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

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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 (W_{255}KR→AAA and R_{290}QQ→AAA), two in the DNA-binding domain (E_{437}E→AA and V_{466}VV→AAA), and a carboxy-terminal truncation of STAT5A (STAT5A/Δ53C) 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, Δ53C. Stable expression of either the WKR or Δ53C 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.

SIGNAL TRANSDUCERS and activation of transcription (STAT) family members comprise a group of latent transcription factors involved in cytokine, hormone, and growth factor signaling.[1,2] STAT5, originally described as mammary gland factor for its essential role in mediating prolactin-induced gene expression,[3] 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,[4-6] the IL-2/IL-7/IL-15 family,[7-10] erythropoietin,[9,11] thrombopoietin,[12] granulocyte colony-stimulating factor (G-CSF),[13,14] epidermal growth factor,[15] leptin,[16] and insulin.[17] 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.[6,18] 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,[19] 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*$^{-/-}$,[19] *stat5b*$^{-/-}$,[20] and *stat5a*$^{-/-}$ *stat5b*$^{-/-}$ double knockout mice[21] 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,[21] whereas granulocytic cells from *stat5a*$^{-/-}$ mice have defects in GM-CSF–induced proliferation.[22] 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.[23] 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,[24,25] but stable expression of one of these mutants in the IL-3–dependent myeloid cell line 32D did not affect

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proliferation or survival.[25] A recently described constitutively active mutant of murine STAT5 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* oncogene,[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 lines[32,33] 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 mmol/L glutamine, and nonessential amino acids. 32D Cl3 cells[35] were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin/streptomycin, 2 mmol/L glutamine, and 10% WEHI-3B–conditioned medium (WEHI-CM)[36] as a source of IL-3.

Construction of STAT5A mutants. A hemagglutinin (HA) epitope tag was added to the NH_2-terminus of murine STAT5A subcloned in pUC19 (New England Biolabs, Beverly, MA) by inverse polymerase chain reaction (PCR) and verified by dideoxy 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.[37] 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 GCG; for STAT5A/RQQ, codons 290-292 were changed from CGG, CAG, and CAG to GCG; 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 GCG, 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 of each 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 described[41] from approximately 1×10^7 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 OD_{595} (Bio-Rad Protein assay; Bio-Rad Laboratories, Hercules, CA), were subjected to immunoprecipitation by 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 electrophorectically 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 ^{32}P-labeled double-stranded FcγRI 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-HA (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 described[41,46] from 1×10^7 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 5×10^5 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 cytospin and Wright/Giemsa staining every 24 hours. FACS analysis was performed by staining 10^5 fresh cells with biotinylated rat antimouse 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 FACScan 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 prestimulated[47] 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 ciprofloxicin, 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 1×10^7 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,[48] the cells were then cocentrifuged with virus at 1,000*g* 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 removed, washed twice in PBS, and plated in triplicate at a density of 1×10^5 cells per 3.5-cm plate in Methocult M3230 medium (StemCell Technologies, Vancouver, British Columbia, Canada) containing 1 mg/mL (absolute) G418 and 100 ng/mL recombinant human G-SCF. 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 cytospin.

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 NH_2-terminus, designated STAT5A/WKR (W_{255}KR→AAA) and STAT5A/RQQ (R_{290}QQ→AAA), and two were located in the putative DNA-binding domain, STAT5A/EE (E_{437}E→AA) and STAT5A/VVV (V_{466}VV→AAA). An HA epitope tag was added at the NH_2-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.[34]

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 coimmunoprecipitate 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-

