The STAT1 transcription factor is organized into several highly conserved domains, each of which has been assigned a function with the exception of the linker domain. We previously characterized a mutant in the linker domain of STAT1 that gave normal DNA binding using a standard probe in an electrophoretic mobility assay but failed to activate transcription in response to interferon γ. We now report the mechanistic basis for the inactivity of this STAT1(K544A/E545A) mutant. Rather than failing to attract transcriptional coactivators, the STAT1(K544A/E545A) mutant has a subtle biophysical defect, which prevents accumulation of the activated protein on chromatin in vivo; the mutant has comparable $K_d$ with greatly increased $k_{off}$ for DNA binding. The increase in both on-rate and off-rate of DNA binding results in a substantially reduced residence time of STAT1(K544A/E545A) on STAT binding sites. We find a similar correlation between off-rate and transcriptional potency for STAT1(N460A), which bears a mutation in the DNA binding domain. These results yield insight into the rate of complex assembly involving STAT1 that leads to transcriptional stimulation.

The STATs (signal transducers and activators of transcription) are latent cytoplasmic transcription factors activated by tyrosine phosphorylation in response to a wide variety of extracellular signaling proteins (1). The modular structure of the STAT proteins includes an SH2 domain that engages in a reciprocal phosphotyrosine-SH2 interaction to hold DNA binding dimers together and a DNA binding domain that is separated from the SH2 domain by a linker domain of unknown function (1–3).

We previously described the failure to stimulate transcription by a mutant in the linker domain of STAT1, STAT1(K544A/E545A) (4). This mutant protein was normally tyrosine- and serine-phosphorylated, accumulated in the nucleus, and bound a high affinity STAT site in an electrophoretic mobility shift assay (EMSA). This mutant also participated normally in transcriptional responses to IFNγ, which involves formation of STAT1:STAT2 heterodimers that cooperates with a third protein, ISGF2/p48/IRF-7. Yet, STAT1(K544A/E545A) failed to trigger transcriptional activation of either reporter or endogenous genes in response to IFNγ, which requires a STAT1: STAT1 dimer (STAT1 homodimer). Therefore, we concluded that the linker domain might have a role in recruiting transcriptional coactivators to IFNγ-responsive start sites.

We have now expanded our analysis of the ability of STAT1(K544A/E545A) to bind DNA. We find that, instead of playing a role in recruiting transcriptional co-activators, the linker mutation affects binding to different STAT1 binding sites to different degrees. By in vitro measurement, the off-rate of the mutant protein is much higher than wild type, which likely determines whether the STAT molecule can activate transcription inside cells. Although it was hypothesized, based on structural analysis of STAT1, that the linker domain might communicate SH2-phosphotyrosine engagement to the DNA binding domain (2), we find that other mutations in the linker domain do not necessarily impair DNA binding. Finally, we show that, despite strong equilibrium binding to certain STAT binding sites, STAT1(K544A/E545A) cannot activate transcription in response and that the phosphorylated mutant protein cannot accumulate on chromatin. From these results, we suggest a time-dependent role of bound STAT molecules in the generation of an effective assembly of proteins to initiate regulated transcription.

EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, and Plasmids—U3 cells and derivatives were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum (Cosmic calf serum; HyClone) at 37 °C and 5% CO2. Stable transfectants were maintained in 400 μg/ml of G418 per ml, and the same STAT1Δc and STAT1(K544A/E545A) clones were used for all experiments. Transient transfections were performed with SuperFect reagent (Qiagen) using 5 μg of total DNA and 5 μl of SuperFect per well of a 6-well plate. IFNγ was used at a final concentration of 5 ng/ml, in the case of transient transfections, usually 24 h after the introduction of DNA.

Immunoprecipitation, supershifting, and Western blotting of STAT1 was performed with an anti-STAT1 C-terminal polyclonal antibody (Santa Cruz Biotechnology). Activated STAT1 was detected with a polyclonal antibody raised to a peptide containing phosphotyrosine 701 (New England BioLabs). FLAG epitopes were detected with M2 monoclonal antibody (Sigma Chemical Co.). c-Eyk was detected with polyclonal antisera provided by Daniel Besser.

