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Transcription factor activity of STAT proteins: structural requirements and regulation by phosphorylation and interacting proteins

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Abstract. The seven mammalian members of the signal transducer and activator of transcription (STAT) family share a common core structure which reflects their shared mechanism of activation, dimerization, and DNA binding. By contrast, the STAT C termini containing the sequences required for transcriptional activation are much less homologous, suggesting different ways by which individual STATs activate their target genes. This paper describes several important discoveries linked to mechanistic aspects of STAT transcription factor function. These include regulated serine phosphorylation of the transactivating domain, promoter-dependent interactions of STATs with each other, or of STATs with other transcription factors, and with transcriptional co-activators. The basis, background, and implications of these molecular events will be summarized and discussed.

Key words. STAT; transcription; phosphorylation; gene; promoter.

STAT target genes and their promoters

To be regulated by signal transducers and activators of transcription (STATs), the promoter of a gene must contain at least one small palindromic DNA sequence, represented by TTN_5AA or TTN_6AA consensus nucleotides and designated a GAS element after the prototype sequence originally identified in the promoter of the interferon (IFN)-regulated GBP gene [1, 2]. The optimal binding sites as determined by in vitro selection procedures are very similar for all STATs: 5′TTCCNGGAA3′ for STATs 1, 3, 4, and 5, or TTCCNNGGAA for STAT6 [reviewed in refs. 3–5]. Genes induced by IFN-α and IFN-β are the only STAT-regulated genes known to employ a non-STAT DNA-binding subunit contained in the ISGF3 complex, and as a consequence, a direct-repeat element, the ISRE (RRTTTCNNTTTCY; see the contribution by C. Schindler and S. Brutsaert) mediates DNA association. Around 100 potential STAT target genes have been described so far [listed in part in ref. 5]. These comprise genes activated by all signals causing STAT activation, originating from class I and II cytokine receptors, tyrosine kinase receptors, and at least some G-protein-coupled receptors. Functionally, there is no common denominator between STAT target genes. They fall into different categories such as cytokines and cytokine receptors, cell cycle regulators like cyclins and cyclin-dependent kinase (CDK) inhibitors, growth-factor-responsive immediate early genes, signal regulators, or gene products required for or produced by differentiated cells such as enzymes, acute-phase reactants, milk proteins, or immunoglobulins.

In the case of IFN-induced genes, STAT1 dimer or ISGF3 binding to their promoter target sequences appears to be the rate-limiting step in transcriptional activation. This is suggested by in vivo footprinting and nuclear run-on data showing a strict correlation between the binding of STAT1 dimer or ISGF3 and the onset of transcription [6]. However, activated, i.e. tyrosine-phosphorylated, STAT dimers do not always cause transcriptional responses [7, 8]. In fact, as discussed below, STAT activation has in a few cases been linked to gene repression. These findings may indicate a requirement for additional signals or interacting proteins that convert STAT dimers into either active

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transcription factors or transcriptional repressors. Ultimately, the confirmation of a bona fide STAT target gene requires an analysis of gene expression in STAT knockout mice. Several target genes for the different STATs have been verified this way, but cases where the knockout did not produce the expected effect are also known [9–16].

As pointed out above, the optimal binding sequence is very similar for most STATs when determined by selex procedures in vitro. Nevertheless, natural promoters have the ability to contribute to the specificity of gene expression by favouring the binding of certain STATs. TTN_4AA sequences select for the binding of STAT3 and TTN_6AA sequences for binding of STAT6. As a rule, sequences conforming to the TTN_5AA consensus bind all STATs except STAT2, but may do so with significantly different affinities [reviewed in ref. 5]. Examples for this are some acute-phase gene promoters or the GAS sequence in the *c-fos* promoter (the SIE) which strongly favour the binding of STAT3 dimer in interleukin (IL)-6 or growth factor responses, respectively, despite the simultaneous presence of STAT1 dimer.

Structural features of STATs and their relevance for transcripitonal activity

The core structures of STAT1 and STAT3 dimers (roughly amino acids 130–710) were recently deduced from co-crystals with their binding sites [17, 18] (see contribution by C. Schindler and S. Brutsaert). In addition, the crystal structure of the STAT4 N terminus has been determined [19]. These data complete a picture that emerged from earlier computer-aided structure predictions and site-directed mutagenesis [20–26]. From the transcriptional activation point of view it is unfortunate that the crystals did not include most of the C terminus beyond the phosphorylated tyrosines Y701 (STAT1) and Y705 (STAT3). The thermodynamic properties of the STAT C termini may in fact preclude their forming a highly ordered array in a crystal and thus the determination of their structure at high resolution.

