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Tyrosine Phosphorylation Is Required for Functional Activation of Disulfide-Containing Constitutively Active STAT Mutants†

Forrester J. Liddle,‡ James V. Alvarez,‡ Valeria Poli,§ and David A. Frank*,‡

Department of Medical Oncology, Dana-Farber Cancer Institute, Departments of Medicine, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115, and Department of Genetics, Biology, and Biochemistry, University of Turin, Via Santena 5 bis, Torino, Italy 10126

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ABSTRACT: Aberrant activation of STAT transcription factors has been implicated in a variety of cancers. Constitutively active forms of STAT1 and STAT3 (STAT1C and STAT3C) have been developed to determine the effects of STAT activation in isolation from other cytokine-stimulated signaling pathways. These mutants were created by engineering cysteine residues into the carboxy terminus of each STAT molecule, allowing a hypothesized disulfide bond to form between two unphosphorylated monomers. To determine whether the presence of cysteine residues is sufficient to allow for functional activation in the absence of tyrosine phosphorylation, we developed STAT1C and STAT3C mutants that are unable to be phosphorylated on the critical tyrosine residue. Without the tyrosine residue, cysteine containing constitutive STAT mutants failed to transactivate STAT target genes. Furthermore, transfection of STAT dominant negative mutants prevented the activation of STAT1C and STAT3C. Cytokine-induced activation of STAT1C and STAT3C was dramatically prolonged when compared to wild-type proteins and led to extended STAT-dependent gene activation. These data show that tyrosine phosphorylation is required for activation of STAT1C and STAT3C. Additionally, these findings suggest the existence of basal phosphorylation that is a dynamic process that involves both phosphorylation and dephosphorylation. The constitutive STAT mutants likely show heightened activity because of the cysteine residues stabilizing these dimers and preventing dephosphorylation, resulting in the accumulation of trancriptionally active STAT dimer complexes.

The development of spontaneously activated STAT molecules has allowed for a number of important advances to occur in understanding the molecular function and biological role of these transcription factors (*1*−*4*). Most importantly, the finding that constitutively activated STAT3 was sufficient to transform fibroblasts has clearly demonstrated the central role that this protein can play in oncogenesis (*1, 2, 5*−*7*). Making use of the crystal structure of a STAT dimer (*8*), these mutants were engineered to have cysteine residues introduced to allow for this so-called STAT3C to dimerize in the appropriate conformation without the need for tyrosine phosphorylation. Thus, the expression of these constructs, without further stimulation, is sufficient for STAT3 DNA binding and gene activation to occur (*1*).

However, spontaneous intracellular formation of disulfide-bonded STAT molecules seems improbable for several reasons. The intracellular milieu is a relatively reducing environment, making spontaneous formation of these bonds unlikely (*9*). Also, given the relatively low concentration of STATs and the high concentration of cysteine side chains from other proteins, the formation of STATC dimers in the correct conformation would seem unfavorable. Recent studies demonstrate that STAT1 molecules exist as dimers prior to activation (*10*−*12*). These dimers appear to have an anti-parallel structure, whose formation appears necessary for dephosphorylation. While in this conformation, the carboxy termini of the two STAT molecules would appear to be unfavorably positioned for a disulfide bond to form spontaneously.

An alternate model can be proposed to explain the clear activity of these proteins without exogenous stimulation. Evidence in a number of systems suggests that low levels of tyrosine phosphorylation may occur in a cell under basal conditions, balanced by dephosphorylation (*13*−*16*). It can be hypothesized that the disulfide bond formed between cysteine residues preserves these transient phosphorylation events, thereby allowing apparent spontaneous activity of these STAT mutants.

In this study, we examine the requirement for tyrosine phosphorylation in these cysteine-containing mutants of STAT1 and STAT3. We find that these constitutively active STATs are tyrosine-phosphorylated in the absence of exogenous stimulation and that this phosphorylation is necessary for their function. Furthermore, the cysteine-containing

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* To whom correspondence should be addressed: Dana-Farber Cancer Institute, Room M552B, 44 Binney Street, Boston, MA 02115. Telephone: (617) 632-4714. Fax: (617) 632-6356. E-mail: david_frank@dfci.harvard.edu.

‡ Department of Medical Oncology, Dana-Farber Cancer Institute, Departments of Medicine, Harvard Medical School and Brigham and Women's Hospital.

§ University of Turin.

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mutants show enhanced DNA binding compared to wild-type STATs, and this increased interaction with DNA is associated with slowed dephosphorylation and prolonged activation of a reporter gene under the control of a STAT-dependent promoter. Thus, cysteine-containing STAT mutants show enhanced and constitutive function through the stabilization of spontaneously occurring tyrosine phosphorylation.

