Signal Transducer and Activator of Transcription 5b (Stat5b) Serine 193 Is a Novel Cytokine-induced Phospho-regulatory Site That Is Constitutively Activated in Primary Hematopoietic Malignancies*

Abhisek Mitra†, Jeremy A. Ross†, Georgialina Rodriguez‡, Zsuzsanna S. Nagy§, Harry L. Wilson**, and Robert A. Kirken††

From the †Department of Pathology, Providence Memorial Hospital, El Paso, Texas 79902 and the ‡Department of Biological Sciences and Border Biomedical Research Center, The University of Texas, El Paso, Texas 79968 and the §Department of Pathology, Providence Memorial Hospital, El Paso, Texas 79902

Background: Stat5 transcription factors regulate genes required for cell growth, survival, and differentiation. Results: A novel cytokine-induced Stat5b phospho-regulatory site was identified and found to be constitutively activated in hematopoietic cancers. Conclusion: This newly defined phosphorylation site acts to regulate Stat5b DNA binding and transcriptional activity. Significance: Therapeutic strategies that disrupt Stat5 serine phosphorylation may be important for controlling hematopoietic diseases such as cancer.

Signal transducer and activator of transcription 5b (Stat5b) is a critical node in the signaling network downstream of external (cytokines or growth factors) or internal (oncogenic tyrosine kinases) stimuli. Maximum transcriptional activation of Stat5b requires both tyrosine and serine phosphorylation. Although the mechanisms governing tyrosine phosphorylation and activation of Stat5b have been extensively studied, the role of serine phosphorylation remains to be fully elucidated. Using mass spectrometry and phospho-specific antibodies, we identified Stat5b Ser(P)-193 as a novel site of cytokine-induced phosphorylation within human Stat5b. Stat5b Ser(P)-193 was detected in activated primary human peripheral blood mononuclear cells or lymphoid cell lines in response to several γ common (γc) cytokines, including interleukin (IL)-2, IL-7, IL-9, and IL-15. Kinetic and spatial analysis indicated that Stat5b Ser-193 phosphorylation was rapid and transient and occurred in the cytoplasmic compartment of the cell prior to Stat5b translocation to the nucleus. Moreover, inducible Stat5b Ser-193 phosphorylation was sensitive to inhibitors of mammalian target of rapamycin (mTOR), whereas inhibition of protein phosphatase 2A (PP2A) induced phosphorylation of Ser-193. Reconstitution assays in HEK293 cells in conjunction with site-directed mutagenesis, EMSA, and reporter assays indicated that Ser(P)-193 is required for maximal Stat5b transcriptional activity. Indeed, Stat5b Ser-193 was found constitutively phosphorylated in several lymphoid tumor cell lines as well as primary leukemia and lymphoma patient tumor cells. Taken together, IL-2 family cytokines tightly control Stat5b Ser-193 phosphorylation through a rapamycin-sensitive mechanism. Furthermore, constitutive Ser-193 phosphorylation is associated with Stat5b proto-oncogenic activity and therefore may serve as a novel therapeutic target for treating hematopoietic malignancies.

Signal transducers and activators of transcription (Stats) are a family of latent transcription factors that upon activation mediate multiple cellular processes, including proliferation, differentiation, and survival (1). The Stat protein family is composed of seven members (Stat1–6), including the two closely related Stat5a and Stat5b molecules that share 96% amino acid sequence identity (2, 3). Stat5a and Stat5b are critical for lymphoid, myeloid, and erythroid cell development and function (4, 5). Indeed, Stat5 proteins are activated by multiple cytokines, including IL-2, IL-3, IL-5, IL-7, IL-9, IL-15, and erythropoietin (6–8). Following cytokine stimulation, human Stat5a and Stat5b are phosphorylated on the conserved tyrosine residues Tyr-694 and Tyr-699, respectively, which allows for their dissociation from the receptor complex, formation of hetero- or homodimers, and nuclear translocation to bind specific elements in the promoter of target genes and activate transcription (9). In addition to tyrosine phosphorylation, the activity of Stat5 has been shown to be modulated by serine phosphorylation. Previous studies from our group and others identified a novel phospho-serine site located in a conserved Pro-Ser-Pro (PSP) motif in the transactivation domain at amino acid posi-
tions 726 (Stat5a) and 731 (Stat5b) (10–12). In addition, human Stat5a harbors a unique serine phosphorylation site within a Ser-Pro (SP) motif (Ser-780) (13). These studies indicate that maximum transcriptional activation of Stat5 requires both tyrosine and serine phosphorylation. Most efforts to date have focused on determining the mechanisms that govern tyrosine phosphorylation and activation of Stat5; however, the role of serine phosphorylation remains to be fully elucidated.