Whereas the extreme N and C termini are dispensable for STAT tyrosine phosphorylation in response to cytokines, i.e. the extranuclear signalling function, it appears that all structural elements of the molecule are required for transcription factor activity. The requirement for the DNA-binding domain is obvious, but the firm association of the SH2 domain through the linker segment is crucial to stabilize the interaction of each STAT monomer with DNA. Another important contribution to the stability of DNA association can be made by the N terminus (roughly amino acids 1–120). The crystal structure of the isolated STAT4 N terminus demonstrated formation of eight helices, assembled into a hook-like structure, and forming a hydrophilic patch to interact with similar surfaces of other proteins. Earlier findings had suggested that STAT dimers on separate binding sites can interact to form tetramers (see below) and thus stabilize each other in the DNA-bound state. Mutation of an invariant tryptophan residue (W37) within the predicted interface between STAT N termini reduced transcriptional activity on promoters with two adjacent binding sites and at the same time disrupted the ability of STAT4 to tetramerize, thus assigning this important function to the furthest N terminal STAT domain [19].

Similar to the N domain, the anti-parallel helices of the coiled-coil domain form a hydrophilic interface for potential protein interactions [17, 18]. At this time, the proteins using the coiled-coil domain for connecting to STATs are elusive, but may be among the many transcription factors now known to interact with STATs.

The C-terminal portion of STATs represents a bona fide transactivation domain. Removing the terminal 50 amino acids results in STATs capable of dimerization and DNA binding, but not of transcription activation, as demonstrated for STATs 1, 3, 5 and 6. Strikingly, these C-terminally truncated molecules have the potential to act as dominant-negative alleles when expressed together with wild-type alleles [24, 27–32]. This may be physiologically important, because such variant STATs are generated in various cell types as a result of alternative splicing or proteolytic processing. C-terminally truncated beta forms have been described for STATs 1, 3, 4 and 5; however, their regulatory input into STAT-dependent gene expression remains to be ascertained [20, 33–37].

Individual STAT C termini display fairly little amino acid homology. When assayed in the context of Gal4 fusion proteins, their ability to activate reporter genes may differ significantly, as shown for STAT5 and STAT6 [38], or for STAT1 and STAT2 (D. E. Levy, personal communication). STAT transactivation domain analyses thus suggest major mechanistic differences in the way these domains function.

Modulation of STAT transcription factor activity by serine phosphorylation

Correlative data and the use of serine kinase inhibitors indicated that a serine phosphorylation event might modulate the transcription factor activity of STATs 1 and 3 [39–41]; reviewed in ref. 26]. The occurrence of regulated serine phosphorylation was confirmed for these STATs by phosphoamino acid analyses [39, 42, 43]. A target amino acid for STAT1 and STAT3 serine kinases was uncovered by Darnell's laboratory [43].

Sequence comparison of the STAT1 and STAT3 C termini revealed a conserved serine residue at position 727 within a potential MAP kinase phosphorylation site (fig. 1). S727 of STAT1 was shown to be phosphorylated in response to IFN-γ and weakly in response to platelet-derived growth factor (PDGF). Phosphorylation of STAT3 S727 was strongly induced by PDGF. Mutation of S727 to alanine significantly reduced the ability of STAT1, and to a lesser extent STAT3, to activate reporter and endogenous genes. STAT1 and STAT3 S727A mutations did not affect the binding of the respective homodimers to DNA [43, 44].

The possibility that S727 phosphorylation might impinge on STAT-mediated transcription by influencing tyrosine (de)phosphorylation was considered in several studies. Reconstituting STAT1-deficient cells with wild-type STAT1 or the STAT1 S727A mutation did not reveal a marked influence of the mutation on the intensity or kinetics of tyrosine phosphorylation [45]. Furthermore, the Y701F mutation did not significantly alter the phosphorylation of S727 in response to IFN-γ. These findings with STAT1 mutants were corroborated in macrophages where lipopolysaccharide (LPS) causes pronounced phosphorylation of STAT1 S727 without concomitant tyrosine phosphorylation and primes STAT1 for enhanced transcription factor activity [46]. LPS treatment of macrophages, i.e. induction of the STAT1 S727 kinase, had no apparent effect on subsequent Y701 phosphorylation stimulated by IFN-γ [46]. In conclusion, a two-step activation modus is likely for STAT1 in which the Janus kinase (JAK) (or another tyrosine kinase) triggers dimerization and the ability to bind DNA, whereas a serine kinase acting on S727 regulates the transactivation potential independently of tyrosine phosphorylation.

Unlike STAT1, S727 phosphorylation of STAT3 may impinge on tyrosine phosphorylation. Increased tyrosine phosphorylation of the STAT3 S727A mutant in response to epidermal growth factor (EGF) was observed in transfected Cos cells [47]. In another transfection study, phosphorylation of S727 inhibited tyrosine phosphorylation of STAT3 by an overexpressed Src kinase or by EGF [48]. Both reports thus suggest a negative effect of S727 phosphorylation on STAT3 tyrosine phosphorylation. Whether phospho-S727 contributes to the down-regulation of STAT3-dependent cytokine responses under more physiologic conditions remains to be shown. Assuming that S727 phosphorylation increases the transcriptional potential as suggested by Darnell's laboratory and at the same time acts as a dephosphorylation signal, the net effect of phosphorylated S727 would be to concentrate cytokine-induced gene expression involving STAT3 by increasing the output per time, but at the same time reducing the overall duration of the response.