MATERIALS AND METHODS

Plasmids. All mutagenesis was performed using the Stratagene QuikChange XL site-directed mutagenesis kit. STAT1C was created from a pRC-CMV STAT1 vector using the primer 5′-CCCTGACATCATTCGCAATTACAAAGTCATGGCTTGTGAGTGTATTCCGGAGAATCCCCTGAAGTATCTGTATCC and a 3′ primer with its complementary sequence; two cysteine residues were substituted for N658 and A656. This plasmid was further mutated to contain a Y701F substitution, STAT1C(Y → F), using the primer 5′-GGCCCTAAAGGAACTGGATTTATCAAGACTGAGTTGATTTCTGTGTCTGAAGTTCACCCTTCCAGGCTTCAGACCACAGACAACCTGCTCCCC and a 3′ primer with its complementing sequence. STAT3C (kindly provided by Jacqueline Bromberg, Memorial Sloan-Kettering, New York, NY) was mutated to contain a Y705F substitution using the primer pairs 5′-CCCGAAGCCGACCCAGGTAGTGCTGCCCCGTTCCTAAAGACCAAGTTCATCTGTGTGACACCAACGACC and its complement. pRC-CMV STAT3 was obtained from James Darnell (Rockefeller University, New York, NY), and a pRC-CMV STAT3 containing the Y705F was created using the same primers as above. m67 pTATA TK-luc was kindly provided by J. Bromberg. phRL TK-luc was from Promega (Madison, WI).

Cell Culture, Transfection, and Cytokines. U3A cells, a gift from George Stark (Cleveland Clinic, Cleveland, OH), and STAT3-deleted mouse embryonic fibroblasts (MEFs)[1] (*17*) were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum and 1% *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). STAT3 and STAT3-C with a C-terminal FLAG epitope were cloned into the retroviral vector pLNCX2 (Clontech). The retroviral supernatant was prepared as described (*5*). To infect STAT3 null MEFs (*17*), 1 mL of retroviral supernatant was added to cells on 10 cm tissue culture plates in 4 mL of media supplemented with Polybrene (4 μg/mL). After 4 h, 5 mL of media was added to the cells, and 24 h later, the medium was changed and G418 (500 μg/mL) was added. Cells were selected for 1 week in G418, and resistant cells were pooled and analyzed for STAT3 and FLAG expression.

Transient transfections were done on confluent cells on 6-well plates using LipofectAMINE2000 (Invitrogen) with 2 μg of m67 pTATA TK-luc and 2 μg of the indicated pRC-CMV-expressing STAT plasmid (1 μg of each of two STAT plasmids was simultaneously transfected) along with 0.4 μg of phRL TK-luc.

U3A cells were treated with human interferon (IFN) γ at a concentration of 500 units/mL, and MEFs were treated with murine Oncostatin M (OsM) at a concentration of 25 ng/mL.

Luciferase Expression. U3A cells and MEFs were transfected using lipofectamine 2000 (Invitrogen) with 2 μg of m67-luc reporter construct and 2 μg of the indicated pRC-CMV-expressing STAT1 plasmid along with 0.4 μg of pRC-CMV (Promega) as a transfection control. After 3 h, the transfection mix was removed and adherent cells were resuspended in DMEM containing 10% fetal calf serum, 1% penicillin/streptomycin, and 1% HEPES. These cells were then plated on 96-well plates followed by the indicated cytokine treatment. Luciferase was measured using a dual-luciferase assay kit (Promega) and a Luminoskan Ascent luminometer. Results are standardized to renilla luciferase expression to control for transfection efficiency.

