Stat3β Inhibits γ-Globin Gene Expression in Erythroid Cells*

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We demonstrated previously γ-globin gene inhibition in K562 cells and primary erythroid progenitors treated with interleukin-6. Although several cis-acting elements have been identified in the globin promoters, the precise mechanism for cytokine-mediated globin gene regulation remains to be elucidated. In this report we demonstrate inhibitors of Stat3 phosphorylation abrogate interleukin-6-mediated γ gene silencing in erythroid cells. DNA-protein binding studies established Stat3 interaction in the 5′-untranslated γ-globin promoter region. Furthermore, co-transfection experiments with Stat3β demonstrate γ promoter inhibition in a concentration-dependent manner, which was significantly reversed when the cognate Stat3-binding site in the 5′-untranslated region was mutated. These studies establish a novel mechanism for γ gene silencing through the STAT signal transduction pathway.

Signal transducers and activators of transcription (STATs) play an essential role in regulating gene expression via a variety of pathways involving cytokines, growth factors, and other extracellular stimulants. Protein tyrosine kinases of the Janus kinase (JAK) family activate STAT proteins upon binding of cytokines to their cognate receptors (1, 2). The JAK-STAT pathway mediates the effect of several cytokines (3–5) including interleukin 6 (IL-6), which was previously demonstrated to inhibit γ gene expression (6) and to most likely exert its negative effect at the level of the bi-potential progenitor cell. Although several cis-acting elements have been identified in the globin gene promoters (7, 8), the precise mechanism for cytokine-mediated globin gene regulation during development remains to be clarified.

Receptor-bound IL-6 stimulates glycoprotein 130 homodimerization and ligand-dependent JAK2 activation (9, 10). Activated JAK2 phosphorylates the cytoplasmic domain of glycoprotein 130 creating docking sites for dimerization of Stat1 and Stat3. The phosphorylated dimer molecules translocate into the nucleus and bind IL-6-response elements in target promoters. Stat3β is a naturally occurring splice variant of Stat3c with a deletion of the carboxyl-terminal 55 amino acid residues including Ser-727, which is important for efficient transcriptional activation (11–14). Stat3β efficiently binds to the palindromic IL-6-response element as a homodimer but lacks transcriptional activity alone and is generally considered a dominant negative regulator (15). However, Schaefer et al. (16) demonstrated α-macroglobulin gene activation via Stat3β-c-Jun interactions. Likewise, murine Stat3β has been shown to activate selected promoters in a cell type-specific manner (16).

In this report we demonstrate abrogation of the IL-6-mediated γ gene repression in K562 cells pretreated with the JAK2 and Stat3/Stat5 inhibitors AG490 and piceatannol (PIC), respectively. Moreover, PIC reversed IL-6-mediated γ gene silencing in primary erythroid cells grown from adult and fetal stage progenitors. Western blot analysis confirmed that IL-6 preferentially activated Stat3β in K562 cells. Subsequent experiments completed in a genetic reporter system demonstrated a concentration-dependent Stat3-mediated γ gene silencing. Targeted mutagenesis of the cognate Stat3-binding site in the γ promoter reversed the negative effects of Stat3β on γ gene expression. Our studies establish a novel role for STAT proteins in globin gene regulation and a mechanism whereby Stat3 inhibits γ-globin gene activity.

EXPERIMENTAL PROCEDURES

Cell Lines—K562 erythroleukemia cells were cultured under the following conditions: RPMI 1640 containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) in a humidified incubator at 37 °C, 5% CO2. Cells were treated for 48 h with either IL-6 (100 ng/ml) or sodium butyrate (NaB, 2 mM) (Sigma) alone or following pretreatment with the inhibitors PIC (10 μM) for 30 min, AG490 (25 μM) for 18 h, or UO126 (10 μM) for 1 h. All inhibitors were purchased from Calbiochem.

 Burst-forming Unit-Erythroid (BFU-E) Colony Growth—Peripheral and umbilical cord blood samples were obtained from normal individuals after informed consent was obtained in accordance with the University of South Alabama Institutional Review Board guidelines. Mononuclear cells were isolated by density gradient centrifugation (Histopaque-1077, Sigma) and pretreated for 30 min with PIC (10 μM) where indicated. Erythroid progenitors were cultured in methylcellulose as published previously by the authors (17), and NaB (1 mM) or IL-6 (100 ng/ml) was added on day 0 of culture. BFU-E colonies were counted and harvested on day 14, and fetal hemoglobin (HbF) was measured as a percent of total hemoglobin, normalized to total protein, by alkaline denaturation as published previously (17, 18).

