Elevated Activity of STAT3C due to Higher DNA Binding Affinity of Phosphotyrosine Dimer Rather than Covalent Dimer Formation*5

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Signal transducer and activator of transcription (STAT) proteins are involved in cell proliferation and survival, aspects of tissue differentiation and immune function. STAT3 appears to be fundamentally important for vertebrate organisms, being required for the self-renewal of embryonal stem cells in response to leukemia inhibitory factor signaling and for proliferation of some somatic cell types. Moreover, STAT3 is up-regulated in a range of tumors, and a modified version of STAT3 (STAT3C) has been shown to function as an oncogene, whereas inhibition of STAT3 can suppress tumor cell growth. The constitutive activity of oncogenic STAT3C was reported to depend on spontaneous dimerization directed by disulfide bonds in the absence of tyrosine phosphorylation. In fact, tyrosine phosphorylation consequent upon cytokine or mitogen-induced signaling events remains obligatory for STAT3C activation. Instead, the DNA-binding affinity of phospho-STAT3C is elevated resulting in a faster on-rate and slower off-rate. The faster on-rate sensitizes STAT3C to cytokine stimulation, and the slower off-rate protects it from inactivation by nuclear phosphatases. These changes account for the ability of STAT3C to up-regulate persistently the expression of STAT3 target genes and promote cell cycle progression.

Signal transducer and activator of transcription (STAT) proteins constitute a family of multifaceted transcription factors, with members involved in cell proliferation and survival, aspects of tissue differentiation and immune function (1, 2). As well as sharing structural attributes with Rel/nuclear factor κB (NFκB) family members (3), STATs also partly mirror their mode of activation by being resident in the cytoplasm as latent factors and translocating to the nucleus upon activation. According to the established model of activation, STAT monomers interact with tyrosine-phosphorylated cytokine or growth factor receptors through their SH2 domains, and they are themselves phosphorylated on a single tyrosine residue, whereupon they dimerize through reciprocal phospho-tyrosine-SH2 interactions, migrate to the nucleus, and bind to elements in the promoters of target genes (4).

Emerging details have expanded our knowledge of these processes. For example, Love and colleagues (5) recently demonstrated that activated STAT3 moved from cell membrane to perinuclear structures along the endocytotic pathway. In addition, Vinkemeier and colleagues (6, 7) were able to show that nuclear entry was a continuous event involving both phosphorylated and unphosphorylated STAT1, but that, while unphosphorylated STAT1 was immediately exported, phosphorylated STAT1 remained in the nucleus and was protected from dephosphorylation by high affinity, sequence-specific binding to DNA.

Other aspects of the established model, however, have been difficult to substantiate. For example, the existence of STAT monomers has been questioned by numerous reports of latent cytoplasmic STAT dimers and larger cytoplasmic STAT complexes (8–12). Furthermore, a molecular rationale for latent cytoplasmic STAT dimers has been provided by the crystal structures of unphosphorylated STAT1 and STAT5α (13, 14).

Among STAT proteins, STAT3 has the most fundamental importance for vertebrate organisms. In mice, deletion of the STAT3 gene results in early embryonal lethality (15). In addition, STAT3 is required for ES cell renewal (16, 17), and STAT3 inactivation is necessary for differentiation in the PC12 cell model (18). Conversely, cell transformation by oncogenic Src alleles requires the function of STAT3 (19, 20), and indeed, a constitutively active version of STAT3 has been generated that functions as an oncogene (21). The prediction that cysteines introduced at residues 662 and 664 in a loop of the SH2 domain in STAT3 would create a constitutively active protein (STAT3C) was supported experimentally, but a propensity for dimer formation through inter-chain disulfide bridges in the absence of tyrosine phosphorylation was not formally demonstrated. This proposed mechanism made a number of assumptions, most important of which was that STAT3C activated target gene expression independently of mitogen or cytokine signaling. In addition it assumed that that STAT3C experienced an intracellular environment allowing stable, inter-chain disulfide bond formation.

Our studies of STAT3 function led us to question the mechanism proposed for the behavior of STAT3C. We found that

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The abbreviations used are: STAT, signal transducer and activator of transcription; SH2, Src homology 2; FCS, fetal calf serum; EGF, epidermal growth factor; IL-6, interleukin-6; DTT, dithiothreitol; SIE, serum-inducible element; HA, hemagglutinin.