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared by resuspending cells in hypotonic buffer [10 mM Tris at pH 7.4, 10 mM NaCl, 6 mM $MgCl_2$, 1 mM β-mercaptoethanol (βME), 1 mM sodium orthovanadate, 10 μg/mL PMSF, 2 μg/mL pepstatin, 2 μg/mL leupeptin, and 10 mM aprotinin] followed by incubation on ice for 5 min. Cells were centrifuged for 10 s at 12000*g*, resuspended in hypotonic buffer, and disrupted using a Dounce homogenizer (Type B pestle, 30 strokes; Wheaton, Millville, NJ). The nuclei were collected by centrifugation for 10 s at 12000*g* and washed once with hypotonic buffer. The nuclear pellet was resuspended in 1 pellet volume of high salt buffer [20 mM HEPES at pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 10 μg/mL phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, and 1 mM βME] and incubated for 30 min at 4 °C followed by centrifugation for 3 min at 12000*g*. A total of 2 μL of nuclear extract was incubated with a double-stranded ^{32}P-labeled oligonucleotide (1 ng) *sis*-inducible element (SIE), (5′-TCGAGTCGACATTTCCCGTAAATCGTCGA-3′ and its complement) in 10 μL of binding buffer [25 mM HEPES at pH 7.9, 100 μM ethylene glycol tetraacetic acid (EGTA), 200 μM $MgCl_2$, 500 μM dithiothreitol, 1 μg/mL bovine serum albumin (BSA), 0.2 μg/μL poly dI:dC, 1% Ficoll, and 0.1 μg/μL salmon sperm DNA] for 15 min at room temperature. For supershift analysis, the nuclear extracts were incubated with 1 μL of anti-FLAG antibody during the binding reaction. To determine the off rate of STATs bound to DNA, an unlabeled SIE probe was added in 100-fold molar excess. Protein−DNA complexes were loaded onto a nondenaturing gel immediately after incubation with an unlabeled probe, and a 500 V field was applied for 5 min, whereupon the field was decreased to 100 V until the next complex was loaded. After all of the complexes were loaded, a 500 V field was applied until the free probe migrated to the bottom of the gel. Protein−DNA complexes were detected by autoradiography and quantified using a phosphoimager and Kodak *Quantity One* software. Values were measured in counts/mm^2, and a local background subtraction method was used to normalize for background intensity.

Western Blot Analysis. Cells were lysed in a radioimmunoprecipitation assay buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS] containing 1 mM PMSF, 1 μg/mL pepstatin, and 1 mM

[1] Abbreviations: MEF, mouse embryo fibroblast; βME, β-mercaptoethanol; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SIE, *sis*-inducible element; EGTA, ethylene glycol tetraacetic acid; BSA, bovine serum albumin; IFN, interferon; OsM, oncostatin M; DN, dominant negative.