In addition to the physiological role of Stat5 in hematopoietic cell development, dysregulation of the Stat5 signaling pathway plays a role in oncogenesis and leukemogenesis (14, 15). Specifically, Stat5 has been shown to be constitutively activated in several forms of lymphoid, myeloid, and erythroid leukemias (16–21). Indeed, the introduction of constitutively active Stat5 mutants into hematopoietic cells is sufficient to induce multilineage leukemia in mice (22, 23). Persistent tyrosine phosphorylation and activation of Stat5 was found to be a result of deregulated cytokine signaling (24) or the presence of onco- 

genic tyrosine kinases. Stat5 proteins are critical targets for many oncogenic tyrosine kinases, including Bcr-Abl (breakpoint cluster region-abelson), Tel-Jak2, mutated forms of Flt3 (fms-like tyrosine kinase receptor-3) and c-Kit, and the Jak2 V617F mutant (19, 23, 25–28). Although the aberrant regulation of Stat5 tyrosine phosphorylation is well established, the significance of Stat5 serine phosphorylation is less clear. The present study was initiated in an attempt to fully define the Stat5 serine phosphorylation sites and determine their role in normal and proto-oncogenic Stat5 activity. We provide novel evidence that Stat5b undergoes cytokine-induced phos-

phorylation at Ser-193 through a rapamycin-sensitive mecha-

nism. Furthermore, Ser-193 phosphorylation was found to play an important role in Stat5b DNA binding and transcriptional activity. Indeed, Stat5b Ser-193 was constitutively phosphory-

lated in several lymphoid tumor cell lines as well as primary leukemia and lymphoma patient tumor cells. These insights provide further evidence regarding the importance of serine phosphorylation in Stat5 function and dysfunction.

EXPERIMENTAL PROCEDURES

Cell Culture and Patient Samples—The human YT, MT-2, HUT-78, HUT-102, and HEK293 cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum (Atlanta Biologicals), 2 mM L-glutamine, and penicillin-strepto-


cillin. HUT-78, HUT-102, and HEK293 cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum

H9262 (Atlanta Biologicals), 2 mM L-glutamine, and penicillin-strepto-

ncillin. Anti-Phospho-serine 726/731 Stat5a (α-Stat5a (P)–726/731 Stat5a) polyclonal antibodies were applied as described previ-

ously (12). The anti-Phospho-tyrosine 694/699 Stat5a (α-Phos-

pho-Tyr Stat5a) and anti-Phospho-tyrosine (α-Phospho-Tyr) 

monoclonal antibodies were purchased from Millipore. The anti-Stat5 polyclonal antibodies (α-Stat5) were purchased from Santa Cruz Biotechnology. Kinase and phosphatase experi-

ments employing PD98059 (New England Biolabs), wortman-

nin (Calbiochem), rapamycin (Calbiochem), calyculin A (CA) (Sigma-Aldrich), okadaic acid (OA) (Sigma-Aldrich), fostriecin (FOS) (Sigma-Aldrich), or tautomycin (TAU) (Sigma-Aldrich) were performed at 37 °C with the concentrations and time points indicated.

Cell Lysis, Immunoprecipitation, Western Blot, and Mass Spectrometry Analysis—Cells were pelleted, lysed, and sub-

ected to immunoprecipitation and Western blot analysis as reported previously (32). For all samples, total protein was determined by the bicinchoninic acid method (Pierce). When reblotting, PVDF membranes were incubated with stripping buffer (100 mm β-mercaptoethanol, 2% SDS, 62.5 mm Tris-

HCl, pH 6.7) at 55 °C for 30 min, blocked, and then reprobed. Liquid chromatography-tandem mass spectrometry analysis was performed by the Taplin Biological Mass Spectrometry Facility (Harvard University) or the Biomolecule Analysis Core Facility, a component of the Border Biomedical Research Center (University of Texas at El Paso) following their standard procedures.

