Constitutive Activation of STAT5 by a Point Mutation in the SH2 Domain*

We previously identified a constitutively active form of STAT (signal transducer and activator of transcription) 5A by polymerase chain reaction-driven random mutagenesis followed by retrovirus-mediated expression screening, which had two point mutations in the DNA-binding and transcriptional activation domains, and was designated STAT5A*6. STAT5A*6 showed markedly elevated DNA binding and transactivation activities with stable tyrosine phosphorylation and nuclear accumulation, and conferred autonomous cell growth on interleukin 3-dependent Ba/F3 cells. We now report another constitutively active mutant, STAT5A-N642H which has a single point mutation (N642H) in its SH2 domain, identified using the same strategy as that used to identify STAT5A*6. STAT5A-N642H showed identical properties to those of STAT5A*6 both biochemically and biologically. Interestingly the mutation in STAT5A-N642H resulted in restoration of the conserved critical histidine which is involved in the binding of phosphoryrosine in the majority of SH2-containing proteins. Introduction of an additional mutation (Y694F) to STAT5A-N642H, which disrupted critical tyrosine 694 required for dimerization of STAT5, abolished all the activities manifested by the mutant STAT5A-N642H, which indicates that dimerization is required for the activity of STAT5A-N642H as was the case for the wild-type STAT5A. The present findings also show that different mutations rendered STAT5A constitutively active, through a common mechanism, which is similar to that of physiological activation.

The STAT1 protein is a transcription factor which is activated upon stimulation with various cytokines, and plays a central role in cytokine signaling (1–3). The STAT family consists of seven known members, including closely related STAT5A and STAT5B. Once ligands bind to their cognate receptors, Janus kinases (JAKs) and STATs are phosphorylated successively. The phosphorylated STAT protein forms homo- or heterodimer through intermolecular interaction between the SH2 domain and the phosphoryrosine of the STAT. The dimerized STAT then translocates into the nuclei and binds to promotor regions of target genes to activate transcription. Since phosphorylated STAT is rapidly dephosphorylated, transactivation of gene expression by STAT is generally transient (4). On the other hand, it was reported that human leukemias were frequently associated with the constitutive activation of STATs (5–8), albeit the role of activated STATs in leukemogenesis being unknown.

Although gene targeting is a powerful strategy in analyzing biological roles of the gene product, redundancy of functional genes occasionally masks the phenotype of the null mutation of the gene. In the case of STAT5A and STAT5B- doubly disrupted mice, fetal anemia and apoptosis of erythroid progenitors occurred. However, no gross abnormalities were found in hematopoietic systems of adult mice (9–12). Therefore, biological functions of STAT5 in hematopoietic cells have remained to be elucidated.

Our group identified a constitutively active STAT5A mutant (STAT5A*6) by polymerase chain reaction (PCR)-driven random mutagenesis followed by retrovirus-mediated expression screening (13). STAT5A*6 harbors two point mutations, one in the transactivation domain (S710F) and the other in the DNA-binding domain (H298R). The interleukin-3 (IL-3)-dependent murine pro-B cell line Ba/F3 can proliferate autonomously in the absence of IL-3 after transduction with STAT5A*6. We recently found that STAT5A*6 also provoked differentiation and apoptosis in Ba/F3 cells upon IL-3 stimulation with prolonged expression of growth-suppressive genes induced by STAT5 (14). We have now identified and characterized another constitutively active mutant, STAT5A-N642H, which harbors a point mutation on or very close to the phosphotyrosine-binding site in the SH2 domain and has the identical phenotype to that of STAT5A*6. In addition, substitution of Tyr694, the phosphorylation of which is required for dimerization and activation of STAT5, abolished the constitutive activity of STAT5A-N642H. These findings indicate that activation of these mutant STAT5s mimicked the physiological activation of STAT5, an event not caused by gain-of-function mutations.

**Experimental Procedures**

Construction of the STAT5A Mutants—Mutations were introduced into the mouse STAT5A sequence by PCR-driven random mutagenesis (13, 15, 16). The pMX-STAT5A DNA was used as a template, and a 5′ vector primer, pMX3′ (5′-CCCGGGGTTGGACCCATCTCT-3′), and a 3′ vector primer, pMX3′ (5′-CCCTTTTTCTGGAGACT-3′), were used to

