Aberrant Stat3 Signaling by Interleukin-4 in Malignant Glioma Cells: Involvement of IL-13Rα2

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Abstract

Interleukin (IL)-4 exhibits antitumor activity in rodent experimental gliomas, which is likely mediated by the actions of IL-4 on a variety of immune cells present in and around the tumor masses. Here, we show that IL-4, which activates Stat6 in normal human astrocytes and in a variety of other cells, induces an aberrant activation of Stat3 in glioblastoma multiforme (GBM) cells but not in normal human astrocytes. Previously, we have shown that autocrine IL-6 signaling induces a persistent activation of Stat3. Now, we show that Stat3 is further activated by IL-4 stimulation of GBM cells. Expression of IL-13Rα2, a decoy receptor for IL-13 that partly blocks IL-4–mediated activation of Stat6 in GBM cells, up-regulates the activation of Stat3 as shown by a small interfering RNA–mediated inhibition of IL-13Rα2 expression. In addition, transient expression of the IL-13Rα2 transgene in 293T cells increases the IL-4–mediated activation of Stat3 and subsequent expression of Stat3-targeted gene. Coimmunoprecipitation results reveal that IL-13Rα2–mediated activation of Stat3 does not require a direct physical interaction between Stat3 and IL-13Rα2. Chromatin immunoprecipitation assay employing anti-Stat3 antibody confirms the *in vivo* binding of activated Stat3 to the promoters of genes that encode antiapoptotic proteins Bcl-2, Bcl-x_L, and Mcl-1. IL-4 significantly up-regulates of the steady-state levels of Bcl-2, Bcl-x_L, and Mcl-1 in GBM cells. These results indicate that IL-4/IL-13 receptor-mediated Stat3 signaling may contribute to the pathogenesis of GBM cells by modulating the expression of the Bcl-2 family of antiapoptotic proteins. (Cancer Res 2005; 65(7): 2956-63)

Introduction

Gliomas are the most common primary tumors of the central nervous system, which originate from astrocytes or their precursor cells, and are clinically classified into four grades (1–4). Glioblastoma multiforme (GBM), the grade 4 tumors, are the most aggressive among the malignant gliomas, with a median survival of 9 to 12 months (3). The conventional modalities of treatment for GBM, which include surgery, radiation, and chemotherapy, remain ineffective, because the tumor cells are highly invasive and resistant to radiation and chemotherapeutic agents (1–4). Patients bearing malignant gliomas and animals bearing experimental gliomas suffer from the suppression of systemic as well as local immunity, which is largely attributable to the tumor cell production of immune suppressant molecules that include transforming growth factor-β, interleukin (IL)-10, prostaglandin E_2, and Fas ligand (ref. 5 and references therein). This suggests that growth of malignant gliomas takes advantage of an immune suppressed environment (5). Further, this notion is supported by results of an international population-based case-control study, which reveal a reciprocal association between gliomas and allergic diseases that result from aberrant activations of the immune system (6). Several cytokines that include IL-2, IL-4, IL-12, and granulocyte-macrophage colony-stimulating factor have been tested for their ability to activate the immune suppressed system of glioma-bearing animals (7–9). IL-4 shows the most promising results in rodent glioma models (8). However, the translation of animal studies to humans has not been successful for reasons not known yet. IL-4 is produced by type II T helper cells, mast cells, and basophils, which are recognized as the key effector cells in the pathobiology of allergic disorders (10, 11). Although IL-4 induces the differentiation and growth of B and T lymphocytes (10, 11), it inhibits the proliferation of astrocytes that are derived from nonneoplastic cortex or low-grade gliomas but not from high-grade gliomas (12–14).

IL-4 normally activates two intracellular signaling pathways: the IRS-phosphatidylinositol 3-kinase (PI3K) pathway that promotes the growth of target lymphocytes and the Jak-Stat6 pathway that induces the expression of IL-4-responsive genes (10, 11, 15). IL-4 initiates transmembrane signaling in nonhematopoietic cells by activating its type II receptor complex composed of IL-4Rα and IL-13Rα1 that are constitutively associated with Jak1 and Jak2 or Tyk2, respectively (10, 11, 15, 16). We have shown previously that despite all the components of the type II receptor are expressed in GBM cells, unlike normal astrocytes, GBM cells fail to activate Stat6 in response to IL-4 stimulation, which is in part attributable to the expression of the IL-13 decoy receptor IL-13Rα2 in GBM cells (17).

Now, we show herein that IL-4 activates Stat3 in GBM cells but not in normal astrocytes. Stat3, on activation by aberrant cytokine or growth factor signaling, contributes to a variety of human cancers by enhancing cell proliferation, cell survival, and angiogenesis and suppressing the innate and adaptive immune responses to tumor cells (18–21). Recently, we have shown that GBM tumors and cell lines contain persistently activated Stat3 (22). Here, we address how IL-4 further induces the activation of Stat3, and IL-13Rα2 plays a role in this process.

Materials and Methods

Cell lines and reagents. GBM cell lines U251, T98G, and A172 were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FCS (Life Technologies, Inc., Rockville, MD), 2 mmol/L glutamine, and 50 mg/L penicillin and streptomycin. Normal human astrocytes (NHA) were purchased and maintained in specific growth medium AGM bullet kit (Clonetics BioWhittaker, Walkersville, MD). 293T cells were grown in DMEM containing 10% serum. For electrophoretic mobility shift assay (EMSA) supershift, polyclonal antibodies for Stat1, Stat3, and Stat5

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were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Bcl-x_L antibody was purchased from Transduction Laboratories (Franklin Lakes, NJ). Anti-V5 and anti-Myc antibodies were purchased from Invitrogen (Carlsbad, CA), and anti-Flag antibody was from Santa Cruz Biotechnology. Antibodies for Bcl-2, Mcl-1, actin, pAKT, pErk, and Erk were also obtained from Santa Cruz Biotechnology. AG490 and LY294002 were purchased from Calbiochem (Darmstadt, Germany), and U0126 was from Promega (Madison, WI).

Plasmid construction. Human IL-4Rα cDNA was cloned into the expression vector pKCR in which the SV40 early gene promoter drove the transcription (17, 23). Human IL-13Rα2 cDNA was cloned into pSecTagC (Invitrogen; ref. 17). Erythropoietin receptor (EPOR)-IL-4Rα chimeric receptor construct was cloned into pcDNA3.1 as described (24). Human Stat3 cDNA was cloned into the expression vector pcDNA3.1(+) that contained cytomegalovirus promoter and hygromycin-resistant gene (Invitrogen). The deletion mutants of IL-13Rα2 (17) and Δ-CD-chi were constructed by PCR technique using specific primers and the IL-13Rα2 cDNA or EPOR-IL-4Rα chimeric plasmid, respectively, as templates. PCR-mediated mutagenesis was carried out to generate Δ-box-1-chi and to introduce a phenylalanine substitution for tyrosine employing the Stratagene Quick Change kit (La Jolla, CA). Truncated EPOR-IL-4Rα chimeric receptors were made by introducing stop codon at desired positions using the same kit. All constructs were verified by nucleotide sequencing.