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constitutive activity is only apparent and that cytokine or mitogen-induced signaling events and consequent tyrosine phosphorylation of STAT3C are obligatory for its activation. Our data show that the DNA binding affinity of phospho-STAT3C is elevated with respect to that of phospho-STAT3, resulting in a faster on-rate and slower off-rate. The faster on-rate sensitizes STAT3C to cytokine stimulation, and the slow off-rate protects it from inactivation by phosphatases. This increase in affinity underlies the ability of STAT3C to persistently up-regulate STAT3 target genes, including those that promote cell cycle progression.

MATERIALS AND METHODS

Cell Culture and Extract Preparation—HEK293 and COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. Cells were transfected by DNA–calcium phosphate co-precipitations as described previously (22). Unless otherwise stated cells were stimulated, respectively, with 10 ng ml⁻¹ IL-6 or 50 ng ml⁻¹ EGF.

For immunoblotting experiments, lysates were prepared in TNEN buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) supplemented with protease inhibitors (1 mM Na₂VO₃, 10 mM Na₃P₂O₇, 10 mM NaF, 5 mM EGTA, 10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin). Lysates were cleared by centrifugation at 16,000 × g for 10 min and used directly for immunoprecipitations or stored at −20 °C for further use.

For electrophoretic mobility shift assays, cells were maintained in medium containing 0.5% FCS overnight before application of appropriate stimuli. Nuclear extracts were prepared as described previously (23). Extracts were incubated with 32P-labeled oligonucleotide duplex (m67SIE) in buffer containing 15 mM HEPES, pH 7.9, 82 mM NaCl, 1 mM DTT, 0.43 mM EDTA, 60 μg ml⁻¹ poly(dI/dC), and 10% glycerol. Protein–DNA complexes were separated by electrophoresis on 5% polyacrylamide gels containing 2.5% glycerol in 0.5× Tris-borate-EDTA (TBE) buffer. Gels were fixed, dried, and analyzed with a FLA2000 phosphorimaging device (Fuji) and Aida software. For supershift analyses of SIE–STAT3 complexes, extracts were preincubated with anti-STAT3 antisera in binding buffer containing 0.05% Nonidet P-40 at room temperature for 1 h. Kinetic studies were performed according to established protocols (24–26). For on-rate experiments, binding reactions were applied directly to gels under current. For off-rates, complexes were allowed to form for 15 (wt and 3C) or 30 min (C712A) before addition of a 20-fold excess of unlabeled homologous competitor. Binding analyses were performed with GraphPad version 4.0. The Kₘₒ and Kₒₒ values were obtained separately from one-phase exponential association and decay.

RESULTS

Phosphorylation of Tyrosine 705 Is Essential for STAT3C Function—If STAT3C were able to form constitutive dimers, bind DNA, and activate transcription in the absence of tyrosine phosphorylation, substitution of tyrosine 705 with phenylalanine should have no effect on STAT3C-dependent gene expression. However, as shown in Fig. 1A, Y705F substitution in STAT3C (STAT3C-Y/F) ablates the activation of a STAT3-dependent reporter gene, as it does in native STAT3 (STAT3Y/F), regardless of cytokine stimulation.

In line with the failure of STAT3Y/F and STAT3C-Y/F to activate transcription, neither mutant bound to the optimized c-fos serum-inducible element (m67SIE) (Fig. 1B, lanes 7, 8, 15, and 16). However, although STAT3 binding to the SIE in the absence of IL-6 was almost undetectable (lanes 1 and 2), binding by STAT3C in unstimulated cells was clearly present (lanes 9 and 10), which correlates with the constitutive activity ascribed to STAT3C (Fig. 1A). Immunoblotting revealed no difference between the overall levels of STAT3, STAT3C, and the corresponding mutants in these experiments (Fig. 1C, lower panel), although IL-6-induced phospho-STAT3C was reproducibly lower (upper panel, compare lane 6 with lane 2). The weak phospho-STAT3 signals seen with the Y/F mutants can be attributed to low levels of endogenous STAT3 in HEK293 cells.
Detection of Latent STAT3 Dimers by Co-immunoprecipitation—Several independent experimental approaches have indicated that latent STATs are present in cells as dimers or multimeric complexes (8–12, 27). To assess dimerization by STAT3, we first performed co-immunoprecipitation assays with epitope-tagged STAT proteins. Although complexes with differentially tagged STAT1 proteins or containing STAT1 and STAT3 were barely detected in the absence of tyrosine phosphorylation (Fig. 2A, lanes 2 and 4), their formation was stimulated by...