Plasmids, Site-directed Mutagenesis, and Transfection of H9293 Cells—The pcDNA3.1/GS human IL-2Rγ and IL-2Rβ expression plasmids were purchased from Invitrogen. The human Jak3 and β-casein-luciferase reporter plasmids were used as described previously (33). The pCMV-β-galactosidase (β-gal) expression plasmid was kindly provided by Dr. Hallgeir Rui and described in Ref. 34. Human Stat5b cDNA (OriGene) was PCR-amplified and subcloned into the pCMV-Tag2 (Stratagene). Mutant forms of Stat5b were prepared using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The primers used for Stat5b mutation were as follows: S193A (5′-GCTGGCCCCAGCTGGCCCCC-3′); S193E (5′-CCGCTGGCCCCAGCTGGAC-A-3′). All subclones and mutations were verified by DNA sequencing. Transient transfections of H9293 cells were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Luciferase and β-Galactosidase Assays—Subconfluent H9293 cells in 10-cm dishes were transfected with the following plasmids: β-casein-luciferase reporter (3 μg), pCMV-β-gal (0.3 μg), Jak3 (0.5 μg), IL-2Rβ (6 μg), IL-2Rγ (6 μg), and wild-type or mutant Stat5b (3 μg). At 48 h after transfection, cells were stimulated with or without 1,000 IU/ml IL-2 for 6 h. The cells were then harvested, and luciferase/β-gal activities were measured with the Dual-Light luciferase assay reporter system (Applied Biosystems) according to the manufacturer’s instruc-
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Immunofluorescent Confocal Microscopy—Cells were cytocentrifuged onto glass slides, fixed, permeabilized, and immunostained using the indicated antibodies as described previously (30). Immunofluorescent confocal microscopy was performed at the Analytical Cytology Core Facility, a component of the Border Biomedical Research Center (University of Texas at El Paso). The cells were visualized using a Zeiss LSM 700 confocal microscope using a 40× oil immersion objective (Carl Zeiss) in the multitrack scanning mode with excitation wavelengths set at 488 (argon laser), 543, and 633 nm (He-Ne lasers); emission wavelengths were 505–530 nm and >560 nm for detection of the Cy2 and Cy3 coupled secondary antibodies, respectively. Images were captured using the ZEN 2009 (version 5.5) software and exported in a 12-bit TIFF RGB format to Adobe Photoshop and Illustrator CS4 for processing.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared and assays were conducted as published previously (35). Oligonucleotides corresponding to the β-casein gene promoter element for Stat5 (5′-AGATTCTA-GGAATTCAATCC-3′) were obtained from Santa Cruz Biotechnology.

RESULTS

Identification of a Novel IL-2-induced Serine Phosphorylation Site in Human Stat5b—In an attempt to fully define the Stat5 phospho-regulatory sites, mass spectrometry analysis of activated Stat5 was performed. Stat5b was immunoprecipitated from YT cells stimulated without (-) or with (+) IL-2 for 15 min, and Stat5b proteins were immunoprecipitated (IP) from soluble cell lysates with α-Stat5b antibodies. Two sets of immunoprecipitations were separated by SDS-PAGE. One set was Coomassie Blue-stained (A), and the other was Western blotted (WB) with α-phospho-Tyr (α-pY), α-phospho-Ser (pS726/731), or α-Stat5b antibodies. HC, heavy chain; LC, light chain. C, tandem mass spectra of a monophosphorylated peptide showing site localization of Ser-193, as indicated by asterisks. D, amino acid sequence alignment of the region surrounding Ser-193 (asterisk) from each human Stat protein using the ClustalW program (progressive alignment) (31). E, domain architecture of human Stat5 with known and newly identified (asterisk) serine and tyrosine phosphorylation sites. Numbers indicate amino acid residues of human Stat5 (a/b).
phosphorylation site. The tandem mass spectrum for the identified peptide R1IQAFQPLAPQTPEPQER, which contains the phosphorylated Stat5b Ser-193 residue (underlined), is shown in Fig. 1C. To determine the extent of Ser-193 sequence conservation among other Stats, human Stat5a and Stat5b protein sequences were aligned with other human Stats (Stat1, Stat2, Stat3, Stat4, and Stat6) (Fig. 1D). Human Stat3 and Stat6 do possess a serine residue at the aligned Stat5 Ser-193 position; however, the surrounding amino acid sequence is not well conserved. Specifically, Stat5 Ser-193 is C-terminally flanked by a proline residue, which is not conserved in the other Stats. It is important to note that the Stat5a Ser-193 phospho-peptide was not detected by LC-MS/MS analysis; thus, the functional studies described herein focused on Stat5b. The location of Ser-193 in the coiled-coil domain of Stat5 is shown along with previously identified sites in the transactivation domain (Fig. 1E).