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**Fig. 1. Interleukin-6 inhibits γ-globin expression via the JAK-STAT pathway.** K562 cells were treated with IL-6 alone or pretreated with anti-IL-6 antibody, AG490 or PIC. RPA was performed with a Huy probe yielding a 170-bp protected fragment and GAPD as an internal control. A, representative gel for the different conditions is shown. B, the quantitative data obtained from PhosphorImager analysis is depicted in the bar graph. Data are shown for uninduced (white bar), IL-6-treated (black bars), or sodium butyrate (gray bar)-treated K562 cells. The fold increased γ mRNA synthesis was calculated as a ratio to GAPD. The uninduced levels were normalized to 1. The data represent the mean ± S.E. Ud, untreated; Ab, IL-6 antibody; A, AG490; P, piceatannol. C, peripheral blood mononuclear cells were isolated and grown in methylcellulose culture for 14 days. Cellular RNA and proteins were isolated and analyzed by RPA and alkaline denaturation, respectively, as described under “Experimental Procedures.” The effect of IL-6 or NaB on BFU-E colony growth (white bars), γ mRNA synthesis (black bars), and fetal hemoglobin (gray bars) levels for the different experimental conditions was analyzed. Untreated progenitor cells were normalized to 1. D, cord blood mononuclear cells were isolated and grown in methylcellulose culture as described under “Experimental Procedures.” BFU-E colonies (white bars) and fetal hemoglobin levels (gray bars) are as shown for the different experimental conditions. Data represent the mean ± S.E.

250, Bio-Rad) as described previously (19). γ-Globin mRNA was normalized to that of GAPD.

**Preparation of Cytosolic Protein Extracts**—K562 cells were lysed in 500 μl of ice-cold lysis buffer (75 mM NaCl, 50 mM NaF, 20 mM HEPES, 2.5 mM MgCl2, 15 mM EDTA, 2 mM EDTA, 1% Triton X-100, 0.1 mM Na3PO4, 0.5 mM dithiothreitol, 4 μg of leupeptin, 200 μg of phenylmethylsulfonyl fluoride, pH 7.4). Cellular debris was removed by centrifugation, and protein concentrations were determined with the Bradford assay (Bio-Rad). Aliquots of 25 μg were frozen at −70°C until used to avoid the adverse effects of freeze-thaw cycles on protein function.

**Western Blot**—Cytosolic protein extracts (25 μg) were resolved by electrophoresis using 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blocked with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% dry milk for 1 h. The membranes were probed for 12 h at 4°C with total Stat3 antibody diluted 1:250 in TBS-T or phopho-Stat3 antibody diluted 1:500 in TSB-T (Santa Cruz Biotechnology, Santa Cruz, CA). Both antibodies allow simultaneous measurement of Stat3α and Stat3β levels. The membranes were washed and then incubated for 1 h with anti-rabbit IgG-horseradish peroxidase (diluted 1:5000) in TBS-T for chemiluminescent detection. The same membranes were stripped and probed with an actin antibody (Santa Cruz Biotechnology) to control for any variations in the amount of protein loaded per well that may have occurred. The intensity of each band was measured using imaging software (SigmaGel, Jandel Scientific, Chicago, IL).

**Reporter Constructions**—Reporter plasmids were constructed with the pGL3-Basic luciferase plasmid (Promega, Madison, WI). The γLuc plasmid was established by subcloning an AluI γ promoter fragment (~299 to +36), from a plasmid containing a genomic clone, into the Smal site of pGL3-Basic. HS2-Luc, a kind gift from Dr. Townes, contains the same promoter fragment as γ-Luc downstream of the 1.9-kb hypersensitive site 2 (HS2) enhancer from the β-globin locus control region. Plasmid DNA was extracted using the alkaline lysis method and affinity purification (Qiagen Maxiprep System, Valencia, CA).