All mammalian expression constructs were derived from pRC/CMV (Invitrogen). The point mutations (W555A, W557A, K336A, E421A, N460A, K336A/E421A/N460A) were prepared using a QuikChange site-directed mutagenesis kit (Stratagene) and verified by sequencing. The STAT1Δc and STAT1(K544A/E545A) constructs were previously de-
responded to IFN-γ/H9251 RT-PCR of target genes introduced into U3 cells and selected as stable clones. As measured by constructs (data not shown).

The EMSA decay in EMSA activity was quantitatively measured by a PhosphorImager and normalized against the gel shift activity at $t = 0$.

Recombinant STAT1 Variants

Preparation of Internally FLAG-tagged STAT1 and Production of Recombinant STAT1 Variants—The internal FLAG-tagged STAT was provided by Albert Deisseroth (5). The 4xm67-tk luciferase construct as well as RC/CMV c-Eyk constructs were a gift of Daniel Besser (6). The CMV-driven enhanced green fluorescent protein (EGFP) was a gift of Marc Fuccillo.

Preparation of Internally FLAG-tagged STAT1 and Production of Recombinant STAT1 Variants—The internal FLAG-tagged STAT was prepared by a two-part PCR strategy and resulted in a FLAG epitope inserted between helix 1 and 2 of the STAT1 coiled coil domain (“FA12” tagged). In the first step, two PCR fragments were generated using RC/CMV STAT1α amplified by Vent Polymerase (New England Biolabs) with two different sets of primers: 1) an upstream primer in the RC/CMV backbone (5′-TCTATATAAGCAGCTCTCTG-3′) and a FLAG antisense/STAT1 chimeric primer (5′-CTTGGTCGTACGTCATT-TTGATGTTTCTGTAGGATCTGTTAAGTGAGCAGAAGCGA- GGCGCTGTTACTCAAGAAG-3′); and an antisense primer in the STAT1 chimeric primer (5′-GACTACAAAGACGATGACGACAAGCAA-TGTAGTCTTTCTGATCACTCTTTGCCACAC-3′); 2) a FLAG sense/STAT1 linker domain. The resulting two fragments were annealed to each other and amplified using the outside primer pairs. The resulting fragment was cut with restriction site “dIII” and swapped with the “dIII” fragment with the corresponding DNA binding site and either STAT1(K544A/E545A)FA12 or STAT1(K544A/E545A)FA12 to measure off-rates of DNA binding. d, the resulting protein was equilibrated with the 32P-labeled oligonucleotide at room temperature. DNA complexes were loaded on the EMSA gel. Radiolabeled DNA complexes were then separated by 4% polyacrylamide gel electrophoresis at 4 °C (400 V, 0.25 × Tris borate-EDTA). To measure dissociation rates of STAT-oligo complexes, a 400-fold excess of cold probe was added. After the stated incubation period at room temperature, the residual STAT-DNA complexes were loaded on the EMSA gel. Radiolabeled oligonucleotides were prepared by T4 polynucleotide kinase phosphorylation of oligonucleotides corresponding to STAT binding sites. The STAT binding sites (m67, Ly6E, IRF-1).

Western blotted by the indicated antibodies. b, EMSAs were performed with three dilutions of recombinant FLAG-tagged STAT1 molecules and three different STAT binding sites (m67, Ly6E, IRF-1).

Electrophoretic Mobility Assay—The indicated amount of extract or protein was equilibrated with the 32P-labeled oligonucleotide at room temperature for 15 min as previously described (4). DNA-protein complexes were then separated by 4% polyacrylamide gel electrophoresis at 4 °C (400 V, 0.25 × Tris borate-EDTA). To measure dissociation rates of STAT-oligo complexes, a 400-fold excess of cold probe was added. After the stated incubation period at room temperature, the residual STAT-DNA complexes were loaded on the EMSA gel. Radiolabeled oligonucleotides were prepared by T4 polynucleotide kinase phosphorylation of oligonucleotides corresponding to STAT binding sites. The STAT binding sites (m67, Ly6E, IRF-1).
sense strand of the oligos were: m67, 5′-GATCCATTTCCCGTAAATCAGATCG-3′; IRF-1:5′-CTATTTCCCAGGAAATGGA-3′; Ly6E: 5′-GATCTTTATGCTAAATTTCTGTAA-3′ (GAS sites are underlined, and italics indicate artificial sequence). Quantitation was performed on a Molecular Dynamics Storm PhosphorImager.

