START

HEMATOPOIESIS

Dominant Negative Mutants Implicate STAT5 in Myeloid Cell Proliferation and Neutrophil Differentiation

By Robert L. Ilaria Jr, Robert G. Hawley, and Richard A. Van Etten

STAT5 is a member of the signal transducers and activation of transcription (STAT) family of latent transcription factors activated in a variety of cytokine signaling pathways. We introduced alanine substitution mutations in highly conserved regions of murine STAT5A and studied the mutants for dimerization, DNA binding, transactivation, and dominant negative effects on erythropoietin-induced STAT5-dependent transcriptional activation. The mutations included two near the amino-terminus (W256KR and R259Q), two in the DNA-binding domain (E427F and V466V), and a carboxy-terminal truncation of STAT5A (STAT5A/D53C) analogous to a naturally occurring isoform of rat STAT5B. All of the STAT mutant proteins were tyrosine phosphorylated by JAK2 and heterodimerized with STAT5B except for the WKR mutant, suggesting an important role for this region in STAT5 for stabilizing dimerization. The WKR, EE, and VVV mutants had no detectable DNA-binding activity, and the WKR and VVV mutants, but not EE, were defective in transcriptional induction. The VVV mutant had a moderate dominant negative effect on erythropoietin-induced STAT5 transcriptional activation, which was likely due to the formation of heterodimers that are defective in DNA binding. Interestingly, the WKR mutant had a potent dominant negative effect, comparable to the transactivation domain deletion mutant, D53C. Stable expression of either the WKR or D53C STAT5 mutants in the murine myeloid cytokine-dependent cell line 32D inhibited both interleukin-3-dependent proliferation and granulocyte colony-stimulating factor (G-CSF)-dependent differentiation, without induction of apoptosis. Expression of these mutants in primary murine bone marrow inhibited G-CSF–dependent granulocyte colony formation in vitro. These results demonstrate that mutations in distinct regions of STAT5 exert dominant negative effects on cytokine signaling, likely through different mechanisms, and suggest a role for STAT5 in proliferation and differentiation of myeloid cells.

© 1999 by The American Society of Hematology.

SIGNAL TRANSDUCERS and activation of transcription (STAT) family members comprise a group of latent transcription factors involved in cytokine, hormone, and growth factor signaling. STAT5, originally described as mammary gland factor for its essential role in mediating prolactin-induced gene expression, has been implicated in a diverse range of signal transduction pathways induced by the interleukin-3 (IL-3)/granulocyte-macrophage colony-stimulating factor (GM-CSF)/IL-5 family, the IL-2/IL-7/IL-15 family, erythropoietin, thrombopoietin, granulocyte colony-stimulating factor (G-CSF), epidermal growth factor, leptin, and insulin. STAT5 is expressed in a variety of tissues and, like other STAT family members, binds to specific phosphotyrosine-containing sequences on cytoplasmic receptors via its Src homology 2 (SH2) domain. STAT5 exists in two principal forms, STAT5A and STAT5B, which are encoded by separate genes. Although they differ in their C-terminal regions, STAT5A and STAT5B are highly homologous with each other and across species. Upon receptor activation, a highly conserved tyrosine near the carboxy-terminus (C-terminus) of STAT5 becomes phosphorylated by Janus kinase (JAK) family members, resulting in dissociation of STAT5 from the receptor, and STAT5A and 5B homodimerization or heterodimerization by mutual SH2 domain-phosphotyrosine interactions. STAT5 dimers then translocate to the nucleus and bind DNA at specific sequences, inducing gene expression.

The biological effects of activation of STAT5 are incompletely understood. Female mice with homozygous inactivation of the stat5a gene exhibit a failure of postpartum mammary gland differentiation and lactation, demonstrating that STAT5A is required for mammopoiesis and lactogenic signaling. Given the prominent activation of STAT5 in response to hematopoietic cytokines such as erythropoietin and IL-3, it was anticipated that STAT5 might also be required for aspects of hematopoiesis, but the stat5a–/–, stat5b–/–, and stat5a–/–stat5b–/– double knockout mice show no overt defects in the blood system. Although STAT5 is apparently not required for hematopoiesis, it may still play a role in the antiapoptotic and proliferative responses to hematopoietic cytokines. Thymocytes from stat5a–/–stat5b–/– double knockout mice fail to proliferate in response to IL-2, whereas granulocytic cells from stat5a–/– mice have defects in GM-CSF–induced proliferation. A mutant of STAT5B with a large deletion in the C-terminus exhibited dominant negative effects on STAT5-dependent transcription and a modest inhibitory effect on IL-3–dependent proliferation in the murine B-lymphoid cell line Ba/F3. Subsequently, STAT5A mutants with smaller C-terminal truncations of a basic transactivation domain have also been demonstrated to have dominant negative effects on STAT5-dependent transcription. But stable expression of one of these mutants in the IL-3–dependent myeloid cell line 32D did not affect...
proliferation or survival.23 A recently described constitutively active mutant of murine STAT5A has been shown to partially substitute for IL-3 for survival and proliferation of Ba/F3 cells.26 However, because this mutant was originally selected for on the basis of these properties, the involvement of endogenous STAT5 in these processes remains unresolved.

STAT5 activation may also play a role in the pathogenesis of human leukemia. Constitutive activation of diverse STAT proteins is found in a variety of primary human leukemia cells.27,28 In particular, prominent activation of STAT5 is consistently observed in cell lines from patients with chronic myeloid leukemia (CML) and in IL-3–dependent cell lines transformed by the BCR/ABL oncoprotein.29-31 likely due to direct phosphorylation by the Bcr/Abl tyrosine kinase.29 Because Bcr/Abl can substitute for IL-3 for survival and growth of IL-3–dependent hematopoietic cell lines32,33 and both IL-3 and Bcr/Abl activate STAT5, it is plausible that STAT5 activation may contribute to the pathogenesis of human CML.