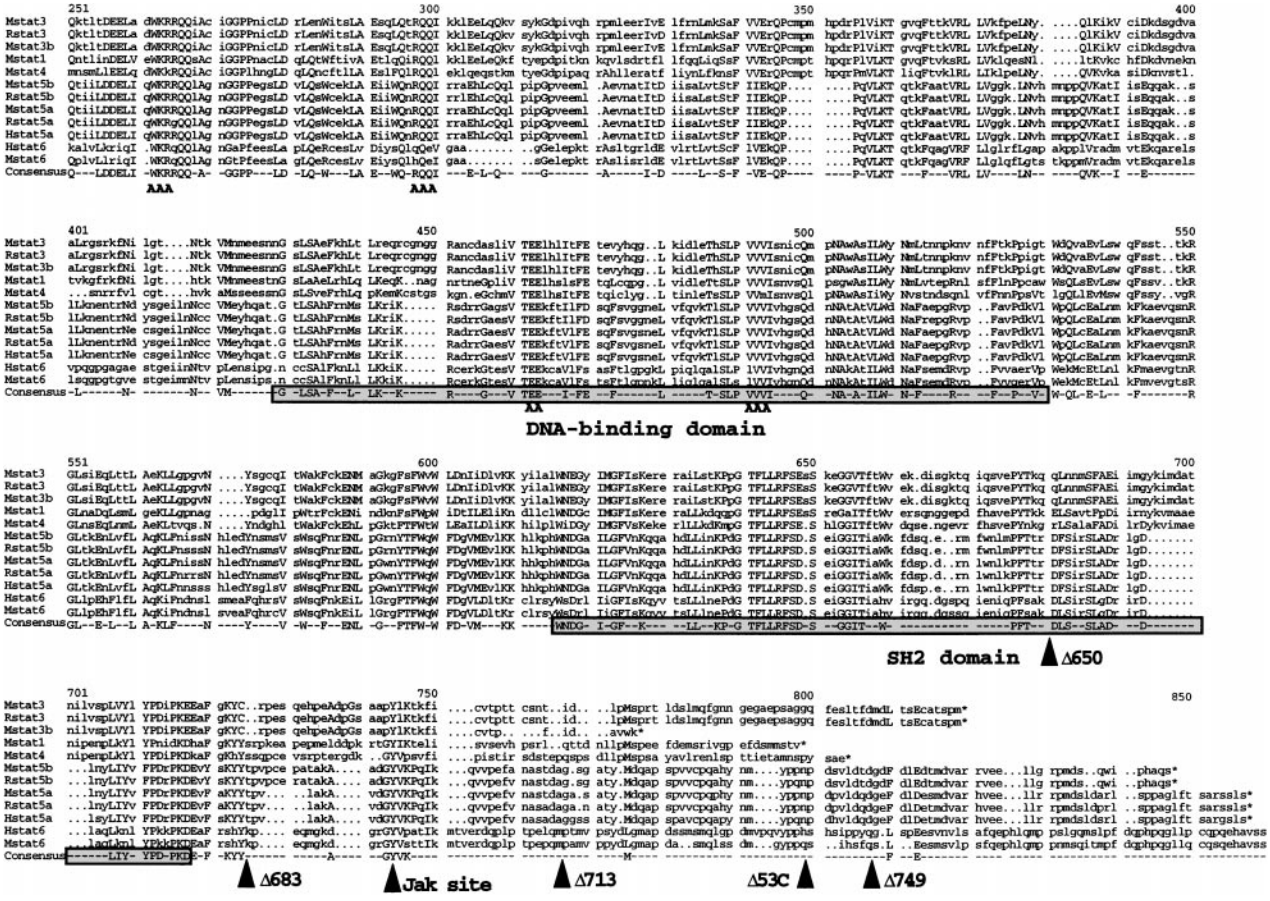

Fig 1. Mutagenesis of conserved regions of murine STAT5A. Amino acid sequence alignment of the central region of several mammalian (H, human; M, mouse; R, rat) STAT family members is shown. Numbering is from the amino terminus, and alignment was performed by the program PRETTY.PILEUP (GCG, University 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 positions of the canonical Src homology 2 (SH2) domain and the putative DNA-binding domain as defined by Horvath et al[57] 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 Δ53C (this report), Δ683,[23] Δ749,[24] Δ713,[25] and Δ650[49] are shown by arrowheads.

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 $F_c\gamma$RI 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

Table 1. Summary of Biological Properties of STAT5A Mutants

	293T Cells					32D Cells	
STAT5A Mutant	Tyrosine PO_4	Dimerization*	DNA Binding	Transactivation	DN Activity†	Inhibition of IL-3–Dependent Proliferation	Inhibition of Neutrophil Maturation
STAT5A WT	++	++	++	++	−	−	−
STAT5A/WKR	+	−	−	−	++	+	++
STAT5A/RQQ	++	++	++	++	−		
STAT5A/EE	++	++	−	++	−		
STAT5A/VVV	+	+	−	−	+		
STAT5A/Δ53C	++	++	+++	+/−	++	+	++

*JAK2-dependent heterodimerization with STAT5B.

†Inhibition of erythropoietin-induced STAT5-dependent luciferase induction.

