[Frontiers in Bioscience 16, 3043-3056, June 1, 2011]

Serine phosphorylation of the Stat5a C-terminus is a driving force for transformation

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1. ABSTRACT

Persistent tyrosine phosphorylation of Stat3 and Stat5 is associated with oncogenic activity. Phosphorylation of the conserved tyrosine residue (pTyr) was long believed to be the only essential prerequisite to promote activation and nuclear translocation of Stat proteins. It has become evident, however, that post-translational protein modifications like serine phosphorylation, acetylation, glycosylation as well as protein splicing and processing constitute further regulatory mechanisms to modulate Stat transcriptional activity and to provide an additional layer of specificity to Jak-Stat signal transduction. Significantly, most vertebrate Stat proteins contain one conserved serine phosphorylation site within their transactivation domains. This phosphorylation motif is located within a P(M)SP sequence. Stat transcription factor activity is negatively influenced by mutation of the serine to alanine. Moreover, it was shown for both Stat3 and Stat5 that their capacity to transform cells was diminished. This review addresses recent advances in understanding the regulation and the biochemical and biological consequences of Stat serine phosphorylation. In particular, we discuss their role in persistently activated Stat proteins for cancer research.

2. INTRODUCTION

The Stat3, Stat5a and Stat5b genes are located in close vicinity on mouse chromosome #11 and human chromosome #17, respectively (1). They encode transcriptional regulators, which act as major mediators of cytokine/growth factor signaling (2, 3). All three are critical for hematopoiesis, proper organ function, immunity and inflammatory responses (4, 5). Last but not least they have been found to act as oncogenes or tumor suppressors in many different cancer types (6, 7).

Protein phosphorylation is one of the major mechanisms by which cells convert extracellular signals into intracellular responses. A common feature of cytokine-stimulated Stat activation is the requirement of tyrosine phosphorylation, which in the case of Stat5a and Stat5b induces homo- and heterodimerization followed by nuclear translocation and subsequent target gene transcription (8). Importantly, Stats are also regulated by serine phosphorylation (9-11), which, however, appears to be more specific for individual Stats and their different activators. While the impact of tyrosine kinases has been intensively investigated in case of Jak-Stat signal transduction and for the development of cancer (2, 4, 12),

the roles of serine/threonine kinases have been examined to a lesser extent (13-17).

In Jak-Stat signaling, both types of receptor cascades—with and without intrinsic tyrosine kinase activity—have been shown to interact with serine/threonine kinase pathways to induce maximal activation (18). Here, mitogen-activated protein kinase (MAPK) and PKCdelta kinase pathways play important roles (13). Biochemical and biological functions of Stat serine phosphorylation include DNA binding (11, 19), cofactor association (20), immune responses (16, 21), signal transduction (22), mitochondrial function (23) as well as transformation (14, 15, 24). Serine/threonine phosphorylation of Stat1 and Stat3 has also been implicated in the etiology of certain human cancers and immunodeficiencies (16, 18, 21, 25-27). Studies of activated Stat3 and Stat5 proteins have shown that serine phosphorylation plays an important role for transformation (14, 15). Thus, understanding the biological relevance of serine/threonine phosphorylation for oncogenic Stat functions and the regulation of their activity is vital for the development of potential novel cancer therapeutics which specifically recognize the activated proteins.

3. STAT SERINE PHOSHPHORYLATION

The common comprehension of the regulation of the activity of transcription factors of the Stat family was originally that they are controlled by the Jak-catalyzed phosphorylation of a single conserved, carboxy-terminal tyrosine residue. According to current knowledge this might only be true for Stat2. All other Stat proteins, however, have been found to additionally undergo serine phosphorylation on at least one serine residue in their transactivation domain upon various stimuli, which may modulate cytokine responses (3, 13, 28, 29). Conserved phosphorylation sites are contained in a PMSP motif (Ser727 in Stats 1 and 3 and Ser721 in Stat4), a PSP motif (Ser725 in Stat5a; Ser730 in Stat5b), and a SSPD motif (Ser756 in Stat6) (Figure 1A) (30). Stat1 and Stat5a possess at least one additional serine phosphorylation site in their transactivation domain, Ser708 and Ser779, respectively. In HEK 293 cells, ectopic expression of Stat5a and ERBB4 identified novel additional serine phosphorylations in the N-terminus of Stat5a at Ser127/Ser128, which were required for ERBB4-induced activation of Stat5a (31).

Maximal transcriptional activity of Stat1, Stat3, Stat4, and Stat6—unlike that of Stat5a and Stat5b—has been demonstrated to be substantially modulated by serine phosphorylation (10, 13, 32-34). Additionally, mutation of the C-terminal serine residue to alanine has been shown to prolong tyrosine phosphorylation and DNA binding of Stat5a (19) and to cause both a decrease in transcriptional activity and sustained tyrosine phosphorylation of Stat3 (10, 35, 36). Additional studies suggest that some Stat1-dependent apoptotic responses require phosphorylation of Ser727 but not tyrosine (37). Interestingly, mice expressing a Stat1 S727A mutant exhibit defective IFN-gamma-mediated innate immunity (21). Likewise, IL-12-induced production of IFN-gamma is impaired in Stat4 S721A expressing T-cells, while IL-12-induced proliferation remained unaffected (38).