Because deletion mutagenesis may have multiple effects on STAT function, we introduced specific point mutations in highly conserved regions of murine STAT5A and studied their effect on heterodimerization, DNA binding, and transactivation. STAT5A point mutants were also evaluated for their ability to exert a dominant negative effect on erythropoietin-induced STAT5-dependent transcriptional activation. The point mutants chosen for study included two located in the amino-terminus and two in the putative DNA-binding domain. In addition, we generated a novel carboxy-terminal STAT5A truncation mutant that is similar to a naturally occurring isoform of rat STAT5B.34 Three of the STAT5A mutants function as dominant negative mutants and interfere with STAT5-dependent transcription, likely through different mechanisms. Another of the mutations implicates the STAT5A amino terminus in stabilization of SH2-dependent dimerization. Two of the mutants inhibited IL-3–dependent proliferation and G-CSF–induced neutrophil differentiation upon stable expression in the myeloid cell line 32D and decreased G-CSF–dependent granulocytic colony formation after transduction of primary murine bone marrow. These results suggest a role for STAT5 in growth and differentiation of myeloid cells.

**MATERIALS AND METHODS**

**Cells and cell culture.** 293T cells were grown in Dulbecco's modified Eagle's (DME) medium supplemented with 10% heat-inactivated fetal calf serum, penicillin/streptomycin, 2 mM/L glutamine, and nonessential amino acids. 32D CI3 cells35 were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin/streptomycin, 2 mM/L glutamine, and 10% WEHI-3B–conditioned medium (WEHI-CM)9,10 as a source of IL-3.

**Construction of STAT5A mutants.** A hemagglutinin (HA) epitope tag was added to the NH2-terminus of murine STAT5A subcloned in pUC19 (New England Biolabs, Beverly, MA) by inverse polymerase chain reaction (PCR) and verified by dyeoxy DNA sequencing. Point mutations were then introduced into HA-tagged murine STAT5A by enzymatic inverse PCR using mutagenic primers containing a new Kpn I restriction site.77 All point mutations involved substitution of the respective amino acid with alanine. For STAT5A/ WKR, codons 255-257 were changed from TGG; AAG, and CGG to GCC; for STAT5A/ RQQ, codons 290-292 were changed from CGG, CAG, and CGA to GCC; for STAT5A/EE, codons 437 and 438 were changed from GAG to GCG; and for STAT5A/ VVV, codons 466-468 were changed from GTG, GTC, and GTT to GGC, GCC, and GCT, respectively. Positive clones were identified by the creation of a new Kpn I site and confirmed by DNA sequencing. The STAT5A mutants were then digested with Kpn I, blunted with T4 DNA polymerase, and self-ligated. Mutant clones were identified by loss of the Kpn I site and restoration of the reading frame confirmed by DNA sequencing. The STAT5A/Δ53C mutant was generated by inverse PCR using a mutagenic oligonucleotide introducing a stop codon (TGA) immediately after the asparagine at amino acid number 740, along with a new Kpn I restriction site. The resulting clone, lacking the last C-terminal 53 amino acids of murine STAT5A, was identified by digestion with Kpn I and confirmed by DNA sequencing. Wild-type and mutant forms of STAT5A were subcloned into the vector pcDNA3 (Invitrogen, San Diego, CA) for transient expression studies.

**Transient transfection and β-casein-luciferase transactivation assay.** 293T cells were transfected by a modified calcium phosphate protocol as previously described.38 To assess tyrosine phosphorylation and heterodimerization, cells were cotransfected with 2.5 µg STAT5A wild-type or mutant, 2.5 µg STAT5B expression construct, and 5 µg of pEFBos/JAK2. For transactivation assays, sets of 4 plates were transfected with 0.25 µg each of STAT5A mutant, cotransfected with 0.5 µg of pEFBos/JAK2 or parental pEFBos; one set was transfected with 1.0 µg of a wild-type β-casein luciferase reporter and the other was transfected with 1.0 µg of a mutant β-casein luciferase construct with point mutations in both STAT5 binding sites.39 Twenty-four hours posttransfection, cell lysates were prepared and luciferase activity was determined using an AutoLumat LB953 luminometer (EG&G Berthold/ Wallac, Inc, Gaithersburg, MD), as described.40 To correct for differences in transfection efficiency, all transfections included 0.1 µg of a human growth hormone (hGH) expression plasmid, RSV-hGH. hGH levels in culture medium from transfected cells were measured by radioimmunoassay (Nichols Institute, San Juan Capistrano, CA), compared with a standard curve, and this value was used to normalize the level of luciferase activity between different transfections. To assess dominant negative effects, cells were cotransfected with 1.0 µg of pXM190 murine erythropoietin receptor expression construct, 0.25 µg of wild-type STAT5A, and either 1.0 or 2.0 µg of STAT5A mutant, along with the wild-type β-casein luciferase reporter and RSV-hGH expression construct for normalization. Twenty-four hours posttransfection, the cells were stimulated with 10 U/mL recombinant human erythropoietin (Amgen, Thousand Oaks, CA). Cell lysates were prepared 7 to 8 hours poststimulation, and luciferase activity was measured and normalized as described above.