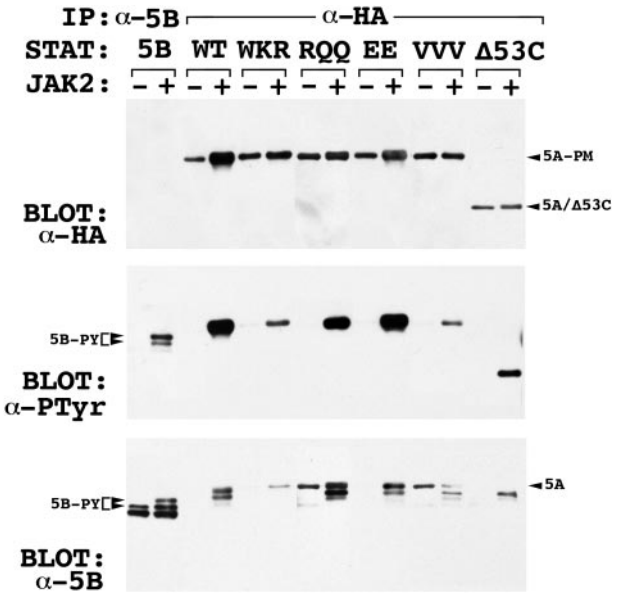

Fig 2. The STAT5A mutants are tyrosine-phosphorylated by JAK2, but the STAT5A/WKR mutant fails to heterodimerize with STAT5B. Wild-type or mutant STAT5A was coexpressed in 293T cells with STAT5B with or without JAK2; in the left two lanes, STAT5B alone was expressed with and without JAK2. Proteins were immunoprecipitated with anti-HA (α-HA) or anti-STAT5B (α-5B) antibodies and blotted with anti-HA (top panel), anti-phosphotyrosine (middle panel), or anti-STAT5B (bottom panel) antibodies. Note that the anti-STAT5B antibody also recognizes STAT5A and the STAT5A mutants with the exception of Δ53C, which is lacking the C-terminal epitope recognized by this antisera. The positions of the STAT5A and STAT5B proteins are indicated by the arrowheads at right. The STAT5A point mutants (5A-PM) migrate more slowly than STAT5B due to additional amino acids at the C-terminus and the HA epitope tag at the N-terminus. When coexpressed with JAK2, STAT5B migrates as three distinct forms (left 2 lanes, bottom panel), of which the two most slowly migrating forms are tyrosine phosphorylated (5B-PY, middle panel).

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/Δ53C 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.[24] 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/Δ650,[49] 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.[39] 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[39] 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

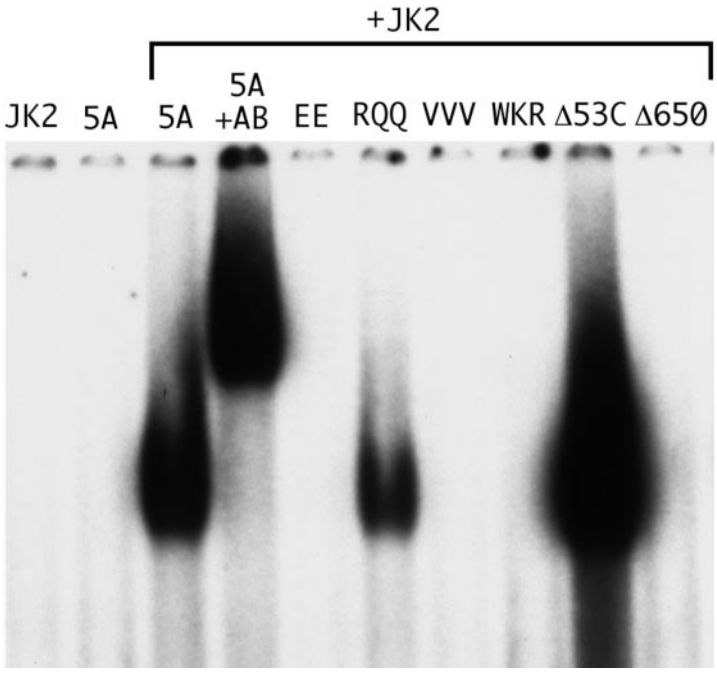

Fig 3. The STAT5A/WKR, EE, and VVV mutants lack FcγRI GAS DNA-binding activity. Nuclear extracts were prepared from 293T cells transfected with JAK2 and the indicated murine STAT5A mutants and analyzed by EMSA using a ^{32}P-labeled FcγRI-derived GAS probe. 293T cells transfected with either parental STAT5A (5A) alone or JAK2 (JK2) alone are shown at left. STAT5-specific GAS DNA-binding activity was confirmed by incubation with anti-STAT5A antibody (5A+JK2+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).