**Site-directed Mutagenesis**—The A/Stat3 site was mutated (mtAγ) by using an ExSite PCR-based Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). Briefly, a forward primer from nucleotide +9 to +30 in the γ promoter containing the desired mutation 5′-TTTGGAAAGGGTCT-GAGATTAC-3′ and a 5′-phosphorylated reverse primer from −14 to +8, 5′-GGCGAGTGTGGAATTGCTGAA-3′, were synthesized for the PCR, using the wild-type HS2-Luc plasmid as template. The HS2-Luc plasmid was digested from the PCR product with DpnI, followed by end polishing using Pfu DNA polymerase. The integrity of the PCR product was verified on an agarose gel followed by T4 ligation and transformation into Epicurian Coli® XL1-Blue Supercompetent cells (Stratagene).

The mutant carrying the desired base substitution was confirmed by direct sequencing.

**Transient Transfections**—Two reporter plasmids γLuc and HS2-Luc were analyzed in K562 cells. Each reporter plasmid (10 μg) was added to K562 cells (5 x 10⁵) and incubated at room temperature for 15 min followed by electroporation at 260 V and 975 microfarads (GenePulser, Bio-Rad). Transfected cells were cultured as described above with the addition of IL-6 (100 ng/ml) or NaB (2 mM) alone or in combination with signaling pathway inhibitors followed by chemical inductions. Cells were harvested after 24 h and extracts prepared according to the manufacturer’s instructions using a reporter lysis buffer (Promega).
Protein extracts (20 μl) were combined with 100 μl of enzyme reagent, and luciferase activity was measured (TD Luminoimeter, Promega). Total reporter gene activity was normalized to total protein. The relative luciferase activity was calculated as a ratio to that of untreated controls. The pGL3 control vector was transfected as a control with each trans-activator. The total reporter gene activity was normalized to total protein. The relative luciferase activity was calculated by comparing the different treatments to untreated (Ud) K562 cells. Significance levels were established by comparing the different treatments to untreated (Ud) K562 cells. A representative Western blot probed with Stat3-specific polyclonal antibody that detects total Stat3 (pS3α) and Stat3β levels are shown after IL-6 alone or after pretreatment with PIC (P/IL-6) or AG490 (A/IL-6). The representative Western blot probed with Stat3-specific polyclonal antibody that detects total Stat3α (t-Stat3α) and Stat3β (t-Stat3β) protein levels in response to treatment with IL-6 (100 ng/ml) alone or after pretreatment with PIC (P/IL-6) or AG490 (A/IL-6). Significance levels were established by comparing the different treatments to untreated (Ud) K562 cells. A representative Western blot probed with Stat3-specific polyclonal antibody that detects total Stat3α (t-Stat3α) and Stat3β (t-Stat3β) protein levels in response to treatment with IL-6 (100 ng/ml) alone or after pretreatment with PIC (P/IL-6) or AG490 (A/IL-6).

**Fig. 2.** *Interleukin-6 preferentially induces Stat3β synthesis in K562 cells.* A, schematic of wild-type Stat3α and the naturally occurring splice variant Stat3β, with a deletion in the carboxyl-terminal trans-activation domain, is shown. The various DNA domains and the major sites of phosphorylation are as indicated. SH2, Sre homology 2; Y, tyrosine residue 705; S, serine 727. B, Western blot analysis was performed on K562 cells to determine Stat3α and Stat3β protein levels in response to treatment with IL-6 (100 ng/ml) alone or after pretreatment with PIC (P/IL-6) or AG490 (A/IL-6). Significance levels were established by comparing the different treatments to untreated (Ud) K562 cells. A representative Western blot probed with Stat3-specific polyclonal antibody that detects total Stat3α (t-Stat3α, 89 kDa) and Stat3β (80 kDa) is shown. The graph shows the quantitative data obtained for the band intensities measured by SigmaGel Imaging software. Stat3α (white bars) and Stat3β (black bars) levels are shown after IL-6 alone or after pretreatment with PIC (P/IL-6) or AG490 (A/IL-6).
RESULTS

Interleukin-6 Is Unique among Megakaryocytic Specific Cytokines in Its Ability to Inhibit γ-globin—We have demonstrated previously (6) that IL-6 reduces the steady-state level of γ-globin mRNA in BFU-E colonies and K562 cells. This effect is evident in a concentration-dependent manner and is concomitant with an increased level of glycoprotein IIb mRNA. To gain further insight into the role of other megakaryocytic specific cytokines in γ gene regulation, we treated K562 cells with thrombopoietin (25–300 ng/ml) or IL-11 (25–100 ng/ml) for 72 h and found that neither cytokine had a significant effect on steady-state γ-globin mRNA levels. This suggests that IL-6 is unique among the megakaryocytic specific cytokines in regulating γ globin.