**Immunoprecipitation and Western blot.** Lysates in RIPA buffer were prepared as previously described41 from approximately 1 × 107 transfected 293T cells or 32D cells stably expressing mutant STAT5A. 32D cells were starved of IL-3 for 3 hours and then stimulated with 10% (vol/vol) WEHI-CM or 100 ng/mL recombinant human G-CSF (Amgen) for 15 minutes at 37°C. Protein lysates, normalized by OD295 (Bio-Rad Protein assay; Bio-Rad Laboratories, Hercules, CA), were subjected to immunoprecipitation with antisera to STAT3, 5A, or 5B (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-HA monoclonal antibody (Berkeley Antibody Co, Richmond, CA) for 4 to 6 hours, were resolved by 5% to 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and were electrophoretically transferred to a nitrocellulose membrane. Western blot was performed with either anti-HA, anti-STAT, or anti-phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY) antibody and detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

**Electromobility shift assay (EMSA).** Nuclear extracts were prepared from 293T cells transfected with either wild-type or mutant STAT5A, with or without JAK2, as previously described.42 Six micrograms of nuclear extract protein was incubated with 0.2 ng of a 32P-labeled double-stranded FcγRII GAS-based oligonucleotide probe 29,43.
for 20 minutes at room temperature. Supershift analysis was performed by incubating nuclear extracts with 1:15 dilutions of either anti-STAT5A (Santa Cruz Biotechnology) or anti-CHA (Berkeley Antibody Co) antibody for 30 minutes at 4°C. The DNA-protein complexes were resolved on 4% tris-acetate-EDTA (TAE) polyacrylamide gels and detected by autoradiography.

Expression of dominant-negative STAT5A mutants in 32D cells. The HA-tagged STAT5A/WKR and STAT5A/Δ53C mutants were cloned into the 5’ position of MINV, an MSCV-based retroviral vector containing an internal ribosome entry site from encephalomyocarditis virus between a 5’ multiple cloning site and a 3’ neomycin resistance gene.44 Helper-free high-titer retroviral supernatant was prepared using the kat transient transfection system,45 as described.38 32D cells were transduced with retroviral stocks of the two STAT5 mutants and a vector expressing the neomycin resistance gene alone by 24 hours of cultivation in the presence of virus-containing supernatant, WEHI-CM, and 6 µg/mL polybrene. Transduced cells were selected 24 hours posttransduction in 1.0 mg/mL G418 (Geneticin; Life Technologies, Grand Island, NY) and IL-3, and the G418-resistant populations were used for studies of gene induction, proliferation, and differentiation immediately thereafter.

RNA isolation and Northern blot. G418-resistant populations of 32D cells transduced with the neomycin-containing virus or with STAT5A/WKR and STAT5A/Δ53C mutants were starved of IL-3 for 6 hours and then stimulated with 10% WEHI-CM as a source of IL-3. RNA was prepared as described41,46 from 1 × 107 cells at 0, 30, 60, and 120 minutes poststimulation; fractionated by formaldehyde agarose gel electrophoresis; transferred to nylon membranes by capillary blot; and probed with radioactive probes from the CIS, PIM-1, and OSM genes.23

Assessment of IL-3-mediated proliferation and G-CSF–induced differentiation. Within 96 hours of retroviral transduction, G418-resistant populations of 32D cells transduced with neomycin-containing virus or with the STAT5A/WKR and STAT5A/Δ53C mutants were seeded in duplicate at 5 × 103 cells in 10 mL of medium containing 10% WEHI-CM but without G418 in a T-25 flask. Viable cells were counted daily by trypan blue exclusion. For some time points, genomic DNA was isolated from populations and DNA fragmentation was assessed by agarose gel electrophoresis to exclude apoptosis. For G-CSF–induced neutrophil differentiation, cells were washed twice in phosphate-buffered saline (PBS) and resuspended in medium containing 0.25% (vol/vol) WEHI-CM and 100 ng/mL recombinant human G-CSF (Amgen). Aliquots were removed for fluorescence-activated cell sorting (FACS) analysis or cytoplasm and Wright/Giemsa staining every 24 hours. FACS analysis was performed by staining 104 fresh cells with biotinylated rat anti mouse CD11b (clone M1/70) or a biotinylated rat IgG2b/κ isotype control antibody (both from PharMingen, San Diego, CA), followed by phycoerythrin-conjugated streptavidin. Cells were analyzed on a FACSscan flow cytometer (Becton Dickinson, San Jose, CA) with CellQuest software.

Retroviral transduction of primary murine bone marrow and granulocyte colony-forming unit (CFU-G) analysis. Bone marrow was harvested from 8- to 10-week-old Balb/c mice (Taconic Farms, Germantown, NY) and prestimulated47 for 24 hours in medium containing DME, 15% (vol/vol) inactivated fetal calf serum, 5% (vol/vol) WEHI-3B-conditioned medium, penicillin/streptomycin, 1.0 µg/mL ciprofloxacin, 200 µmol/L L-glutamine, 6 ng/mL recombinant murine IL-3, 10 ng/mL recombinant murine IL-6, and 70 ng/mL recombinant murine stem cell factor (SCF; all from PeproTech, Rocky Hill, NJ). For transduction, the three retroviral stocks were normalized by appropriate dilutions to a titer of 1 × 106 G418-resistant CFU/mL, assessed on NIH 3T3 cells. After prestimulation, equal numbers of viable cells were transduced with either MINV/STAT5A/WKR, MINV/STAT5A/Δ53C, or MINV retrovirus alone in the presence of 10 mmol/L HEPES, pH 7.4, and 2 µg/mL polybrene. To increase transduction efficiency,49 the cells were then cocentrifuged with virus at 1,000g for 90 minutes in a Sorvall RT7 centrifuge, and the medium was changed after a 2- to 4-hour adsorption period. Sixteen to 18 hours later, a second round of retroviral transduction with cocentrifugation was performed, and the cells were allowed to recover for 4 to 6 hours at 37°C. Cells were then acetylated, washed twice in PBS, and plated in triplicate at a density of 1 × 103 cells per 3.5-cm plate in Methocult M3230 medium (StemCell Technologies, Vancouver, British Columbia, Canada) containing 1 ng/mL (absolute) G418 and 100 ng/mL recombinant human G-CSF. To assure that colonies were truly G-CSF–dependent, cells were also plated in the same medium lacking G-CSF. The number of G-CSF–dependent colonies was scored on day 4 to 5 and granulocytic morphology was confirmed by cytoxin.