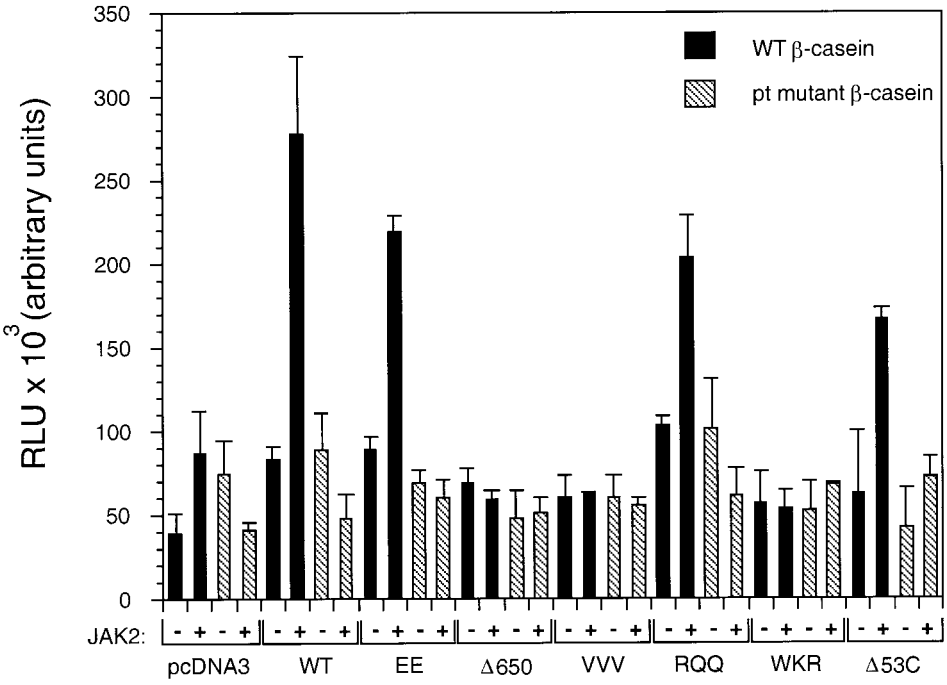

Fig 4. The STAT5A/VVV and STAT5A/WKR mutants are defective in transcriptional activation. 293T cells were transfected with pcDNA3 vector alone, wild-type STAT5A (WT), or the indicated STAT5A mutant in the absence (−) or presence (+) of JAK2 and either wild-type (■) or point mutant (▨) β-casein luciferase reporter. The transcriptional activation of the mutant or wild-type β-casein reporter was assessed by luciferase assay and expressed in relative light units (RLU). To control for variations in transfection efficiency, all samples were cotransfected with an hGH expression plasmid to allow normalization of RLU values to secreted levels of hGH, determined by radioimmunoassay. Error bars represent the standard error based on at least two independent experiments.

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 WKR 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