Interleukin-6 Mediates γ Gene Inhibition via the JAK-STAT Signaling Pathway—To determine the role of signaling proteins in the IL-6-mediated regulation of the γ-globin gene, we analyzed the effect of specific inhibitors of the JAK-STAT pathway. K562 cells were pretreated with AG490 or PIC prior to induction with IL-6. AG490 inhibits the ability of JAK2 to activate multiple downstream STAT proteins, whereas PIC specifically blocks activation of Stat3 and Stat5 (21, 22). Steady-state γ-globin mRNA level remained unchanged in IL-6-induced K562 cells pretreated with either inhibitor suggesting that the repressive effect of IL-6 was mediated through the JAK-STAT pathway (Fig. 1, A and B). The effect of AG490 and PIC was similar to that observed with an anti-IL-6 antibody, which blocked the ability of IL-6 to reduce γ-globin mRNA level by 50–60% (Fig. 1B). The concentrations used for AG490 and PIC have been widely applied to inhibit effectively the JAK-STAT pathway (23). To ensure that the K562 cell line used in these experiments was responsive to γ-globin gene activation, control experiments were performed with NaB, which resulted in a 4.5-fold increase in γ-globin mRNA levels. We conclude from these experiments that JAK2 and Stat3 and/or Stat5 are necessary for the IL-6-mediated repression of the γ-globin gene.

To address the physiological relevance of this finding, we extended the studies with PIC to primary erythroid cells. Adult peripheral blood and umbilical cord blood mononuclear cells were cultured for 14 days in the presence of IL-6 alone or following pretreatment with PIC. Cellular protein was isolated and HbF determined by alkaline denaturation as a percent of total hemoglobin (18). In agreement with previous data from
our laboratory (17, 18, 24) and by others (25, 26), we observed an increase (1.5-fold) in BFU-E colony growth in adult peripheral blood cultures treated with IL-6 (Fig. 1C). However, steady-state γ-globin mRNA levels were reduced by 6-fold \( (p < 0.05) \). The effect on mRNA synthesis was reflected at the protein level by a significant reduction in HbF. In control experiments NaB increased γ mRNA and HbF levels by 2.5- and 4.8-fold \( (p < 0.05) \), respectively. Prior treatment of mononuclear cells with PIC on day 0 abrogated the ability of IL-6 to repress HbF synthesis (data not shown). In contrast to promoting BFU-E colony growth from adult stage progenitors, IL-6 restricted the expansion of BFU-E in cultures established with umbilical cord blood. Fig. 1D shows that IL-6 reduced BFU-E colony number by 5-fold and HbF by 6-fold in umbilical cord blood cultures. Interestingly, on this fetal-globin background there was concordance of the IL-6 effect at the cellular and molecular level. It is worth noting that PIC abolished the effect of IL-6 on both cell proliferation and gene expression (Fig. 1D). The observation for gene control corroborates those observed in K562 cells and further implicates STAT transcription factors as negative regulators of γ-globin gene expression. Our recent findings (24) from studying mechanisms for γ-globin activation by short chain fatty acids show that γ-globin induction is associated with increased Stat3 activation. It is therefore likely that the negative regulation of γ-globin reported here involves a variant of Stat3.

**Interleukin-6 Induces Stat3β Protein Synthesis in K562 Cells**—The potential for Stat3 involvement in γ-globin gene regulation was determined by analyzing the expression of Stat3 proteins in IL-6-treated K562 cells. Cytosolic protein extracts were probed with a polyclonal antibody that recognizes both Stat3α and the truncated variant Stat3β (Fig. 2A). Cells treated with IL-6 alone showed an insignificant increase in Stat3α, which was reduced to 50% below base-line values following pretreatment with PIC (Fig. 2B). By contrast the level of Stat3β,80 kDa increased 5-fold in IL-6-treated K562 cells \( (p < 0.05) \). PIC inhibited Stat3β induction by IL-6 and reduced endogenous Stat3β levels to 80% below steady-state values \( (p < 0.05) \). AG490 had a less dramatic effect on the levels of Stat3 induced by IL-6 generally. These data demonstrate that IL-6 preferentially enhances Stat3β expression in K562 cells.