Electrophoretic mobility shift assay. EMSA was done using 12 to 16 μg whole cell extract (WCE) proteins and 0.2 ng ^{32}P-labeled high-affinity *sis*-inducible element (hSIE) probe (top strand 5′-TCGACATTTCCCGTAAATC-3′) derived from the c-*fos* gene promoter (25). For EMSA supershift, before the addition of radiolabeled probe, WCEs were preincubated with polyclonal antibodies for Stat1, Stat3, or Stat5 for 20 minutes at room temperature.

Transfection of cells. 293T cells were transfected with the indicated expression plasmids by using calcium phosphate method (24). T98G cells were transfected with the IL-4Rα in pKCR and puromycin resistance gene in pBabe by LipofectAMINE Plus, and stable clones were selected by puromycin (0.5 μg/mL) for 3 to 4 weeks. U251 cells in six-well plates without antibiotics were transfected with IL-13Rα2 small interfering RNA (siRNA) formulated into liposomes (OligofectAMINE, Life Technologies, Inc., Rockville, MD) according to the manufacturer's instructions. Cells were harvested for analysis after 48 hours of transfection.

Coimmunoprecipitation and Western blot analyses. For coimmunoprecipitation, extracts were prepared by lysing the cells in ice-cold buffer containing 50 mmol/L Tris (pH 7.9), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 1% NP40, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, 2 μg/mL pepstatin, and 5 μg/mL aprotinin on ice for 30 minutes. The cleared supernatant containing 0.5 mg protein was incubated with 3.0 μg of the indicated antibody immobilized on agarose beads for 16 hours at 4°C. Captured beads were boiled in SDS-PAGE loading buffer and released proteins analyzed by Western blot as described (24).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were done as described (26). Briefly, U251 cells were cross-linked with formaldehyde (1% final concentration) for 10 minutes at room temperature. Nuclei from these cells were sonicated to generate chromatin particles of average lengths of 500 to 800 bp. Sonicated nuclei were isolated by a CsCl step gradient followed by dialysis against TE buffer [10 mmol/L Tris-HCl (pH 8.0)/1 mmol/L EDTA/0.5 mmol/L EGTA/10% glycerol]. Purified chromatin particles were precleared with a mixture of protein A-Sepharose and protein G-Sepharose beads that were blocked with 1 mg/mL salmon sperm DNA and 1 mg/mL bovine serum albumin. Twenty-five percent of the precleared chromatin were kept for input control and the rest was immunoprecipitated with either anti-Stat3 rabbit polyclonal antibody (sc-482, Santa Cruz Biotechnology) or anti-actin polyclonal antibody. Immunoprecipitation, treatments with RNase A and proteinase K, de-cross-linking, and isolation of DNA were done in accordance with the methods described by Takahashi et al. (26). Purified DNA was subjected to PCR using primers that were specific for regions spanning the Stat3 binding sites in the promoters of *bcl-2*, *bcl-x*, and *mcl-1*. PCR products were resolved on a 2.5% agarose gel and stained with ethidium bromide.

Luciferase assay. 293 cells were seeded in six-well plates and transfected the next day by calcium phosphate method (24). Cells were cotransfected with expression plasmids for IL-13Rα2, Stat3, and luciferase reporter that contained three copies of the Stat binding site from the IRF-1 promoter in a minimal thymidine kinase promoter (GAS3-Luc; ref. 27). After 48 hours of transfection, cell lysates were prepared and luciferase activity was measured following the manufacturer's instructions (Promega).

Results

Interleukin-4 activates Stat3 in glioblastoma multiforme cells but not in normal astrocytes. Previously, we showed that all the GBM cell lines examined were refractory to IL-4– and IL-13–mediated activation of Stat6, which was in part attributable to the expression of IL-13Rα2 (17). We also found that Stat3 was persistently activated in GBM tumors and cell lines (22). Here, we show that IL-4 markedly induced the activation of Stat3 in GBM cell lines T98G, U251, and A172 but not in NHAs, which activated Stat3 in response to IL-6 (Fig. 1). Moreover, the level of Stat3 activation by IL-4 was comparable with that of Stat3 activation by IL-6 in A172 cells. A SIE probe derived from the c-*fos* promoter binds to three *sis*-inducible factors (SIF), termed SIF-A (Stat3 homodimers), SIF-B (Stat1-Stat3 heterodimers), and SIF-C (Stat1 homodimers; ref. 25). Using radiolabeled SIE probe in an EMSA, we observed that SIF was further activated by IL-4 in GBM cells. The maximum activation of Stat3 occurred at 30 minutes after the IL-4 treatment (10-25 ng/mL) of T98G cells, which sustained for 48 hours in the continuous presence of the cytokine (data not shown). To determine the identity of the SIF complexes activated by IL-4, we did EMSA supershift assay employing antibodies that were specific for Stat1 or Stat3. Stat5 antibody was used as a negative control. Stat3 homodimers were found to be predominant in IL-4-treated GBM cell lines (Fig. 1). No significant amount of Stat1 homodimers (SIF-C) was detectable after IL-4 treatment (data not shown). These results clearly show that IL-4 can up-regulate the activation and hence DNA binding activity of Stat3 in GBM cells.

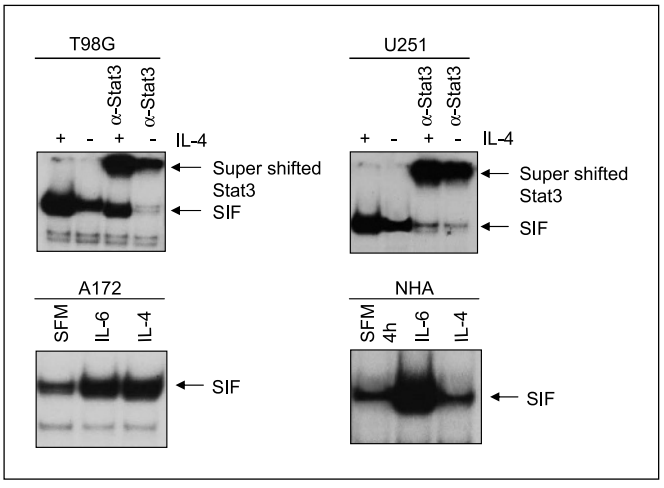

Figure 1. IL-4 activates Stat3 in glioblastoma cells but not in normal astrocytes. T98G, U251, and A172 cells and NHAs were treated with IL-4 (20 ng/mL) for 30 minutes or left untreated. A172 cells and NHAs were treated with IL-6 (25 ng/mL) for 30 minutes. Cells were maintained in serum-free medium for 4 to 16 hours before harvesting for protein extraction. Stat3 activation was measured by EMSA using 15 μg WCE protein and 0.2 ng ^{32}P-labeled hSIE probe that binds to SIF. WCEs from GBM cell lines were also preincubated with the Stat-specific antibodies (anti-Stat1, anti-Stat3, and anti-Stat5) followed by EMSA analysis employing the hSIE probe as described above. *Arrowheads,* SIF and the supershifted SIF complexes.