Serine phosphorylation of the Stat6 transactivation domain does neither affect tyrosine phosphorylation, nor dimer formation, nor the ability to translocate to the nucleus in IL-4-stimulated cells. However, gene transcription mediated by Stat6 requires the convergence of tyrosine and multiple serine/threonine kinase pathways (28). IL-4/IL-13-induced serine/threonine phosphorylation in the Stat6 C-terminal transactivation domain has been shown to be important for full transcriptional activity of Stat6 (28, 39, 40), though phosphorylation of multiple serine residues in the Stat6 transactivation domain has also been reported to abrogate DNA binding activity possibly by inducing conformational changes in Stat6 dimers. Thus, serine phosphorylation might also negatively regulate the expression of IL-4-responsive genes (41). Interestingly, serine phosphorylation of Stat5 does not enhance its transcriptional activity in response to IL-2 (42) but affects growth hormone (GH)-stimulated transcription in a promoter-dependent manner (43). These results suggest that the functional implications of the phosphorylation of serine residues are Stat-, cell type-, target gene- as well as stimulus dependent (10, 13, 38, 43).

According to current literature, all cytokines causing Stat tyrosine phosphorylation also result in serine phosphorylation (37). Additionally, basal serine phosphorylation of Stat proteins was observed (13). Upon a number of extracellular signals Stats become only phosphorylated on their serine residues without concomitant tyrosine phosphorylation (13, 37). Serine phosphorylation can also negatively regulate cytokine-induced tyrosine phosphorylation in the case of Stat3 (35). For Stat1 and Stat3, transcriptional activity has been reported even in the absence of tyrosine phosphorylation (37, 44, 45) which might result from protein interaction of Stats with other transcriptional regulators. In addition, recent ChIP-seq (chromatin immunoprecipitation followed by parallel sequencing) analyses of Stat3, Stat4 and Stat6 transcription factors in T cell subsets revealed that a large fraction of target genes are regulated without induction of the Stat proteins by cytokines (and thus without pTyr) (46, 47). These results suggest that Stats are able to exert biological activity even if they are phosphorylated on serine only. Since Stat nuclear translocation and DNA binding activity, with the exception of Stat6, are generally not affected by serine phosphorylation it is likely that serine phosphorylation modulates the intrinsic transcriptional potential of Stat proteins (43). Their transcriptional activity is mediated largely through the C-terminal transactivation domain adjacent to the serine phosphorylation site (3), which has a regulatory role in protein stability (30, 48). An attractive explanation how Stat serine phosphorylation modulates transcription would be provided by coactivator molecules associating with Stats in a serine phosphorylation-dependent manner. The conserved C-terminal serine phosphorylation sites might be able to direct the recruitment of coregulators. Stats are known to recruit

A

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hStat1      .DNL..LPMSPCC...FDCMC
hStat2            not found
hStat3      .D....LPMSPRT...LDSLS
hStat4      QSPSDLLPMSPSA.YAVLRE.
hStat5a     MDQA...P.SP.....VVCP.
hStat5b     MDQA...P.SP.....VVCP.
hStat6      QPQEHAVSSPDPLLCSDVTMV
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B

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human           MDSLDSRLSPPAGLFTSARGSLS
orangutan       MDSLDSRLSPPAGLFTSARGSLS
chimpanzee      MDSLDSRLSPPAGLFTSARGSLS
rhesus macaque  MDSLDSRLSPPAGLFTSARGSLS
marmoset        MDSLDPRLSPPAGLFTSARGSLS
horse           MDSLDPRLSPPAGLFTSARGSLS
panda           MDSLDPRLSPPAGLFTAARGSLS
dog             MDSLDPRLSPPAGLFTAARGSLS
mouse           MDSLDARLSPPAGLFTSARSSLS
rat             MDSLDPRLSPPAGLFTSARSSLS
pig             MDSLDPRLSPPAGLFASTRGSLS
sheep           MDSLDPSLSPPAGLFTPARGSLS
bovine          MDSLEPSLSPPTGLFTPGRGSLS
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C

	phosphorylated Stat	consensus sequence
Proline-directed kinases		
- MAPK		(P)-(L)-P-X-S/T-P-(P)
ERK1/2	Stat3, Stat5	P-X-S/T-P
p38	Stat1, Stat3, Stat4, Stat6	P-Q-S-P
JNK	Stat3, (Stat1)	L-P-M-S-P
- Cyclin dependent kinases (CDK) 1-5	Stat3	(K)- S/T-P-X-K/R or (K)-S/T-P-(K/R)
- Nemo-like kinase (NLK)	Stat3	F-S/T-P-G
- Glycogen synthase kinase-3 (GSK-3)	Stat3, Stat5	S-X-X-X-S-P
Protein kinase A (PKA)	not examined	R-R-X-S/T-P
p21 activated kinase 1 (PAK1)	Stat5a	K/R- R-X-S
Casein kinase	not examined	S/T-X-X-S/T
Protein kinase C delta (PKCδ)	Stat1, Stat3	R-K-G-S/T
Protein kinase C epsilon (PKCε)	Stat3	R-K/R/Q-G-S/T
Calmodulin-dependent protein kinase-2 (CaMK2)	Stat1	R-X-X-S/T
mTOR	Stat3	F-S/T-Y
IKKε	Stat1	S-X-X-X-S
PIM1–3	not examined	R-X-R-X-X-S/T