Furthermore, the level of activated Stat3β (phosphorylated) was greater than activated Stat3α at steady state. In time course experiments we observed a transient deactivation of both Stat3 variants by IL-6 for up to 4 h. This was replaced by a rapid rise in the level of activated Stat3β reaching a maximal value at 24 h, and remaining above base-line values for up to 48 h (data not shown). In contrast, deactivation of Stat3α was progressive throughout the study period. Consequently, the ratio of activated Stat3β/Stat3α at 48 h was higher compared with untreated cells. This indicates that Stat3β is the primary signaling protein mediating the effect of IL-6 in K562 cells.

**Stat3 Binds a Consensus Sequence in the γ Promoter**—A computer-aided search revealed a Stat3-like binding sequence (TTCTGGAA) in the Aγ and Gγ promoters, in the interval between nucleotides +9 to +16 relative to the cap site, which is similar in structure to the Stat3 consensus sequence TTTN₅₋₆AA (27, 28). DNA sequence alignment of the β-like globin genes showed that the embryonic (e) globin gene lacked the terminal AA dinucleotide critical for Stat3 binding (Fig. 3A). In the two adult globin genes (δ and β) the octamer sequence terminates with a CA dinucleotide (Fig. 3A). We investigated potential DNA-protein interactions in this region for each globin promoter using oligonucleotide probes spanning nucleotides +1 to +22. We observed two complexes of variable intensities with each radiolabeled probe and nuclear extract from K562 cells (Fig. 3B). Competition experiments with self and consensus Stat3 oligonucleotides suggest each promoter, e (lanes 2 and 3), β (lanes 5 and 6), and γ (lanes 7–9), may interact with Stat3. However, the motif in the γ-globin promoter is the most closely related to the Stat3 consensus, and in competition experiments a consensus Stat3 oligonucleotide was most effective against the γ probe (lane 9).

DNA-protein interaction with the γ-globin probe (AγSTAT3) was competed by a Stat1 consensus oligonucleotide as well (Fig. 4A, lanes 1–3) suggesting that Stat1 and Stat3 might bind as a heterodimeric complex to this region in the γ-globin promoter. Unlabeled AγSTAT3 oligonucleotide successfully competed for binding with consensus Stat3 and Stat1 probes (Fig. 4A, lanes 5–10). The Stat1 probe formed two major and two variable minor complexes (lane 8). AγSTAT3 specifically abolished the Band 2 DNA-protein complexes formed with the Stat3 and Stat1 probes. In addition, we observe a new faster mobility complex in competition experiments between the Stat1 probe and AγSTAT3 cold competitor (Band 3, lane 10). Subsequent experiments to confirm Stat3 binding were performed using untreated K562 nuclear extract and anti-Stat3 or anti-Stat1 antibodies (see ‘‘Experimental Procedures’’). In both instances the addition of antibodies reproducibly abolished formation of the Band 2 complex (Fig. 4, lanes 1–3). This confirms that Stat1 and Stat3 bind to the interval +1 to +22 containing a Stat3-like element in the γ-globin gene.

To determine whether IL-6 treatment altered STAT protein interactions in the γ-globin promoter, we performed EMSA with nuclear extracts from K562 cells induced with IL-6 alone or following pretreated with AG490 or PIC. Treatment with IL-6 alone produced a consistent increase in binding of nuclear proteins to the Band 2 complex (lanes 1 and 4). This effect was reduced significantly by PIC (lane 6) and AG490 to a lesser degree. Mutation analysis showed a requirement for an intact Stat3-like motif in the AγSTAT3 probe for nuclear protein binding.
binding. Formation of the Stat3-specific DNA-protein complex (Band 2) as well as a slower migrating complex was abolished in a mutant A/H9253 STAT3 oligonucleotide containing a CTG→TGA substitution (nucleotides 11 to 13) (Fig. 5). An identical modification of the consensus Stat3 oligonucleotide produced an identical result. Collectively, the JAK-STAT pathway inhibitor and DNA-protein binding experiments suggest a role for Stat3 in γ-globin gene regulation.