Identification of the kinases phosphorylating STAT1/3 S727 in various situations is of crucial importance to understanding the regulation of STAT transcription factor activity. The conservation of a PMSP motif at an identical position in the STAT1 and STAT3 C termini suggested that both proteins might be regulated by the same serine kinase. This original assumption might still be true for some situations; however, in many experimental situations, STAT1 and STAT3 S727 phosphorylation can be uncoupled. For example, signals from the colony-stimulating factor (CSF)-1 receptor tyrosine ki-

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Stat1       GYIKTELISV.........SEVHPSRLQTT.DNL..LPMSPEE...FDEMSRI...VGPEF.
Stat3       PYLKTKFICV.........TPTTCS..NTI.D....LPMSPRT...LDSLMQFG.NNGEGAE
Stat4       GYVPSVFIPI.........STI.RSDSTEPQSPSDLLPMSPSA.YAVLRE.....NLSPTTI
Stat5a      GYVKPQ.I.KQVVPEFVNASTDAGAS.ATYMDQA...P.SP.....VVCP.QPHYNMYPPNP
Stat5b      GYVKPQ.I.KQVVPEFANASTDAGSG.ATYMDQA...P.SP.....VVCP.QAHYNMYPPNP
Consensus   GYVK---I-V---------ST---S---T--D----LPMSP-----VV---Q---N--P---

Stat1       DSMMSTV
Stat3       PSAGGQFESLTFDMDLTSECATSPM
Stat4       ETAMNSPYSAE
Stat5a      DPVLDQDGE..FDLDESMDVARHVEELLRRPMDSLDARLSPPAGLFTSARSSLS
Stat5b      DSVLDTDGD..FDLEDTMDVARRVEELLGRPMDSQWIPHAQS
Consensus   DS---------FD-D-TMDVAR-VEELL-RPMDS-------------------
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Figure 1. Serine phosphorylation sites within STAT C termini. The STAT4 phosphorylation site is deduced from its homologous position to the others; however, we have no knowledge of firm experimental evidence for phosphorylation of STAT4 S722.

nase cause no phosphorylation of STAT1 S727 in macrophages, while leading to robust phosphorylation of STAT3 S727 [46; P. Kovarik and T. Decker, unpublished data]. Conversely, IFN-γ causes phosphorylation of STAT1 S727 while not affecting STAT3. Thus, STAT3 must be a substrate for serine kinase(s) not affecting STAT1 and vice versa. Several laboratories have reported that serine kinase inhibitors differentially affect phosphorylation of the same STAT in different cytokine responses. For example, STAT3 S727 phosphorylation in response to EGF, IL-2, or T cell receptor signalling can be inhibited by the MEK inhibitor PD98059, but not the inhibitor H7, whereas the exact opposite is true for STAT3 S727 phosphorylation in response to IL-6 or IFN-α [47, 49]. Likewise, STAT1 S727 phosphorylation in response to IFN-γ is inhibited by PD98059, but not the p38MAPK inhibitor SB203580, whereas S727 phosphorylation in response to LPS has the opposite sensitivity to these inhibitors [R. Kovarik and T. Decker, unpublished data]. Thus, it appears that there are several STAT serine kinases, or signal transduction paths activating such kinases, and at least some of these are selective for either STAT1 or STAT3.

An original notion that the MAP kinase ERK2 might be a STAT1 S727 kinase was fuelled by a study demonstrating activation of ERK2 by the IFN-α receptor, an association between STAT1 and ERK2 as well as an interference of a dnERK2 allele with IFN-α-induced transcription [50]. More recently, expression of a dominant-negative allele of the Pyk2 tyrosine kinase in fibroblasts was reported to cause parallel inhibitory effects on STAT1 serine phosphorylation and ERK2 activation by IFN-γ [51]. These data suggest a link between ERK2 and STAT1 serine kinase activity; however, there is also experimental evidence arguing against a role for ERK2 in STAT1 S727 phosphorylation. ERK2 activity and STAT1 S727 phosphorylation can be uncoupled in physiological responses [46, 52]. Moreover, the STAT1 C terminus is a poor substrate for ERKs in vitro [47; R. Kovarik and T. Decker, unpublished data]. Preliminary biochemical evidence was recently provided for a non-ERK STAT1 kinase by Darnell's laboratory. This partially purified kinase specifically phosphorylated STAT1 S727, but its regulation by the appropriate cell surface stimuli could not be demonstrated [45].

While these experiments cast some doubt on a simple enzyme-substrate relationship between ERKs and STAT1, considerable evidence has been produced in favour of ERK2 as a STAT3 kinase in several situations. Importantly, STAT3 S727 is phosphorylated when ERKs are active and ERK2 phosphorylates the recombinant STAT3 C terminus in vitro [47–49]. Thus ERKs may mediate the PD98059-sensitive phosphorylation of STAT3 S727.