Figure 1. Amino acid sequence comparison of Stat C-termini. (A) Comparison of the C-terminal region of human Stat proteins containing the conserved P(M)SP serine phosphorylation site. The P(M)SP motif is highlighted by the gray area. (B) Inter-species comparison of the Stat5a amino acid sequence harboring the conserved unique C-terminal LSPP serine phosphorylation motif at the position 779/780. (C) Table of serine/threonine kinases which are potentially involved in the phosphorylation of Stat5 C-terminal serine residues (references: (3, 13, 119)).

chromatin-modifying enzymes through their transactivation domains (3). Indeed, coactivators like CBP/p300 (32) or MCM (mini-chromosome maintenance) complexes (37, 49), NcoA (50) and Oct-1 (51) bind to the Stat3 and Stat5 C-termini (Figure 2). All Stats likely bind to p300 and CBP (8), thus influencing acetylation responses within chromatin. Additionally, interaction with chromatin remodeling complexes (52-55) and histone acetyl transferase enzymes has been implicated in the modulation of Stat transcriptional responses. In particular NcoA-1 was shown to interact with the transactivation domain of Stat3, a short FDLD motif within the transactivation domain of

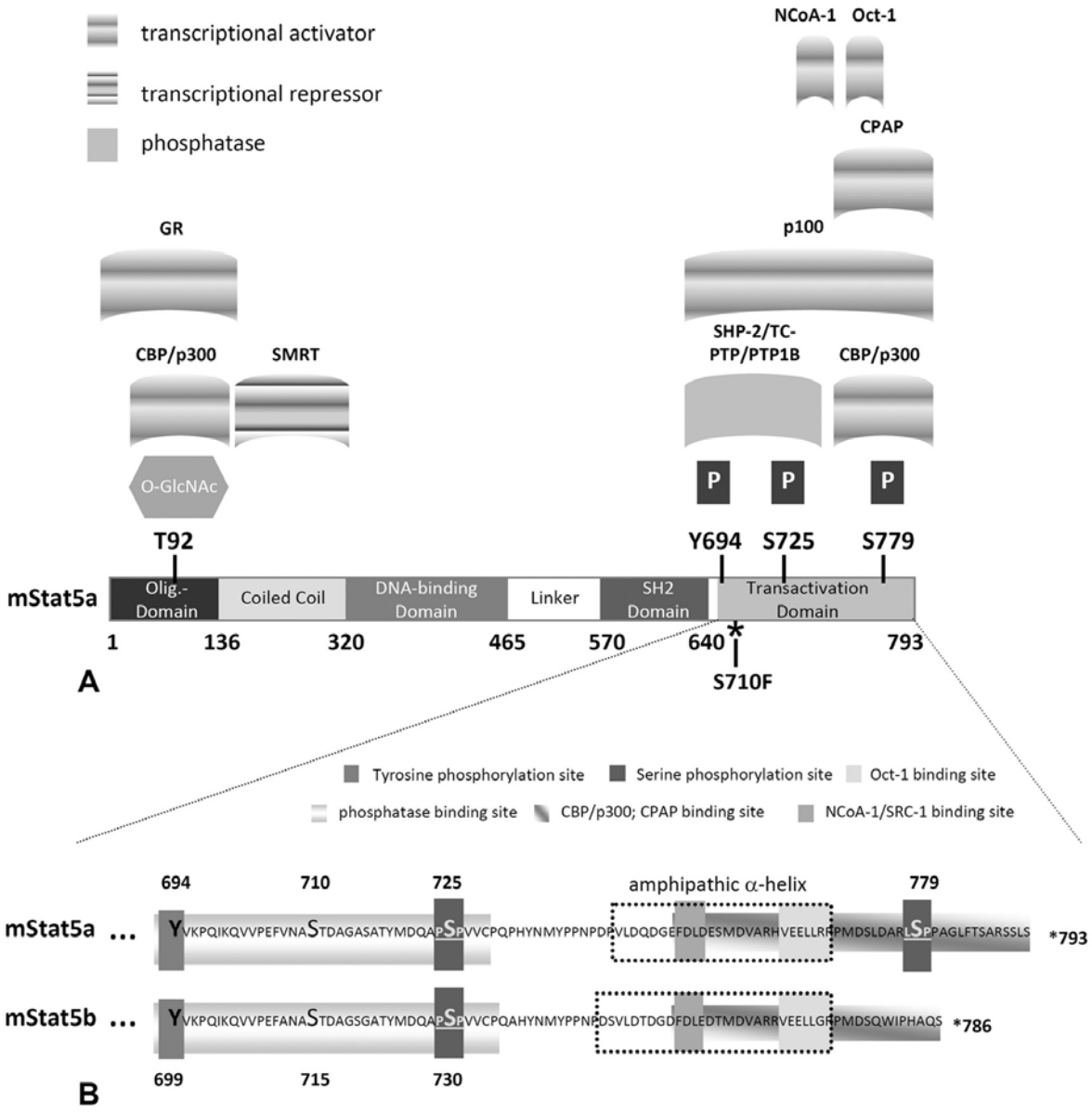


Figure 2. Coregulator molecules associating with Stat5a. (A) Interaction of Stat5a with transcriptional activators (50, 51, 75, 77, 120, 121), transcriptional repressors (122), and phosphatases (101-103). The domain structure of Stat5a is depicted schematically. Post-translational protein modifications (O-GlcNAc, P-Ser, and P-Tyr) which may affect the binding of coregulators are indicated. (B) Comparison of the C-terminal amino acid sequences of mStat5a and mStat5b with regard to protein-protein interaction adjacent to the serine phosphorylation motifs (references: (30, 50, 51, 75, 102, 120)).

Stat5, and an LXXLL motif within the C-terminus of Stat6 (56, 57). The global gene regulation of chromatin bound Stat molecules identified by the powerful new techniques of ChIP-seq therefore starts to alter our understanding of the function of these transcription factors.

4. SPECIFICITY OF JAK-STAT SIGNAL TRANSDUCTION IS INFLUENCED BY SERINE PHOSPHORYLATION

Serine phosphorylation can be elicited by a variety of different stimuli and signaling pathways (13). Therefore, Stat C-termini contribute to the specificity of cellular responses by the difference in the dependence on serine phosphorylation at different target gene promoters and the kinase pathways used to phosphorylate these residues (58).

Serine phosphorylation of one Stat protein can occur through different signal transduction pathways. For example, Stat1 Ser727 phosphorylation in macrophages is sensitive to SB203580 (inhibitor of p38-MAPKs) in stress of LPS responses, but insensitive to this inhibitor in the IFN-gamma response (59). Similarly, Stat3 Ser727