The Inhibitory Effect of IL-6 Is Mediated through the Minimal γ Promoter—The functional importance of Stat3 to γ-globin gene regulation was directly tested by transient transfection assays. In initial experiments we found that IL-6 reduced the luciferase activity from an enhancerless construct, γLuc, containing the minimum γ-globin promoter (−299 to +36) by 6-fold in transfected K562 cells after 24 h of IL-6 treatment. This effect was abrogated when transfected cells were pretreated with PIC prior to IL-6 treatment (data not shown). To accurately gauge the magnitude of the repressive effect on the γ promoter, subsequent experiments were performed with a construct containing the erythroid-specific enhancer HS2 of the β-globin locus control region upstream of the −299 promoter (HS2-Luc). As expected, the luciferase activity from HS2-Luc was 10-fold higher compared with that of the enhancerless construct. IL-6 reduced luciferase activity from the HS2-Luc reporter 8-fold (p = 0.05) (Fig. 6B). This result demonstrates that the powerful HS2 enhancer failed to avert the repressive effect of IL-6 on the γ promoter. Inhibition of Stat3 activation in transfected K562 cells with PIC, however, restored luciferase activity.

FIG. 6. Interleukin-6 mediates γ gene repression through the proximal promoter. A, schematic diagram of the luciferase reporter constructs studied. B, K562 cells were transfected with the γLuc (white bar) or HS2γLuc (black bar) reporter constructs followed by IL-6 (100 ng/ml) treatment for 24 h (striped bars) in the absence or presence of PIC (10 µg/ml) or UO126 pretreatment for 30 min. Total protein was isolated and luciferase activity was performed as outlined under “Experimental Procedures.” The base plasmid PGL3-Basic was used as a positive control for transfection efficiency in all experiments. Luciferase activity for untreated (Ud) K562 cells was normalized to 1. C, K562 cells were transfected with the HS2γLuc plasmid alone (black bar) or followed by treatment with IL-6 alone or pretreatment with Pias1 (P1) or Pias3 (P3) antibody (10 µg/ml) for 2 h and then IL-6 for 24 h (striped bars). Total protein was isolated and luciferase assay performed as outlined under “Experimental Procedures.” D, K562 cells were transfected with the HS2γLuc plasmid followed by NaB induction alone or pretreatment with P1 or P3 antibody for 2 h and then NaB (striped bars) for 24 h. Significance levels were established by comparing the different experimental conditions to untreated (Ud) K562 cells. Data represent the mean ± S.E.

FIG. 7. Stat3 proteins directly modulate γ promoter activity. Transient transfections were completed in K562 cells transfected with the HS2γLuc reporter alone or with the control base plasmids pSG5 and pDNA3.1. Experimental samples containing the expression vectors pStat3β (pS3β) or pStat3α (pS3α) either alone or with the mutant pStat3α (pmS3α) vector were analyzed. Total DNA added was held constant at 30 µg. Transfected cells were grown for 24 h in the absence (−, white bars) or presence of IL-6 (+, black bars), and then total protein was harvested for luciferase assays as described under “Experimental Procedures.” Luciferase activity was normalized for total protein, and PGL3 control activity was monitored with each transfection. The mean ± S.E. is shown.
activity to base-line values. In control experiments, U0126, an extracellular receptor kinase pathway inhibitor, showed no effect on IL-6-mediated γ promoter repression. These data provide evidence for the involvement of Stat3 in γ gene regulation.

**Stat3 Inhibition by PIAS3 Antibody Reverses γ Gene Repression by IL-6**—We next sought to inhibit specifically the Stat3 transcription factor with protein inhibitors of activated STAT-3 (PIAS3) antibodies. PIAS are naturally occurring antibodies that directly repress phosphorylated STAT proteins (30, 31). Transfected K562 cells were pretreated with PIAS1 or PIAS3 antibody (10 ng/ml) for 2 h followed by IL-6 treatment. The reduction in luciferase activity from the HS2γ-Luc construct in the presence of IL-6 was unaffected by PIAS1 (Fig. 6C). In contrast, PIAS3 abolished the negative effects of IL-6 on the HS2γ-Luc reporter and restored luciferase activity to base-line levels. This result provides further evidence for the importance of Stat3 in silencing γ-globin gene expression. Interestingly, in control experiments we found that PIAS3 reduced the luciferase activity in cells treated with NaB suggesting a requirement for Stat3 by NaB to augment γ promoter activity (Fig. 6D). These diaminic effects from general inhibition of Stat3 are plausible given that the α and β variants have opposing outcomes on gene expression.

**Stat3β Mediates γ Promoter Silencing through Its Cognate Downstream Binding Site**—Co-transfection experiments were performed with the HS2γ-Luc reporter and expression vectors for Stat3α (pS3α) and Stat3β (pS3β) to provide a direct analysis of each transcription factor on γ promoter activity. Transfected K562 cells were cultured either in normal medium or in medium supplemented with IL-6 for maximal phosphorylation of Stat3 transcription factors.