Interleukin-4 increases the steady-state levels of Bcl-2, Bcl-x_L, and Mcl-1 in glioblastoma multiforme cells. Previously, we have shown that Stat3 activation up-regulates the expression of antiapoptotic proteins Bcl-2, Bcl-x_L, and Mcl-1 in GBM cells (22). To determine whether expression of these proteins was further up-regulated by IL-4, we treated T98G cells with IL-4 for varying lengths of time and measured steady-state levels of these proteins by Western blot analysis. The expression levels of Bcl-2, Bcl-x_L, and Mcl-1 were markedly up-regulated at 6 to 12 hours of IL-4 treatment (Fig. 2). IL-4 normally activates the Jak-Stat6 and the IRS-PI3K-AKT pathways; however, in some cases, it also activates the mitogen-activated protein kinase (Erk1/Erk2) pathway (10, 11, 15, 28–30). T98G cells contained persistently activated Erk1/Erk2, which was not further increased by IL-4 stimulation but completely ablated by U0126 that inhibits mitogen-activated protein kinase (Erk1/Erk2) kinase (Fig. 3*C*). It is reported that activation of both PI3K-AKT and Jak/receptor tyrosine kinase/Stat3 pathways can regulate the expression of Bcl-2 family of antiapoptotic proteins depending on cellular context and cytokine/growth factor involved (29–31). To evaluate the relative contributions of these two pathways, we determined the effects of inhibition of (*a*) Jak activity by AG490 and (*b*) PI3K activity by LY294002 on the IL-4–dependent expression of Bcl-2, Bcl-x_L, and Mcl-1. AG490 treatment almost completely inhibited the IL-4–mediated activation of Stat3 as expected, whereas LY294002 had little effect on it (Fig. 3*B*). However, LY294002 inhibited IL-4–mediated activation of AKT, whereas AG490 did not (Fig. 3*A*). Inhibition of Stat3 activation by AG490 was consistent with inhibition of Bcl-2 and Mcl-1 expression in IL-4-stimulated cells (Fig. 3*A*). AG490 inhibited Bcl-x_L expression by 16.5% and 25.5% at 0.5 and 6 hours, respectively, whereas at 15 hours the inhibition was reduced to 6.3%. LY294002 did not inhibit the Bcl-x_L expression (Fig. 3*A*). Inhibition of the PI3K pathway had little effect on IL-4–mediated up-regulation of Bcl-2, Bcl-x_L, and Mcl-1expression (Fig. 3*A*). Taken together, these findings indicate that whereas IL-4–mediated up-regulation of Bcl-2, Bcl-x_L, and Mcl-1 in GBM cells is mainly dependent on the activation of Stat3, activated PI3K-AKT pathway also has a minor contribution to the up-regulation of these genes. To show the binding of activated Stat3 to the promoters of *bcl-2*, *bcl-x*, and *mcl-1* genes in GBM cells, we did ChIP assays using anti-Stat3 antibody. Data clearly indicate that activated Stat3 directly binds to the promoters of *bcl-2*, *bcl-x*, and *mcl-1* genes *in vivo* (Fig. 3*D*). Collectively, these results show that Stat3 signaling through an aberrant activation of the IL-4 receptor complex induces the expression of *bcl-2*, *bcl-x*, and *mcl-1* in GBM cells.

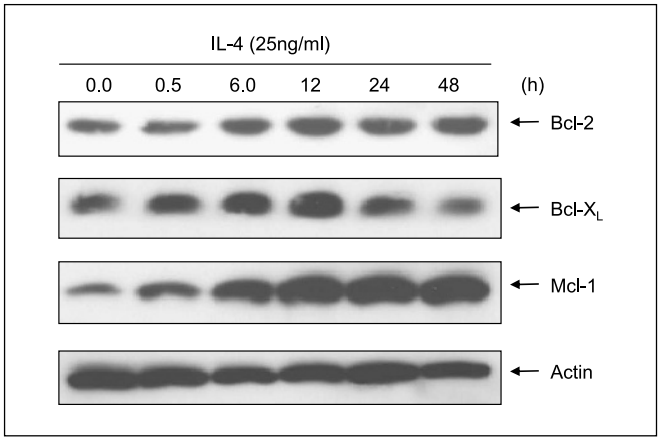

Figure 2. Up-regulation of Bcl-2 family proteins by IL-4 in GBM cells. T98G cells were treated with IL-4 for the indicated lengths of time and steady-state levels of expression of Bcl-2, Bcl-x_L, and Mcl-1 were determined by immunoblot analyses using 50 μg proteins of cell lysate. Blots were stripped and reprobed with anti-actin antibody to ensure equivalent protein loading. *Bottom,* representative Western blot for actin.