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amplify the full-length sequence of STAT5A. PCR was run for 35 cycles (1 min at 94 °C, 2 min at 58 °C, and 3 min at 72 °C) with recombinant Taq DNA polymerase (Perkin-Elmer), under standard conditions, except that the deoxynucleotide triphosphate concentration was 400 μM. The average frequency of point mutations ranged from 1/600 to 1/1200 under these conditions (data not shown). The constitutively active STAT5As were identified by the ability to induce IL-3-independent growth of IL-3-dependent Ba/F3 cells in retrovirus-mediated expression screening, as described (13). One such mutant harboring a point mutation (N642H) in the SH2 domain was designated STAT5A-N642H. We introduced an additional mutation to STAT5A-N642H by site-directed mutagenesis using a high fidelity DNA polymerase (Pyrococcus furiosus) to acquire amino acid substitution from Tyr694 to Phe (STAT5A-N642H/Y694F) (17). STAT5B-N642H was constructed by PCR-based site-directed mutagenesis. DNA sequences of all the constructs were confirmed by sequencing.

**Cells—**Ba/F3 cells were maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 2 ng/ml murine IL-3 (mIL-3) (pro-vided by DNAX Research Institute). A granulocyte-macrophage colony stimulating factor (GM-CSF)-dependent human leukemic cell line, TF-1 (18), was maintained in RPMI 1640 medium containing 10% FCS and 5 ng/ml human GM-CSF (R & D Systems). An ectropic retrovirus packaging cell line, BOSC23 (19), was maintained in Dulbecco’s modified Eagle’s medium containing 10% FCS and 2% v/v horse serum. Cells were transfected by electroporation at 960 microfarads and 300 V with 10 μg of a reporter plasmid consisting of a luciferase gene under the control of the β-casein promoter harboring STAT5-binding sites, 3 μg of Rous sarcoma virus long terminal repeat-driven β-galactosidase plasmid to monitor transfection efficiency, and 10 μg of each effector plasmid (STAT5A wild type, STAT5A-N642H, STAT5B-N642H) at room temperature. After leaving the cells for 12 h in the presence of 10% FCS and IL-3, the cells were divided into two groups, one group was maintained in RPMI 1640 medium with 0.5% bovine serum albumin in the absence of IL-3 for 12 h, and the other was stimulated with 4 ng/ml IL-3 without FCS for the last 6 h after 6 h starvation. Cell lysates were then prepared and subjected to luciferase and β-galactosidase assays. Transfection efficiency was normalized with the β-galactosidase activity. Each experiment was done three times.

**Northern Blotting—**Total RNA was isolated from Ba/F3 cells before and after IL-3 stimulation using RNeasy kits (Qiagen). Thirty μg of total RNA was denatured in 50% formamide at 60 °C for 15 min, separated on 1% agarose with 6% formaldehyde gel by electrophoresis, and blotted onto Hybond-N membrane (Amersham Pharmacia Biotech). The membrane was probed with the randomly primed (Stratagene) ^32P-labeled cDNA fragment at 42 °C in solution containing 50% formamide, 3 × Denhardt’s solution, 5 × SSC, 1% SDS, and 200 μg/ml denatured salmon sperm DNA. After hybridization, the membrane was washed in 0.1 × SSC, 0.1% SDS at room temperature, and autoradio- graphed. The fragments of mouse glyceraldehyde-3-phosphate dehydrogenase were used as probes (14).

**TUNEL Assays—**Cells were pelleted and fixed for 30 min at room temperature in 3% paraformaldehyde. TUNEL assays (Takara) were performed according to the manufacturer’s instructions and analyzed on a FACScan flow cytometer (Becton Dickinson).

**RESULTS**

**Identification of a Constitutively Activated STAT5A Mutant Harboring a Point Mutation in the SH2 Domain—**We identified several STAT5A mutants that induced IL-3-independent growth of Ba/F3 cells, using the method described under “Experimental Procedures” (13). Among them, STAT5A-N642H has a single point mutation in the SH2 domain, which results in amino acid substitution from Asn412 to His (Fig. 1). To confirm that this mutation alone was sufficient to cause constitutive activation of STAT5A, we introduced this point mutation into STAT5A and cloned in the pMX vector (15). High titer retroviruses harboring STAT5A-N642H were produced with a transient retrovirus packaging cell line, BOSC23 (19), and Ba/F3 cells were infected with these retroviruses (20). The infection efficiencies of Ba/F3 cells in the experiments were 20–30%, as assessed by simultaneous experiments using a control vector pMX-EGFP. Twenty-four hours after the infection, the cells were deprived of IL-3 to determine the potential to induce factor-independent growth of Ba/F3 cells. The cells transduced with pMX STAT5A-N642H survived and proliferated well in the absence of IL-3, while those transduced with
pMX STAT5A WT (wild-type) or pMX STAT5A-N642H/Y694F did not (Fig. 2A). In addition, STAT5A-N642H induced factor-independent growth of a GM-CSF-dependent human leukemic cell line TF-1 after retroviral infection (Fig. 2B). Thus a single point mutation in the SH2 domain was sufficient to render Ba/F3 and TF-1 cells factor-independent. The difference in the growth rate between IL-3-driven and STAT5A-N642H-driven Ba/F3 cells (Fig. 2A) and that between GM-CSF-driven and STAT5A-N642H-driven TF-1 cells (Fig. 2B) can be explained by the absence of adequate Ras-Raf-MAPK signal in the latter cells (13). We also introduced the same point mutation to STAT5B (23) to acquire the amino acid substitution from Asn640 to His. Ba/F3 cells transduced with this STAT5B mutant also proliferated in the absence of IL-3 (data not shown).