FIGURE 1. Tyrosine phosphorylation is essential for STAT3C function. A, HEK293 cells were transfected with a m67SIE luciferase reporter and expression plasmids for STAT3, STAT3Y/F, STAT3C, and STAT3C-Y/F. Cells were serum-starved for 24 h or starved and stimulated with IL-6 for 7 h before harvesting and analysis of luciferase expression. Values were normalized against expression from a co-transfected β-galactosidase vector and presented as fold induction with respect to uninduced control. Data derive from one of three independent experiments with duplicate points (error bars = ± S.D.). B, nuclear extracts were prepared from HEK293 cell transfected with vectors for STAT3 proteins, starved, and stimulated with IL-6 for 30 min as indicated. STAT3 DNA binding to a single m67SIE was confirmed by supershift with an antibody specific for STAT3 (even lanes). C, tyrosine phosphorylation of STAT3 protein in lysates of HEK293 cell transfected with vectors for STAT3 proteins, starved, and stimulated with IL-6 for 30 min as indicated.

FIGURE 2. STAT3 interactions in the absence of tyrosine phosphorylation or disulfide bond formation. A, HEK293 cells were transfected with vectors for HA-STAT1 (lanes 1–5), FLAG-STAT3 (lanes 2, 3, and 6), FLAG-STAT1 (lanes 4 and 5), HA-STAT3 (lane 6), and vEyk (lanes 3 and 5) and serum-starved for 24 h. FLAG-tagged proteins were immunoprecipitated from cell lysates, and potential dimerization was monitored by immunoblotting with an anti-HA monoclonal antibody (upper panel). Protein expression and tyrosine phosphorylation were monitored in 5% (vol) of cell lysates (lower panels). B, cells were transfected with vectors for HA-STAT1 (lanes 1, 3, 5, and 7), FLAG-STAT3 (lanes 2 and 3), FLAG-STAT3Y/F (lanes 4 and 5), or FLAG-STAT3C (lanes 6 and 7), serum-starved for 24 h, and processed as described above. C, cells were transfected with vectors for FLAG-STAT3 (lanes 1, 2, 5, and 6) or FLAG-STAT3C (lanes 3, 4, 7, and 8), serum-starved for 24 h or starved and treated with IL-6 for 30 min as indicated. Lysates were prepared and resolved on a non-reducing polyacrylamide gel. Monomeric STAT3 detected by immunoblotting with an anti-FLAG monoclonal antibody is indicated.
We compared the susceptibility of STAT3 and STAT3C to agents that reduce inter-chain disulfide bonds. It has been argued that DNA binding by STAT3C dimers is susceptible to agents that reduce inter-chain disulfide bonds. We, therefore, reasoned that a C712A mutation would impair DNA binding when modified by N-ethylmaleimide. In an attempt to detect STAT3C dimers directed by disulfide bond formation we used non-reducing SDS-PAGE. However, no species potentially corresponding to STAT3C dimers was detected that was not also present with STAT3, either in nuclear or cytoplasmic extracts from starved or cytokine-stimulated cells (Fig. 2C). In summary, our data are consistent with the dimer serves as a scaffolding, wherein the two STAT3 proteins interact in the complete absence of tyrosine phosphorylation (data not shown).

In an attempt to detect STAT3C dimers directed by disulfide bond formation we used non-reducing SDS-PAGE. However, no species potentially corresponding to STAT3C dimers was detected that was not also present with STAT3, either in nuclear or cytoplasmic extracts from starved or cytokine-stimulated cells (Fig. 2C). In summary, our data are consistent with the existence of latent STAT3 dimers, and we find no evidence for disulfide bond formation that could contribute to dimer formation by STAT3C.