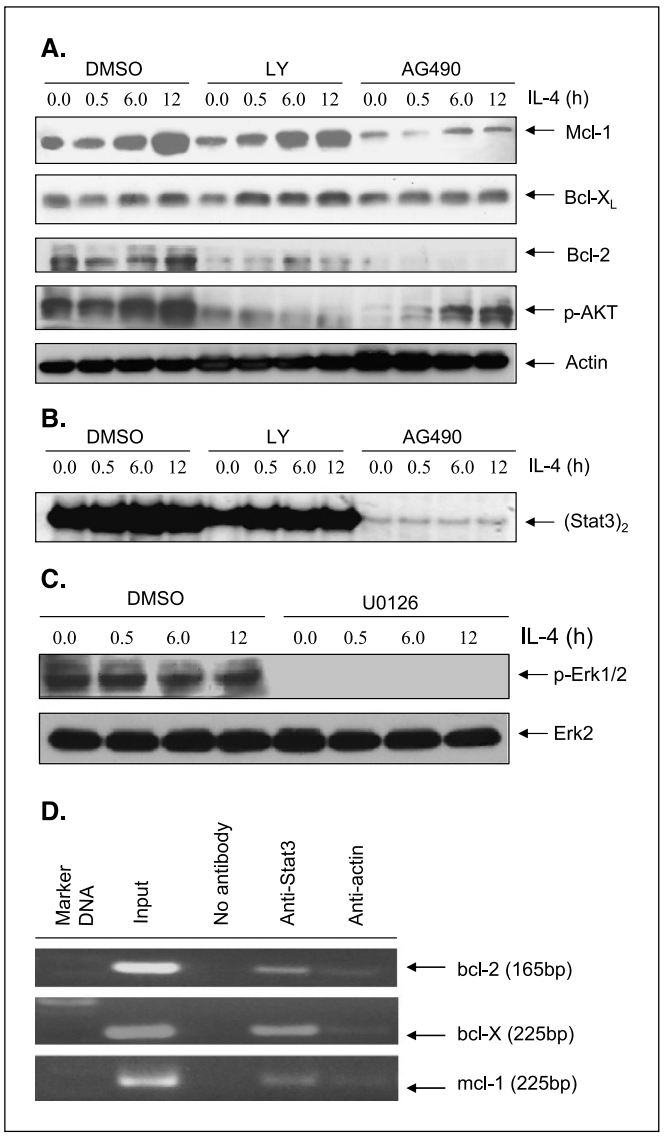

Figure 3. IL-4–mediated up-regulation of Bcl-2, Bcl-x_L, and Mcl-1 depends mainly on Jak-Stat3 pathway and partly on PI3K-AKT pathway. *A,* whole cell lysates were prepared after treatment of T98G cells with IL-4 for the indicated lengths of time and the steady-state expression levels of Bcl-x_L, Bcl-2, Mcl-1, and pAKT were determined by Western blot analyses. Before IL-4 treatment, cells were pretreated with DMSO, LY294002 (20 μmol/L), or AG490 (50 μmol/L) for 16 to 36 hours as indicated. Blots were stripped and reprobed with anti-actin antibody to ensure equivalent protein loading. *B,* Stat3 activation was measured by EMSA using 15 μg WCE protein and 0.2 ng ^{32}P-labeled hSIE probe. *Arrowhead,* Stat3 complexes. *C,* before IL-4 treatment, cells were pretreated with DMSO or U0126 (20 μmol/L) for 16 hours. pErk1/Erk2 and Erk2 were determined by Western blot analyses. *D,* Stat3 binds to the promoters of *bcl-2*, *bcl-x*, and *mcl-1* in GBM cells. ChIP assays were done in U251 cells using either a Stat3-specific antibody, an irrelevant antibody (anti-actin), or no antibody. PCR primers were designed to yield a product, which encompasses the Stat3 binding sites of the *bcl-2*, *bcl-x*, or *mcl-1* promoter. *Input,* total chromatin before immunoprecipitation in ChIP assays that acts as positive control.

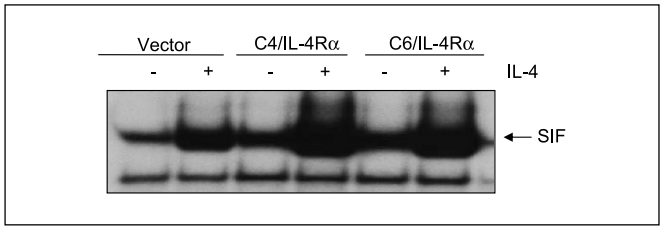

Figure 4. Activation of Stat3 by IL-4Rα chain in GBM cells. T98G cells were cotransfected with human IL-4Rα expression plasmid or the empty vector and pBabe-puro expression plasmid for puromycin resistance gene. Stable clones were isolated by drug selection as described (17). Two clones, C4/IL-4Rα and C6/IL-4Rα, were treated with IL-4 (20 ng/mL) for 30 minutes. WCEs containing 15 μg protein were subjected to EMSA as described above.

Interleukin-4Rα plays a direct role in the aberrant activation of Stat3. The type II IL-4 receptor is composed of IL-4Rα and IL-13Rα1 that are constitutively associated with Jak1 and Jak2 (or Tyk2), respectively (10, 11, 15, 16). In general, binding of either IL-4 to IL-4Rα or IL-13 to IL-13Rα1 leads to the heterodimerization of the receptor chains, which sequentially triggers the transactivation of receptor-associated Jaks, and phosphorylation of multiple tyrosine residues in the cytoplasmic domain of IL-4Rα, which recruits both IRS and Stat6. IL-4 or IL-13 also activate Stat3 in myeloid cells, B lymphocytes, and lung fibroblast cells in which before activation, unlike Stat6, Stat3 is recruited to IL-13Rα1 that contains a Stat3-specific Tyr-X-X-Gln motif (29, 32–34). To examine if IL-4Rα chain has a direct role in the