Like STAT1 and STAT3, STATs 4 and 5 were shown to be targetted by a regulated serine kinase. In natural killer (NK) cells and T lymphocytes both IL-12 and IFN-α cause phosphorylation of STAT4 on both tyrosine 694 and serine [53]. Pharmacological evidence suggests that inhibition of serine phosphorylation in STAT4 reduces its ability to activate transcription. STAT4 contains a $PMS_{722}P$ motif in a homologous position with regard to STAT1/3 S727 and it is likely, but so far unproved, that the phosphorylated serine is S722.

STAT5 contains a conserved PSP motif at positions 725 (STAT5a) and 730 (STAT5b). The hormone prolactin causes STAT5 phosphorylation on tyrosine and on S725 (STAT5a) and S730 (STAT5b) [54–56]. Prolactin-stimulated phosphorylation is resistant to the inhibition of MEK. STAT5 S725A or S730A mutants isolated from prolactin-stimulated cells still contain phosphoserine after stimulation with prolactin, suggesting the presence of at least one more phosphorylated site. According to a report on growth-hormone-induced STAT5 activation by Pircher et al. [57], this second site may be S780 in STAT5a and targeted by ERKs. However, evidence for phosphorylation of this residue in cells has not been presented. Studies in knockout mice suggest that STAT5b is the predominant mediator of growth hormone responses, but S780 is not conserved in STAT5b. Therefore, the physiological significance of S780 phosphorylation and the proposed effect of this event on STAT5-mediated transcription still needs to be ascertained. Yamashita et al. [56] found no significant difference in the ability to stimulate transcription in response to prolactin between wild-type STAT5 and the STAT5a S725A or STAT5b S730A mutants.

In lymphocytes, STAT5 serine phosphorylation occurs in response to IL-2 and is insensitive to MEK, mTOR and PI3 kinase inhibition [58, 59]. The acidic and carboxy-terminal regions within the IL-2 receptor (IL-2R) beta chain were found to be independently capable of mediating activation of the STAT5 serine kinase.

Recently, experimental strategies have been devised to dimerize STATs in cells in the absence of a cytokine-receptor-derived signal, i.e. in the absence of cytokine-induced serine kinase activity. In one case, dimerization of STAT3 was achieved through fusion of gyrase B domains capable of binding the drug coumermycin. Drug-induced dimerization of STAT3 mimicked the activity of IL-10 in inhibiting the proliferation of J774 macrophages [60]. In an alternative approach, a STAT5 gene was subjected to random mutagenesis and individual mutants selected to confer growth factor independence upon IL-3-dependent preB cells. A double point mutation (H299R and S711F) was found to be constitutively phosphorylated on tyrosine, to be located in the cell nucleus and to activate STAT5 target genes [61].

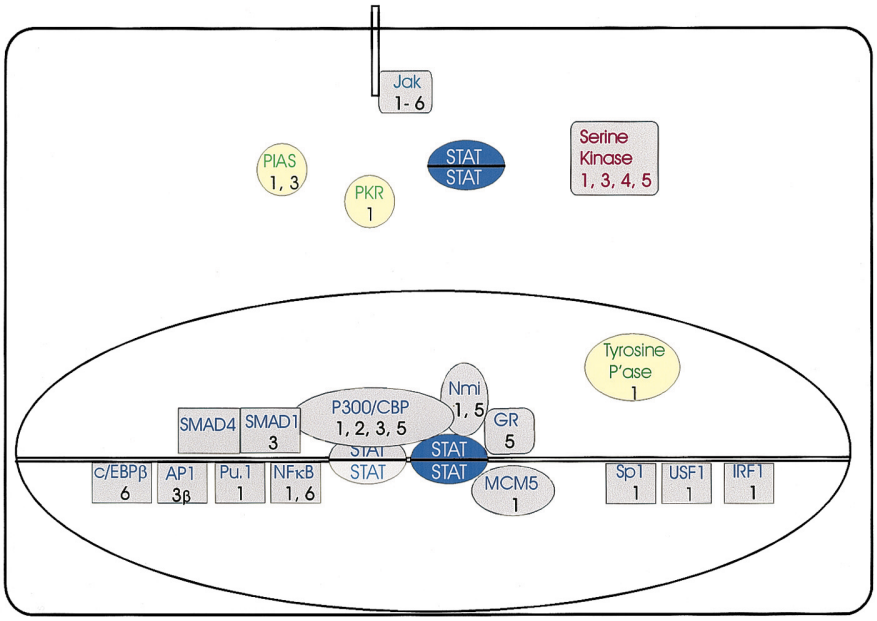

Figure 2. A compilation of proteins with an influence on STAT transcription factor activity. Stimulatory interactions are shown in blue on a grey background, inhibitory interactions are in green on a yellow background. Serine kinases have been implicated in both stimulatory and inhibitory phosphorylation (see text). The numbers below the names of individual proteins indicate for which STAT a functional interaction and/or physical association has been proposed. Among transcription factors, AP1, c/EBP, IRF-1, NF-κB, Pu.1, Sp1 and USF1 co-operate functionally, but direct protein contacts to STATs have not been shown. MCM5 interacts with STAT1 without binding DNA itself and the same may be true for the glucocorticoid receptor (GR) and STAT5. Nmi promotes the interaction between STATs and P300/CBP whereas SMADs can be bridged to STAT3 via P300/CBP. For further information see text.