**Chromatin Immunoprecipitation—**Chromatin immunoprecipitation was performed essentially as described (available at www.upstatebiotech.com/support/protocols/chips.html). Cell lines were stimulated with IFN-γ for 30 min (in our experience the peak accumulation of STAT1 on IRF-1 locus) and then fixed with 1% formaldehyde (final concentration) for 10 min. Following lysis and sonication, an input sample was saved (2.5% of total), the remaining extract was diluted 1:10 in dilution buffer, and 1-2 μg of anti-STAT1 C-terminal antibody was added. After overnight mutation, protein-DNA complexes were recovered with sheared salmon sperm, protein A-agarose (Upstate Biotech), washed extensively, and eluted by sodium bicarbonate, SDS buffer. Cross-link reversal at 65 °C, phenol/chloroform extraction, and precipitation yielded DNA for radioactive PCR. The input and precipitation yielded DNA for radioactive PCR. The input and precipitation yielded DNA for radioactive PCR. The input and precipitation yielded DNA for radioactive PCR. The input and precipitation yielded DNA for radioactive PCR. The input and precipitation yielded DNA for radioactive PCR. The input and precipitation yielded DNA for radioactive PCR.

**Statistical Analysis—**Luciferase and RT-PCR analysis—Luciferase experiments were performed by transfection of U3 cells. In addition to luciferase reporter plasmid and STAT1-variant expression plasmid, EGFP and β-galactosidase were co-transfected in each experiment. Each experimental condition was tested in triplicate. After 4–5 h of treatment with IFN-γ, the cells were lysed and assayed for luciferase activity normalized against β-galactosidase activity. RT-PCR was performed as described previously (4).

**RESULTS**

**Analysis of Purified STAT1(K544A/E545A) Reveals Abnormalities in DNA Binding—**To examine further the properties of the previously described linker domain mutant of STAT1, we designed a new procedure for purifying FLAG-tagged STAT molecules from baculovirus-infected Sf9 cells (see “Experimen- tal Procedures”). An epitope tag was inserted between the first two helices of the STAT1 coiled-coil domain, a location that has the advantage of not interfering with transcription of in vivo gene loci as do many C-terminally tagged variants (data not shown). These “FA12” derivatives of wild type and mutant STAT1α/β were then phosphorylated in vitro as previously described (7). The equal concentration and extent of phosphorylation (Fig. 1a) of purified products was assayed by Western blot using anti-FLAG and anti-pY701 antibodies.

Examination of the relative binding affinity by the purified STAT1 derivatives was accomplished through EMSA analysis of recombinant protein product. As shown in Fig. 1b, the STAT1(K544A/E545A)/FA12 mutant had substantial although reduced (2- to 5-fold) binding compared with wild type STAT1α/FA12 for the high affinity GAS sites m67 and IRF-1. By diluting the proteins in the binding assay we estimated the KD of the STAT1-IRF-1 complex to be ~1.5 × 10^-8 M for wild type and mutant STAT1, a number consistent with previous binding measurements of STAT1 (7, 9). However, binding of the mutant to the low affinity site Ly6E was severely impaired, a surprising finding in that the m67 and Ly6E sites differ by only a single base pair in the center of the GAS (IFNγ activation site) palindrome (−7-CATTCCGGTAAATC−7−→−7-ATAT-TCCTGTAACTG−7−). Statistical analyses of natural STAT binding sites show that an A in the −7− position and non-G/C base pairs in the “0” or middle base pair are extremely rare (6 and 2%, respectively) (10), and a SELEX (in vitro selection of DNA pools followed by amplification) experiment with STAT1 also suggested that T in the center position is unfavored (11). However, the X-ray crystal structure of STAT1 bound to m67 excluded the possibility of direct contact of DNA with the linker domain, and lysine 336, which contacts position “0,” is more distant from K544-E545 than asparagine 460, which contacts positions “3” and “4” (i.e. TT) (2). The importance of the K336-DNA contact is highlighted by the total loss of DNA binding by STAT1 bearing a mutation of this residue (as will be discussed later, Fig. 5b).