Previously, large-scale phospho-proteomic analyses using HeLa (36–38) or Jurkat (39) cell extracts identified Ser-193 as a Stat5b phosphorylation site using discovery-mode mass spectrometry. Accordingly, the present work sought to functionally define Stat5b Ser-193 phosphorylation using site-specific methodologies.

Generation of α-Ser(P)-193 Stat5 Phospho-specific Antibody—To verify that Stat5b is phosphorylated at serine 193 and to investigate the regulatory roles of this phosphorylation site, a phospho-specific polyclonal antibody was generated. Dot blot analysis was performed with the immunizing phospho-peptide and the corresponding nonphosphorylated peptide (see “Experimental Procedures” for sequences) to determine whether the Stat5 phospho-specific antibody cross-reacts with regions distal to the phosphorylated serine. Additionally, a Stat5b Ser-731-containing phospho-peptide and corresponding nonphosphorylated peptide (see “Experimental Procedures” for sequences) were used to determine whether the Stat5 Ser-193 phospho-specific antibody cross-reacts with the other known phosphorylated serines in Stat5b. Increasing amounts of Stat5b Ser-193, Ser(P)-193, Ser-731, or Ser(P)-731 peptides (Fig. 2A) were spotted onto PVDF membranes and immunoblotted with α-Ser(P)-193 Stat5b. The Ser(P)-193 Stat5b antibody primarily recognized the corresponding phosphorylated peptide but not its nonphosphorylated counterpart, nor the Stat5b Ser-731 peptides, indicating that the phospho-specific antibody does not cross-react significantly with the nonphosphorylated peptide nor other proline-flanked serine phosphorylation sites within Stat5b. Multiple studies to determine whether the Ser(P)-193 Stat5b antibody was amenable to SDS-PAGE-separated wild-type protein by Western blot analysis were unsuccessful (data not shown). Therefore, the Ser(P)-193 Stat5b antibody was tested for utilization in immunofluorescent confocal microscopy. For this analysis, YT cells were stimulated with IL-2 for 15 min and stained using α-phospho-Tyr Stat5 and α-Ser(P)-193 Stat5b in the presence of increasing amounts of Ser-193 nonphospho-peptide (lanes a–c) or phospho-peptide (lanes d–f) (Fig. 2B). The Ser(P)-193 Stat5b phospho-antibody was specifically blocked by the phospho-, but not the non-phospho-peptide, thus confirming antibody specificity, as well as its utility in immunofluorescent microscopy (Fig. 2B). Additionally, phospho-peptide inhibition of α-Ser(P)-193 Stat5b was dose-dep-
phosphorylation of Stat5b. To corroborate these results, YT cells were treated with another mTOR inhibitor, PP242 hydrate, which resulted in a similar response at comparative concentrations (Fig. 3B). Taken together, these results suggest that Stat5b Ser-193 is dependent upon IL-2-induced activation of an mTOR-dependent pathway.

**PP2A, but Not PP1, Negatively Regulates Phosphorylation of Stat5b Ser(P)-193**—Our group and others have shown that inhibition of protein phosphatase types 1 (PP1) or 2A (PP2A) attenuates Stat3 (45), Stat5 (32), and Stat6 (45, 46) activity. To determine whether Stat5b Ser-193 phosphorylation is regulated by PP1 or PP2A activity, we tested the ability of the PP1 and PP2A inhibitor CA (47) to induce Stat5b Ser-193 phosphorylation (Fig. 4A). YT cells were left untreated (lanes a and b) or pretreated with either DMSO (lanes c and d) or 50 nm CA (lanes e and f) for 1 h before being stimulated without (−) or with (+) IL-2 for 15 min. Cells stained with α-phospho-Tyr Stat5 (Cy2, green), α-Ser(P)-193 Stat5b (Cy3, red), and DAPI (blue) and overlay images are shown. Immunofluorescent images were captured using PASCAL software on a Zeiss LSM 510 Meta confocal microscope at 63× magnification. Representative data from three independent experiments are shown.