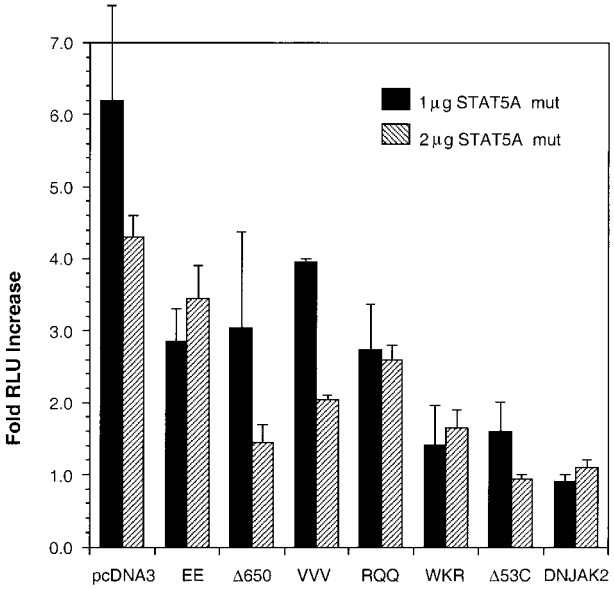

Fig 5. The STAT5A/WKR, STAT5A/VVV, and STAT5A/Δ53C mutants exert dominant negative effects on erythropoietin-induced, STAT5-dependent transcription. The indicated STAT5A mutant in the expression vector pcDNA3 ([■] 1 μg; [▨] 2 μg) was cotransfected into 293T cells together with wild-type STAT5A plasmid (0.25 μg), a murine erythropoietin receptor expression construct, β-casein luciferase reporter, and hGH expression plasmid as an internal control. Cells were stimulated with erythropoietin as described in Materials and Methods, and the increase in luciferase activity relative to unstimulated cells, normalized for transfection efficiency, is shown. Omission of the erythropoietin receptor expression construct from the transfection resulted in background levels of erythropoietin-stimulated luciferase activity in transfected cells (data not shown). pcDNA3 indicates cotransfection with vector alone. Error bars represent the standard error based on at least two independent experiments.

greater than fivefold increase in β-casein luciferase reporter activity. Addition of a dominant negative JAK2 mutant (DNJAK/Δ829)[50] completely blocked this effect. Consistent with a recent report, the mutant STAT5A/Δ53C, lacking most of the STAT5A transactivation domain,[24] 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.[49] 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*[23] 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).

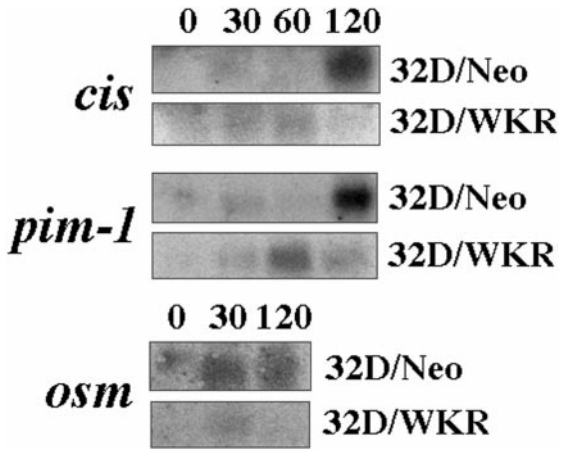

Fig 6. STAT5A/WKR inhibits the expression of the IL-3–responsive genes *pim-1*, *cis*, and *osm* in the myeloid cell line 32D. 32D cells expressing either the neomycin resistance gene alone (32D/Neo) or STAT5A/WKR (32D/WKR) were deprived of IL-3 for 6 hours and then stimulated with 10% WEHI-3B–conditioned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

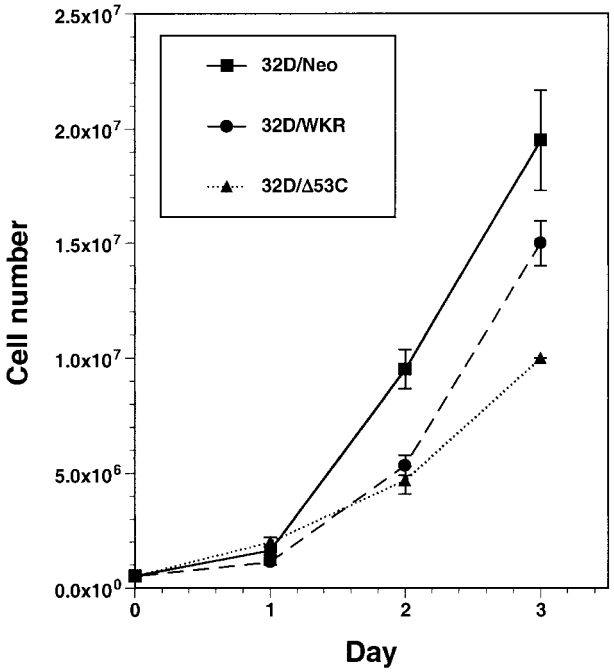

Fig 7. Expression of either STAT5A/WKR or STAT5A/Δ53C in 32D cells inhibits IL-3–dependent proliferation. 32D cells expressing the neomycin resistance gene alone (32D/Neo), STAT5A/WKR (32D/WKR), or STAT5A/Δ53C (32D/Δ53C) were grown in 10% WEHI-3B–conditioned medium without G418 and viable cell number 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).