DNA Binding by STAT3C Is Refractory to Reducing Agents—It has been argued that DNA binding by STAT3C dimers is susceptible to agents that reduce inter-chain disulfide bonds (21). We compared the susceptibility of STAT3 and STAT3C to several reducing agents and obtained conflicting results. STAT3C bound to the m67SIE in the presence of 0.5–50 mM DTT (Fig. 3A, lanes 1, 3, 4, 9, 11, and 12), 100 mM β-mercaptoethanol (lanes 2 and 10) or 5 mM tributylphosphate (lane 15), whether in nuclear extracts from unstimulated or cytokine-stimulated cells. Similar results were obtained when DTT was also included in gels (data not shown). Indeed, the presence of 10–50 mM DTT noticeably enhanced DNA binding by STAT3C (Fig. 3A, compare lanes 1 and 2 with 3 and 4) and, like STAT3, STAT3C failed to bind to the m67SIE in the absence of DTT (supplemental Fig. S1). As shown in Fig. 3B, there was also no detectable difference in the susceptibility of STAT3 and STAT3C DNA binding to N-ethylmaleimide, indicating that the two proteins possess an equal complement of reactive cysteines that interfere with DNA binding when modified by N-ethylmaleimide.

To detect a potentially more subtle contribution of inter-chain disulfide bond formation to DNA binding, the dissociation of radiolabeled m67SIE-STAT3C complexes in the presence of cold competitor DNA, and different concentrations of DTT was followed. As shown in Fig. 3C, the stability of m67SIE-STAT3C complexes was not decreased in the presence of 10 mM DTT; in fact the presence of 10 mM DTT allowed more efficient initial binding (compare lanes 1 and 2 with 7 and 8). In summary, we found no evidence for a contribution of disulfide bond formation to DNA binding by STAT3C.

Mutations in STAT3C Confer an Elevated DNA Binding Affinity—Despite the lack of evidence for inter-chain disulfide bond formation by STAT3C, it remained possible that the mutations introduced into STAT3C might affect DNA binding in another way. Thus we compared the DNA-binding properties of STAT3, STAT3C, and another mutant, STAT3(C712A). Cysteine 712 lies on the C-terminal arm of the phosphorylation loop in STAT3, where intercalation of alternate side chains in the dimer contributes to the dimerization interface (3). We therefore reasoned that a C712A mutation would impair DNA binding by STAT3.

In the experiments shown in Fig. 4, nuclear extracts were prepared from IL-6-stimulated HEK293 cells expressing the proteins, and DNA-binding reactions were set up under optimized binding conditions (see “Materials and Methods”). In Fig. 4A, equivalent levels of phospho-STAT3 for each protein were used, as determined by immunoblotting (data not shown). On-rates determined for the three proteins varied considerably: STAT3C reached saturation within 5 min, STAT3 within 15 min, and STAT3(C712A) took at least 30 min. The on-rate constants estimated from one-phase exponential association were as follows: STAT3C = 1.532, STAT3 = 0.532, and STAT3(C712A) = 0.129 (min⁻¹). Similar experiments were performed to establish off-rates for the three proteins. With amounts of nuclear extract that gave equal levels of m67SIE binding, we followed dissociation in the presence of excess cold competitor over 2 h (Fig. 4B). A similar pattern emerged: STAT3(C712A) dissociated more rapidly from the SIE than STAT3, and the SIE-STAT3C complex was the most stable. The off-rates obtained from these experiments were: STAT3(C712A) = 0.0138, STAT3 = 0.0116, and STAT3C = 0.0082 (min⁻¹). In repeat experiments with differ-
ent protein samples almost identical on- and off-rates were obtained (data not shown). From these data, $K_D$ values for STAT3 and STAT3C could be obtained. For STAT3 $K_{\text{off}}/K_{\text{on}} = 0.0218$ and for STAT3C $K_{\text{off}}/K_{\text{on}} = 0.00532$, indicating that STAT3C has a 4-fold lower $K_D$ than STAT3.

In a third series of experiments we attempted to measure the difference between the dissociation constants for STAT3 and STAT3C. Again it was not possible to determine precise values for each protein, because the concentrations of phospho-proteins could not be determined. Nonetheless, the graph in Fig. 4C shows that the binding curve for STAT3C lies to the left of the one for STAT3 and that half-maximal binding by phospho-STAT3C is achieved with 35% of the protein required for the same level of binding by phospho-STAT3. Taken together these data indicate that STAT3C binds to the SIE with a 3-fold higher affinity than STAT3.