Cytokine Stimulation Was Not Required for the Constitutive Phosphorylation of STAT5A Mutant—We examined tyrosine phosphorylation of STAT5A in factor-independent Ba/F3 cells expressing the Flag epitope-tagged STAT5A-N642H, using immunoprecipitation and Western blot analysis (Fig. 3a). In Ba/F3 cells, STAT5A-N642H-Flag was constitutively phosphorylated on the tyrosine residues in the absence of IL-3, and prolonged hyperphosphorylation of tyrosine residues after IL-3 stimulation was observed as in the case of STAT5A-Flag (13). The degree of tyrosine phosphorylation of STAT5A-N642H-Flag in Ba/F3 cells without IL-3 was nearly as strong as that seen in Ba/F3 cells expressing the wild-type STAT5A-Flag after IL-3 stimulation. Next we asked if Tyr694, which is essential for dimerization, was required for the activity of STAT5A-N642H by introducing the Y694F mutation (17) in STAT5A-N642H-Flag (Fig. 1). STAT5A-N642H/Y694F-Flag did not give constitutive or prolonged phosphorylation of STAT5A. Tyrosine phosphorylation observed in STAT5A-N642H/Y694F-Flag in response to IL-3 stimulation probably reflects that of endogenous STAT5A or B which was co-immunoprecipitated with transduced STAT5A-N642H/Y694F-Flag, or that of residues other than the Y694F in STAT5A-N642H/Y694F-Flag. To test these possibilities, we examined tyrosine phosphorylation of STAT5A in COS-7 cells using an anti-phospho-STAT5A/B(Y694/Y699) antibody after transfection with STAT5A-Flag constructs and human MPL (the receptor for TPO) expression vector for thrombopoietin (TPO) stimulation (Fig. 3b). As in Ba/F3 cells, STAT5A-N642H-Flag, but not STAT5A-N642H/Y694F-Flag was constitutively phosphorylated on the critical tyrosine residues in the absence of TPO stimulation. Phosphorylation of Tyr694/Tyr699 was observed in the immunoprecipitates of the cells expressing wild-type STAT5A-Flag or STAT5A-N642H/Y694F-Flag only in the presence of TPO stimulation. This result indicated that co-immunoprecipitation of the endogenous STAT5A or -B gave rise to tyrosine phosphorylation of Tyr694/Tyr699 observed in the cells expressing STAT5A-N642H/Y694F-Flag. However, we cannot exclude the possibility that the other tyrosines of the STAT5A-N642H/Y694F-Flag are also phosphorylated.

The Mutant STAT5 Was Predominantly Located in Nuclei and Had a Potent Transactivational Ability—Because phosphorylation of STATs is required for the binding of STATs to the promoter elements of target genes (17), we examined whether STAT5A-N642H bound the target sequence without IL-3 stimulation, using Ba/F3 transfectants expressing wild-type and mutant STAT5As. As shown in Fig. 4, the wild-type STAT5A bound the target sequence only in the presence of IL-3, while STAT5A-N642H bound the target sequence even in the absence of IL-3. A supershift experiment confirmed that STAT5A-N642H is involved in the complex formation. We next investigated the intracellular localization of mutants of STAT5A in NIH3T3 cells, using fusion constructs with EGFP. In the absence of IL-3, STAT5A-N642H-EGFP was predominantly localized in the nuclei of NIH3T3 cells, using fusion constructs with EGFP. In the absence of IL-3, STAT5A-N642H-EGFP was mainly localized in the nuclei of NIH3T3 cells (Fig. 5C), while STAT5A wild-type EGFP (Fig. 5A) and STAT5A-N642H/Y694F-EGFP (Fig. 5E) showed predominant nuclear accumulation. To determine whether STAT5A-N642H is transcriptionally active in the absence of IL-3, we examined transactivation of the...
b-casein promoter in Ba/F3 cells, using luciferase assay (17) (Fig. 6). The transcriptional activity induced by STAT5A-N642H was 25-fold higher than that induced by STAT5A wild-type or STAT5A-N642H/Y694F in the absence of IL-3, and was as potent as that induced by STAT5A wild-type in the presence of IL-3. STAT5A-N642H/Y694F did not behave as a dominant negative mutant, rather it had activity comparable to that of the wild type in this assay, suggesting that heterodimerization of STAT5A-N642H/Y694F with endogenous STAT5A or STAT5B had occurred. This result is consistent with that of the Western blot analysis shown in Fig. 3. Thus STAT5A-N642H activated transcription of the target gene without IL-3 stimulation, and Tyr694 required for dimerization of STAT5A was necessary for transactivation in STAT5A-N642H as was the case with the wild-type.