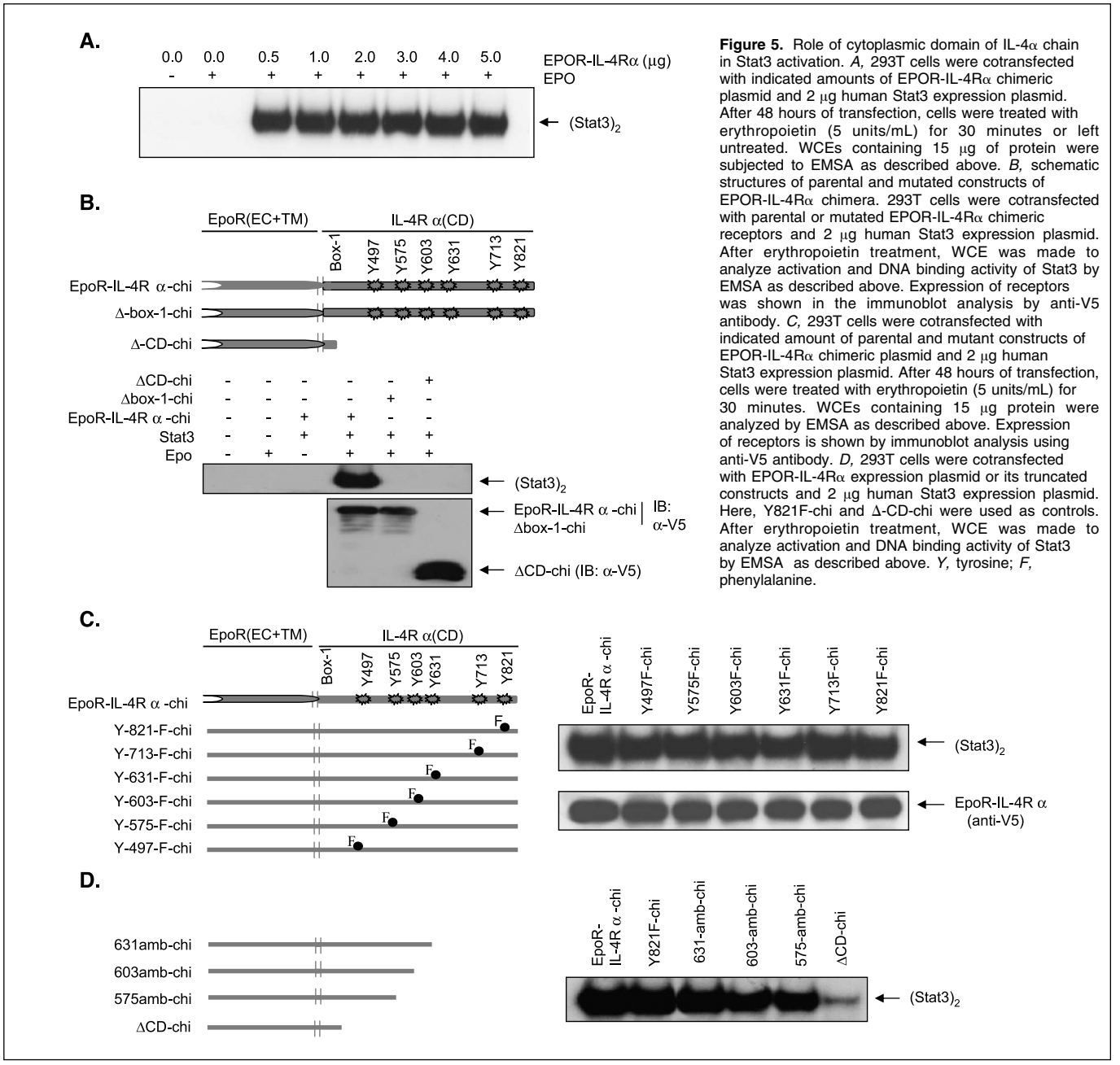

Figure 5. Role of cytoplasmic domain of IL-4α chain in Stat3 activation. *A,* 293T cells were cotransfected with indicated amounts of EPOR-IL-4Rα chimeric plasmid and 2 μg human Stat3 expression plasmid. After 48 hours of transfection, cells were treated with erythropoietin (5 units/mL) for 30 minutes or left untreated. WCEs containing 15 μg of protein were subjected to EMSA as described above. *B,* schematic structures of parental and mutated constructs of EPOR-IL-4Rα chimera. 293T cells were cotransfected with parental or mutated EPOR-IL-4Rα chimeric receptors and 2 μg human Stat3 expression plasmid. After erythropoietin treatment, WCE was made to analyze activation and DNA binding activity of Stat3 by EMSA as described above. Expression of receptors was shown in the immunoblot analysis by anti-V5 antibody. *C,* 293T cells were cotransfected with indicated amount of parental and mutant constructs of EPOR-IL-4Rα chimeric plasmid and 2 μg human Stat3 expression plasmid. After 48 hours of transfection, cells were treated with erythropoietin (5 units/mL) for 30 minutes. WCEs containing 15 μg protein were analyzed by EMSA as described above. Expression of receptors is shown by immunoblot analysis using anti-V5 antibody. *D,* 293T cells were cotransfected with EPOR-IL-4Rα expression plasmid or its truncated constructs and 2 μg human Stat3 expression plasmid. Here, Y821F-chi and Δ-CD-chi were used as controls. After erythropoietin treatment, WCE was made to analyze activation and DNA binding activity of Stat3 by EMSA as described above. *Y,* tyrosine; *F,* phenylalanine.