Stat3β overexpression reduced luciferase activity by 20% in the absence of IL-6 and by a further 50% after activation with IL-6, compared with the levels observed in co-transfection experiments with HS2γ-Luc and the corresponding empty expression vector (pSG5) (Fig. 7). Co-transfection of the reporter vector with pS3β and a mutant Stat3α (pS3α) vector carrying a Y705F mutation blocked the effects of pS3β and restored luciferase activity to approximately base-line levels. Activation of the wild-type pS3α vector blocked the repressive effect of endogenous Stat3β on γ promoter activity, which was observed with the HS2γ-Luc reporter with the empty expression vector (pcDNA3.1) (Fig. 7). The highest level of luciferase activity (5-fold above steady-state levels) was recorded in co-transfection experiments with the wild-type and mutant Stat3α expression vectors.

The diaminic effect of the two Stat3 transcription factors on γ promoter activity was confirmed by dose-response experiments. Fig. 8 shows that increasing Stat3β expression progressively reduced luciferase activity for the wild-type HS2γ-Luc construct. The lowest level of activity (99% inhibition) was obtained by co-transfecting 50 μg of the expression vector pS3β with the reporter vector. In contrast, increasing the Stat3α expression vector to 50 μg stimulated luciferase activity to levels above base line in co-transfection experiments with HS2γ-Luc. Finally, co-transfection experiments with pS3β at a fixed concentration of 15 μg with increasing amounts of pS3α, from 10 to 50 μg (Fig. 8B), were completed. We observed a 10-fold concentration-dependent increase in γ promoter activity in the presence of IL-6 demonstrating the ability of Stat3α to overcome the repressive effect of Stat3β.

Finally experiments were completed to determine whether the Stat3-binding site AγSTAT3 plays an important functional role in Stat3β-mediated γ gene silencing. By using site-directed mutagenesis we constructed a reporter plasmid with the three nucleotides +11 to +13 mutated (CTG → TGA) to create HS2mtAγ-Luc. The identical bases were shown in Fig. 5 to be essential for Stat3 binding to AγSTAT3 by EMSA analysis. Most striking was the loss of IL-6-mediated repression for HS2mtAγ-Luc shown in Fig. 8C. Reporter activity was decreased 20% for the mutant plasmid versus 80% inhibition for the wild-type HS2γ-Luc construct. With increasing amounts of Stat3β, we no longer observed the concentration-dependent repression of γ promoter activity, although some degree of repression at the 20- and 30-μg concentrations did occur. Interestingly, the reporter activity for HS2mtAγ-Luc returned to base-line levels despite the addition of up to 50 μg of Stat3β. This is in contrast to the abolishment of γ promoter activity by 99% for the wild-type HS2γ-Luc reporter (Fig. 8A, black bars). These studies provide direct evidence that the AγSTAT3 site plays an important role in γ-globin silencing by Stat3β.
FIG. 9. Model for Stat3 interactions in the γ promoter target cis-regulatory element. Depicted is the γ promoter sequence contained in the HS2γ-Luc reporter plasmid. Five potential regulatory elements are clustered in the 5′-untranslated region including a Stat3-like sequence, AgStat3, the stage selector element (SSE)-like element, a GATA-1 site, and an A-T-rich sequence. The EMSA analysis presented in this study demonstrated Stat3 and Stat1 binding to the AlSTAT3 site, which overlaps the SSE-like element. After IL-6 induction Stat3 increases and might form homodimers on its binding site to repress the γ promoter. Whether Stat3β can interact with the Stat3-like sequence to form a tetrameric complex and/or the GATA-1 site via Stat3-GATA-1 heterodimers remains to be investigated. The γ-globin promoter is not drawn to scale.

DISCUSSION

We have demonstrated in this study that IL-6 reduces steady-state HbF synthesis in fetal erythroid progenitors thus corroborating our observations made previously (6). Interestingly, IL-6 inhibited the proliferative capacity of fetal-stage progenitors contrary to what we observe routinely in cultures established with adult erythroid progenitors. This likely reflects phenotypic differences among erythroid progenitors from the two stages of development. Umbilical cord blood contains a larger pool of progenitors with a fetal pattern of globin gene expression, higher stem cell density, and engraftment capacity compared with progenitors from adult peripheral blood (32). Our results suggest that IL-6 elicits factors with distinct properties in fetal erythroid cells to regulate cell growth and globin gene regulation.