STAT5A-N642H Highly Induced Expression of Target Genes—Next we studied the effect of STAT5A-N642H expression on induction of target genes by Northern blot analysis (Fig. 7). Since OSM (24), pim-1 (25), bcl-x (26), JAB/SSI-1/ SOCS-1 (27–29), and CIS (30) are target genes of STAT5A, the expression of these genes in Ba/F3 cells in the absence or presence of IL-3 was examined. We also examined the expression of c-myc that is rapidly induced by IL-3 stimulation (31). Ba/F3 cells expressing the wild-type STAT5A or STAT5A-N642H were de-
prived of IL-3, and then stimulated with IL-3. In Ba/F3 cells expressing STAT5A-N642H, pim-1, bcl-x, and c-myc were expressed in the absence of IL-3, while these genes were not expressed in Ba/F3 cells expressing wild-type STAT5A in the absence of IL-3. After IL-3 stimulation, expression of JAB/SSI-1/SOCS-1, CIS, and OSM was more strongly induced in Ba/F3 cells expressing STAT5A-N642H than in Ba/F3 cells expressing the wild-type STAT5A.

**DISCUSSION**

Cytokines have a wide variety of biological activities including proliferation, differentiation, and immune responses. Binding of cytokines to cell surface receptors leads to rapid increases in phosphorylated proteins. Among them, tyrosine phosphorylation of proteins play important roles in signaling systems (32, 33). Various intracellular signaling proteins have

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**FIG. 6.** Transactivational activities of the STAT5A mutants on the β-casein promoter in Ba/F3 cells. Luciferase activities in the lysates of Ba/F3 cells transfected with the pMX neo vector (pMX neo), the pMX neo STAT5A wild-type-Flag (WT), the pMX neo STAT5A-N642H-Flag (N642H), and the pMX neo STAT5A-N642H/Y694F-Flag (N642H/Y694F) were examined before and after IL-3 stimulation as described under “Experimental Procedures.” Transfection efficiency was normalized with the results of a simultaneous β-galactosidase assay. The results shown are averages of three independent experiments, with standard deviations.

**FIG. 7.** Expression and induction of various genes in Ba/F3 cells expressing either STAT5A wild-type-Flag (WT), or STAT5A-N642H-Flag (N642H), before and after IL-3 stimulation. Total RNA was isolated from Ba/F3 cells, and 30 μg of total RNA was separated through 1% agarose, 6% formaldehyde gel. Expression of OSM, pim-1, c-myc, bcl-x, JAB, CIS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was assessed by Northern blot analysis.

**FIG. 8.** IL-3-induced apoptosis in Ba/F3 cells expressing the STAT5A-N642H-Flag. A, phase-contrast microscopy of the cells expressing the STAT5A-N642H-Flag before and after IL-3 stimulation (3 ng/ml for 41 h) is shown. Original magnification was ×200. B, TUNEL assay of the cells expressing the STAT5A-N642H-Flag before (upper right panel) and after (lower right panel) IL-3 stimulation (3 ng/ml for 41 h). The samples of parental Ba/F3 cells cultured with (upper left panel) or without IL-3 for 24 h (lower left panel) are shown as reference. The percentage of TUNEL-positive cells is indicated in each panel.