EMSA is an indirect assay that measures equilibrium binding of a protein with DNA at a given protein:DNA ratio. It is known that DNA-protein complexes, upon initial electrophoresis, enter gel pores together and remain together during the course of electrophoresis. To assess possible differences in kinetics of DNA binding, we tested the stability of STAT1-labeled-DNA complexes by adding excess cold probe and examining remaining complexes after various times (7). As seen in Fig. 1c, the decay in the number of STAT complexes detected by gel electrophoresis occurred much more rapidly for STAT1(K544A/E545A)FA12 than for STAT1α/FA12. Quantitatively, the half-life of the mutant-DNA complex was no more than 1 min or at least 40 times shorter than wild type (Fig. 1d). Given the similar KD values of STAT1α/FA12 and STAT1(K544A/E545A)FA12 (estimated from the number of complexes at a fixed protein:DNA ratio), the off-rate measurement implies a real increase in both koff and kcat for STAT1(K544A/E545A), because kcat = koff/kcat. In other words, STAT1(K544A/E545A) has increases in both the rate of DNA binding and rate of dissociation. This finding was replicated with the m67 binding site (data not shown). In addition, these observations were not dependent upon the FA12 tag, because they were later confirmed in the context of untagged STAT1 constructs (see below).

**Trp-555 and Trp-557 Are Not Critical for DNA Binding by STAT1—**The crystallographic structure of STAT1 revealed a unique configuration of conserved tryptophans 555 and 557 in the linker domain. Specifically, it was suggested that the two tryptophans might couple STAT dimerization to stable DNA binding (2). Because unstable DNA binding is the apparent phenotype of STAT1(K544A/E545A), we examined W555A and W557A mutant proteins for several functions. As shown in Fig. 2a, STAT1(W557A) activation of a luciferase reporter plasmid by IFNγ was greatly decreased compared with wild type protein. In contrast, STAT1(W555A) consistently gave an equivalent induction compared with STAT1α.

The results in transcriptional induction were paralleled by EMSA assays. The W555A from IFNγ-treated cells bound DNA almost as well as wild type, but the W557A hardly bound at all (Fig. 2b). To determine the basis for the failure of the W557A mutant to bind DNA mutants, we prepared extracts from cells that should contain maximally phosphorylated STAT1 derivatives. Either constitutively active or kinase-dead CD8-c-Eyk constructs were transiently transfected along with either STAT1α, STAT1(K544A/E545A), STAT1(W555A), or STAT1(W557A) into U3 cells. The constitutively active CD8-c-Eyk chimeric receptor can phosphorylate STAT1 and thus act as a potent source of STAT1 phosphorylation when overexpressed in mammalian cells (6). When coupled with interferon treatment of the CD8-c-Eyk containing cells, high concentrations of active STAT1 are present in cell extracts. As seen in Fig. 2c, all constructs with the exception of STAT1(W557A) were phosphorylated robustly by IFNγ treatment of the CD8-c-Eyk-transfected cells. This result explains the lack of DNA binding activity by the STAT1(W557A) mutant seen in Fig. 2b, perhaps suggesting some role of the linker domain in physiological SH2 function. Further experiments on this topic have not been carried out.

The activated STAT1 derivatives from Fig. 2c were then used to assess the DNA binding properties of the protein that did become phosphorylated. STAT1(W555A) and STAT1(W557A),
FIG. 2. Mutations in conserved residues Trp-555 and Trp-557 do not impair DNA binding properties of STAT1. a, U3 cells were transfected with 4xm67-luciferase reporter plasmid and RC/CMV expression constructs bearing wild type STAT1, STAT1(W555A), or STAT1(W557A). Following 4–5 h of IFN-γ stimulation, luciferase values were measured, and -fold induction was calculated as described under “Experimental Procedures.” b, U3 cells were transfected with the indicated expression constructs and stimulated with IFN-γ for a half hour. Cells were lysed in whole cell extract buffer, and the STAT dimer was visualized by EMSA with IRF-1 oligonucleotide. Anti-STAT1 C-terminal polyclonal antibody was used to supershift the STAT1 DNA complex. c, indicated STAT1 constructs were transfected into U3 cells with constitutively active (CA) or kinase-dead (KD) CD8-c-Eyk.
FIG. 3. STAT1(K544A/E545A) fails to trigger transcriptional activation on reporter constructs or target loci to which it binds under equilibrium conditions. a, U3 cells were transfected with STAT1 or STAT1(K544A/E545A) as well as a luciferase reporter gene attached to the (−1312 to +7) region of the human IRF-1 promoter. b, same as panel A except that an artificial luciferase promoter was used with four m67 sites upstream of the HSV thymidine kinase promoter. c, clonal, STAT1α, or STAT1(K544A/E545A) stable transfectants of U3 cells were activated with IFN-γ over the indicated time course. Following radioactive RT-PCR with the indicated primer sets, the product bands were separated by 5% polyacrylamide gel and visualized by autoradiography.