Inactivation of STAT3C in Cells Is Delayed—The faster on-rate, lower dissociation constant, and slower off-rate for STAT3C offer an explanation for its increased transcriptional activity and associated transformation potential. First, from these data STAT3C would be predicted to achieve a greater degree of promoter occupation than STAT3 in response to low level stimulation. Second, once activated STAT3C would remain bound to promoter sequences for longer than STAT3. Because DNA binding has been shown to counteract the inactivation of STAT proteins by nuclear phosphatases (7), STAT3C would be more resistant to inactivation. The data presented in Fig. 1 above arguably support the first prediction.
Discussion), so the experiments described below first focused on the second prediction.

Cells expressing STAT3, STAT3C, STAT3C(C712A), or STAT3C(C712A), which contains the cysteines present in STAT3C as well as the alanine substitution of cysteine 712, were treated with IL-6 and harvested at various time points for analysis of STAT3 phosphorylation and DNA binding. Maximal DNA binding was observed 30 min after IL-6 stimulation for all proteins and decayed subsequently over an 8-h period (Fig. 5 A). As anticipated, DNA binding by STAT3(C712A) decreased most rapidly and was undetectable after 4 h (Fig. 5 A, second panel, lane 5, and B). DNA-binding activity by STAT3(C712A) decreased less rapidly than STAT3(C712A), consistent with the notion that the additional cysteines in STAT3C complement for the defect in STAT3(C712A), but more rapidly than either STAT3 or STAT3C. Unexpectedly, DNA binding by STAT3 and STAT3C decreased with almost identical kinetics over the 8-h time course, and this effect was consistent in several independent experiments.

A very similar picture emerged when phosphorylation of tyrosine 705 was monitored (Fig. 5, A and B, right panels). STAT3(C712A) phosphorylation decreased most rapidly, followed by that of STAT3C(C712A). Phosphorylation of STAT3 and STAT3C decreased the slowest and in parallel. Thus under these experimental conditions, the consequences of the decreased binding affinity of the C712A substitution seen in vitro were detected but no direct effect of the increased binding affinity of STAT3C.

One reason for the inability of this experiment to distinguish between STAT3 and STAT3C might be the duration of IL-6 signaling, which, if persistent, might mask a decay in the DNA binding and dephosphorylation of STAT3. In fact cytoplasmic phospho-STAT3 levels remain high in cells exposed to IL-6 for 8 h, whereas if after 30 min the cells are treated with staurosporine, a protein kinase inhibitor, cytoplasmic phospho-STAT3 levels return almost to baseline within 3 h (supplemental Fig. S2). The preceding experiment was therefore modified by the addition of staurosporine to the cells after 30 min. Under these circumstances DNA binding by STAT3 reverted to baseline within 3 h (Fig. 6 A, lower panel, and Fig. 6 B, left panel), whereas binding by STAT3C persisted at almost maximal levels (upper panel). Phosphorylation of nuclear STAT3C also remained elevated with respect to STAT3 under these conditions (Fig. 6, right panels). This is consistent with STAT3C having a higher DNA binding affinity than STAT3 and its slower off-rate results in delayed dephosphorylation by nuclear phosphatases. This effect could account for increased transcriptional activity of STAT3C.

Nuclear Accumulation of STAT3C Does Not Depend Exclusively on DNA Binding—The altered DNA-binding properties of STAT3C may have additional consequences for its distribution in cells. Indeed, it was originally noted that STAT3C was predominantly nuclear (21). We also compared the partitioning of STAT3 proteins between nucleus and cytoplasm prior to and following IL-6 stimulation. As shown in Fig. 7, more STAT3C than STAT3 was detected in the nucleus (upper panels), as reported previously, and, conversely, less STAT3C than STAT3 was present in the cytoplasm (lower panels). Stimulation with IL-6 had no apparent effect on these ratios: similar amounts of nuclear phospho-STAT3C and phospho-STAT3 were detected yet consistently less phospho-STAT3C than phospho-STAT3 was present in the cytoplasm. As phosphorylation occurs exclusively outside the nucleus, this suggests that phospho-STAT3C is retained more efficiently in the nucleus than phospho-STAT3, possibly as a consequence of its enhanced DNA binding. However, the partitioning of STAT3Y/F and STAT3C-Y/F
between nucleus and cytoplasm mirrored that of their counterparts. As the Y/F mutants are severely impaired in their sequence-specific DNA binding, the higher DNA binding affinity of STAT3C alone cannot account for its nuclear accumulation.