phosphorylation in response to EGF, PDGF, insulin, IL-2, BCR or TCR stimulation is sensitive to PD98059 (highly selective inhibitor of MEK1 and MEK2) and, where tested, insensitive to H7 (broader spectrum of protein kinase inhibition), whereas the opposite is true for IL-6 or IFN-alpha stimulation (35, 60-62). Additionally, Ser727 phosphorylation of different Stats by signals from the same cell surface receptor can be routed through distinct signaling pathways. This has been documented for bacterial LPS or for UV-irradiation which cause SB203580-sensitive Ser727 phosphorylation in case of Stat1, but SB203580-insensitive Ser727 phosphorylation in case of Stat3 (59, 63). Moreover, specific effects on gene expression are generated by the dependence of individual Stat target genes on a phosphorylated serine residue (58).

Serine phosphorylation of Stat5 proteins may also serve as a mechanism to differentially modulate the expression of Stat5 target genes. Although many cellular functions of Stat5a and the closely related Stat5b isoform are overlapping both proteins play distinct physiological roles in mediating hormonal responses to prolactin (PRL; Stat5a) and GH (Stat5b) in the mammary gland and liver, respectively, as well as responses to interleukins in lymphoid- and NK cells (64, 65) (reviewed in (2, 4, 6)). The differential properties may not only be attributed to the distinct tissue distribution of the two Stat5 isoforms. Biochemical properties like DNA binding affinity and specificity, intracellular trafficking as well as the duration of pTyr play a role for functional differences (66-68). Moreover, their propensity to bind to DNA as oligomers (69, 70), and important differences in their regulation by serine phosphorylation add to Stat isoform specificity.

5. PHOSPHORYLATION OF C-TERMINAL SERINES IN STAT5

Although no modulatory effects of serine phosphorylation on transcriptional activity have been reported for Stat5a and Stat5b proteins, distinct serine residues were found to be phosphorylated in their carboxy-terminal transactivation domains (19, 34). Serine phosphorylation of Stat5 has been observed in cells and tissues stimulated with Stat5-activating ligands such as GH (43, 71), PRL (72, 73), and IL-2 (42, 74). In PRL-stimulated cells, both Stat5 isoforms are phosphorylated on a serine moiety located within the conserved PSP motif at the positions 725 (Stat5a) and 730 (Stat5b), which correspond in location to the PSMP serine phosphorylation sequences of Stat1/3/4 (Figure 1A) (34). In addition, Stat5a harbors a unique serine residue within an LSPP motif (Ser779) (19), which is the major site of serine phosphorylation of Stat5a (19). Notably, this LSPP motif is conserved between different mammalian species (Figure 1B). Stat5b, which lacks the COOH-terminal peptide sequence corresponding to Ser779, can be phosphorylated at Ser730, in a manner that is inducible by either PRL (34) or GH (43). By contrast, Stat5a has been found constitutively phosphorylated on both serine residues in a variety of cells and tissues (19, 34). It has remained unclear, however, whether both serine residues can be simultaneously phosphorylated in a given Stat5a molecule, leaving open the possibility that phosphorylation of Ser725 may negatively regulate Ser779 phosphorylation and the resulting phospho-Ser779-dependent transcriptional responses.