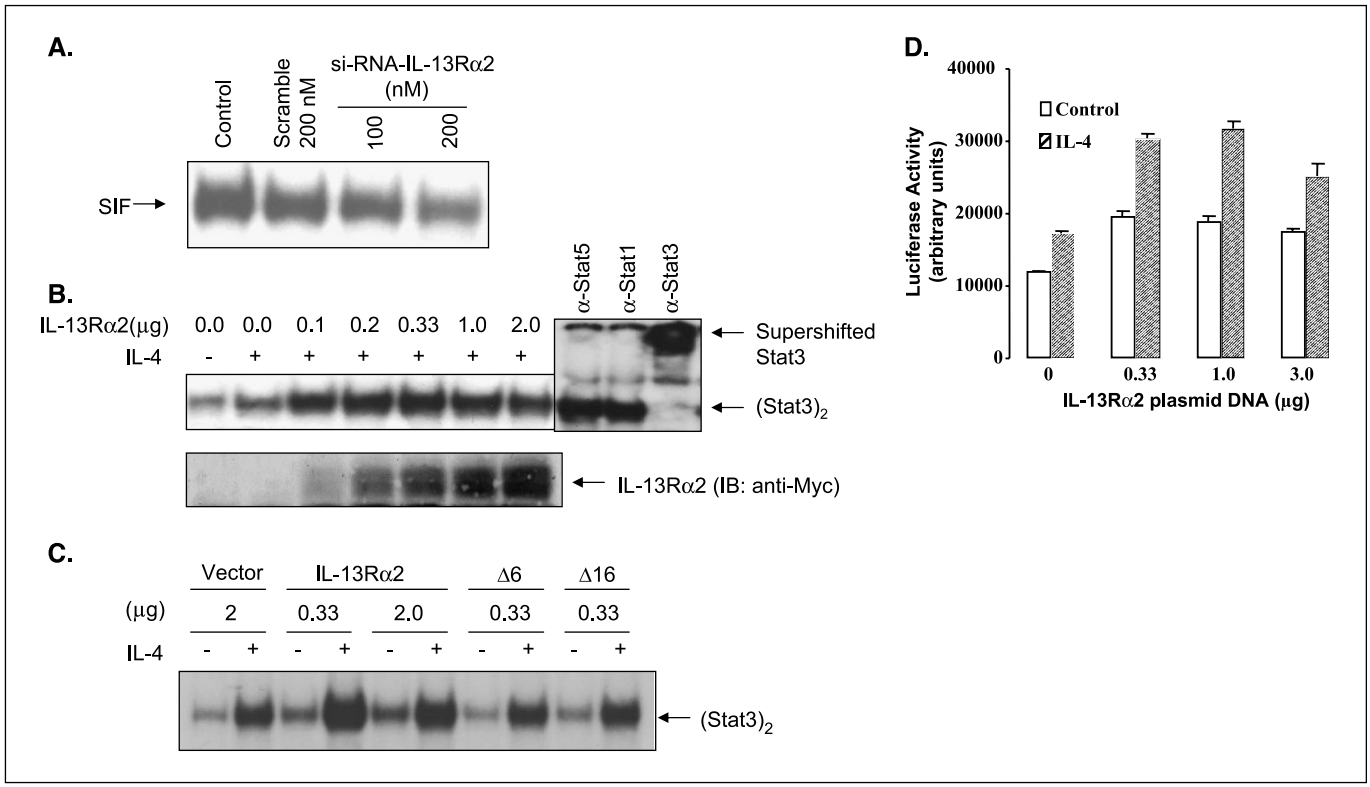

Figure 6. IL-13Rα2 up-regulates the IL-4–mediated activation of Stat3. *A,* synthetic siRNA duplex designed for human IL-13Rα2 was transfected into U251 cells. Cells were harvested for EMSA analysis employing the hSIE probe after 48 hours of transfection. *B,* 293T cells were cotransfected with increasing amounts of IL-13Rα2 expressing plasmid as indicated and 2 μg human Stat3 expression plasmid. After 48 hours of transfection, cells were treated with IL-4 for 30 minutes or left untreated. WCEs containing 15 μg protein were subjected to EMSA as described above. WCEs were also preincubated with the Stat-specific antibodies (anti-Stat1, anti-Stat3 or anti-Stat5) followed by EMSA analyses employing the hSIE probe as described above. *Arrowheads,* SIF and the supershifted SIF complexes (*top*). Expression of IL-13Rα2 receptor chain was shown by the immunoblot analysis using anti-Myc antibody (*bottom*). *C,* 293T cells were cotransfected with parental or truncated IL-13Rα2 receptor chain and 2 μg human Stat3 expression plasmid. After IL-4 treatment, WCE was prepared to analyze activation and DNA binding activity of Stat3 by EMSA as described above. *D,* regulation of Stat3-dependent gene expression by IL-13Rα2. 293T cells were cotransfected with 2 μg human Stat3 expression plasmid, 4 μg Stat3-responsive luciferase reporter, or control plasmid and varying amounts of human IL-13Rα2 expression plasmid as indicated. After 24 hours of transfection, cells were trypsinized and divided into two halves. After 12 hours, IL-4 (20 ng/mL) was added to one half and the other half was left untreated as control. After 15 hours, cells were harvested to measure the luciferase activity. Luciferase activity was normalized based on protein concentration and expressed in arbitrary units. Mean ± SD of three independent experiments.

activation of Stat3 in GBM cells, we generated T98G cell lines that stably expressed the human IL-4Rα transgene. Two such cell lines and a vector control cell line were treated with IL-4 (25 ng/mL) for 30 minutes or left untreated, and Stat3 activation was measured by EMSA. The results show that overexpression of IL-4Rα transgene in T98G cells increases the constitutive as well as IL-4–induced activation of Stat3 (Fig. 4).

To understand the mechanisms underlying the IL-4Rα–mediated activation of Stat3, we used a chimeric receptor composed of the extracellular plus the transmembrane domains of the murine EPOR and the cytoplasmic domain of the human IL-4Rα chain (24). This chimeric receptor (EPOR-IL-4Rα-chi), when coexpressed with Stat3 in 293T cells, became activated by erythropoietin-dependent homodimerization, which in turn induced an activation of Stat3 (Fig. 5*A*). To define the specificity of IL-4Rα–mediated activation of Stat3, we prepared two mutant constructs of EPOR-IL-4Rα-chi (Fig. 5*B*, *top*): Δ-box-1-chi in which Jak1 binding box-1 motif in the membrane proximal region of the cytoplasmic domain of IL-4Rα, was deleted and Δ-CD-chi that lacked the cytoplasmic domain of IL-4Rα containing six tyrosine residues (10). Both the mutant chimeric receptors failed to induce erythropoietin-mediated activation of Stat3 when coexpressed with Stat3 in 293T cells (Fig. 5*B*). Taken together, these results clearly suggest that activation of Stat3 via IL-4Rα chain depends not only on receptor association of Jak1 but also on the presence of the cytoplasmic domain of IL-4Rα chain. Many cytokine receptors are phosphorylated on tyrosine residues in response to specific stimuli, and the phospho-tyrosine residues function as docking sites for Stats and other signaling proteins (34). Cytoplasmic domain of IL-4Rα chain possesses six tyrosine residues located at amino acid positions 497, 575, 603, 631, 713, and 821 (10). However, none of these tyrosine residues matches the Stat3 binding consensus motif Tyr-X-X-Gln (34). To determine the contribution of individual tyrosine residues, we generated mutant constructs of EPOR-IL-4Rα-chi (Fig. 5*C*, *left*) in which each of the six tyrosine residues was changed to phenylalanine individually. We determined the activation level of Stat3 by each mutant receptor in response to erythropoietin stimulation of 293T cells. Results indicated that phenylalanine substitution of each one of the six tyrosine residues did not affect the activation level of Stat3 (Fig. 5*C*, *top right*). Mutant constructs of EPOR-IL-4Rα-chi receptor were expressed at comparable levels in transfected cell as shown by immunoblot analysis (Fig. 5*C*, *bottom right*). However, it was not clear from these experiments whether activation of Stat3 through IL-4Rα chain required one or multiple tyrosine residues. To address this, we generated three mutant EPOR-IL-4Rα-chi receptors by introducing stop codon at

amino acid positions 631, 603, and 575 (Fig. 5*D*, *left*). Analyses of these mutants revealed that presence of a single tyrosine residue (as in construct 575-amb-chi) in the cytoplasmic domain of IL-4Rα chain was sufficient to support the activation of Stat3 through the chimeric receptor (Fig. 5*D*, *right*). Figure 5*C* showed that Tyr^{497} in IL-4Rα was dispensable for the activation of Stat3. Collectively, these data suggest that the presence of at least one tyrosine residue in the cytoplasmic domain of IL-4Rα chain is sufficient for the IL-4–dependent activation of Stat3.

Interleukin-13Rα2 up-regulates the interleukin-4–dependent activation of Stat3 and consequent gene expression. Previously, we have reported that IL-13Rα2 functions as a negative regulator of IL-4–dependent signal transduction and Stat6-responsive gene expression (17). This is possibly mediated by a physical interaction between the intracellular domain of IL-13Rα2 and the cytoplasmic domain of the IL-4Rα chain. IL-13Rα2 is overexpressed in anaplastic astrocytoma and GBM cells but not in normal astrocytes or low-grade glioma cells (35–37). However, the exact biological significance of IL-13Rα2 expression in gliomas remains unclear. To examine the possibility that overexpression of IL-13Rα2 in GBM cells might have a role in IL-4–mediated activation of Stat3, the expression of IL-13Rα2 was inhibited in U251 cells by siRNA-IL-13Rα2. After 48 hours of transfection with siRNA, cells were treated with IL-4 for 30 minutes, and Stat3 activation was measured by EMSA. The results showed that IL-4–dependent activation of Stat3 was markedly inhibited by siRNA-IL-13Rα2 (Fig. 6*A*).