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) and proliferation assays were performed immediately after selection in G418. Compared with 32D cells transduced with virus carrying the neomycin resistance gene alone, cells expressing STAT5A/WKR demonstrated a moderate decrease in proliferation in 10% WEHI-3B–conditioned medium (Fig 7). There was no evidence of apoptosis in STAT5A/WKR-expressing 32D cells, even when grown in minimal (0.25% WEHI-CM) amounts of IL-3 (data not shown). Western blot analysis of the G418-resistant 32D-STAT5A/

WKR 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.[51] Because STAT5 has been shown to be activated by G-CSF treatment,[13,14] 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-GSF 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.[52] 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.[13,14] Interestingly, in 32D/WKR cells, there was somewhat 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,[24] 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 STATs has been demonstrated to impair the ability of STATs to bind to tandem, but not to

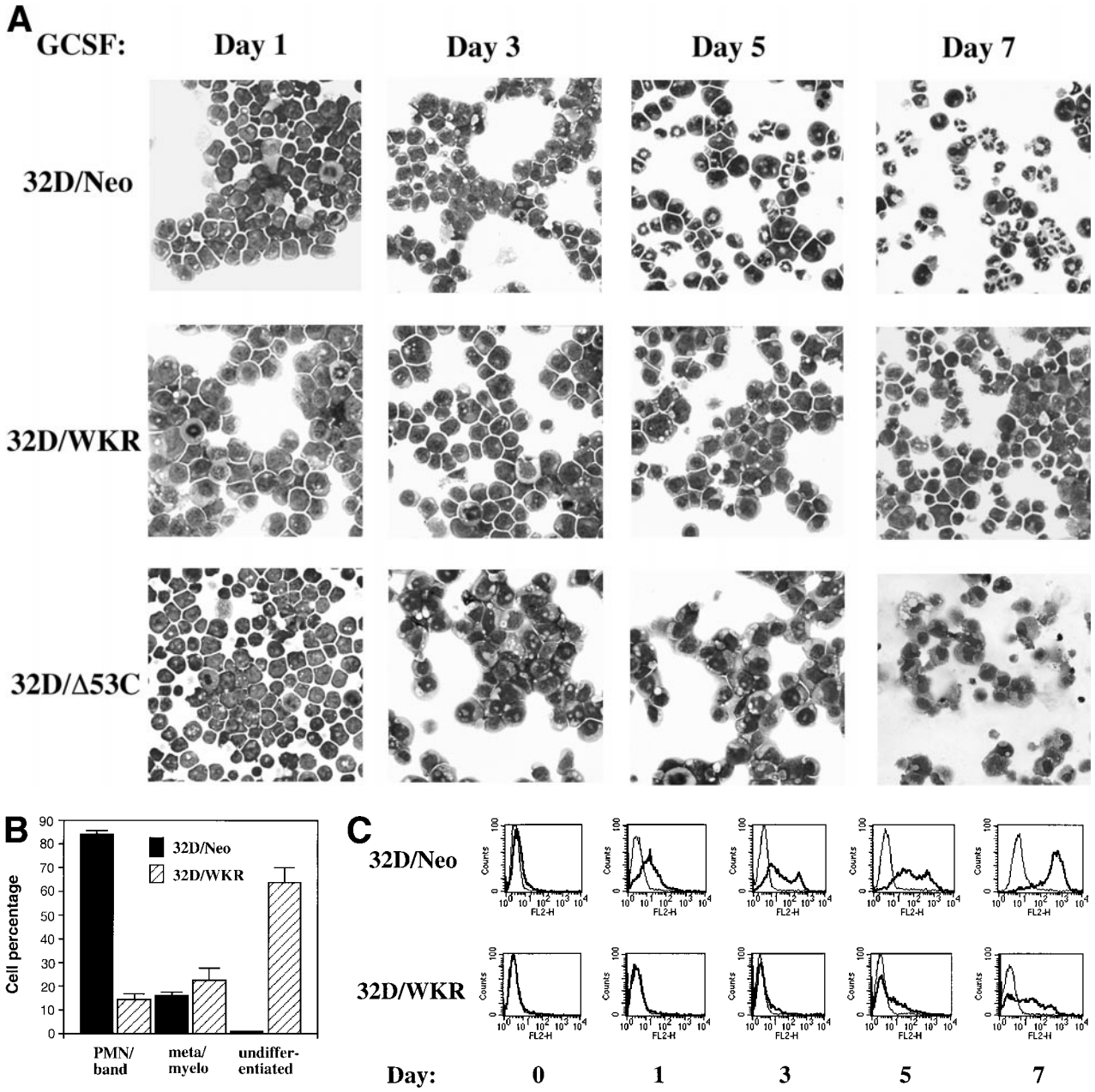

Fig 8. STAT5A/WKR and STAT5A/Δ53C block G-CSF–induced neutrophil differentiation of 32D cells. (A) 32D cells expressing either the neomycin resistance gene alone (32D/Neo), STAT5A/WKR (32D/WKR), or STAT5A/Δ53C (32D/Δ53C) were placed in 100 ng/mL human recombinant G-CSF and minimal IL-3 to permit cell viability throughout the differentiation assay. Wright-Giemsa–stained cytospins were prepared on the indicated days to assess G-CSF–dependent morphologic maturation of 32D cells. The photographs depicted are representative of at least two independent experiments. (B) Cytospins from day 7 of G-CSF treatment from 32D/Neo and 32D/WRK cells were scored for neutrophil differentiation on the basis of morphology, and the results of three differential counts of 100 cells are each depicted with standard error. (C) 32D/Neo and 32D/WKR cells were analyzed by FACS after staining with anti-CD11b monoclonal antibody (thick line) or isotype control antibody (thin line) on the day before (day 0) and the indicated days after initiation of G-CSF treatment.