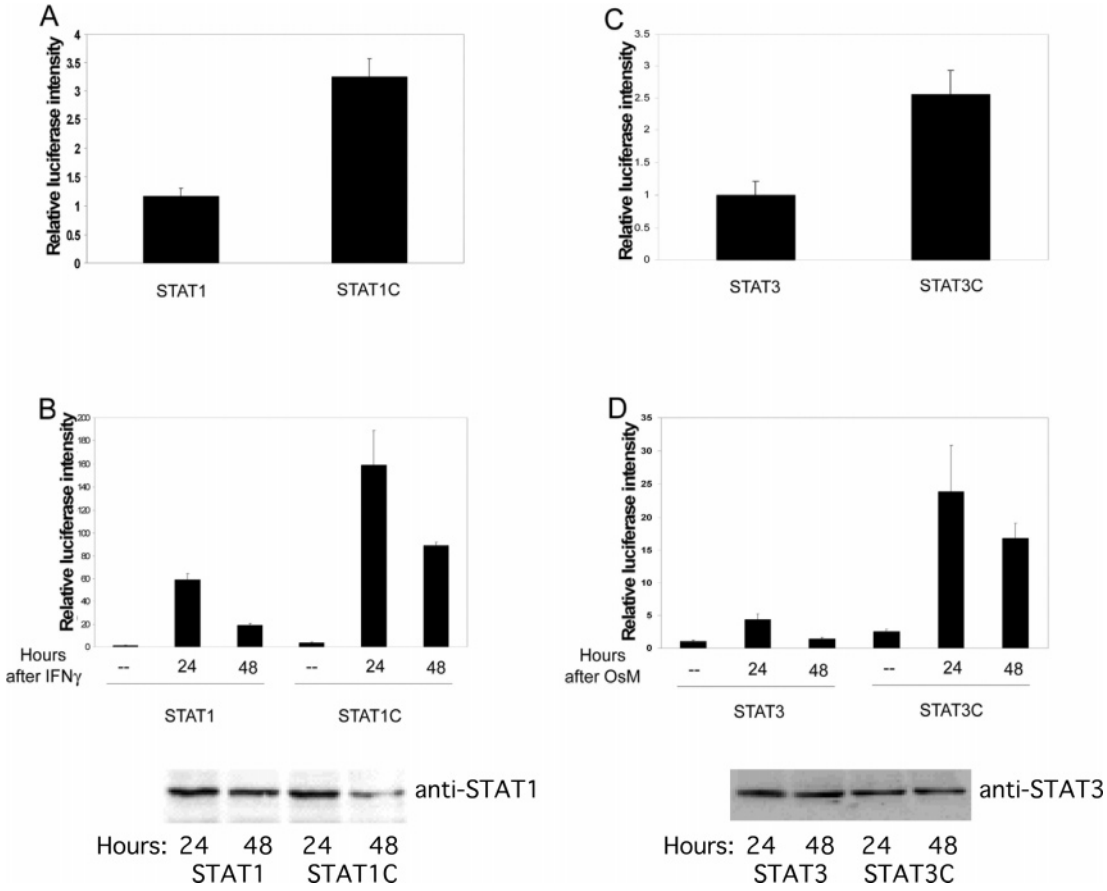

FIGURE 1: STATC mutants show enhanced and prolonged activity. (A) U3A cells were transiently transfected with a STAT responsive (m67) firefly luciferase reporter along with the indicated STAT1 construct and a control plasmid expressing renilla luciferase. Firefly luciferase activity (normalized to renilla luciferase as a transfection control) was measured 24 h after transfections. (B) At 24 h after transfection, U3A cells were treated with IFNγ for 15 min, washed, and incubated in fresh media and luciferase activity was determined at the indicated time points. (C) STAT3 null MEFs stably transfected with STAT3 or STAT3C were transiently transfected with reporter constructs as described above, and luciferase activity was determined 24 h later. (D) At 24 h after transfection, MEFs were treated with OsM for 15 min, washed, and incubated in fresh media. Luciferase activity was measured at the indicated times. Values represent mean fold induction of luciferase. Error bars represent the standard error of the mean (SEM) from five experiments. Western blots for STAT1 (B) and STAT3 (D) indicate a comparable expression of each isoform over the time of the experiment.

sodium vanadate on ice for 15 min. The protein (25 μg) was resolved on 8% SDS−polyacrylamide gels and transferred to nitrocellulose. Blots were probed with antibodies against the FLAG epitope (M2, Sigma, St. Louis, MO), STAT3 (Santa Cruz, Santa Cruz, CA), or phospho-STAT3 (pSTAT3; Cell Signaling, Inc., Beverly, MA)

Immunoprecipitation. Cells were lysed in 0.2 mL of boiling denaturing lysis buffer [0.5% SDS and 50 mM Tris-HCl (pH 7.4)] and boiled for 5 min, after which 0.8 mL of 1.25× RIPA buffer [197.5 mM NaCl, 62.5 mM Tris-HCl (pH 7.4), 1.25% NP-40, and 0.625% sodium deoxycholate] was added. Lysates were incubated for 30 min with 10 μL of P-Tyr-100 antibody (Cell Signaling, Inc., Beverly, MA) and 1 μL each of anti-phospho-STAT3 antibodies [pSTAT3; Cell Signaling, Inc., Beverly, MA, and pSTAT3 as described previously (*18*)]. The lysate antibody mixture was incubated with protein A/G PLUS agarose (Santa Cruz, Santa Cruz, CA) for 30 min, washed 3 times, and eluted with sample buffer including 10% βME.

mRNA Preparation and Quantitative Polymerase Chain Reaction (PCR). STAT3-deleted MEFs (*17*) were transfected with the indicated STAT3 constructs on 3 cm plates using 4 μg of DNA with 10 μL of lipofectamine 2000. Cells were incubated overnight, and RNA was harvested. cDNA was prepared using Superscript First Strand Synthesis system (Invitrogen), and quantitative PCR was performed using SYBR green QPCR mastermix (Stratagene) and analyzed on an ABI 7500 system. Primer concentrations were 50 nM. SOCS3 qPCR primer sequences were 5′-GTTCCTGGATCAGTATGATGC and 3′-CGCTTGTCAAAGGTATTGTCC. Data represent the fold expression over untransfected null MEFs.

RESULTS

Stat1C and STAT3C Are Transcriptionally Active. To study the effects of STAT1 activation in the absence of cytokine stimulation, we generated a STAT1C construct analogous to STAT3C by substituting cysteine for residues N658 and A656. Using a luciferase construct under the control of a STAT-dependent (m67) promoter, we found that cells transiently expressing STAT1C had a modest increase in luciferase expression (Figure 1A), although they did not show activation of the known endogenous STAT1 target gene IRF-1 (data not shown). However, upon IFNγ stimulation, the luciferase expression in cells expressing STAT1C was enhanced and greatly prolonged compared to cells transfected with wild-type STAT1 (Figure 1B). In cells expressing STAT1, 48 h after incubation with IFNγ normalized,

luciferase expression dropped to one-third the value at 24 h, while in cells expressing STAT1C, luciferase levels remained at 60% the 24 h value, which itself was 2.5-fold that seen with STAT1. These results support the observation that STAT1C preserves IFNγ-mediated STAT1 activation (*19*).