RESULTS

The STAT5A mutants are tyrosine phosphorylated by JAK2 and all heterodimerize with STAT5B except for STAT5A/WKR. To assess the impact of STAT5 mutagenesis on heterodimerization, tyrosine phosphorylation, DNA binding, and transcriptional activation, alanine substitution mutations were introduced into murine STAT5A in regions highly conserved among STAT family members (Fig 1). Two point mutants were located near the NH2-terminus, designated STAT5A/WKR (W255KR→AAA) and STAT5A/RQQ (R299QQ→AAA), and two were located in the putative DNA-binding domain, STAT5A/EE (E437E→AAA) and STAT5A/VVV (V466VV→AAA). An HA epitope tag was added at the NH2-terminus to distinguish mutant from wild-type STAT5A. A C-terminal truncation mutant was also constructed by placement of a stop codon, resulting in deletion of the last 53 amino acids (STAT5A/Δ53C), analogous to a naturally occurring rat STAT5B isoform.38

The expression and biological properties of these mutants were assessed in 293T cells (Table 1), which were chosen because of their lack of endogenous STAT5A (data not shown) and high transfection efficiency. All of the STAT5A mutants were readily expressed and, as expected, comigrated with wild-type STAT5A, except for the truncation mutant STAT5A/Δ53C (Fig 2, top panel). Transfection of wild-type STAT5A alone did not result in any tyrosine phosphorylation of STAT5, due to the very low level of endogenous JAK2 (data not shown). Therefore, to evaluate activation of mutant or wild-type STAT5A, 293T cells were cotransfected with or without supplemental JAK2 and analyzed by anti-HA immunoprecipitation and anti-phosphotyrosine Western blot. All of the STAT5A mutants were tyrosine phosphorylated in the presence of JAK2 (Fig 2, middle panel), although the WKR and VVV mutants appeared to be phosphorylated at lower levels than the other proteins. Because 293T cells contain low endogenous levels of STAT5B (data not shown), to assess STAT5 heterodimerization, 293T cells were transfected with HA-tagged mutant or wild-type STAT5A, together with STAT5B and JAK2, and analyzed for the ability of the STAT5A mutants to communoprecipitate STAT5B. STAT5A wild-type, STAT5A/VVV, STAT5A/EE, and STAT5A/RQQ formed heterodimers with STAT5B in a JAK2-dependent manner (Fig 2, bottom panel). Although STAT5A/WKR was tyrosine phosphorylated by JAK2, it failed to heterodimerize with STAT5B, suggesting that this region of STAT5A may be important in stabilizing STAT dimerization. The C-terminal truncation mutant, STAT5A/Δ53C, also re-
tained the ability to heterodimerize with STAT5B, confirming that the transactivation domain is not required for dimerization.

**All STAT5A point mutants lack GAS DNA-binding ability except for STAT5A/RQQ.** The DNA-binding ability of the STAT5A mutants was assessed by EMSA using a GAS probe derived from the FcγRI gene promoter.29,43 Nuclear extracts were prepared from 293T cells transfected with wild-type or mutant STAT5A in the presence or absence of JAK2. Transfection of 293T cells with JAK2 alone, or wild-type or mutant STAT5A in the absence of exogenous JAK2, resulted in no

---

**Fig 1. Mutagenesis of conserved regions of murine STAT5A.** Amino acid sequence alignment of the central region of several mammalian (H, human; M, mouse; R, rat) STAT family members is shown. Numbering is from the amino terminus, and alignment was performed by the program PRETTY.PILEUP (GCC, University of Wisconsin). The consensus sequence is given at the bottom; a capital letter indicates a highly conserved amino acid (at least 8 of 12 members). The numbering at the top of each panel refers to the consensus sequence and because of gaps in the homology is not correct for STAT5A. The locations of the Src homology 2 (SH2) domain as defined by Horvath et al57 are indicated by the shaded boxes, whereas the conserved JAK phosphorylation site is indicated by the arrowhead. The locations of the alanine substitution mutations WKR = AAA, RQQ = AAA, EE = AA, and VVV = AAA are indicated by bold face A characters below the sequence. The positions of insertion of termination codons in the murine STAT5A C-terminal truncation mutants D53C (this report), D683, D749, D713, and D650 are shown by arrowheads.

---

### Table 1. Summary of Biological Properties of STAT5A Mutants

<table>
<thead>
<tr>
<th>STAT5A Mutant</th>
<th>293T Cells</th>
<th>32D Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tyrosine PO4</td>
<td>Dimerization*</td>
</tr>
<tr>
<td>STAT5A WT</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>STAT5A/WKR</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>STAT5A/RQQ</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>STAT5A/EE</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>STAT5A/AAA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>STAT5A/D53C</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*AK2-dependent heterodimerization with STAT5B.
†Inhibition of erythropoietin-induced STAT5A-dependent luciferase induction.
detectable GAS DNA-binding activity (Fig 3). STAT5A-specific DNA binding was confirmed by supershift of the DNA-protein complex with either anti-HA (data not shown) or anti-STAT5A antibody. The point mutant STAT5A/RQQ exhibited GAS DNA binding similar to wild-type STAT5A, whereas STAT5A/D53C demonstrated a DNA-protein complex even more prominent than parental STAT5A, consistent with the observation that the transactivation domain may negatively regulate STAT5A DNA binding. In contrast, the point mutants STAT5A/VVV and STAT5A/EE demonstrated no appreciable GAS DNA-binding activity, demonstrating the importance of these highly conserved regions in the STAT5A DNA-binding domain. STAT5A/WKR also had no detectable GAS DNA-binding activity, likely because of its inability to homodimerize or heterodimerize. Similarly, a STAT5A mutant with a large truncation of the C-terminus, STAT5A/D650, also did not bind DNA, likely due to the absence of the Y694 JAK2 phosphorylation site that is required for dimerization.