single GAS binding sites.[53] In addition, a point mutation at the extreme amino-terminus of STAT1 has been demonstrated to result in its constitutive tyrosine phosphorylation,[54] 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-

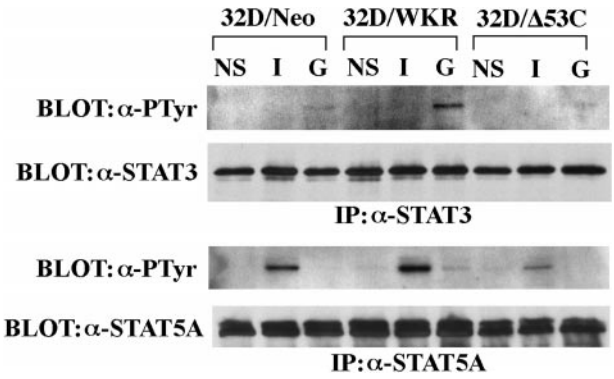

Fig 9. The STAT5A/WKR and Δ53C mutants do not inhibit STAT3 activation. Populations of 32D cells expressing either the neomycin resistance gene alone (32D/Neo), STAT5A/WKR (32D/WKR), or STAT5A/Δ53C (32D/Δ53C) were starved of IL-3 and then stimulated with IL-3 (I), G-CSF (G), or unstimulated (NS). Extracts 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).

phorylation and DNA binding of the mutant STAT5A.[26] We did not observe any evidence of constitutive tyrosine phosphorylation or transactivation with any of our STAT5A mutants. In contrast, the nearby STAT5A/WKR mutation disrupted STAT5 heterodimerization, despite a lack of effect on tyrosine phosphorylation, suggesting that this region of the STAT5A may act to stabilize dimerization. Consistent with its defect in dimerization, STAT5A/WKR completely lacked DNA binding to a FcγRI-derived GAS probe and demonstrated no significant

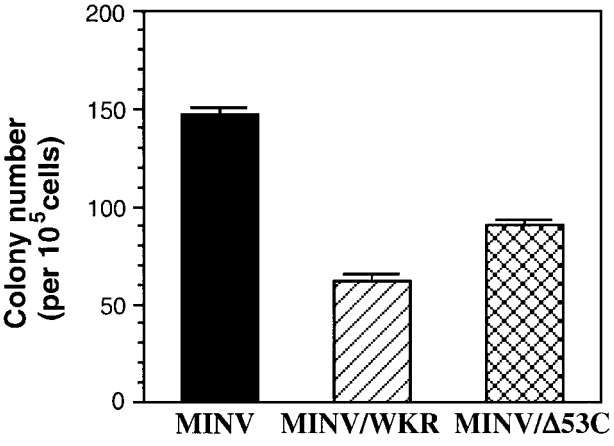

Fig 10. The STAT5A/WKR and Δ53C mutants inhibit G-CSF–dependent granulocytic colony formation in primary murine bone marrow. Primary murine bone marrow was transduced with parental MINV retrovirus (MINV) or MINV retrovirus expressing STAT5A/WKR (MINV/WKR) or STAT5A/Δ53C (MINV/Δ53C) and then plated in triplicate in methylcellulose culture with G418 and exogenous G-CSF. The mean number of granulocytic colonies on day 4 to 5 per 10^5 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.

