-suppressing function (94-97). In contrast, elevated SOCS-3 expression was reported in human breast cancer and melanoma tissues, as well as in a subset of classic Hodgkin's lymphoma cell lines and primary lymphoma cells (98-104). In several studies, SOCS-3 promoted cell growth and proliferation in a number of cancers (100, 103, 105), and elevated levels of SOCS-3 were reported to confer a growth advantage to a melanoma cell line (103). Lymphoma cells with low levels of endogenous SOCS-3 expression succumbed to proliferation arrest whereas cells that endogenously expressed higher levels of SOCS-3 did not undergo arrest (100).

SOCS-3 overexpression has been attributed to constitutive STAT-3 activation in various cancers (101, 106). However, there are few reports describing SOCS-3 expression in glioblastoma. Our studies showed that human glioblastoma tissues expressed higher levels of SOCS-3 than did control brain tissues, and that SOCS-3 promoter activity and mRNA expression is inhibited by ectopic PIAS3 expression.¹ Moreover, constitutive expression of SOCS-3 in human glioblastoma tissues correlated with enhanced cell survival and radioresistance *in vitro* (105). This resistance was dependent on SOCS-3 expression, as evidenced by marked sensitivity to ionizing radiation in SOCS-3^{-/-} mouse embryonic fibroblasts compared with wild-type mouse embryonic fibroblasts (105). Interestingly, U87-MG glioma cells that constitutively express SOCS-3 displayed significant radioresistance that was attenuated by expression of a dominant-negative STAT-3 construct (105). Current results collectively show that constitutive expression of SOCS-3 and the associated radioresistance were both mediated by STAT-3, adding another aspect of tumorigenesis mediated by activated STAT-3 (i.e., radioresistance).

Interestingly, a unique characteristic of glioblastoma cells that are resistant to ionizing radiation is the expression of the cell surface marker, CD133, a hallmark of neural precursor cells. CD133⁺ cells have recently been identified as the tumor-initiating subset of glioblastoma cells (107). Upon treatment with ionizing radiation, growth of glioblastoma cells grown *in vitro* or as grafts in mice was largely inhibited, but the proportion of CD133⁺ cells was greater. In addition, heightened DNA repair responses were detected in CD133⁺ glioblastoma cells (107). Because the expression of SOCS-3 in glioblastoma cells was also shown to correlate with resistance to ionizing radiation (105), it is tempting to speculate that CD133⁺ cells might also express elevated levels of SOCS-3, as well as activated STAT-3. Experiments to examine this parameter of STAT-3 signaling are currently being explored.

Although SOCS-3 is a negative regulator of STAT-3, it is also a target gene of STAT-3 (13); therefore, the simultaneous

overexpression/activation of both of these proteins in glioblastoma is reasonable. It is possible that as a transcriptional target of STAT-3, SOCS-3 is involved in promoting cell growth, cell proliferation, and resistance to radiation. Importantly, however, studies examining the expression and function of SOCS-3 in glioblastoma are limited in number. Future experiments should be aimed at confirming the elevated expression of SOCS-3 in glioblastomas, identifying the cell types expressing SOCS-3 and elucidating the role of SOCS-3 in glioblastoma.

Conclusions

In summary, aberrant expression and activation of the STAT-3 transcription factor has been implicated in glioblastoma pathology both *in vivo* and *in vitro*. Aberrant STAT-3 activation was often associated with signaling initiated by IL-6 cytokine family members or growth factors such as EGF and FGF. These signaling pathways converge at the molecular hub STAT-3, which links various extracellular signals to transcriptional control of cell proliferation, cell cycle, and apoptosis. The central role STAT-3 plays in glioblastoma-associated cell signaling makes it an attractive therapeutic target in the ongoing search for relevant glioblastoma therapies, and studies thus far have revealed significant clinical potential in inhibiting STAT-3.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Rebecca Goldstein for excellent editorial assistance.

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Signal Transducer and Activator of Transcription-3: A Molecular Hub for Signaling Pathways in Gliomas

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Mol Cancer Res 2008;6:675-684.

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