Oestrogen-mediated dimerization of STAT6 was achieved through fusion of an oestrogen-binding domain, and dimerized STAT6 possessed transcriptional activity when assayed with appropriate reporter genes [62]. These results with constitutively dimerized STATs may be taken to indicate that cytokine-regulated serine phosphorylation is not required for STATs 3, 5 and 6 to express their biological effects. Alternatively, the artificially dimerized STATs may have adopted more powerful transactivating domains, or serine kinases induced by serum factors may have phosphorylated their C termini. Data obtained in STAT1-deficient cells, reconstituted with the STAT1 S727A mutant, are in striking contrast to the assumption that serine phosphorylation has little impact on the biological activity of STATs [63, 64]. Such cells were found to be unresponsive to the anti-proliferative and anti-viral effect of IFN-γ. Consistently, transcriptional induction of IRF-1, an IFN-γ target gene, was severely impaired. Interestingly, responses to IFN-α were found to be intact, indicating that STAT1 S727 phosphorylation is required in the context of the STAT1 dimer, but not in the context of the STAT1-STAT2-p48 ISGF3 complex where the transactivating function appears to be provided by STAT2.

All facts considered, the overall importance of STAT serine phosphorylation for the biological response to cytokines is currently hard to judge, but may differ among individual STATs. A clear answer will be provided once STAT knockout mice have been reconstituted with transgenes of serine-alanine mutants or the wild-type version replaced with an alanine mutant in a knock-in experiment. Another important and unsolved question concerns the mechanistic implications of STAT serine phosphorylation and its connection to transcription factor activity. A likely assumption is the existence of protein interactions which depend on the presence of phosphoserine and which regulate transcription factor activity of STATs.

Interactions between STATs and other transcription factors

STATs have been shown to interact physically with a variety of transcription factors. Mechanistically, these interactions appear to fall into two distinct categories, those that stabilize DNA binding and those that increase transcription factor activity without affecting the association with DNA (fig. 2).

STAT1 or STAT5 dimers in binding equilibrium readily dissociate from their template DNA due to a high off-rate of binding [65; F. Barahmand-Pour and T. Decker, unpublished data]. Destabilization of STAT5 dimers by disrupting one of the two ptyr-SH2 domain interactions drastically shifts the binding equilibrium towards the unbound state [66]. These findings suggest that dimer stability contributes to the rate of association with DNA, but that even stable dimers may not be effective in activating several rounds of transcription because they do not remain bound to their template long enough. This is especially true for weak STAT-binding sites whose sequence deviates from the consensus element.

Several target promoters for STATs 1, 4 and 5 have been shown to co-operatively bind STAT dimers to two or more adjacent GAS sequences through tetramerization and thus stabilize association with individual dimers [65, 67–71]. Where investigated, tetramerization occurs through the interaction of the N domains, i.e. the interface formed by the helices of the 130 N-terminal amino acids, and requires the conserved tryptophan residue homologous to position 37 in STAT1 [65, 69, 71]. The ability of adjacent sites to support tetramerization depends on their orientation in a tandem array, because the N domains must be positioned facing each other [65]. The distance between sites that bind STAT tetramers is flexible to some extent, but a minimal distance (> 5 bp) must be maintained. Tetramerization can cause the occupation of weak STAT-binding sites which on their own would be incapable of STAT association; even a half-site of a palindrome may be enough for co-operative binding [70]. Since weak STAT-binding sites appear to display a higher potential of discriminating between individual STAT dimers, occupation of two or more weak sites through co-operative binding, driven by oligomerization of individual dimers on DNA, may be a way of increasing the specificity of gene expression. A rather extreme example for this is found within the first intron of the IFN-γ gene which contains an IL-12 response element, constituted of several weak GAS elements. These bind one or more STAT4 tetramers interacting through their N domains [69]. In this and similar situations, the STAT N domains thus provide additional specificity of gene regulation through their intrinsic preference for partner N domains. To date only homotypic interactions have been described.