To test whether STAT3C prolongs cytokine stimulation, we utilized STAT3 null MEFs that had been stably transfected with either wild-type STAT3 or STAT3C. Cells were transiently transfected with the STAT-responsive m67 luciferase construct and a control renilla luciferase plasmid. As has been reported previously, STAT3C is active in the absence of cytokine stimulation (Figure 1C). Upon stimulation with OsM, luciferase expression was enhanced in cells expressing STAT3C compared to cells expressing wild-type STAT3 (Figure 1D). Furthermore, in cells expressing wild-type STAT3, luciferase levels after 48 h dropped to 32% of the measurements after 24 h, while luciferase levels in cells expressing STAT3C remained at 70% of their initial level after 48 h.

STAT3C Shows Increased Binding to DNA. To determine the mechanism by which constitutive STATs preserve cytokine-induced gene activation, we sought to test whether the STAT3C mutation affects the binding affinity of STAT3 for DNA using an EMSA. Employing STAT3 null MEFs stably transfected with either STAT3 or STAT3C, we induced STAT tyrosine phosphorylation by treating the cells with OsM. Nuclear lysates were prepared and incubated with a ^{32}P-labeled SIE DNA probe that contained consensus STAT3-binding sites. After 15 min of initial incubation, a 100-fold excess of unlabeled probe was added to the binding mixtures and samples were obtained and separated by electrophoresis at discrete intervals.

The intensity of the initial STAT3C−DNA complex was much greater than that seen with wild-type STAT3 (Figure 2A), consistent with the difference in luciferase activity (Figure 1D). While the higher mobility complexes, which contain STAT1−STAT1 dimers, showed similar kinetics between the two lysates, the rate of decay was significantly slower for the STAT3C−DNA complex than for the wild-type STAT3−DNA complex (Figure 2A). Quantification of these complexes indicates that STAT3C−DNA complexes persist with roughly double the half-life of STAT3−DNA complexes (Figure 2B). These data suggest that STAT3C forms a more stable complex with DNA, allowing for prolonged target gene transcription.

STAT1C and STAT3C Display Prolonged Phosphorylation. An increase in binding affinity between the constitutive STAT molecules and DNA may result in a delay of STAT deactivation. Specifically, unloading from DNA has been hypothesized to be necessary for STAT dephosphorylation (*20*, *21*). To test whether STAT3C molecules are resistant to dephosphorylation, we compared STAT3 null MEFs stably transfected with either wild-type STAT3 or STAT3C. Cells were stimulated with OsM for 15 min, washed, and placed in fresh media at intervals prior to harvesting. Western analysis using phospho-specific STAT3 antibodies showed similar levels of phosphorylation of STAT3 15 min after stimulation. The tyrosine phosphorylation of wild-type STAT3 decreased to near basal levels after 2 h, whereas prominent phosphorylation of the constitutive mutant remained 6 h after stimulation (Figure 3). These data are consistent with previous findings, demonstrating that the rate of dephosphorylation of STAT1 is greater than that of STAT1C in cytokine-stimulated cells (*19*).