Studies in transgenic mice indicate that γ-globin gene silencing in the adult stage is achieved primarily through competition between the β- and γ-globin genes for activation by the locus control region. Promoter elements with a more active role in globin gene silencing containing cognate binding motifs for GATA-1 and YY1 have been described in the e-globin gene at −208 and −289 (33, 34). Interestingly, overexpression of GATA-1 in transgenic mice carrying the β-globin locus in a yeast artificial chromosome results in e-globin repression in both embryonic and adult erythroid cells (35). This suggests there is overlap in stage-specific and developmental stage-stable silencing mechanisms. A consensus YY1 site at −1086 (36), two direct repeat elements in the distal CCAAT box (37), and a GATA-1 site in the 5′-untranslated region have been identified in the γ-globin gene (29); however, their role in γ gene silencing has not been fully characterized. In addition to these regulatory elements we have identified a Stat3 motif (TTCTGGAAG) in the γ-globin 5′-untranslated region that is structurally similar to the IL-6-response element in the αγ-macroglobin gene (28). EMSA analysis in this study supports binding of a heterodimeric Stat1-Stat3 complex to AlSTAT3 during steady-state γ gene activity.

The observation that Stat3-like binding sites are located in the analogous positions in the e-γ and β-globin genes lends further evidence for a physiologic role for Stat3 proteins in globin gene regulation. In the β-globin 5′-untranslated region an important regulatory element called the downstream core element is located at nucleotide +22, which binds transcription factor IID to augment transcription initiation (38). Naturally occurring mutations identified in this region produce a β-thalassemia phenotype in humans (39). Whether STAT proteins alter transcription factor interactions in this regulatory region remains to be elucidated.

A detailed analysis of the γ-globin promoter was completed to determine whether the repressive effect of IL-6 occurs at the structural gene or cellular levels. The fact that Stat3β is activated at steady state and that IL-6 preferentially induced and phosphorylated this variant suggests Stat3β serves as a more physiologically relevant repressor of γ-globin. This conclusion was supported by the Western blot data as well, which showed higher level activation for Stat3β in untreated K562 cells. Direct evidence that both Stat3α and Stat3β are capable of altering γ promoter activity was provided by co-transfection experiments. Stat3β completely silenced γ promoter activity in a concentration-dependent manner for the wild-type HS2γ-Luc plasmid. Mutating the AlSTAT3 site resulted in a loss of the concentration-dependent γ promoter repression by Stat3β at higher concentrations. The fact that inhibition was seen at the lower Stat3β concentrations suggests that other regulatory elements are required to accomplish complete γ gene silencing. Finally we observed an opposite regulatory effect for Stat3α, which enhanced γ promoter activity to overcome repression. This observation is consistent with the transfection data for NAB-induced γ promoter activity, which was inhibited in the presence of PIAS3 antibody. This supports the ability of NAB to induce Stat3α to activate γ gene expression. Therefore, both Stat3 variants have physiologically relevant roles in γ gene regulation.

Possible mechanisms for γ gene silencing by STAT proteins are the formation of Stat3α/Stat3β heterodimers or Stat3β homodimers (11) that could serve as a dominant negative regulator when bound to AlSTAT3 (Fig. 9). Caldenhoven et al. (11) demonstrated an increased affinity of Stat3β over Stat3α for binding to the IL-6-response element to produce a net negative effect on gene expression even in the presence of lower levels of the former variant. Another potential mechanism for γ gene silencing is through protein-protein interactions. Although Stat3-like elements exist at position +9 in all globin promoters, the γ and AlSTAT3 sites are unique in that a second Stat3-like site is present between bases −6 and +3. Furthermore, a GATA-1 site is located at position +25 to +31, which has been
shown to mediate γ gene repression (29). Preliminary EMSA analysis confirmed two DNA-protein complexes are established with the upstream Stat3-like sequence, similar to that observed for the consensus Stat3-binding site (data not shown). Stat3β has been shown to interact with the AP-1 transcription factor to activate α2-macroglobulin gene activity in humans despite the absence of a transactivation domain (16). In the γ promoter 5′-untranslated region although no AP-1 sites are present, Aγ-ung-translation region although no AP-1 sites are present, Aγ-ung-

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