**FIGURE 2. Phosphorylation of Stat5b Ser-193 displays rapid kinetics and is inducible by multiple cytokines.** A, a phospho-specific polyclonal Stat5b Ser(P)-193 antibody was generated and tested by dot blot analysis using increasing amounts of Stat5b Ser-193 (CLAQLSPQERL), Ser(P)-193 (CLAQL(pS)PQERL), Ser-731 (KDQAPSPAVCP), and Ser(P)-731 (KDQAP(pS)PAVCP) peptides spotted onto PVDF membrane (where pS indicates phospho-serine). IB, immunoblotting. B, YT cells stimulated with IL-2 for 15 min were fluorescently labeled using α-phospho-Tyr (α-pY) Stat5 (Cy2, green), α-Ser(P)-193 (α-pS193) Stat5b (Cy3, red), and DAPI (blue) and visualized by confocal microscopy. The overlay (bottom panel) shows co-localization of phospho-Tyr Stat5 and Ser(P)-193 Stat5b. For peptide competition analysis, the polyclonal Ser(P)-193 Stat5b antibody was preblocked with increasing amounts of non-phospho-peptide (lanes a–c) or phospho-peptide (lanes d–f) for 1 h at 4 °C followed by staining of YT cells treated with IL-2 for 15 min. C, YT cells were stimulated without (−) (lane a) or with IL-2 (+) from 0 to 60 min (lanes b–e) and fixed with cold methanol. Cells were then stained with α-phospho-Tyr Stat5 (Cy2, green), α-Ser(P)-193 Stat5b (Cy3, red), and DAPI (blue) and visualized by confocal microscopy. The overlay (bottom panel) shows co-localization of phospho-Tyr Stat5 and Ser(P)-193 Stat5b. Insets show a higher magnification view of α-Ser(P)-193 Stat5b (Cy3, red)-stained YT cells treated with IL-2 for the indicated time points. D, quiescent PHA-activated human PBMCs were stimulated with medium (−) (lane a) or IL-2 (lane b), IL-7 (lane c), IL-9 (lane d), or IL-15 (lane e) for 15 min and fixed with cold methanol. Confocal images using α-phospho-Tyr Stat5 (Cy2, green), α-Ser(P)-193 Stat5b (Cy3, red), and DAPI (blue) and overlay images are shown. Immunofluorescent images were captured using PASCAL software on a Zeiss LSM 510 Meta confocal microscope at 63× magnification. Representative data from three independent experiments are shown.
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A

\[\alpha-pY \text{Stat5} \]

\[\alpha-pS193 \text{Stat5b} \]

DAPI

Overlay

- + + 10 50 100

\[\text{IL-2 (15 min)} \]

- + + + + +

\[\text{Rapamycin: (nM)} \]

b, d, and f) or calyculin A (lane e) resulted in a significant induction of Stat5b Ser-193 phosphorylation (Fig. 4A). To further define the phosphatase involved, PP1- or PP2A-specific inhibitors were administered, including OA and FOS, which selectively block PP2A, and TAU, which preferentially inhibits PP1 (32). For this analysis, YT cells were treated with inhibitory concentrations of 150 nM OA (lanes c and d), 25 nM FOS (lanes e and f), or 1 μM TAU (lanes g and h) for 60 min prior to stimulation without or with IL-2 for 15 min (Fig. 4B). When compared with untreated controls (lanes a and b), blockade of PP2A by OA and FOS showed constitutively phosphorylated Stat5b Ser-193 at levels comparable with that of IL-2-induced phosphorylation (lane b), whereas no change was observed following treatment with the PP1 inhibitor TAU (lanes g and h) (Fig. 4B). Collectively, these data suggest that PP2A negatively regulates Stat5b Ser-193 phosphorylation.

Phosphorylation of Ser-193 Is Required for Maximum Stat5b Transcriptional Activity—To determine the functional role of Stat5b Ser-193 phosphorylation, a HEK293 reconstitution system was employed using phospho-deletion (S193A) and phospho-mimetic (S193E) mutations of Stat5b. Plasmids encoding Jak3, IL-2Rβ, and IL-2Rγ were co-transfected into HEK293 cells with wild-type (WT) (lanes a and b), S193A (lanes c and d), or S193E (lanes e and f) forms of Stat5b. At 48 h after transfection, cells were stimulated in the absence (−) or presence (+) of IL-2 for 15 min. Western blot analysis of Stat5b immuno-
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Figure 4. PP2A, but not PP1, negatively regulates Stat5b Ser-193 phosphorylation in YT cells. A, cells were pretreated without (lanes a and b) or with DMSO (lanes c and d) or 50 nM CA (lanes e and f) for 1 h before stimulation without (lanes a, c, and e) or with IL-2 (lanes b, d, and f) for 15 min at 37°C. The cells were fixed and stained with α-phospho-Tyr Stat5 (Cy2, green), α-Ser(P)-193 (α-pS193) Stat5b (Cy3, red), and DAPI (blue) and visualized by confocal microscopy. The overlay (bottom panel) shows co-localization of phospho-Tyr Stat5 and Ser(P)-193 Stat5b and visualized by confocal microscopy. Representative data from three independent experiments are shown.