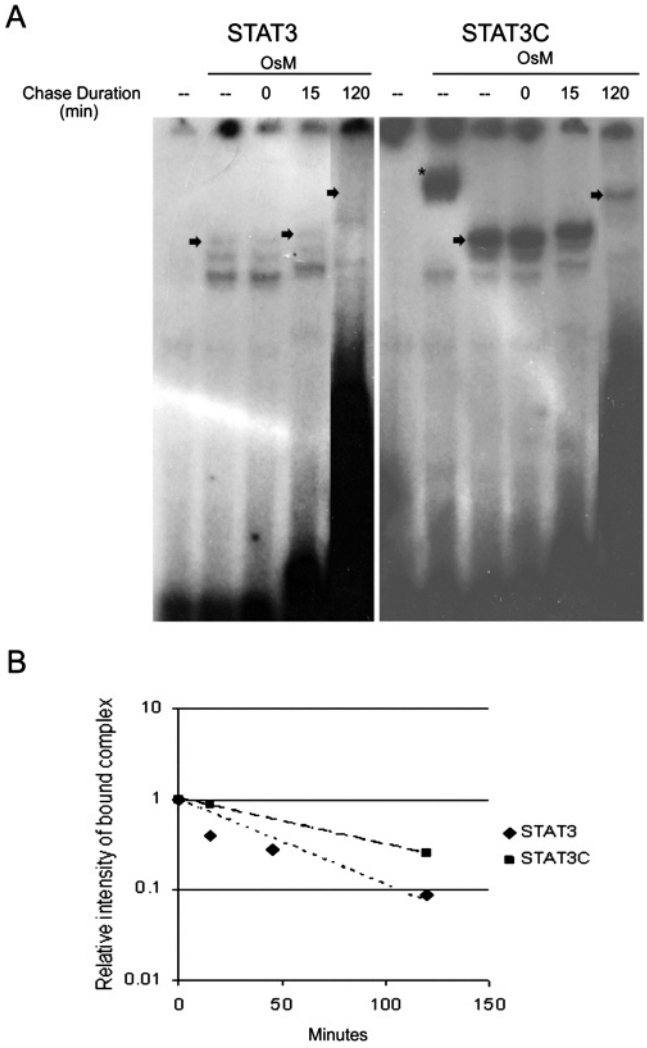

FIGURE 2: STAT3C has an enhanced binding affinity for DNA. (A) STAT3−DNA binding from cells treated with OsM was examined by an EMSA using a radiolabeled SIE probe. After a 15 min binding reaction, an unlabeled probe was added in 100-fold excess to the indicated binding reactions. DNA−protein complexes were loaded onto a separating gel after the indicated additional incubation times. Because electrophoresis was occurring prior to the loading of the latter time points, the relative mobilities of these complexes is somewhat reduced. Arrows indicate STAT3/STAT3C−DNA complexes. The asterisk indicates a supershift with an anti-FLAG antibody. A longer exposure was used for the 120 min time point to improve visualization of complexes. (B) Intensity of DNA−protein complexes from a representative experiment were measured using a phosphorimager, and normalized values were plotted on a semi-log plot.

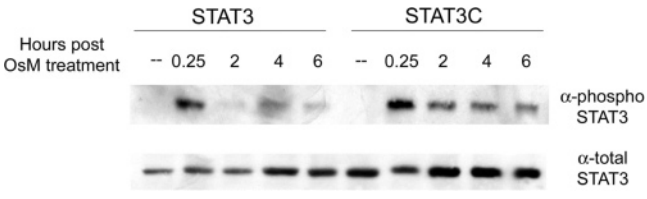

FIGURE 3: STAT3C preserves cytokine-mediated tyrosine phosphorylation. STAT3 null MEFs stably transfected with the indicated STAT3 construct were treated with OsM for 15 min, washed, and incubated in fresh media. At the indicated time points, protein lysates were prepared and analyzed by Western blot with the indicated antibodies.

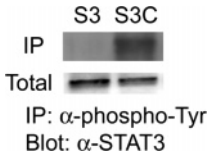

FIGURE 4: STAT3C is a tyrosine-phosphorylated absent stimulation. Lysates from untreated MEFs stably expressing either STAT3 or STAT3C were immunoprecipitated with anti-phosphotyrosine antibodies. Western analysis was performed with anti-STAT3 antibodies (top). As a control for the amount of each STAT3 form, equal aliquots from each cell lysate were taken prior to immunoprecipitation for anti-STAT3 Western blot (bottom).

To determine whether STAT3C is phosphorylated in the absence of stimulation, we performed immunoprecipitation experiments. We have found that immunoprecipitating with an antibody to phosphotyrosine followed by Western blotting for STAT3 is the most sensitive approach for detecting low-level phosphorylation (data not shown). Thus, extracts from untreated cells expressing either STAT3 or STAT3C were immunoprecipitated with anti-phosphotyrosine antibodies, and the presence of tyrosine-phosphorylated STAT3 was detected by anti-STAT3 Western blot. The cell lysates were prepared under denaturing conditions to ensure that any STAT3 that was precipitated was not merely associated with another tyrosine-phosphorylated protein. A significant portion of STAT3C was immunoprecipitated by the anti-phosphotyrosine antibody compared to wild-type STAT3, indicating that STAT3C displays prominent basal tyrosine phosphorylation in untreated cells (Figure 4). While it is probable that there is a low level of phosphorylation of wild-type STAT3, it is likely that in the absence of disulfide bonds the steady-state level is insufficient for detection. These data directly demonstrate that STAT3C is phosphorylated under basal conditions.

Phosphorylation Is Required for Functional Activation. To test whether tyrosine phosphorylation is necessary for formation of functionally active STAT1C, we generated mutant forms of STAT1C in which the critical tyrosine 701 residue necessary for phosphorylation and activation of the wild-type protein was substituted with phenylalanine. Using a luciferase reporter construct containing a STAT-sensitive promoter, we transfected wild-type STAT1, STAT1C, or STAT1C Y701F(Y → F) into U3A cells, which lack endogenous STAT1 (*22*). Whereas STAT1C led to the prominent induction of the reporter construct, neither STAT1C-(Y → F) nor wild-type STAT1 displayed any activity despite similar expression of the STAT1 protein (Figure 5A). These results indicate that the tyrosine 701 residue is necessary for constitutive function, suggesting a requirement for phosphorylation.