Furthermore, transient expression of the IL-13Rα2 transgene in 293T cells increased the IL-4–mediated activation of Stat3 in a dose-dependent manner (Fig. 6*B*). The human IL-13Rα2 is a 380–amino acid transmembrane protein with 341–amino acid extracellular domain and a short cytoplasmic tail that contains only 17 amino acids (38). To examine if the cytoplasmic tail of IL-13Rα2 plays a role in Stat3 activation, we prepared mutant IL-13Rα2 constructs that lacked the carboxyl-terminal 6 or 16 amino acids. The mutant IL-13Rα2 proteins (Δ6-IL-13Rα2 and Δ16-IL-13Rα2) when coexpressed with Stat3 in 293T cells failed to induce the activation of Stat3 in IL-4-treated 293T cells (Fig. 6*C*). In Caki-1 renal cell carcinoma that expresses IL-13Rα2, IL-4 treatment also up-regulated the activation of Stat3 (data not shown).

To determine whether IL-13Rα2 expression regulates the transcription of Stat3-responsive genes, a GAS3-Luc reporter construct and the IL-13Rα2 expression plasmid (at varying amounts) were cotransfected into 293T cells along with a Stat3 expression plasmid. The results showed that IL-13Rα2 induced the IL-4–mediated up-regulation of luciferase activity in 293T cells (Fig. 6*D*), which was consistent with the EMSA results (Fig. 6*B*). These results provide an evidence for the first time that expression of IL-13Rα2 positively regulates IL-4R/IL-13R–mediated activation of Stat3 in GBM cells.

To address how the short cytoplasmic domain of IL-13Rα2 confers the IL-4–mediated activation of Stat3, we carried out a protein-protein interaction assay. 293T cells were cotransfected with the IL-13Rα2 (tagged with Myc epitope) and Stat3 (tagged with Flag epitope) expression constructs. After 48 hours of transfection, cell lysates were prepared and subjected to immunoprecipitation with either anti-Myc or anti-Flag antibody followed by immunoblot analyses with either anti-Flag or anti-Myc antibody. The results showed that IL-13Rα2 did not coimmunoprecipitate Stat3 when coexpressed in 293T cells (Fig. 7). These data suggest that IL-13Rα2-mediated activation of Stat3 does not occur via a direct physical interaction of this receptor with Stat3. Further investigation is required to precisely define the role of IL-13Rα2 in the regulation of IL-4–dependent Stat3 signaling in GBM cells.

Discussion

IL-4 exhibits antitumor activity in rodent experimental glioma models (8). This raises the key question: What are the underlying cellular and molecular mechanisms? Binding of IL-4 to its receptor on the surface of the target cells normally activates two intracellular signaling pathways: the Jak-Stat6 pathway that activates the expression of Stat6-targeted genes and the IRS-PI3K-AKT pathway that promotes growth of the recipient cells (10, 11, 15, 16). IL-13, another T-cell-derived cytokine that shares the type II IL-4 receptor, also activates the same signaling pathways in target cells that exclude B and T cells in mice and T cells in human (10, 11, 15, 16). *In vivo* IL-4 may act on GBM cells and immune cells that are present in and around the tumor masses and/or the other resident cells of the brain tissues.

Here, we choose to focus on the direct action of IL-4 on GBM cells because they express the cognate receptor, and IL-4 is used for gene therapy in rodent glioma models (8). Previously, we have shown that unlike normal astrocytes GBM cells are refractory to IL-4– and IL-13–dependent activation of Stat6 (17). GBM cells, but not NHA or low-grade astrocytoma cells, express IL-13Rα2, a high-affinity receptor for IL-13 on the cell surface, which does not transduce any intracellular signals and thus functions as a decoy receptor for IL-13. This explains why GBM cells fail to activate Stat6 in response to IL-13 stimulation. We found that IL-13Rα2 also acts as a negative regulator of Stat6 activation by IL-4 in GBM cells (17). IL-4 reduces the proliferation of cells derived from normal cortex or low-grade glioma but not high-grade glioma cells, and p21-dependent elevation of p27 level is required for the IL-4–mediated growth arrest in the low-grade glioma cells (39). This raises the question: Does IL-13Rα2 prevent the IL-4–mediated growth arrest in GBM cells? In this context, we have made an important observation that in response to IL-4 stimulation, unlike NHA, GBM cells activate Stat3, another

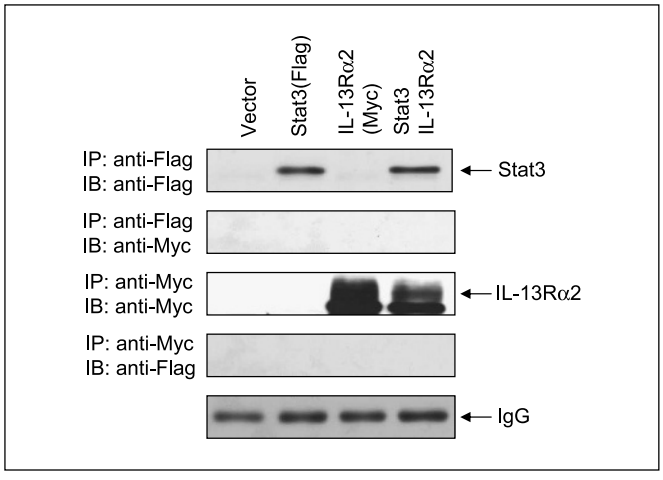

Figure 7. IL-13Rα2 does not physically interact with Stat3. 293T cells were cotransfected with expression plasmids for IL-13Rα2 (tagged with Myc-epitope) and Stat3 (tagged with Flag-epitope). After 48 hours, cell lysates were prepared and subjected to coimmunoprecipitation followed by Western blot analysis using antibodies to anti-Myc and anti-Flag. IgG band was shown as loading controls.