d,8 g of whole cell extract from IFN-γ-treated, c-Eyk CA-transfected cells from panel c were incubated with EMSA probes for m67 (M), IRF-1 (I), or Ly6E (L) STAT binding sites. e, EMSA was used to estimate the dissociation rate between STAT1 isoforms and the IRF-1 STAT binding site. Minutes after addition of excess cold probe are indicated.

After stimulation of the CA-transfected cells with IFN-γ for 30 min, the cells were lysed in whole cell extract buffer. 20 µg of each extract was separated by SDS-PAGE and blotted with the indicated antibody. The asterisk indicates the slower mobility, tyrosine-phosphorylated STAT1 isoform. d, 8 µg of whole cell extract from IFN-γ-treated, c-Eyk CA-transfected cells from panel c were incubated with EMSA probes for m67 (M), IRF-1 (I), or Ly6E (L) STAT binding sites. e, EMSA was used to estimate the dissociation rate between STAT1 isoforms and the IRF-1 STAT binding site. Minutes after addition of excess cold probe are indicated.
although weakly phosphorylated, bound all three GAS sites tested, including the Ly6E site to which STAT1(K544A/E545A) failed to bind at all (Fig. 2d). The limited amount of binding by STAT1(W557A) is consistent with the extent of phosphorylation shown in Fig. 2c. When off-rate measurements were made, neither STAT1(W555A) or STAT1(W557A) exhibited the rapid off-rate seen with STAT1(K544A/E545A) (Fig. 2e). Thus, mutations in conserved residues of the linker domain of STAT1 do not necessarily impair DNA binding kinetics.

STAT1(K544A/E545A) Is Incapable of Supporting Transcription on Chromosomal or Reporter Genes—Our original experiments with STAT1(K544A/E545A) relied upon a luciferase construct with three Ly6E sites driving a thymidine kinase promoter (4). However, we have shown above that STAT1(K544A/E545A) fails in EMSA assays to bind Ly6E sites effectively but does bind to IRF-1 and m67 sites. Therefore, we repeated transfection assays using IRF-1- or m67-driven reporter constructs to test the in vivo activity of STAT1(K544A/E545A) (Fig. 3, a and b, respectively). In both cases, the STAT1(K544A/E545A) failed to activate transcription in response to IFNγ despite strong induced EMSA activity for these binding sites.

Our original RT-PCR experiments on complemented, stable U3 transfectants used only a single time point to score transcriptional induction. Given the altered DNA binding kinetics for STAT1(K544A/E545A), we tested the ability of this mutant to respond to IFNγ on chromosomal genes over a wider time period. As shown in Fig. 3c, RT-PCR examination at several time points did not reveal significant accumulation of either GBP1 or IFR-1 mRNA in cells containing STAT1(K544A/E545A). Thus there is no detectable transcriptional activation by STAT1(K544A/E545A) on chromosomal or on transfected genes.

STAT1(K544A/E545A) Does Not Accumulate on the IRF-1 Locus in Vivo—Because STAT1(K544A/E545A) has an accelerated dissociation from DNA but did bind oligonucleotides from the IRF-1 promoter, we determined whether a significant amount of this mutant bound to the chromosomal template of the IRF-1 gene. Chromatin immunoprecipitation (ChIP) using anti-STAT1 antisera was carried out in complemented U3 cell lines that had been stimulated for 30 min with IFNγ. In three separate experiments, we observed STAT1 accumulation on the IRF-1 promoter for wild type complemented cells but not for STAT1(K544A/E545A) complemented or U3 parental cells. This difference occurred despite significant amounts of phosphorylated STAT protein in the nuclei of the two complemented U3 cell lines (Fig. 4c). Thus, we conclude that the STAT1(K544A/E545A) mutant fails to activate transcription, because it never accumulates on promoter STAT binding sites, consistent with the altered DNA binding kinetics of the STAT1(K544A/E545A) mutant.