To determine whether phosphorylation is required for the activity of STAT3C, we constructed a mutant of STAT3C, substituting tyrosine 705 with phenylalanine. When transfected into STAT3−/− MEFs, STAT3C showed a 14-fold induction of luciferase expression compared to wild-type STAT3. In contrast, STAT3C(Y → F) showed a complete absence of activity (Figure 5B). To determine whether endogenous STAT3 target genes showed a similar requirement of Y705 for transcriptional induction, we transiently transfected STAT3 mutants into STAT3 null MEFs. While STAT3C increased the expression of the known STAT3 target SOCS3 by 70% compared to wild-type STAT3, STAT3C(Y → F) failed to induce the expression of this gene (Figure 6). RT-PCR (data not shown) and Western analysis (Figure 5B) showed a comparable expression of the STAT3C and STAT3C(Y → F) constructs. When these data are taken together, they indicate that the critical tyrosine residue is necessary for STAT-dependent transcription of both model reporter genes and endogenous target genes.

Dominant Negative (DN) Mutants Block STATC Activation. While these experiments indicate that the critical tyrosine residue is necessary for the activity of the STATC proteins, they do not exclude the possibility that the Y → F mutations alter the proteins in some other way that inhibits their function. While this is unlikely based on structural considerations, we took a second approach to examine this possibility. We used mutant forms of STAT1 or STAT3 in which the critical tyrosine residue had been changed to phenylalanine. These proteins act in a DN fashion by suppressing tyrosine phosphorylation of wild-type STATs. We thus cotransfected U3A cells with DN STAT1 as well as STAT1C in a 1:1 ratio and determined the expression of the STAT-dependent luciferase reporter construct. DN STAT1 completely suppressed transcriptional activation mediated by STAT1C (Figure 7A). In contrast, cotransfection

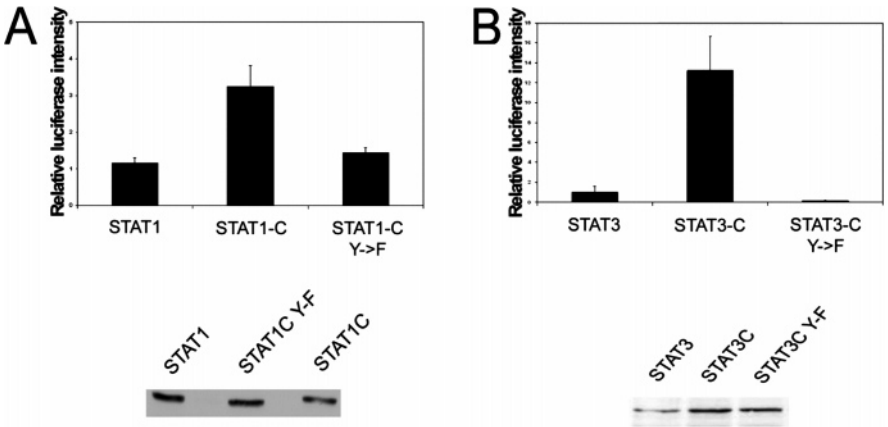

FIGURE 5: Tyrosine phosphorylation is required for STATC activity. U3A (A) or STAT3-deleted MEFs (B) were transiently transfected with a STAT-dependent luciferase reporter construct and the indicated STAT construct. At 24 h later, lysates were prepared and analyzed by luminometry (top). Parallel extracts were analyzed by Western blot to STAT1 (A) or STAT3 (B) to confirm equal expression of each STAT form. Results indicate the average fold induction of luciferase activity normalized to the renilla luciferase activity of five experiments. Error bars indicate SEM.

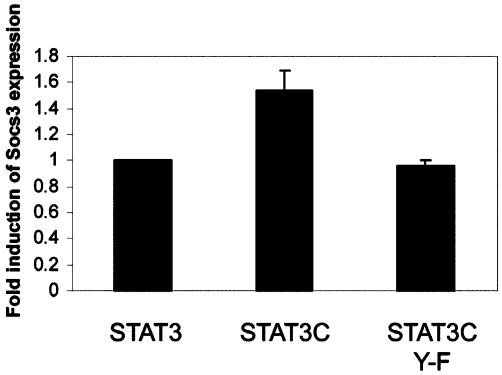

FIGURE 6: Tyrosine phosphorylation is required for STAT3-mediated expression of SOCS3. STAT3 null MEFs were transiently transfected with the indicated STAT3 construct. Cells were incubated overnight; RNA was harvested; and mRNA levels of SOCS3 were determined using quantitative RT-PCR. Values represent the mean of three experiments. Error bars represent SEM.