**Ba/F3 Cells Expressing STAT5A-N642H Underwent Apoptosis after IL-3 Stimulation—**We earlier reported IL-3-induced apoptosis of Ba/F3 cells expressing STAT5A1*6. Prolonged expression of JAB/SSI-1/SOCS-1 by STAT5A1*6 was found to be responsible for the IL-3-induced apoptosis (14). Consistent with the finding of prolonged expression of JAB/SSI-1/SOCS-1 after IL-3 stimulation in Ba/F3 cells expressing STAT5A-N642H (Fig. 7), the cells showed apoptotic appearance within 41 h after IL-3 addition, as shown in Fig. 8A. The TUNEL assay detected *in situ* fragmented DNA through fluorescent end labeling of fragmented DNA in intact nuclei as fluorescein isothiocyanate positive cells by a flow cytometer. The proportion of TUNEL-positive cells in Ba/F3 cells expressing STAT5A-N642H was increased from 0.1% without IL-3 to about 25% at 41 h after IL-3 addition (Fig. 8B).
Src homology 2 (SH2) domains which play critical roles in activation and localization of intracellular molecules by specifically binding to their partners with distinct phosphorylated tyrosine residues. Such specific bindings occur between activated receptors and direct downstream signaling molecules or adapter molecules. These protein-protein interactions are critical to transmit activated intracellular signals (34). In most cases, specificity of the binding derives from the specific interaction between the phosphorytrosine (Tyr(P))-containing peptide sequence and the Tyr(P)-binding site of the SH2 domain. In particular, the Tyr(P) and the neighboring C-terminal three amino acids are important for primary specificity of SH2 interactions (35), and the pocket structure of the SH2 domain interacts with the prolined structure of the Tyr(P) (36). The binding pocket of the SH2 domain contains positively charged critical amino acids to catch negatively charged Tyr(P) (36–42). In the present report, we focus on a constitutively active mutant of an SH2-containing transcription factor STAT5, STAT5A-N642H, that harbors a point mutation in its SH2 domain.

STAT5A-N642H was constitutively phosphorylated and activated in the absence of cytokine stimulation. The mutation of STAT5A-N642H is localized to an important residue (βD4) in the 4th β-sheet of the SH2 domain (Fig. 9), which is very close to the Tyr(P)-binding loop in the three-dimensional structure (43, 44). It is noteworthy that Tyr(P) is known to interact with positively charged residues at positions of αa2, βb5, βb4, and βb6 of the SH2 domain (36, 39–42). Although the amino acid residue at βD4 dose not match to the concensus sequence of the SH2 domain in the wild-type STAT5A, the N642H mutation results in restoration of the prototype structure of the SH2 domain with critical four basic amino acids to interact with Tyr(P). The enhanced activity of STAT5A-N642H by the N642H mutation is reminiscent of the enhanced activity of c-Src by the H201R mutation at the corresponding site (36, 43). Interestingly, introduction of two cysteine residues (A662C and N664C) within the C-terminal loop of the SH2 domain of STAT3 (designated STAT3-C) has recently been reported to result in spontaneous dimerization and constitutive activation of STAT3 (47). In this case, double mutations contribute to dimerization of STAT3 without tyrosine phosphorylation through sulfhydryl bonds between monomers. It was also reported that tyrosine phosphorylation of STAT molecules was not required for activation of STAT6-estrogen receptor fusion protein and the STAT3-gyrase B chimera that can be inducibly activated by 4-hydroxytamoxifen and coumamycin, respectively (48, 49). In the present study, however, an additional mutation of Y694F revealed the necessity for the critical tyrosine residue (17) which is required for dimerization of STAT5A-N642H; STAT5A-N642H/Y694F did not support proliferation of Ba/F3 cells nor activate transcription in the absence of IL-3. These findings indicate that tyrosine phosphorylation is a prerequisite for efficient or stable dimerization of STAT5A-N642H, which in turn leads to activation of the molecule. This is in sharp contrast to the constitutively active mutant STAT3-C (47), STAT6-ER (48), and STAT3-gyrase B (49), of which dimerization was achieved through rather artificial ways without significant tyrosine phosphorylation. In this context, activation of STAT5A-N642H which requires tyrosine phosphorylation of the molecule is more physiological.

STAT5 regulates many genes associated with cell proliferation and differentiation. pim-1 and bcl-xL, which are involved in anti-apoptotic effects and inducing cell proliferation (50–53), were expressed even in the absence of IL-3 in Ba/F3 cells expressing STAT5A-N642H, and this would induce IL-3-independent cell growth of the Ba/F3 cells as was the case in Ba/F3 cells expressing STAT5A1*6 (14). Dysregulation of STAT5 signaling is implicated in certain stages of tumorigenesis, including leukemogenesis (5–8, 54–59). Constitutively activated STAT5 mutants will lead to a better understanding of molecular mechanisms of STAT5 functions under the physiological and pathological conditions, and prospects for treatments of some diseases can be devised.

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