Off-rate Correlates with Transcriptional Activity for Mutations in the DNA Binding Domain—The crystal structure of STAT1 bound to an m67 oligonucleotide suggested three major contact points between STATs and DNA: lysine 336, glutamate 421, and asparagine 460 (2). For comparison with the STAT1(K544A/E545A) mutant, STAT1 constructs with single or triple mutation of these residues were prepared and tested. As seen in Fig. 5a, only STAT1(E421A) retained transcriptional activity. However, STAT1(N460A) and STAT1(E421A) both gave clear EMSA activity when phosphorylated by c-Eyk (Fig. 5b); Western blotting also indicated that both constructs were tyrosine-phosphorylated upon treatment with IFNγ (data not shown). Because the N460A mutant phenotype resembled that for STAT1(K544A/E545A), we tested its off-rate for DNA bind-

**Fig. 4.** STAT1(K544A/E545A) fails to accumulate on the IRF-1 promoter in vivo despite nuclear gel shift activity to the corresponding STAT binding site. a, diagram of a fragment of the human IRF-1 locus detected by chromatin immunoprecipitation. Relative position of the GAS site to transcription start site is indicated. b, U3 cells or stable transfectants bearing STAT1α or STAT1(K544A/E545A) were treated with IFNγ for 30 min and then subjected to chromatin immunoprecipitation with anti-STAT1 C-terminal polyclonal antibody. Input and precipitated DNA were then amplified by radioactive PCR followed by polyacrylamide gel separation and autoradiography. c, EMSA of 4.5 μg of nuclear extract from three stable cells used in panel b with m67 and IRF-1 probes. The supershift was performed with anti-STAT1 C-terminal antibody.

**DISCUSSION**

Electrophoretic mobility shift assays (EMSAs) have gained widespread acceptance as a measure of protein-DNA interaction (9). However, the limitations of the assay are not widely appreciated: The assay is indirect, and due to the "caging" phenomenon (effectively irreversible protein-DNA association when the two interacting species enter gel pores together during electrophoresis), true binding kinetics become obscured. Both these limitations are clearly demonstrated by the present series of experiments. Although STAT1(K544A/E545A) binds a variety of DNA probes as detected by EMSA, it fails to be detected on genes by chromatin immunoprecipitation. And although the apparent affinity for IRF-1 and m67 binding sites is close to wild type, the mutant exhibits a greatly accelerated off-rate for the interaction with these probes. In fact, the 40-fold increase in off-rate probably also accounts for the failure to detect STAT1(K544A/E545A) accumulation by chromatin precipitation (ChIP). The rate of formaldehyde cross-link formation during the chromatin precipitation procedure is known to be a slow process, occurring over minutes rather than seconds (12, 13). The decreased residence time by the STAT1(K544A/E545A) mutant on the IRF-1 promoter may therefore be insufficient to allow for efficient cross-linking. To our knowledge, the STAT1(K544A/E545A) mutant is the first DNA binding factor
FIG. 5. Off-rate kinetics correlate with transcriptional activity for mutants in the STAT1 DNA binding domain. a, U3 cells were transfected with 4xm67 reporter plasmid and either wild type, single (K336A, E421A, N460A), or triple (K336A/E421A/N460A) point mutant constructs of STAT1. Experiments were worked up according to the protocol described in Fig. 2a. b, U3 cells were transfected with CD8-c-Eyk, EGFP, and one of five STAT1 constructs: wild type, K336A, E421A, N460A, or the triple mutant (K336A/E421A/N460A). After stimulation with IFNγ for 0.5 h, whole cell extracts were made and 8 μg of extract was used in an EMSA with radioactive m67 probe. c, EMSA was used to estimate the dissociation rate between wild type or mutant STAT1 and the IRF-1 GAS site. The extracts from panel b were the source of phosphorylated STAT1 molecules. Minutes after addition of excess, cold probes are indicated.
to demonstrate so severe a discordance between EMSA activity and in vivo binding. However, such studies require cells in which wild type protein is missing so that the function or lack of function of mutant proteins can be examined after permanent introduction.