In the case of STAT5, the ability to form tetramers may be a distinguishing feature between the very closely related a and b isoforms. This is suggested by promoter analysis of the STAT5-responsive Cis gene [70]. It contains four GAS sequences arranged in two pairs, each pair being capable of forming a complex suggested to contain a STAT tetramer. In each case, the putative tetramer contains exclusively STAT5a whereas STAT5b can form dimers or, if provided in sufficient excess, pairs of dimers that do not interact and thus do not bind in a co-operative fashion. Thus, a distinct ability to form tetramers may selectively influence the ability of the two STAT5 isoforms to act upon target genes. If proven correct, functional differences between the STAT5 isoforms may thus contribute to the significantly different phenotypes of STAT5a and STAT5b knockout mice. Contrasting this view, STAT5a-deficient mammary gland tissue could be functionally restored by increasing expression of STAT5b [72], suggesting expression levels as a major determinant of the relative activities of STAT5a and STAT5b.

Non-STAT DNA-binding proteins can similarly interact with and display co-operativity with STATs. The C-terminally truncated form of STAT3, STAT3β, interacts with c-Jun and co-operates transcriptionally with AP1 (Jun/Fos heterodimer) bound to an adjacent promoter site. It is not known whether this results from co-operative binding to DNA. However, in the case of the MHCII activator CIITA gene, a weak GAS element in the promoter is positioned adjacent to an E box motif, a binding site for the ubiquitous transcription factor USF-1. STAT1 binding in response to IFN-γ requires the concomitant presence of USF-1. In vitro, the two transcription factors associate with DNA in a co-operative fashion [73]. This may result from either physical interaction or alteration of the DNA template by USF-1. Important functional interactions in transcriptional activation have also been reported for STAT1 and Sp1 (ICAM-1 promoter, IFN-γ response), STAT1 and the ets family member Pu.1 (myeloid-specific expression of the FcγRI gene in response to IFN-γ [74]), or STAT6 and c/EBPβ (transcription of the germline Igε gene in response to IL-4 [75]). Interactions between the proinflammatory transcription factors NF-κB and IFN-γ-activated STAT1 were described for the IRF-1 and RANTES promoters; however, mechanistically this is not yet understood [76, 77]. These examples most likely represent the tip of an iceberg of complexity describing the mechanism of STAT activity on different target gene promoters.

In the examples described so far, STAT-interacting proteins must associate with DNA and, in many cases, affect the DNA binding of STATs to GAS sequences. However, there are a few known cases of proteins that may co-operate with STATs primarily through an influence on the transactivation potential of the DNA-bound STAT. The first such example reported in the literature is the glucocorticoid receptor (GR). Studies, prompted by the enhancing effects of glucocorticoids on the induction of milk proteins by the STAT5-activating hormone prolactin in mammary gland epithelium, demonstrated a direct physical interaction as well as transcriptional synergy between STAT5 bound to the

GAS sequence and GR [78, 79]. Groner's laboratory demonstrated that, in principle, STAT5 can functionally co-operate with a GR containing a mutated DNA-binding domain. However, it is currently a matter of debate whether STAT5-GR interaction additionally requires, or is enhanced by, binding of GR to GRE half-sites [80]. It appears clear that the observed enhancement of STAT5-mediated transcription by GR is brought about by the powerful GR transactivating domain, a finding in line with the result that a functional complex with GR can be formed by STAT5 with a deleted C terminus.

Other proteins involved in transcriptional activation by STATs are p300 and CBP. Originally described as proteins interacting with the adenovirus E1a oncoprotein and serving as co-activators for the cAMP-responsive transcription factor Creb [81], these proteins are now implied in multiple signalling paths affecting transcriptional regulation [82–84]. They were recently shown to exert histone acetylase activity and to be part of multiprotein complexes containing other co-activator proteins and/or histone acetylases [85–88]. The C termini of both STAT1 and STAT 2 were shown to interact with a cysteine-histidine-rich domain (C/H3) of p300 and CBP [89–91]. Additionally, the N terminus of STAT1 also associates with the KIX domain of p300/CBP, the region identified to bind serine133-phosphorylated CREB [90]. The importance of p300 and CBP for STAT1-mediated transcription in vivo was documented by the inhibitory effect of microinjected antibodies to both proteins on gene induction by IFN-γ [92].

The utilization of p300/CBP by multiple families of transcription factors raises the possibility that the co-activators may become limiting for transcriptional responses in cases where several different signalling paths are active. To account for this situation, a model was proposed according to which integration of transcription factor activities linked to different signalling paths occurs through their intrinsic affinity to p300/CBP. Several experimental results are consistent with this assumption. For example, inhibition of scavenger receptor gene expression in macrophages by IFN-γ may occur through competition between STAT1 and AP1/ets domain proteins for limited cellular amounts of p300/CBP, because it could be relieved by CBP over-expression [92]. Similarly, STAT1 may be down-regulated through competition for CBP or other co-activators by the peroxisome proliferator co-activator-γ (PPRC-γ). PPRC-γ ligands like the prostaglandin metabolite 15d-PGJ2 down-regulate macrophage activation [93, 94]. Consistently, PPRC-γ co-expression reduces the transcriptional activity of STAT1, AP-1 and NF-κB [93], which suggests a promoter-independent mechanism of repression.