However, functional studies of the effects of serine phosphorylation on the transcriptional activity particularly of the two Stat5 isoforms have not provided a consistent picture (19, 43). Whereas no difference in PRL-stimulated Stat5 reporter gene activity was seen when cells were transfected with serine to alanine mutant forms of Stat5b (S730A) or Stat5a (S725A, S779A, or the S725A/S779A double mutant) compared with the corresponding wild-type Stat5 forms (19, 34), mutation of Stat5b Ser730 was shown to modulate Stat5b's transcriptional activity in a promoter-dependent manner upon GH stimulation (43). While transcriptional activity was found to be decreased for the hepatic *ntcp* bile acid transporter gene upon mutation of Ser730, transcription of the *beta-casein* milk gene was increased under the same stimulating conditions (43). This increase was suggested to be associated with a higher level of Stat5 DNA-binding activity in the case of the Ser730-mutated Stat5b. The increased DNA-binding activity of Stat5b S730A compared with wild-type Stat5b was suggested to be at least partly due to increased expression and/or stability of the Ser730-mutated Stat5 protein. This raises the possibility that phosphorylation of Ser730 in the wild-type protein may enhance the turnover of Stat5b. The increase in transcriptional activity could additionally involve a decrease/delay in the rate of Stat5b tyrosine dephosphorylation, as it was suggested for Stat5a mutated at serine 725 and/or 779 in cells stimulated with PRL (19) and GH (43), respectively, which resulted in prolonged DNA binding (19). Thus, the phosphorylation of Stat5a and Stat5b on Ser725/730 is not only subject to differential regulation (constitutive phosphorylation of Stat5a vs. inducible phosphorylation of Stat5b), but leads to modulatory effects on gene transcription of specific target genes, which has been reported for Stat5b only. In the case of Stat5a, such a modulatory effect might require additional phosphorylation on Ser779 (43).

The promoter-intrinsic differences in the effects of Stat5b serine mutation on transcription support the assumption that the functional consequences of Stat5 serine phosphorylation may also be cell type and target gene-specific. If the activating ligand exerts additional influence remains an open question. Moreover, serine phosphorylation may modulate interactions between Stat5 and other transcription factors bound to the same promoter, or perhaps it may influence the recruitment of Stat5-interacting coactivator and corepressor proteins (75, 76), which is likely to occur in a promoter-dependent manner. Stat5 forms complexes with different transcriptional regulators, including the glucocorticoid receptor (4, 77) and p300/CREB (75). Complex formation of these coactivators with Stat5 might be controlled by serine phosphorylation in combination with other posttranslational modifications such as the reported glycosylation (threonine 92 is O-GlcNac modified; (78)) or differential splicing/proteolytic processing (30, 68, 79, 80). Splicing, proteolytic processing

as well as differential use of ATG initiation codons for the RNA polymerase II holoenzyme within the Stat5a/b locus particularly changes the N- and C-terminal domains of Stat5 proteins, which constitute the interface for coactivator and corepressor binding (30, 68, 79-81). The post-translational modification of nucleocytoplasmic proteins with O-linked 2-acetamido-2-deoxy-D-glucopyranose (O-GlcNAc) can occur multiple times in the course of the Stat5 protein lifetime. In addition, there is a dynamic interplay between the O-GlcNAc modification and phosphorylation on serine/threonine residues (82, 83). O-GlcNAc modification is sometimes found in a reciprocal relationship with proline-directed phosphorylation, but this has not yet been reported for Stat transcription factors. However, several other transcription regulators such as c-myc, Sp1, p53, FoxO, mSin3A or EWS-FLI1 have been identified as being modified with the monosaccharide (84), which influences phosphorylation events on these proteins.

Yet, the understanding of the regulation and importance of serine phosphorylation of Stat5 has remained limited and the biological significance of Stat5 serine phosphorylation has long been underestimated (13). In the last couple of years, however it has become apparent that the role of serine phosphorylation in Stat5 function is more complex than originally assumed. More recently, an essential role in hematopoietic transformation has been attributed to Stat5a serine phosphorylation (14).