STAT5A/VVV and STAT5A/WKR are defective in transcriptional activation of a β-casein-luciferase reporter. The transactivation ability of wild-type and mutants STAT5A proteins was assessed using a construct containing a minimal cytomegalovirus (CMV) promoter and tandem β-casein DNA elements upstream of a luciferase reporter gene. 293T cells were transfected with this reporter and each of the STAT5A mutants with or without JAK2, and the level of transactivation was compared with wild-type STAT5A by quantitation of luciferase activity in cell extracts. A parallel experiment using a β-casein luciferase reporter with point mutations in the STAT5 binding sites was used to control for nonspecific transactivation. None of the STAT5A species caused significant transcriptional activation of the β-casein luciferase reporter in the absence of JAK2 (Fig 4). When coexpressed with JAK2, wild-type STAT5A induced a fourfold activation of the luciferase reporter, whereas

Fig 3. The STAT5A/WKR, EE, and VVV mutants lack FcγRI GAS DNA-binding activity. Nuclear extracts were prepared from 293T cells transfected with JAK2 and the indicated murine STAT5A mutants and analyzed by EMSA using a 32P-labeled FcγRI-derived GAS probe. 293T cells transfected with either parental STAT5A (5A) alone or JAK2 (J2K) alone are shown at left. STAT5-specific GAS DNA-binding activity was confirmed by incubation with anti-STAT5A antibody (5A+J2K+AB). The positions of the STAT5 GAS DNA-binding complex (lower arrowhead) and the supershifted complex (upper arrowhead) are indicated. Similar supershifts using anti-HA antibody were observed for the STAT5A/RQQ and STAT5A/Δ53C complexes (data not shown).
the carboxy-terminal truncation mutant STAT5A/Δ650 completely lacked transactivation ability. Although STAT5A/Δ53C demonstrated a significant decrease in β-casein transcriptional activation, it retained some transactivation ability, suggesting that this deletion did not eliminate the entire STAT5A transactivation domain. The VVV and WKl point mutations completely abolished STAT5A transactivation ability, reflecting their potent inhibitory effect on DNA binding. The STAT5A/RQQ mutant, on the other hand, demonstrated little impairment in transactivation, consistent with its preserved DNA-binding ability and intact transactivation domain. Interestingly, STAT5A/EE exhibited relatively normal β-casein transcriptional activation despite the lack of DNA-binding activity by EMSA. None of the STAT5A proteins significantly stimulated transcription of the mutant β-casein luciferase reporter, demonstrating that transcriptional activation in this assay is dependent on STAT5 binding sites in the promoter.

The point mutants STAT5A/WKR and STAT5A/VVV and the transactivation domain deletion mutant STAT5A/Δ53C exert a dominant negative effect on erythropoietin-induced STAT5-dependent transcriptional activation in 293T cells. Our STAT5A mutants demonstrated defects in various aspects of STAT5A function, including dimerization, DNA binding, and transactivation. To determine whether any of these mutants could interfere with wild-type STAT5 signaling, an erythropoietin receptor expression system was used. 293T cells (which lack erythropoietin receptors) were transfected with wild-type STAT5A, murine erythropoietin receptor, and each of the STAT5A mutants, and dominant negative activity was assessed by inhibition of erythropoietin-induced activation of the β-casein luciferase reporter (Fig 5). Neither wild-type nor mutant STAT5A demonstrated significant activation of the reporter in the absence of erythropoietin (data not shown). In the absence of any STAT5A mutant, erythropoietin stimulation resulted in a
greater than fivefold increase in β-casein luciferase reporter activity. Addition of a dominant negative JAK2 mutant (DNJAK/Δ829) completely blocked this effect. Consistent with a recent report, the mutant STAT5A/Δ53C, lacking most of the STAT5A transactivation domain, severely inhibited β-casein reporter activity. The point mutant STAT5A/WKR also demonstrated a potent dominant negative effect, similar to the Δ53C mutant. The other four STAT5A mutants were intermediate in their ability to block transactivation by wild-type STAT5A. STAT5A/Δ650 and STAT5A/VVV exhibited dose-dependent dominant negative activity consistent with their lack of intrinsic transcriptional activation and with previous observations. The STAT5A/RQQ or STAT5A/EE mutants decreased transactivation by wild-type STAT5A, but this inhibitory activity was not dose-dependent, suggesting these latter mutants do not function significantly as dominant negatives, consistent with their preserved transactivation ability.

STAT5A/WKR expression in 32D cells inhibits the activation of the IL-3-responsive genes cis, pim-1, and osm. To confirm a dominant negative effect on transcription in hematopoietic cells, STAT5A/WKR was expressed in the murine hematopoietic factor-dependent cell line 32D and activation of the IL-3-responsive genes cis, pim-1, and osm was analyzed by Northern blot. Control 32D/Neo cells showed evidence of cis and pim-1 gene activation as early as 30 minutes after IL-3 stimulation (Fig 6 and data not shown), with a maximum transcriptional induction at 120 minutes. In 32D cells expressing STAT5A/WKR, cis gene activation was severely impaired, with cis mRNA levels remaining at barely detectable levels for up to 120 minutes after IL-3 stimulation (Fig 6, upper panel). Pim-1 gene transcription was also suppressed in STAT5A/WKR-expressing cells, with only a slight increase at 60 minutes after IL-3 stimulation (Fig 6, middle panel). In 32D/Neo cells, osm gene transcription peaked at 30 minutes and was readily detectable for up to 2 hours; however, in 32D cells expressing STAT5A/WKR, osm mRNA levels only reached barely detectable levels 30 minutes after IL-3 stimulation and were undetectable thereafter (Fig 6, lower panel). Similar results were also observed upon expression of the STAT5A/Δ53C mutant (data not shown).