Competition for p300/CBP association was also sought as an explanation for the interplay between the adenovirus E1A oncoprotein and establishment of the antiviral state in response to IFNs. IFN treatment can inhibit the formation of early adenoviral RNAs which depends on E1A, but once enough E1A is present in a cell it inhibits the formation of ISGF3 [95–97]. Since STAT1 and STAT2 (and ISGF3) both interact with the C/H3 domain of p300/CBP, i.e. the E1A-binding site, both phenomena can be explained by direct competition between STATs 1/2 and E1A for binding to p300/CBP [89]. Notably however, a different view of the situation emerged from a recent report demonstrating E1A inhibition of STAT1 function in the absence of STAT1 interaction with P300/CBP, suggesting other or additional means by which E1A can suppress the IFN response [98]. The recent finding that E1A acts as an inhibitor of histone acetyl transferases (HAT) [99] may provide an explanation for the non-competitive mode of E1A inhibition of STAT-dependent transcription.

Different transcription factors require distinct CBP-containing co-activator complexes [88, 91]. For example, CREB, retinoic acid receptor (RAR/RXR dimer) and STAT1, all of which can directly associate with p300/CBP, recruit different multi-protein complexes, or at least use different components of the same multiprotein complexes containing p300/CBP for transcriptional activation. Whereas RAR/RXR requires the co-activator/histone acetylase components p/CIP, NCoA-1/SRC-1, p/CAF and p300, CREB functions without the NCoA-1/SRC-1 component, and IFN-γ-activated STAT1 will activate transcription in the absence of NCoA-1/SRC-1 and p/CAF. RAR/RXR does not require the HAT activity of p300/CBP, but CREB and STAT1 do. These findings suggest that STAT-containing transcriptional activator complexes modify the chromatin structure of their target promoters. They are further in line with the assumption that adenovirus E1A protein inhibits STAT1-dependent reporter gene expression through a suppression of P300 HAT activity [99]. Importantly, an E1A variant interacting with P300, but lacking the domains required for HAT repression did not inhibit STAT1 transcription factor activity. Together with the data of Look et al. [98], these findings suggest that competition for P300 is neither necessary nor sufficient to cause inhibition of Stat1 target gene transcription.

Whereas binding to CBP/p300 may be the basis of negative cross-regulation in the examples described above, a different picture emerged for the interaction between STAT3 and SMAD1. Leukaemia inhibitory factor (LIF) and the transforming growth factor (TGF)-β family cytokine BMP2 act in synergy to cause astrocyte differentiation from fetal neuronal progenitor cells. While LIF activates STAT3 in these cells, BMP2

causes the phosphorylation of SMAD1 which forms a transcriptionally active complex with the common mediator SMAD4. By associating with STAT3 and SMAD1, p300 causes the simultaneous recruitment of both transcription factor complexes to target promoters like that of the glial fibrillary acidic protein gene [100]. The resulting enhancement of transcription is thought to be instrumental in mediating the observed biological synergy between LIF and BMP2.

STAT5 also interacts with CBP [101] and this interaction can be enhanced by a third protein. Yeast two-hybrid analysis with parts of the STAT5b N terminus as a bait revealed association with the N-Myc interactor (Nmi) protein [102]. Nmi is induced by cytokines and also binds to STAT1 and possibly other STATs. Zhu et al. [102] demonstrated an increased formation of STAT5-p300/CBP complexes in the presence of Nmi, thus proposing an anchor function for the protein increasing transcription factor activity of STAT5.

Transcription factor CREB requires phosphorylation of S133 to bind to the p300/CBP KIX domain [103]. An attractive hypothesis linking p300/CBP transcriptional enhancement with STAT serine phosphorylation assumed a similar requirement for STATs. However, at least for STAT1 this is not the case. The STAT1 N terminus binds to the KIX domain, and the C-terminal association with the C/H3 domain occurs equally well with bacterially expressed STAT1 which is not phosphorylated on S727 [90].

Evidence for a protein interaction enhanced by S727 phosphorylation of STAT1 was provided by a biochemical approach using the STAT1 C terminus as an affinity matrix which identified human MCM5 as an interacting protein [104]. MCM5 had previously been associated with the initiation and elongation of DNA replication [reviewed in ref. 105]. The S727A and L724A mutants associated less well with MCM5. In further support of a role for MCM5 in STAT1-dependent functions, the transcription factor activity of the protein during the cell cycle paralleled the levels of nuclear MCM5 which increase in G1 and decrease during S phase.

Besides MCM5, the Darnell laboratory identified other proteins whose association with a S727A-mutated STAT1 C terminus was reduced, indicating they might similarly explain the effect of S727 phosphorylation on transcription [104]. The question whether other STATs associate with MCM5 is currently not answered.