B, YT cells were left untreated (lanes a and b) or pretreated with 150 nM OA (lanes c and d), 25 nM FOS (lanes e and f), or 1 μM TAU (lanes g and h) for 1 h before stimulation without (−) or with (+) IL-2 for 15 min at 37°C. The cells were fixed and analyzed using α-Ser(P)-193 Stat5b and DAPI as described above. Immunofluorescent images were captured using PASCAL software on a Zeiss LSM 510 Meta confocal microscope at 63× magnification. Representative data from three independent experiments are shown.

cipitates using α-phospho-Tyr Stat5 indicated that when compared with WT, S193A and S193E mutations did not affect IL-2-induced tyrosine phosphorylation (Fig. 5A). Reprobing this blot with α-Stat5b confirmed that similar amounts of Stat5b protein were immunoprecipitated and measured (Fig. 5A). Upon tyrosine phosphorylation, Stat5 proteins dimerize, translocate to the nucleus, and bind specific promoter elements that stimulate transcription of target genes that control cell growth and differentiation (48, 49). Therefore, EMSA were utilized to determine whether Ser-193 phosphorylation was important for Stat5 DNA binding activity. For these assays, nuclear extracts were isolated from reconstituted HEK293 cells treated without (−) or with (+) IL-2 for 30 min and incubated with radiolabeled probe corresponding to the Stat5 binding element in the β-casein gene promoter. This complex was separated within a nondenaturing gel to determine its DNA binding activity. When compared with WT (lanes b and c), the S193A mutation (lanes d and e) displayed significantly decreased IL-2-induced Stat5b-DNA binding activity (Fig. 5B). In addition, the S193E mutation (lanes f and g) recovered IL-2-induced Stat5b DNA binding activity (Fig. 5B). Stat5b-DNA supershift assays were performed with an amino-terminal Stat5 antibody (lane h) to confirm the presence of Stat5 in the complex, whereas normal rabbit IgG (lane i) was used as a negative control. Thus, phosphorylation of Stat5b Ser-193 is required for optimal IL-2-induced DNA binding activity. To correlate the reduced DNA binding activity with decreased transcriptional activity, Stat5b luciferase reporter assays were performed in the HEK293 reconstitution system. Reconstituted HEK293 expressing WT (lanes c and d), S193A (lanes e and f), and S193E (lanes g and h) Stat5b proteins were co-transfected with a β-casein-firefly luciferase reporter construct harboring three Stat5 response elements in tandem (Fig. 5C). The cells were also transfected with a second β-gal reporter gene under the control of a constitutive CMV promoter to control for transfection efficiency. In these experiments, each luciferase activity was normalized...
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Stat5 activation is tightly controlled by a complex interplay between tyrosine and serine/threonine kinases and phosphatases. Although significant effort has established the importance of Stat5 tyrosine phosphorylation, the role of serine phosphorylation in Stat5 function and dysfunction remains to be fully elucidated. In the present study, a novel cytokine-induced

first to the β-gal activity and second to WT Stat5b activity. Upon IL-2 stimulation, the S193A mutant Stat5b (lane f) showed a 40% decrease in Stat5b luciferase reporter activity relative to WT Stat5b (lane d) (Fig. 5C). Additionally, the Stat5b S193E (lane h) mutant displayed normal IL-2-induced transcriptional activity (Fig. 5C). As a control, cell lysates were immunoblotted with α-phospho-Tyr Stat5 to confirm equal expression (data not shown). These data suggest that Ser-193 phosphorylation is required for maximum Stat5b transcriptional activity.