32D cells expressing either STAT5A/WKR or STAT5A/Δ53C demonstrate decreased IL-3-dependent proliferation in vivo. Because STAT5A/WKR and STAT5A/Δ53C exerted dominant negative effects on STAT5-dependent erythropoietin-induced transcriptional activation and the activation of several IL-3-responsive genes, further studies were performed to determine their impact on IL-3-dependent proliferation. Because of the possibility that dominant negative STAT5 expression might have deleterious effects on proliferation, STAT5A/WKR and STAT5A/Δ53C were introduced into 32D cells by retroviral gene transfer (with an transduction efficiency of 25% to 30%; data not shown).}

Fig 6. STAT5A/WKR inhibits the expression of the IL-3-responsive genes pim-1, cis, and osm in the myeloid cell line 32D. 32D cells expressing either the neomycin resistance gene alone (32D/Neo) or STAT5A/WKR (32D/WKR) were deprived of IL-3 for 6 hours and then stimulated with 10% WEHI-3B-conditioned medium. Total RNA was prepared at the indicated time periods and analyzed by Northern blot using 32P-labeled PIM-1, CIS, or OSM probes. All lanes had equal loading as judged by ethidium bromide staining of ribosomal RNAs (data not shown).

Fig 7. Expression of either STAT5A/WKR or STAT5A/Δ53C in 32D cells inhibits IL-3-dependent proliferation. 32D cells expressing the neomycin resistance gene alone (32D/Neo), STAT5A/WKR (32D/WKR), or STAT5A/Δ53C (32D/Δ53C) were grown in 10% WEHI-3B-conditioned medium without G418 and viable cell number was determined daily. Data points represent the average of at least two independent experiments performed in duplicate and error bars represent the calculated standard error. The day-2 cell number for 32D/Neo cells was significantly higher than that of either 32D/WKR or 32D/Δ53C (P < .01, t-test).
DOMINANT NEGATIVE STAT5 MUTANTS

WKIR population confirmed expression of STAT5A/WKR at levels about threefold higher than endogenous STAT5A when compared with neo-transduced cells (data not shown). STAT5A/Δ53C also exerted a dominant negative effect on IL-3–dependent 32D cell proliferation (Fig 7), again without evidence of apoptosis (data not shown).

STAT5A/WKR and STAT5A/Δ53C block the G-CSF–dependent differentiation of 32D cells. In addition to IL-3–dependent growth, 32D cells differentiate into mature neutrophils upon treatment with exogenous G-CSF. STAT5 has been shown to be activated by G-CSF treatment, the effect of dominant negative STAT5 expression on the G-CSF–induced differentiation of 32D cells was evaluated. 32D cells expressing neomycin resistance alone, STAT5A/WKR, or STAT5A/Δ53C were stimulated with 100 ng/mL recombinant human G-CSF in the presence of minimal IL-3 (to prevent apoptosis) and were examined for morphological evidence of myeloid differentiation. 32D cells transduced with the neomycin vector alone (32D/Neo) differentiated into neutrophils by day 7 of G-CSF treatment, whereas the 32D cells expressing the STAT5A/WKR and STAT5A/Δ53C mutants showed only limited morphologic maturation (Fig 8A). When the number of mature and undifferentiated cells were quantitated, 32D/STAT5A/WKR cells demonstrated an approximately sixfold decrease in the number of neutrophils and band forms compared with 32D/Neo cells (Fig 8B). Virtually all 32D/Neo cells exhibited morphologic differentiation by day 7, whereas approximately 65% of 32D/STAT5A/WKR cells remained undifferentiated, resembling non–G-CSF–treated 32D cells. Consistent with a delay in morphologic maturation, 32D/STAT5A/WKR cells also showed a higher percentage of myelocytes and metamyelocytes compared with 32D/Neo cells (22% and 16%, respectively). G-CSF–dependent induction of the myeloperoxidase gene, another molecular marker of neutrophil differentiation, was intact in 32D/WKR- and 32D/Δ53C-expressing cells but delayed by about 1 day relative to 32D/Neo cells (data not shown), indicating that STAT5 is not required for some transcriptional responses in the maturation pathway induced by G-CSF. Coincident with the block in morphological neutrophil maturation, induction of the myeloid cell surface antigens Mac-1 (CD11b; Fig 8C) and Gr-1 (Ly-6G; data not shown) was inhibited to a similar extent in 32D/WKR cells relative to 32D/Neo cells. Overexpression of wild-type STAT5A in 32D cells had no effect on proliferation or differentiation (data not shown). These results suggest that STAT5 contributes to proliferation and is required for neutrophil differentiation in 32D cells.

STAT5A/WKR and STAT5A/Δ53C do not interfere with G-CSF–induced STAT3 activation in 32D cells. Expression of a dominant negative allele of STAT3 in a murine myeloid cell line has been shown to block G-CSF–induced neutrophil maturation. To exclude the possibility that our dominant negative STAT5 mutants might block neutrophil differentiation through inhibition of STAT3, we examined STAT3 activation in 32D cells stably expressing the dominant negative STAT5A mutants (Fig 9). STAT3 was activated by G-CSF but not IL-3 in parental 32D/Neo cells, and there was no appreciable decrease in G-CSF–induced tyrosine phosphorylation of STAT3 in 32D/WKR and 32D/Δ53C cells (Fig 9, top panel). In parental 32D/Neo cells, STAT5 was activated prominently by IL-3 and to a lesser extent by G-CSF (Fig 9, bottom panel), as reported. Interestingly, in 32D/WKR cells, there was some what increased tyrosine phosphorylation of STAT5 in response to both IL-3 and G-CSF, likely from phosphorylation of the overexpressed STAT5A/WKR protein, which is recognized by the anti-STAT5A antisera and comigrates with endogenous STAT5. In 32D/Δ53C cells, there was decreased tyrosine phosphorylation of STAT5 in response to both cytokines, likely due to specific interference from the truncated STAT5A/Δ53C protein, which is not immunoprecipitated by the anti-STAT5A antisera. Although anti-HA immunoprecipitates confirmed expression of both dominant negative STAT5 proteins in the respective cell lines, immunoprecipitation with this antibody was inefficient and cytokine-induced tyrosine phosphorylation of the mutant STATs was not detected under these conditions (data not shown). These results demonstrate that the dominant negative effects of the STAT5A mutants are restricted to STAT5 and do not interfere with activation of STAT3, implying that STAT5 also plays an essential role in the differentiation of neutrophils.