These results are most easily understood by revisiting the familiar equations $K_d = k_{off}/k_{on}$ and $k_{off} = \ln(2)/t_{1/2}$ (for STAT DNA ↔ STAT + DNA) (14). This means that off-rate and on-rate for STAT-DNA dissociation can both increase while keeping the $K_d$ constant. Thus the 40-fold increase in $k_{off}$ (40-fold decrease in half-life) with a similar $K_d$ for the STAT1(K544A/E545A) dimer suggests that the mutant protein binds and dissociates from DNA more rapidly than wild type protein (i.e. $k_{on}$ and $k_{off}$ increase). The crystal structure of STAT1 bound to DNA ("pliers grip") is in accord with this idea, because wild type STAT1 dimers clearly envelope their target DNA sequences (2). Given the strong reciprocal phosphotyrosine-SH2 interaction plus the total envelopment of the DNA by the dimer (15), it is reasonable to infer that STAT1 (possibly STATs in general) may undergo a limited conformational change in binding and dissociating from DNA. Any such conformational change would quite probably involve the linker and/or DNA binding domains. The K544-E545 residues and the linker domain as a whole may assist this process by providing enough flexibility to allow binding but also enough rigidity to ensure a reasonable residence time for STAT1. Although the surface-exposed K544-E545 residues do not appear to have any contact with residues directly involved in DNA binding, local structural perturbations in the linker domain might increase the "flexibility" of the linker domain enough to account for increases in both association and dissociation rates. These perturbations apparently leave intact the residues that contact the phosphate backbone and major groove and that provide the majority of the binding energy between STAT and DNA, because STAT1(K544A/E545A) can still bind GAS sites with affinities in the range of wild type. Although similar findings have been reported for other types of protein interactions (16), we have been unable to find any other mutational study that has likewise altered protein-DNA binding kinetics without significantly changing binding affinity.

Lastly, our data may provide some of the first indications about the rate of transcriptional complex formation by the STATs in vivo. Although it is still possible that STAT1(K544A/E545A) may have defective co-activator recruitment, there seems little question that the primary defect in this mutant is its abnormal DNA binding kinetics and inability to accumulate on an in vivo template. Fusion proteins of Gal4 DNA binding domain and the STAT1 C terminus are active under the same conditions as Fig. 3 (a and b), and the Gal4 DNA binding domain has an off-rate of ~20–30 min under physiologic conditions.2 Given that STAT1(K544A/E545A) still bears the C-terminal activation domain, it is not unreasonable to entertain the notion that STAT1(K544A/E545A) would be able to activate transcription were it to reside for a sufficiently long time on a GAS-bearing promoter. Interpreted in this light, transcriptional activation by STAT1 simply takes more time than the residence time of the mutant on DNA ($t_{1/2} = 1$ min). In support of this idea, the correlation between a rapid off-rate and transcriptional effectiveness was observed for both STAT1(N460A) and STAT1(K544A/E545A).

Signal-regulated transcription factors have been shown to act over different time scales. On the one hand, CHIP data for estrogen-responsive promoters argues for nearly instantaneous recruitment of RNA polymerase II in response to activator accumulation (17). This fact is consistent with estrogen receptor’s off-rate of 1–2 min (18), and observations of glucocorticoid receptor DNA binding dynamics in vivo, which suggest extremely rapid on/off-rates (19). On the other hand, many activators have been demonstrated to accumulate on chromatin hours before RNA polymerase accumulation and maximal transcription rates (20, 21). This time-lag between activator binding and transcription may suggest a time-dependent accumulation of co-activators and components of the basal transcription machinery. In vitro studies of transcription support this idea, because transcription from the adenovirus major late promoter takes 10–20 min to reach maximal activity in crude extracts (22). Although preliminary, our data suggest that STAT1 must reside at transcriptional start sites for a period somewhere between the residence time of mutant and wild type, or 1–40 min. Given the fact that active STAT dimers accumulate in the nucleus within 5 min of ligand activation (8), this situation would imply that co-activator assembly is potentially a rate-limiting component of transcription by the STATs.

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