Association of STATs with transcriptional repression

Although investigated in a small number of situations, repressive effects of activated STATs on transcription are very likely. Co-transfected STAT5a or STAT5b were shown to repress prolactin stimulation of a transfected IRF-1 promoter while enhancing transcription of the β-casein promoter under the same conditions [106]. The repressive effect of STAT5 was seen in the absence of DNA binding, but required the C domain of the protein. The physiological relevance of this report is suggested by the analysis of sexually dimorphic gene expression in the livers of both STAT5b knockout mice and STAT5a/b double-knockout mice [16, 107]. For example, the P450 family CYP2A4 gene encoding testosterone 15α hydroxylase is suppressed in the livers of normal male mice, but expressed at levels comparable to female mice upon targetted disruption of both STAT5 genes. Similarly, the lack of suppression of 20-α-hydroxysteroid dehydrogenase in corpora lutea and the resulting degradation of progesterone in female STAT5a/b knockout mice may contribute to the infertility of these animals.

STAT6, activated by IL-4, was found to exert a repressive effect on the activation of the IRF-1 promoter in response to IFN-γ [108]. Repression requires the STAT6 C terminus, possibly as a means to recruit inhibitory proteins. In another report, the suppression of tumour necrosis factor (TNF)-α-induced E-selectin gene expression in vascular endothelial cells was shown to result from STAT6-mediated inhibition of NF-κB binding to the E-selectin promoter which contains overlapping STAT6 and NF-κB binding sites [109]. These studies demonstrate a dual function of STATs as activators and suppressors of cytokine-induced gene expression and emphasize the influence of the specific promoter context on STAT action.

Inactivation of STAT transcription factor activity

Three possibilities to inactivate STATs have been described. These are proteasome-mediated degradation, tyrosine dephosphorylation, and association with inhibitory proteins.

Ubiquitinated species of tyrosine-phosphorylated STAT1 were found in the nuclei of IFN-γ-treated HeLa cells. Furthermore, the ability of a proteasome inhibitor to prolong the response to IFN-γ was taken as evidence for a major contribution of proteasome-mediated STAT1 degradation to the negative regulation of IFN-γ signalling [110]. This view was challenged by experiments demonstrating that the effect of the proteasome inhibitor requires continuous signalling by the IFN-γ receptor [111]. Therefore, it is possible that IFN-γ receptors are normally down-regulated by a proteasome-dependent mechanism and the inhibitor thus prolongs the IFN-γ response by stabilizing a pool of activated receptors on the cell surface.

A major contribution of tyrosine phosphatases to the inactivation of STATs was originally suggested by the ability of the tyrosine phosphatase inhibitor vanadate to cause and prolong STAT tyrosine phosphorylation [112]. Pulse-chase experiments during which a pool of STAT1 molecules was followed through an IFN-γ response convincingly demonstrated that within 4 h very little STAT1 was lost and that a previously tyrosine-phosphorylated, nuclear STAT1 could reappear in the cytoplasm dephosphorylated [111]. Thus, it is likely that a nuclear tyrosine phosphatase plays a major role in shutting off the transcriptional activity of STAT1 and, possibly, other STATs. The identification and characterization of this phosphatase will add another important piece to the JAK-STAT puzzle.

Structurally, two regions of STATs have been implicated in negative regulation. Removing the C terminus causes increased constitutive tyrosine phosphorylation and prolonged signal persistence for STATs 3 and 5, but not for STAT1 [24, 28, 30]. In the case of STAT5, kinetic studies suggested that the C-terminally deleted species were dephosphorylated at a lower rate [30]. However, studying a reconstituted signalling path in yeast suggested that C-terminal deletion also increases the rate of JAK2-mediated phosphorylation [66]. Therefore, the role of the C terminus in enhancing the interaction of STATs with phosphatases needs to be further clarified. Removal of the STAT1 N terminus (60 amino acids) similarly caused a prolongation of IFN-γ-induced tyrosine phosphorylation, and this could be recapitulated by mutation of a single amino acid, R31A [113]. While the authors interpret their data at the level of tyrosine dephosphorylation, an effect of the mutation on the rate of JAK-mediated tyrosine phosphorylation cannot be ruled out.

Repression of STATs by inhibitory association with protein inhibitors of activated STATs [114, 115] or protein kinase R [116] are described in the contributions by D. Hilton, and C. Schindler and S. Brutsaert, and will not be reiterated here.

Perspective

A framework has been set to understand the principles governing the function of STATs. However, many questions remain open concerning mechanistic aspects of STAT-mediated transcriptional activation. It is likely that a large number of interactions of the STAT N- and C-terminal domains with other transcription factors or transcriptional co-activators have not been uncovered. The role of serine phosphorylation in these interactions is largely unclear as are the biological consequences of serine phosphorylation for most STATs. Future experiments will also need to clarify the relationship between higher-order complexes influencing transcription and STATs. Among these are the complexes mediating nucleosome displacement, histone acetylation, or binding to RNA polymerase. The excitement in this field will not be over for some time.

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