6. ROLE OF STAT5 SERINE PHOSPHORYLATION IN HEMATOPOIETIC TRANSFORMATION

The C-terminus of several Stat proteins and phosphorylation of the conserved C-terminal serine residue control transcriptional activity. Additionally, a role in the progression of several human cancers, including chronic lymphocytic leukemia, breast tumors, and prostate cancer as well as the transformation of rodent fibroblasts and human breast epithelial cells was attributed to constitutively activated and/or serine phosphorylated Stat3 and Stat1 (18, 24-27, 85). Moreover, Stat3 has been found to be important for Ras-induced transformation in a serine—but not tyrosine—phosphorylated form, which augmented oxidative phosphorylation in mitochondria (15, 23). Surprisingly, transformation was independent of tyrosine phosphorylation and thus DNA binding activity, as well as dimerization through the SH2-domain. Strikingly, the presence of the C-terminal domain—especially the intact serine residue (Ser727)—was identified to be the driving force for oncogenic Ras-induced transformation (15).

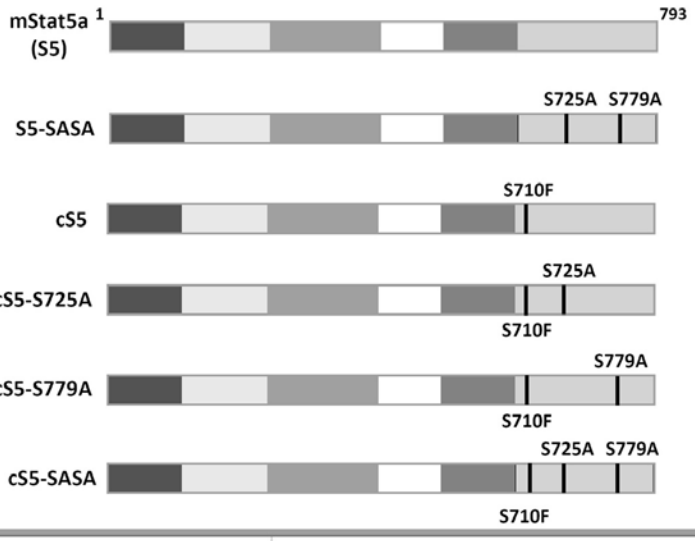
Furthermore, in familial medullary thyroid carcinoma Stat3 was reported to be constitutively phosphorylated on Ser727 in addition to Tyr705 phosphorylation via the activation of a canonical Ras/ERK1/2 pathway by FMTC-RET mutants, which plays an important role in cell mitogenicity and transformation (86).

Constitutive activation of Stat5 is known to be directly involved in oncogenic transformation (2, 4, 87). It has been demonstrated in several studies that the transforming capacity of oncogenic Stat5 in hematopoietic cells includes cytoplasmic functions in addition to its role as a transcription factor in the nucleus. Oncogenic Stat5a was demonstrated to localize in the cytoplasm and to form a signaling complex with PI3K and the scaffolding protein Gab2, which resulted in Akt activation (88-90). In concert with Stat5, Gab2 was also shown to act on many of the key stem cell functions (91, 92). This cytoplasmic role links Jak/Stat signaling to the activation of the PI3K/Akt/mTOR signaling pathway which, in turn, contributes to leukemogenesis (93, 94). Therefore, Akt/mTOR signaling has been proposed as a therapeutic target for inhibiting distinct Stat5-mediated survival signals in myeloproliferative neoplasms (95). Moreover, Stat5 was identified to bind to a consensus sequence within the Akt1 locus in a PRL-dependent manner to initiate transcription of mammary gland-specific Akt1 mRNA (96) and acts as a repressor of miR-15/16 to control Bcl-2 expression (97).

In addition to pTyr, serine phosphorylation might also be able to modulate the function of constitutively active Stat5 proteins. The original oncogenic Stat5 mutant caStat5a1*6 contains two independent mutations, namely S710F plus H299R in the DNA binding domain (98). In a number of manuscripts we have used the single Ser710 mutant (cS5) since—in contrast to caStat5a1*6—it was able to genetically complement $Stat5^{null}$- and $Stat5^{deltaN}$ cells (e.g. (14, 88, 89, 93, 95, 97, 99, 100)). The oncogenic cS5 mutant was similarly hyperactivatable by cytokines, displayed prolonged activation, and conferred IL-3-independent growth to Ba/F3 cells like caStat5a1*6. Conceivably, Ser710 is not phosphorylated like Stat5 Ser725/730 and Ser779. Stat5a harboring a mutation of serine 710 to alanine biochemically and functionally behaved like wild type Stat5a and did not cause persistent activation (100). Most likely, the persistently active mutation S710F induces a conformational change which might hinder tyrosine phosphatases which interact with Stat5 sequences adjacent to Ser710 (Figure 2) from docking to the C-terminal region (101-103). Alternatively, the critical tyrosine 694 in Stat5a might be more accessible to tyrosine kinases. Despite that we know that the cS5 mutant is persistently active, we have no detailed structural information yet on activated Stat5 molecules and the exact mechanism of action remains illusive.