transcriptional activation of a β-casein reporter. Interestingly, this mutant had a potent dominant negative effect on erythropoietin-induced STAT5-dependent transcriptional activation, suggesting that STAT5A/WKR acts by blocking wild-type STAT binding to the intracytoplasmic domain of the erythropoietin receptor. This is also the likely mechanism used by a previously described dominant negative STAT5A mutant, STAT5A/Δ650,[49] which is defective in tyrosine phosphorylation, heterodimerization, DNA binding, and transcriptional activation.

Although STAT proteins lack classical DNA binding domains and transactivation motifs, recent studies have provided considerable insight into regions important for STAT activation and DNA binding.[55-58] The STAT5A/EE and STAT5A/VVV mutants, located in the putative STAT DNA-binding domain,[57,58] were generated because of the striking sequence homology among different STATs family members within this region (Fig 1). Both of these mutations completely eliminated FcγRI GAS DNA-binding as assessed by EMSA, despite preservation of tyrosine phosphorylation and dimerization. These results are consistent with a previous study in which the same mutations in STAT3 significantly impaired binding to an M67-derived oligonucleotide.[57] Interestingly, in the context of STAT3, the VVV→AAA mutant still had some detectable DNA-binding activity, likely reflecting subtle differences in the manner that different STAT proteins bind to distinct oligonucleotide sequences. STAT5A/VVV was also unable to transcriptionally activate a β-casein luciferase reporter; however, STAT5A/EE, also lacking FcγRI-DNA-binding activity, still transactivated a β-casein reporter almost as well as wild-type STAT5A, showing that DNA-binding as assessed by EMSA is not always predictive of transcriptional activation in vivo. When assessed for possible dominant negative activity, STAT5A/EE had little effect on the ability of erythropoietin to activate STAT5-dependent transcription even when transiently expressed at an eightfold excess, suggesting that its preserved transactivation ability could compensate for its relative defect in DNA binding. This finding differs from the behavior of an EE→AA mutation in STAT3, which does function as a dominant negative.[59] In contrast, STAT5A/VVV exerted a dominant negative effect on erythropoietin-induced STAT5-dependent transcription that increased with increasing levels of expression of the mutant STAT protein. Because of its preserved heterodimerization ability, STAT5A/VVV may exert its dominant negative effect by forming heterodimers that are defective in DNA binding. It is also likely that some of the dominant negative effect of STAT5A/VVV is due to competition for binding sites on the cytoplasmic portion of the erythropoietin and IL-3 receptors.

Previous studies of dominant negative STAT5 mutants have focused on deletion mutagenesis of the carboxy-terminal portion of the protein. Some STAT5 mutants have involved large carboxy-terminal deletions, including the tyrosine (Y694) phosphorylated by JAK family members,[23,49] whereas others were constructed by deletion of a small C-terminal basic region implicated in transcriptional activation.[24,25] We generated a similar mutant, STAT5A/Δ53C, by analogy to a naturally occurring isoform of rat STAT5B,[34] which also has dominant negative properties (H. Baumann, personal communication, January 1998). As expected, the STAT5A/Δ53 mutant exhibited JAK2-dependent tyrosine phosphorylation, heterodimerization,

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.[24] 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 STAT5A 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[23,26] 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.[25] The reason for the discrepancy between these results and our observations are 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 antiapoptotic signal in response to activation of the gp130 receptor,[60] we and others[23,25] 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 antiapoptotic 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 myelopoeisis. Unlike other gene products, such as C/EBPα,[61] 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 about a 50% reduction in G-CSF–dependent granulocytic colony formation relative to wild-type marrow,[21] 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 lead to tyrosine phosphorylation and activation of STAT3, STAT1, and STAT5.[13,14] 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.[62,63] Whereas efficient activation of STAT3 requires the presence of at least one of four conserved tyrosine residues present in the distal region of the G-CSF-R,[13,64] activation of STAT5 by G-CSF appears to be independent of G-CSF-R phosphorylation.[13] Expression of dominant-negative STAT3 mutants in myeloid cell lines blocked G-CSF–induced neutrophil differentiation[52] and IL-6–induced macrophage differentiation,[59] 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[13,23,25] nor sufficient to induce differentiation.[30,65] 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.

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