Stat5 Ser-193 Is Constitutively Phosphorylated in HTLV-1-transformed T-cell Lines and Primary Hematopoietic Tumor Cells—Elevated levels of Stat5 tyrosine phosphorylation and transcriptional activity have been observed in a number of primary tumors and tumor cell lines (50–52). However, the significance of serine phosphorylation for the proto-oncogenic function of Stat5 remains unclear. To correlate hyperactive Stat5 with constitutive Stat5 Ser-193 phosphorylation, human T lymphotrophic virus type-1 (HTLV-1)-transformed cell lines and primary hematopoietic tumors were examined by α-phospho-Tyr Stat5 and α-Ser(P)-193 Stat5b-directed immunofluorescent confocal microscopy. In the absence of IL-2 stimulation, Stat5b was not tyrosine- or Ser-193-phosphorylated in naive (lane c) or quieted PHA-activated PBMCs (lane b) (Fig. 6A). However, similar to IL-2-stimulated PHA-activated PBMCs (lane a), MT-2 (lane d) and HUT-102 (lane e) cells displayed Stat5b tyrosine and Ser-193 phosphorylation in the absence of IL-2 stimulation (Fig. 6A). Interestingly, non-HTLV-1-transformed HUT-78 cells (lane f) did not display constitutive tyrosine or Ser-193 Stat5b phosphorylation (Fig. 6A). To substantiate these results, Stat5b Ser-193 and tyrosine phosphorylation was analyzed in primary patient tumor cells of hematopoietic origin. Indeed, immunofluorescent confocal microscopy analysis of tumor cells from patients diagnosed with T-cell acute lymphocytic leukemia (T-ALL) (lane d), acute myeloid leukemia (AML) (lane e), and B-cell lymphoma (lane f) showed constitutive phosphorylation of both Stat5 Tyr-694/699 and Ser-193 residues (Fig. 6B). The phosphorylation status of Stat5b Tyr-699 and Ser-193 was examined in a total of nine primary patient tumors and two control samples and is summarized in Table 1. Taken together, these data indicate that Stat5b is constitutively tyrosine-phosphorylated and Ser-193-phosphorylated in HTLV-1-transformed T-cell lines and primary hematopoietic tumor cells.

**DISCUSSION**

Stat5 activation is tightly controlled by a complex interplay between tyrosine and serine/threonine kinases and phosphatases. Although significant effort has established the importance of Stat5 tyrosine phosphorylation, the role of serine phosphorylation in Stat5 function and dysfunction remains to be fully elucidated. In the present study, a novel cytokine-induced

in A were treated without (−) or with (+) IL-2 for 6 h. Control cells were transfected with Stat5b alone (lanes a and b). At 48 h after transfection, the cells were lysed, and luciferase activities were measured and normalized to β-galactosidase activity. Statistical significance was determined using analysis of variance (*, p < 0.05). Representative data from three independent experiments are shown. Error bars indicate S.D.
serine phosphorylation site in Stat5b (Ser-193) was identified using mass spectrometry and further characterized with a site-specific phospho-specific antibody (Figs. 1 and 2). Stat5b Ser-193 was determined to be rapidly and transiently phosphorylated in response to IL-2 in YT and primary human PBMCs. Additionally, other γc cytokines, including IL-7, IL-9, and IL-15, similarly activated the phosphorylation of Stat5b Ser-193 in primary human PBMCs. Further evidence was provided that indicates phosphorylation of Stat5 Ser-193 is positively regulated by the serine/threonine kinase, mTOR, and negatively regulated by the protein phosphatase PP2A (Figs. 3 and 4). To evaluate the functional importance of Stat5b Ser-193 phosphorylation, WT, S193A, and S193E Stat5 variants were examined for their ability to bind DNA and activate transcription using EMSA and luciferase reporter assays, respectively. The S193A phospho-deletion mutation resulted in reduced IL-2-stimulated Stat5b DNA binding and transcriptional activity, which could be rescued by the S193E phospho-mimetic muta-

FIGURE 6. Stat5b Ser-193 is constitutively phosphorylated in HTLV-1-transformed tumor T-cell lines and primary leukemia and lymphoma patient tumor cells. A, immunofluorescent confocal microscopy was utilized to detect phospho-Tyr Stat5 (α-pY Stat5) and Ser(P)-193 Stat5b (α-pS193 Stat5b) in normal PHA-activated quiescent human PBMCs stimulated with IL-2 or without for 15 min (lanes a and b), non-PHA-activated PBMCs cells (lane c), MT-2 (lane d), HUT-102 (lane e), and HUT-78 (lane f). B, immunofluorescent confocal microscopy was utilized to detect phospho-Tyr Stat5 and Ser(P)-193 Stat5b in primary tumor cells isolated from patients diagnosed with ALL (lane d), AML (lane e), and B-cell lymphoma (lane f). Normal PHA-activated quiescent human PBMCs stimulated with IL-2 or without IL-2 for 15 min (lanes a and b) and non-PHA-activated PBMCs cells (lane c) served as controls. Immunofluorescent images were captured using PASCAL software on a Zeiss LSM 510 Meta confocal microscope at 63× magnification. Representative data from three independent experiments are shown.
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Clinical information and Stat5 activation status of primary human samples

Pre-existing, remnant primary human leukemia, and lymphoma cells were obtained from peripheral blood or lymph node biopsies at the time of diagnosis. Control samples were taken from healthy volunteer donors. The diagnoses were made according to French-American-British (FAB) criteria. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AMML, acute myelomonocytic leukemia; AMoL, acute monocyctic leukemia; n.a., not available; WBC, white blood cell count.