While Stat5a serine phosphorylation at residues 725 and 779 has been ascribed only a minor role in normal hematopoiesis, it was found to be essential for the transformation of hematopoietic cells in the context of oncogenic (cS5) variants of Stat5a (Figure 3) (14). Interestingly, the C-terminally mapped serines of Stat5a (Ser725/779) were found to be phosphorylated in human bcr/abl+ CML samples, human lymphoma cell lines, primary leukemia patient samples, and Ba/F3 cells overexpressing different oncogenic fusion tyrosine kinases. Moreover, loss of Stat5a serine phosphorylation had a strong impact on constitutively active Stat5a-induced leukemia development in a bone marrow transplant model. This suggests that Stat5a serine phosphorylation has an essential role in hematopoietic transformation (14), which

A



B

	S5	S5-SASA	cS5	cS5-S725A	cS5-S779A	cS5-SASA
Tyrosine phosphorylation	+	+	+	+	+	+
DNA binding	+	+	+	+	+	+
Tetramerization	+	+	+	+	+	+
T-cell proliferation	+	+	+	+	+	+
Mast cell proliferation	+	+	+	n.d.	n.d.	n.d.
Lineage repopulation	+	+	+	n.d.	n.d.	n.d.
Factor indep. proliferation	-	n.d.	+	-	-	-
Transformation	-	-	+	+/-	+/-	-
Myeloid neoplasm	-	-	+	+/-	+/-	-

Figure 3. Phosphorylation of Stat5a C-terminal serine residues determines the transforming capacity. (A) Schematic representation of mutant Stat5a proteins. The oncogenic Stat5a mutant cS5 contains a Ser to Phe mutation at position 710, which renders it constitutively active. The Stat5a mutants S725A, S779A, and SASA carry single or double Ser to Ala substitutions. (B) Biochemical, biological, and transforming properties of the Stat5a variants.

represents the first major effect attributable to Stat5a serine phosphorylation.

The reduced transforming capacity of cS5 serine mutants might be explained by alterations in the transcriptional profile. The serine phosphorylation sites are in close vicinity to the amphipathic alpha-helix within the transactivation domain of Stat5a, which interacts with factors that coregulate gene expression cell specifically (Figure 2B) (68). The interaction with cofactors or other transcription factors might be modulated by serine phosphorylation at the C-terminal tail of Stat5 proteins. Alternatively, unknown factors such as different cellular location of Stat5a (15, 23, 88, 89, 93) or altered accessibility for tyrosine phosphatases (101, 103-105) might contribute to the reduced transforming capacity of serine mutants. Lately, Stat5 proteins have developed into promising targets for cancer therapeutics due to their ability to regulate the transcription of genes involved in cellular proliferation and survival (106, 107). Furthermore, Stat5 proteins play fundamental roles in hematopoietic and solid cancers being activated by mutated or fused tyrosine kinases in these cancers (12). Thus, the implication of serine phosphorylation in the transforming capacity of oncogenic Stat5 could potentially be translated into a therapeutic target for hematologic malignancies since overactivated transcription factors are less numerous than upstream activators and are at a focal point in the deregulated pathway (108). Stat5a Ser779 is not conserved in Stat5b and its phosphorylation was detected in primary AML, ALL, and CML patient samples in contrast to healthy individuals (14). The region of Stat5a encompassing Ser779 might, therefore, provide a particularly attractive domain for future therapeutic

strategies for cancer patients. Hence, identification of the kinase(s) responsible for phosphorylating Ser779 in Stat5a would be the next essential step toward the development of a suitable kinase inhibitor.

7. SERINE KINASES POTENTIALLY IMPLICATED IN THE PHOSPHORYLATION OF STAT5 C-TERMINI

The specific kinase phosphorylating a Stat protein in a given cell is depending on the stimulus and promoter context thereby creating the above mentioned important diversity to Stat-mediated gene transcription. The serine phosphorylation sites of Stat1, Stat3, Stat4, and Stat5 are contained in a conserved P(M)SP motif that confers a potential consensus phosphorylation sequence for proline-directed kinases (Figure 1C), particularly mitogen-activated protein (MAP) kinases. Therefore, the role of MAP family kinases in regulation of Stat phosphorylation has been extensively studied. Several Stat serine kinases have already been identified through the use of inhibitors, dominant-negative alleles, and *in vitro* kinase assays (3, 13, 37). They include p38 MAPK (Stats 1, 3, 4), ERK (Stat3, 5), and JNK (Stat3). Extracellular signal-regulated kinases (ERK) are mediating the serine phosphorylation of Stat3 upon epidermal growth factor stimulation. p38 MAPK has been shown to mediate the LPS and cellular stress-induced phosphorylation of Ser-727 in Stat1, and the IL-12-induced phosphorylation of Ser-721 of Stat4 (33, 59). Furthermore, p38 MAPK activity is required for optimal Stat1-mediated transactivation of interferon gamma-activated site and interferon-stimulated response element promoters without an effect on IFN-gamma-induced phosphorylation of Ser-727 of Stat1 (109, 110). The IL-6-induced transcriptional activation of Stat3 has also been shown to be dependent on IL-6-stimulated p38 MAPK activity in hepatocytes. (111) Also for the regulation of Stat6-mediated transcriptional activation a direct role for p38 MAPK was shown (40). Yet, since the transactivation domain of Stat6 is serine/threonine rich (39) and contains multiple phosphorylation sites, it is likely that other serine/threonine kinases play a role (40).