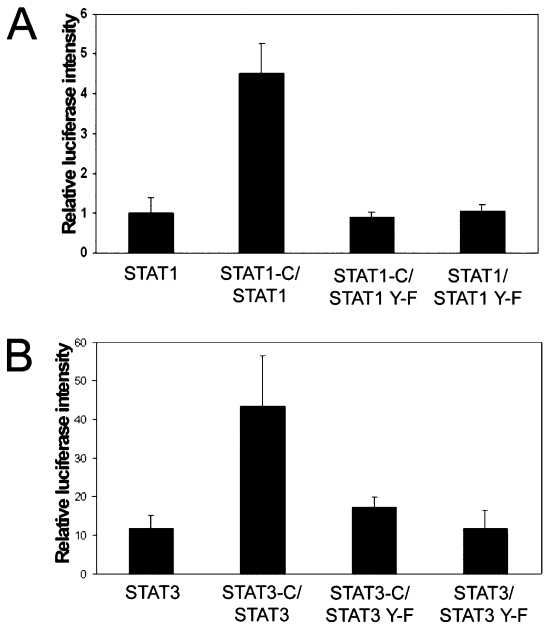

FIGURE 7: DN STAT constructs inhibit the activity of STATC. U3A (A) or STAT3 null MEFs (B) were transiently transfected with a STAT-dependent luciferase reporter and the indicated STAT constructs. Combinations of STAT constructs were transfected at a 1:1 ratio. At 24 h later, lysates were prepared and luciferase was measured. Results represent the average fold induction of luciferase activity normalized to the renilla expression of five experiments. Error bars represent SEM.

of STAT1C with wild-type STAT1 led to the prominent activation of the luciferase reporter. Parallel experiments in STAT3 null MEFs using STAT3C also showed complete suppression of activation by DN STAT3 but not wild-type STAT3 (Figure 7B). These experiments confirm that at least some level of tyrosine phosphorylation is necessary to trigger the activation of these constitutively active cysteine-containing STAT forms.

DISCUSSION

These experiments indicate that tyrosine phosphorylation is necessary for the activity of the constitutive STAT1C and STAT3C mutants. This model suggests that within the cell, under unstimulated conditions, STATs are phosphorylated and dephosphorylated at a low but continuous rate. This supports previous reports, which indicated that phosphatase inhibitors can induce STAT activation (*13*−*16*). The presence of cysteine residues in constitutive STAT mutants inhibits dephosphorylation, increases the binding affinity of the STAT dimer with DNA, and results in elevated expression of STAT target genes.

It is unclear which kinase(s) may be necessary for this low-level phosphorylation to occur. Jak family members and Src family members, both of which can phosphorylate STATs, may have low levels of phosphorylation and kinase activation under basal conditions, and this may be sufficient to trigger STATC dimerization in various cell types. Variations of this low-level phosphorylation may be responsible at least in part for the variations observed in the activity of STAT1C and STAT3C in different cell lines (data not shown). Thus, these cysteine-containing STAT mutants may be a sensitive tool for detecting transient tyrosine phosphorylation events that had previously been below the level of detection.

These results are consistent with the hypothesis that unactivated STAT dimers exist in an antiparallel conformation (*11*, *12*). The hypothesized structure of these dimers suggests that the carboxy termini of STAT molecules would not come into close contact until after the phosphorylation-mediated reorganization of the STAT dimer. This is consistent with the results reported here, which suggest that the presence of cysteine residues within the carboxy termini of STAT molecules is insufficient for spontaneous oxidation and dimer formation.

The finding that cysteine-containing STAT molecules have a decreased rate of dissociation from DNA suggests a mechanism for the prolonged phosphorylation and activity of these mutants. A disulfide bridge between the carboxy terminus of two STAT molecules could lead to a greater stability of the interaction of the STAT dimers with DNA. This result is consistent with the hypothesis that dephosphorylation occurs after STAT molecules release from DNA and suggests that modest changes in the binding affinity of STATs for DNA can substantially affect the duration and intensity of STAT-dependent gene transcription.

Cysteine-containing STAT mutants, particularly STAT3C, have been instrumental in dissecting the STAT function and the contribution of STATs to tumor pathogenesis. The stabilization of phosphorylation and DNA-binding characteristics of these mutants provide new insight into mechanistic aspects of STAT-modulated gene expression. The finding that these mutants require tyrosine phosphorylation for functional activation provides a basis for their activity and confirms the presence of previously undetected basal phosphorylation of STAT1 and STAT3. This model of activation reconciles our understanding of the function of these mutants with recent data suggesting the presence of antiparallel STAT dimers prior to tyrosine phosphorylation.

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