A Mutation That Destabilizes DNA Binding Reverses the Activation of STAT3C—The accelerated on-rate for STAT3C suggested it might be hypersensitive to low level signaling, which could contribute to its DNA binding and transcriptional activity observed in unstimulated cells (Fig. 1). In fact, in dose-response analyses comparing STAT3 and STAT3C, the basal activity of STAT3C exceeded that of IL-6-stimulated STAT3 activity in HEK293 cells (supplemental Fig. S3; see also Figs. 1A and 8B). However, in COS1 cells unstimulated STAT3C activity is relatively low, presumably reflecting low, basal signaling, so responses to EGF stimulation could be compared. Under these circumstances, STAT3C showed a 3-fold higher sensitivity to sub-optimal concentrations of EGF than STAT3 (Fig. 8A), consistent with the notion that it is hypersensitive to low level signaling.

If the transcriptional potency of STAT3 is directly related to its DNA binding affinity, a further prediction from the analyses described in Figs. 5 and 6 is that the activity of STAT3C should be reduced by the C712A substitution. This was found to be the case in HEK293 cells stimulated with IL-6 and in COS-1 cells stimulated with EGF. As shown in Fig. 8 (B and C), reporter gene activation by STAT3(C712A) was impaired, in line with its DNA binding, whereas activation by STAT3C/C712A, was substantially reduced in comparison with STAT3C. In both cell types the activity of STAT3C/C712A was very similar to that of native STAT3. That the positive contribution of the additional cysteines in STAT3C was negated by the C712A substitution is consistent with the notion that STAT3C is hyperactive by virtue of the higher DNA binding affinity of its phosphorylated form, rather than an ability to form constitutive dimers in the absence of phosphorylation.

DISCUSSION

A role for STAT3 in breast carcinoma and other cancers has long been suspected, and the generation of an oncogenic form of STAT3 contributed significantly to the argument that constitutive activation of STAT3 was sufficient to transform cells (21). However, the mechanism proposed for STAT3C, involving constitutive dimer formation through disulfide bonds in the absence of tyrosine phosphorylation, implied that STAT3C acted independently of upstream signaling events. Here we have reappraised the biochemical parameters of STAT3C function and found no evidence of disulfide bond formation or of DNA binding and transcriptional activation in the absence of tyrosine phosphorylation. Instead the data support a model in...
which activation of STAT3C is obligatory and occurs readily, whereas inactivation by nuclear phosphatases is reduced due to its higher sequence-specific DNA binding affinity.

**STAT3 Activation and Switching between Dimer Interfaces**—The crystal structure of STAT3 bound to the m67SIE revealed significant surface interactions in addition to the classic phosphotyrosine motif-SH2 interactions (3). One such contribution is made by the juxtaposed phosphotyrosine loops, in which alternate side chains (Thr-708, Phe-710, and Cys-712) from each monomer intercalate, followed by residues 713–716, which anchor the carboxyl end of the phosphotyrosine loop to the body of the parent monomer. C-terminal truncations of STAT3 that remove threonine 714 and beyond are phosphorylated but unable to bind DNA (29), whereas contributions to dimerization by individual side chains in the phosphotyrosine loop are illustrated by the C712A mutant described here. Substitution of Cys-712 with an isosteric serine causes a less severe impairment of STAT3 activity. The dimerization interface also extends to peripheral loops of the SH2 domain, including interactions in the DB loops involving Gln and Asn residues (3) and potentially the BG loops, which appear closer in the STAT1 structure (30) and into which the cysteine substitutions in STAT3C were engineered (21). Although the cysteine residues ostensibly allowed inter-chain disulfide bond formation, we found no evidence to support this conclusion. Precautions were taken to prevent possible re-oxidation of disulfide bonds due to reductant depletion in our assays, yet the reducing agents used, if anything, enhanced DNA binding by STAT3 and STAT3C alike, while non-reducing gel electrophoresis revealed no propensity for STAT3C to form covalent dimers when compared with STAT3 (Fig. 2C).