member of the Stat family. Unlike other members of this family of transcription factors, Stat3 deficiency via gene targeting in mice results in embryonic lethality (40). Stat3 is activated by IL-6 family of cytokines and several growth factors that include epidermal growth factor, transforming growth factor-α, hepatocyte growth factor, platelet-derived growth factor, and vascular endothelial growth factor leading to the transcriptional activation of genes that are involved in diverse and seeming opposing cellular functions like cell proliferation, differentiation, apoptosis, survival, and acute-phase responses (18, 41–43). One acquired capability that all cancer cells share is the self-sufficiency in growth signals (44). Recently, we found that some GBM cell lines produce IL-6, whereas others harbor activated epidermal growth factor receptor leading to the persistent activation of Stat3.[5] IL-4 further activates Stat3 in GBM cells but not in NHA. Previously, we have shown that blockade of Stat3 activation by Jak inhibitor AG490 or dominant mutant Stat3 protein reduced the steady-state levels of the prosurvival proteins that included Bcl-2, Bcl-x_L, and Mcl-1 resulting in the induction of spontaneous apoptosis in GBM cells (22). Here, we show for the first time that IL-4 increases the steady-state levels of these proteins, and importantly, activated Stat3 binds to the promoters of these three prosurvival genes of the *bcl-2* family as analyzed by ChIP employing anti-Stat3 antibody followed by PCR. The IL-4–mediated up-regulation of Bcl-2, Bcl-x_L, and Mcl-1 in GBM cells also depends in part on the PI3K-AKT pathway (Figs. 3 and 8). These results suggest that IL-4 gene therapy for malignant gliomas may produce adverse effects. Of note, Mcl-1 does not only suppress the apoptosis of cancer cells but also confers resistance to chemotherapy and radiation (45, 46).

The molecular events underlying the IL-4–dependent activation of the PI3K-AKT pathway are well defined (10, 11, 15, 16). However, the mechanisms for the IL-4–dependent activation of Stat3 remain poorly understood. Here, we report for the first time that level of IL-4Rα chain expression in GBM cells and homodimerization of IL-4Rα in 293T cells can directly modulate IL-4–mediated activation of Stat3 (Figs. 4 and 5). Mutational analyses of the cytoplasmic domain of IL-4Rα chain show that individual tyrosine residues are redundant for Stat3 activation. Signaling via homodimerization of IL-4Rα chain has been shown previously (23, 47). However, a biological role for the IL-4Rα homodimers remains to be explored.

Does IL-13Rα2 have a role in IL-4–mediated activation of Stat3 in GBM cells? Previously, we have shown that the short intracellular domain of IL-13Rα2 physically interacts with the cytoplasmic domain of the IL-4Rα chain that harbors the Stat docking sites (17). Now, we show that inhibition of IL-13Rα2 expression by siRNA reduces the IL-4–mediated activation of Stat3 in U251 cells. Conversely, transient expression of the IL-13Rα2 transgene in 293T cells increases the IL-4–mediated Stat3 activation and consequent gene expression. Because Stat6 binding is restricted to three conserved tyrosine residues on cytoplasmic domain of IL-Rα chain (10), it may be possible that overexpression of IL-13Rα2 inhibits Stat6 activation by masking its docking sites on the receptor. Individual tyrosine residues in IL-Rα chain can be used in a redundant fashion for Stat3 activation, which may provide an explanation of why IL-13Rα2 does not inhibit IL-4–mediated Stat3 activation. Coimmunoprecipitation assay reveals that IL-13Rα2-mediated activation of Stat3 does not require a direct physical interaction between Stat3 and IL-13Rα2. We have shown previously that IL-13Rα2 physically interacts with IL-4Rα (17), which may cause conformational changes in the receptor complex favoring the recruitment and activation of Stat3.

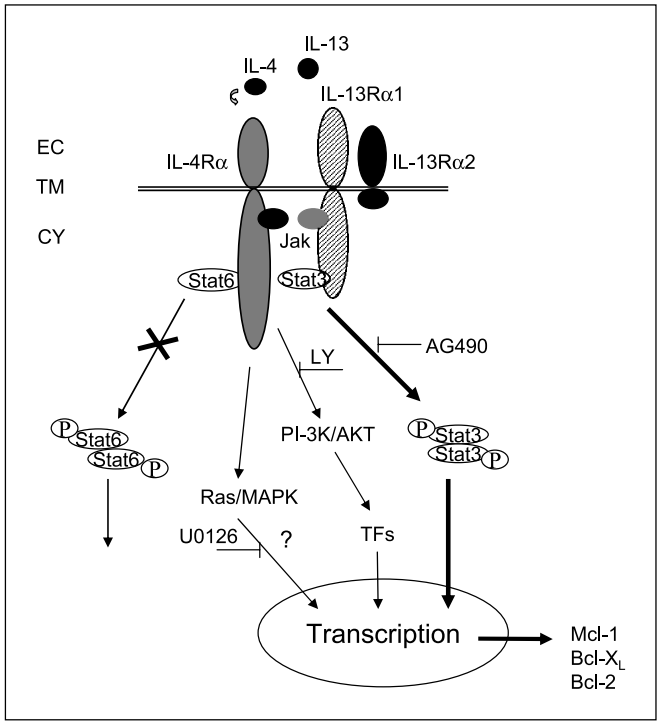

Figure 8. Signal transduction pathways for IL-4–mediated up-regulation of Bcl-2 family of antiapoptotic proteins in GBM cells. IL-4 stimulation in GBM cells causes activation of Jak-Stat3 and PI3K-AKT pathways. It does not activate Stat6 and mitogen-activated protein kinase pathways in GBM cells. Inhibition of signaling pathways by specific inhibitors has indicated that IL-4–mediated up-regulation of Bcl-2, Bcl-x_L, and Mcl-1 is largely dependent on the Jak-Stat3 pathway and partly on the PI3K-AKT pathway. IL-13Rα2 plays a role in IL-4–mediated Stat3 signaling via a novel mechanism. *EC*, *TM*, and *CY*, extracellular, transmembrane, and cytoplasmic regions of the receptors, respectively.

Several solid tumors express IL-4 receptors and IL-4 inhibits the growth of several cancer cells *in vitro* (48–50). An antitumor activity that IL-4 exhibits in rodent models is believed to be mediated by T cells, macrophages, and eosinophils, supporting a potential use of IL-4 in cancer immunotherapy (8). However, human clinical trial using IL-4 in both hematologic and nonhematologic cancers has shown unsatisfactory results, producing minor or negligible effects or even leading to a possible increase in the number of malignant cells (51–54). A recent study shows that tumor Stat3 activity can mediate immune evasion by regulating both innate and adaptive immune responses (20, 21). In conclusion, we provide evidence that IL-4 can directly act on GBM cells and increase the expression of antiapoptotic proteins, and this may explain why IL-4 fails to exhibit antiproliferative activity in GBM cells.

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[5] M.K. Ghosh, S.O. Rahaman, S.J. Haque, unpublished data.

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