STAT5A/WKR and STAT5A/Δ53C inhibit G-CSF–dependent granulocytic colony formation in primary murine bone marrow. Although 32D cells are frequently used to model granulocyte development, a cell line may not accurately reflect normal myeloid cell maturation. Therefore, we introduced the STAT5A/WKR and STAT5A/Δ53C mutants into primary murine bone marrow by retroviral transduction and analyzed their effect on G-CSF–dependent granulocytic colony formation in vitro. Primary marrow cells were transduced with parental virus (MINV) expressing neomycin resistance gene alone and with viruses coexpressing either STAT5A/WKR or STAT5A/Δ53C with the neomycin resistance gene via an internal ribosome entry site (MINV/WKR and MINV/Δ53C, respectively). After plating in semisolid medium containing G418 and G-CSF, we observed a 40% to 60% reduction in granulocytic colony formation after transduction with the DN-STAT5A mutants, relative to vector-transduced cells (Fig 10). No colonies were observed in mock-infected cultures or in the absence of G-CSF (data not shown). These results suggest that STAT5 contributes to granulocytic differentiation during normal myelopoiesis.

DISCUSSION

We have introduced point mutations in highly conserved regions of murine STAT5A to gain insight into their impact on STAT5 heterodimerization, tyrosine phosphorylation, DNA binding, and transactivation. Two point mutants in the amino-terminal region of STAT5A, STAT5A/WKR and STAT5A/RQQ, and two in the DNA-binding domain, STAT5A/EE and STAT5A/VVV, were chosen for study. Because deletions in the transactivation domain of STAT5A have been demonstrated to interfere with wild-type STAT5A signaling, a novel carboxy-terminal truncation mutant, STAT5A/Δ53C, was also constructed.

The STAT amino-terminal domain is a highly conserved region that may play a role in signaling specificity and regulation. Recently, a deletion of approximately 83 amino acids from the amino-terminus of STAT5 has been demonstrated to impair the ability of STATs to bind to tandem, but not to
In addition, a point mutation at the extreme amino-terminus of STAT1 has been demonstrated to result in its constitutive tyrosine phosphorylation, suggesting that this region may be important for the interaction with a negative regulator such as a phosphatase. The point mutants STAT5A/WKR and STAT5A/RQQ are located in highly conserved regions of unknown function just C-terminal to this area. STAT5A/RQQ mutation had no effect on STAT5 heterodimerization, DNA binding, or JAK-dependent transactivation and, as expected, did not have a dominant negative effect on erythropoietin-induced STAT5-dependent transcriptional activation. Interestingly, a point mutation in an adjacent residue (H299R) that is not highly conserved, in conjunction with a second point mutation in the C-terminus, induces constitutive tyrosine phos-
The mean number of granulocytic colonies on day 4 to 5 per 105 cells plated is shown and is representative of three independent experiments. Error bars indicate the standard error. No colonies were plated is shown and is representative of three independent experiments.

DOMINANT NEGATIVE STAT5 MUTANTS

Fig 9. The STAT5A/WKR and Δ53C mutants do not inhibit STAT3 activation. Populations of 32D cells expressing either the neomycin resistance gene alone (32D/Neo), STAT5A/WKR (32D/WKR), or STAT5A/Δ53C (32D/Δ53C) were starved of IL-3 and then stimulated with IL-3 (l), G-CSF (G), or unstimulated (NS). Extracts were precipitated with anti-STAT3 (top 2 panels) or anti-STAT5A (bottom 2 panels) antibodies, blotted to nitrocellulose, and hybridized with anti-phosphotyrosine antibody (top panel of each pair). Membranes were then stripped and reprobed with anti-STAT3 or anti-STAT5A antibodies, respectively (bottom panel of each pair). Expression of STAT5A/Δ53C (not recognized by STAT5A antibody) was confirmed by anti-HA Western blot (data not shown).

Fig 10. The STAT5A/WKR and Δ53C mutants inhibit G-CSF-dependent granulocytic colony formation in primary murine bone marrow. Primary murine bone marrow was transduced with parental MINV retrovirus (MINV) or MINV retrovirus expressing STAT5A/WKR (MINV/WKR) or STAT5A/Δ53C (MINV/Δ53C) and then plated in triplicate in methylcellulose culture with G418 and exogenous G-CSF. The mean number of granulocytic colonies on day 4 to 5 per 105 cells plated is shown and is representative of three independent experiments. Error bars indicate the standard error. No colonies were observed in sham-infected cultures selected in G418 or upon omission of G-CSF from the medium.
and DNA binding, with an apparent increase in the efficiency of DNA binding by EMSA, consistent with the suggestion that the STAT5A C-terminus might also negatively regulate DNA binding. Interestingly, we found that the Δ53C truncation diminished, but did not completely eliminate, transcriptional activation by the mutant STAT, suggesting that this mutation has not completely abolished the transactivation function of STAT5A. Despite this, the STAT5A/Δ53C mutant functioned fairly efficiently as a dominant negative, particularly when expressed at higher levels. This suggests that, whereas some of the dominant negative action of STAT5A/Δ53C is through the formation of heterodimers with wild-type STAT5 that are defective in transcriptional activation, the precise mechanism of interference with wild-type STAT5 function is more complicated.