A configuration for latent STAT3 dimers may be inferred from those determined for unphosphorylated STAT1 and STAT5a (13, 14, 31), which differ considerably from the DNA-bound structure and involve an antiparallel arrangement of the two monomers with an extensive interface involving both the four helical bundle and β-barrel domains. A second interface comprises the N-terminal domains, which were absent from earlier structures, as predicted earlier (32) and in addition to their role in promoting cooperative binding to tandem DNA elements (33). Our co-immunoprecipitation experiments readily detected homotypic associations by inactive STAT3 and, equally, by STAT3-Y/F mutants (Fig. 2B), which presumably correspond to this latent conformation. Nonetheless, reciprocal SH2-phosphotyrosine interactions remain critical for STAT activation, because Y/F substitutions inactivate the proteins completely and generate dominant inhibitory versions (23).

Because the conformations of STAT1 monomers in both dimer structures are virtually superimposable, tyrosine phosphorylation must induce a change in the spatial alignment of STAT proteins rendering them competent for DNA binding. The cysteines introduced into STAT3C are unlikely to cause collapse of the dimer structures are virtually superimposable, tyrosine phosphorylation must induce a change in the spatial alignment of STAT proteins rendering them competent for DNA binding. The cysteines introduced into STAT3C are unlikely to cause collapse of the dimeric STAT3 dimer insofar as it lies outside its interface (32). The chance that they might form inter-chain disulfide bonds spontaneously also seems remote given that they reside at opposite ends of the latent dimer. Instead, based on the negative effect of substituting alanine for cysteine 712, we infer that the additional cysteines in STAT3C most likely serve to increase buried surface area at the phospho-dimer interface, in particular once DNA binding has occurred.

**An Increase in DNA Binding Affinity Suffices to Explain STAT3C Phenotype**—Biochemical analysis of STAT3C revealed improved DNA-binding parameters with respect to native STAT3. All experiments were carried out with pro-

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1 L. Li and P. E. Shaw, unpublished observation.
teins expressed in IL-6-stimulated HEK293 cells and in the context of nuclear extracts. Although this had the disadvantage that the absolute concentrations of STAT3 proteins were not known and thus true rate and dissociation constants could not be calculated, we could be confident that the proteins were in their native conformation and modification states. Protein levels were ascertained by immunoblotting and DNA binding measurements were performed with normalized phospho-protein levels, because all our data indicated that unphosphorylated STAT3 proteins do not bind DNA.

In our model we propose the retarded rate of dissociation of STAT3C from DNA complexes to be the predominant change responsible for elevated gene expression levels and, by extension, oncogenic transformation. Thus, as with STAT3, gene activation would be contingent upon upstream signaling events, but once stimulated, STAT3C binding to promoter elements would endure, resulting in protracted signaling events, but once stimulated, STAT3C binding to promoter elements would endure, resulting in protracted DNA.

Elevated DNA Binding Affinity and Nuclear Accumulation— Whereas unphosphorylated STAT1 shuttles continuously between cytoplasm and nucleus, phospho-STAT1 is retained in the nucleus by high affinity DNA binding, which serves to shield it from nuclear tyrosine phosphatases (7, 37). A similar mechanism may regulate STAT3, because the observed decay in DNA binding and loss of tyrosine phosphorylation broadly paralleled each other for each of the different STAT3 mutants (Fig. 5). The more rapid initial decay of phosphorylation can be attributed to an excess of phospho-STAT3 dimers over high affinity binding sites in the genome, which would be immediately susceptible to nuclear phosphatases. However, it has also been argued in the case of STAT1 that the phosphodimer must undergo conformational change for efficient dephosphorylation to occur (31).

Other factors are also likely to play a role in the intracellular distribution of STAT3. Our data confirm that the distribution of STAT3C is skewed toward the nucleus (21) and show that it occurs independently of DNA binding, because STAT3C-Y/F is similarly affected. Unphosphorylated STAT3 appears to exit from the nucleus by two routes: an energy-independent pathway and a leptomycin B-sensitive CRM1-mediated pathway that requires a leucine-rich nuclear export signal (38, 39). Interestingly, in the case of STAT1, modification of a single cysteine residue by N-ethylmaleimide was found to diminish nuclear export, implicating the residue in nuclear transport (39). Thus the introduction of cysteine residues into the DG loop of STAT3 may have affected the balance between import and export of STAT3C resulting in net nuclear accumulation. This uncertainty can only be resolved by further experiment.

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