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TABLE 1

Clinical information and Stat5 activation status of primary human samples

Pre-existing, remnant primary human leukemia, and lymphoma cells were obtained from peripheral blood or lymph node biopsies at the time of diagnosis. Control samples were taken from healthy volunteer donors. The diagnoses were made according to French-American-British (FAB) criteria. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AMML, acute myelomonocytic leukemia; AMoL, acute monocytic leukemia; n.a., not available; WBC, white blood cell count.
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Many studies have focused on the mechanisms driving Stat phosphorylation and activation; however, much less is known about its negative regulation. In addition to protein tyrosine phosphatases, serine/threonine phosphatases play an important role in the regulation of Stat activation. Recently, our group and others have reported that the serine/threonine phosphatase PP2A plays a role in regulating Stat3 (58), Stat5 (32), and Stat6 (45, 46) activity. To investigate PP2A regulation of Stat5 Ser(P)-193, attenuation of serine/threonine phosphatase activity by CA treatment of YT cells revealed that dephosphorylation of Stat5b Ser-193 occurred through a PP1- or PP2A-mediated process (Fig. 4A). To delineate the specific phosphatase, YT cells were treated with pharmacological inhibitors that preferentially target PP1 (TAU) or PP2A (OA and FOS). In accordance with our previous findings, phosphorylation of Stat5b Ser-193 was induced by OA and FOS, but not TAU, indicating that PP2A was the primary phosphatase responsible for regulating Stat5b Ser-193 phosphorylation (Fig. 4B).

Constitutive activation of Stat5 has been shown to be directly involved in oncogenic transformation (14). Moreover, Stat5 serine phosphorylation was recently shown to be a prerequisite for hematopoietic transformation (59, 60). To correlate hyperactive Stat5b with constitutive Stat5b Ser-193 phosphorylation, HTLV-1-transformed cell lines and primary hematopoietic tumors were examined. HTLV-1-transformed T-cells (MT-2, HUT-102) harbor constitutively tyrosine-phosphorylated Stat5b (61). Indeed, immunofluorescent confocal microscopy analysis showed that Stat5b is constitutively phosphorylated at both Tyr-699 and Ser-193 in MT-2 and HUT102 cells (Fig. 6A). To substantiate the clinical significance of Stat5b Ser-193 phosphorylation, primary patient tumor cells from multiple hematopoietic origins were analyzed (Fig. 6B). Stat5b was found to be constitutively tyrosine- and Ser-193 phosphorylated in various tumor cell types, including precursor T-ALL, AML-M0, AML-M4, large B-cell lymphoma, precursor B-cell ALL, and hairy cell leukemia (Table 1). Interestingly, in contrast to IL-2-stimulated Stat5b that translocates to the nucleus upon activation (Fig. 2), oncogenic Stat5b localizes primarily to the cytoplasm in primary hematopoietic tumor cells (Fig. 6). This suggests that the transforming capacity of Stat5b is not limited to its role as a transcription factor in the nucleus. Indeed, recent studies have demonstrated that oncogenic Stat5 localizes to the cytoplasm and plays an important role in the activation of the PI3K-Akt-mTOR signaling pathway via association with Gab2 (62–64). Extending this model, the results presented herein suggest that upon activation, mTOR serine phosphorylates Stat5b and allows for its maximal transcriptional activation. Therefore, the Stat5-mTOR axis may play an important role in driving hematopoietic malignancy.

In summary, we have identified Stat5b Ser-193 as a novel site of cytokine-mediated phosphorylation. A phospho-antibody that specifically recognized this residue confirmed that Stat5b Ser-193 is rapidly and transiently induced in YT and primary human PBMCs. Using pharmacological inhibitors directed toward particular kinases and phosphatases, we show that phosphorylation of Stat5b Ser-193 is regulated by a mTOR/PP2A-dependent mechanism. Mechanistically, Ser-193 phosphorylation was shown to positively regulate the DNA binding and transcriptional activity of Stat5b. Lastly, Stat5b Ser-193 was found to be constitutively phosphorylated in several lymphoid tumor cell lines as well as primary leukemia and lymphoma patient tumor cells. Thus, drugs targeting the Stat5-mTOR axis could provide an alternative strategy for hematopoietic malignancies that display proto-oncogenic Stat5 activation.

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