To determine the effects of inhibiting STAT5-dependent transcription in hematopoietic cells, we expressed the STAT5A/WKR and STAT5A/Δ53C mutants in the IL-3-dependent myeloid cell line 32D. Attempts to express these STAT5A mutants by low-efficiency transfection of cells with plasmids capable of high level expression, such as pCDNA3, were uniformly unsuccessful, whereas overexpression of wild-type STAT5A was easily achieved (data not shown), suggesting that high-level expression of these dominant negative STAT5 mutants was deleterious to the proliferation or survival of these cells. We therefore turned to a double-cistron retroviral vector with an internal ribosome entry site, which permitted the efficient transduction and rapid selection of a population of cells expressing both a neomycin resistance gene and the dominant negative STAT. Using this system, we were able to obtain populations of G418-resistant 32D cells expressing the dominant negative STAT proteins at severalfold over the level of endogenous STAT5A. When tested immediately after transduction and selection, we observed significant inhibition of IL-3-induced STAT5-dependent transcription (Fig 6) and proliferation (Fig 7) in cells expressing either dominant negative STAT5 allele. These results confirm earlier observations and suggest that a STAT5-dependent pathway contributes to the proliferative response induced by IL-3 in hematopoietic cell lines. Others have failed to observe an effect of a similar C-terminal truncation mutant of STAT5 on proliferation upon stable expression in 32D cells. The reason for the discrepancy between these results and our observations is not clear, but the cells in the prior study were selected for a cotransfected drug resistance marker for 2 weeks followed by subcloning by limiting dilution, lengthy procedures that may select for clones that can compensate for the loss of STAT5 activity for proliferation. Although STAT3 has been implicated in the generation of an antipapoptotic signal in response to activation of the gp130 receptor, we and others observed no increase in cell death in cells expressing dominant negative STAT5 mutants, suggesting that STAT5 does not play a major role in the IL-3-dependent antipapoptotic response in these cells.

Interestingly, we found that expression of both our dominant negative STAT5 mutants profoundly inhibited G-CSF–induced neutrophil differentiation of 32D cells, suggesting a novel role for STAT5 in myeloid cell differentiation as well as proliferation. Because 32D cells are an immortalized cell line, it is possible they do not faithfully model the granulocytic differentiation process in bone marrow. Importantly, we also observed a reproducible and significant impairment of G-CSF–dependent granulocytic colony formation upon expression of the dominant negative STAT5 mutants in primary murine bone marrow, confirming a role for STAT5 activation in neutrophil maturation. At first glance, these results appear to be in conflict with the phenotype of stat5a−/− stat5b−/− double knockout mice, which have apparently normal steady-state myelopoiesis. Unlike other gene products, such as C/EBPα, STAT5 is clearly not absolutely required for granulocyte development when it is lacking from the moment of fertilization. However, we note that marrow from stat5a−/− stat5b−/− double knockout mice exhibits a 50% reduction in G-CSF–dependent granulocytic colony formation relative to wild-type marrow, which is also consistent with a contribution of STAT5 to neutrophil development.

G-CSF receptor stimulation has been shown to activate JAK1 and JAK2 and to tyrosine phosphorylation and activation of STAT3, STAT1, and STAT5. Studies of G-CSF receptor (G-CSF-R) mutants demonstrate that G-CSF–induced differentiation signals require the distal (C-terminal) region of the intracytoplasmic domain of G-CSF-R, distinct from the membrane-proximal region necessary for activation of JAK kinases and for G-CSF–induced proliferative effects. Whereas efficient activation of STAT3 requires the presence of at least one of four conserved tyrosine residues present in the distal region of G-CSF-R, activation of STAT5 by G-CSF appears to be independent of G-CSF-R phosphorylation. Expression of dominant-negative STAT3 mutants in myeloid cell lines blocked G-CSF–induced neutrophil differentiation and IL-6–induced macrophage differentiation, suggesting a functional role for STAT3 in cytokine-induced myeloid differentiation. Our results suggest that STAT5 activation is also required for the G-CSF–induced neutrophil maturation process. The involvement of STAT5 in both proliferative and maturation responses is interesting, because differentiation is intimately coupled with growth arrest in hematopoietic cells, so that proliferation and maturation are to some degree intrinsically antagonistic processes. However, it is very clear that both growth and differentiation are complex processes involving multiple signaling pathways, and activation of STAT5 is neither absolutely required for proliferation nor sufficient to induce differentiation. Complete understanding of myeloid cell growth and maturation as well as myeloid leukemogenesis will require the identification of STAT5-induced genes involved in these processes, which should be facilitated by the use of the mutants we have described here.

ACKNOWLEDGMENT

The authors thank Dr Alan D’Andrea for providing the STAT5A Δ650 mutant before its publication; Dr Don Wojchowski for the gift of the dominant negative JAK2 mutant; Dr Mark Showers for the gift of the erythropoietin receptor expression plasmid and for reading the manuscript; Dr Bernd Groner for gift of the luciferase plasmids; Dr Jim Griffin for gift of 32D C13 cells; Dr Alice Mui for the gift of probes for CIS, PIM-1, and OSM; Vinay Kumar and F.V. Sivakumar for assistance with FACS analysis; and Dr Heinz Baumann for helpful advice.

REFERENCES


3. Wakao H, Gouilleux F, Groner B: Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. EMBO J 13:2182, 1994


13. Tian SS, Tapley P, Sincich C, Stein BB, Rosen J, Lamb P: Multiple signaling pathways induced by granulocyte colony-stimulating factor involving activation of Jak5s, STATs, and/or STAT3 are required for regulation of three distinct classes of immediate early genes. Blood 88:4435, 1996


Dominant Negative Mutants Implicate STAT5 in Myeloid Cell Proliferation and Neutrophil Differentiation

Robert L. Ilaria, Jr, Robert G. Hawley and Richard A. Van Etten