The P(M)SP motif may also be phosphorylated through pathways not involving MAPK, which include CDKs (Stat3), PKCdelta (Stat1, Stat3), mTOR (Stat3), NLK (Stat3), and CaMKII and IKKepsilon (Stat1) (reviewed in (3, 13, 37) (Figure 1C). One such example is Stat3 S727 phosphorylation in response to IL-6 (35, 36). A role for these kinases has however only been confirmed by gene knock-out or knockdown in a limited number of cases (3). Yet, *in vitro* kinase activity is difficult to evaluate with regard to P(M)SP kinase activity *in vivo* (13). Inconsistent results have been obtained with different experimental protocols concerning the phosphorylation of Stats by different MAPK *in vitro* or with regard to the effects of particular signaling pathways on serine phosphorylation of and transcriptional activation by Stats (13).

Stat5 serine phosphorylation may in part be mediated by the MAPK cascade, as suggested by binding interactions between Stat5a and the MAP kinases ERK1 and ERK2 (112). Additionally, inhibitor studies indicate a role for a MAPK-like activity in the constitutive phosphorylation of Stat5a on Ser725, but not for the PRL-inducible phosphorylation of Stat5a Ser725 and of Stat5b on the corresponding Ser730 (34). In other studies carried out in a different cell model, phosphorylation of Stat5a at Ser779 was not blocked by inhibitors of MAPK or PI3K (19). Interestingly, Ser779 occurs within a sequence (RLSPPA) that corresponds to a consensus motif for phosphorylation by PKA as revealed by computer analysis (113). There are some other kinases that may control Stat5a serine phosphorylation, including stress kinases, cyclin-dependent kinases (CDKs), p21-activated kinase (Pak) 1, and Pim kinases. Pim-1 to -3 belong to a family of serine/threonine kinases involved in the control of cell growth, differentiation and apoptosis (114). Deletion of all Pim kinases resulted in phenotypes that are remarkably similar to the deletion of Stat5 genes in lymphocytes, myeloid, and liver epithelial cells. (115). However, the consensus binding sites of these kinases does not seem to overlap with the respective Stat5a sequences (116). By contrast, Pak1, which has important functions in mammary gland development, was shown to directly interact with Stat5a and phosphorylate it at Ser779 (117, 118).

As yet, precisely which of the more than 400 serine/threonine kinases catalyze the constitutive phosphorylation of Stat5a on Ser725 and Ser779 and the signaling pathways that lead to the inducible phosphorylation of Stat5b on Ser730 in response to GH or PRL stimulation remains enigmatic (43). Moreover, the kinase(s) involved in hyperactivating Stat5 and thus contribute(s) to Stat5a-mediated transformation remain(s) to be identified. Elucidating the impact of eliminating Stat5a Ser779 phosphorylation in transformed cells might, therefore, be a next step in the development of a novel therapeutic concept for hematopoietic malignancies that has no major side effects on normal hematopoiesis and liver as well as immune cell function.

8. ACKNOWLEDGEMENTS

This article was supported by grant Sonderforschungsbereich (SFB) F28 from the Austrian Basic Research Funds (FWF) to the authors K.F., A.H., V.S., R.M., Wiener Wissenschafts-, Forschungs- und Technologiefonds (WWTF) grant LS07-037 to V.S., Association de la Recherche contre le Cancer (ARC) grant to F.G., and grant NIH R01DK059380 to KDB.

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Abbreviations: Ala: alanine, ALL: acute lymphoid leukemia, AML: acute myeloid leukemia, BCR: B cell receptor, CDK: cyclin dependent kinase, ChIP-seq : chromatin immunoprecipitation followed by parallel sequencing, CaMK: Ca2+/calmodulin-dependent protein kinase, CML: chronic myeloid leukemia, CPAP: centrosomal P4.1-associated protein, EGF: epidermal growth factor, ERK: Extracellular signal-regulated kinase, FMTC: familial medullary thyroid carcinoma, GH: growth hormone, GR: glucocorticoid receptor, IFN: interferon, IKK: IkappaB kinase, IL: interleukin, Jak: Janus kinase, JNK: c-Jun kinase, LPS: lipopolysaccharide, MAPK: mitogen activated protein kinase, MCM : mini-chromosome maintenance, NLK : Nemo-like kinase, PAK1: p21-activated kinase 1, PDGF: platelet derived growth factor, Phe: phenylalanine, PKC: protein kinase C, PRL: prolactin, PTPB: phosphotyrosine phosphatase B, pTyr: tyrosine phosphorylation, Ser: serine, Stat: Signal transducer and activator of transcription, TC-PTP: T cell phosphotyrosine phosphatase, TCR: T cell receptor, Thr: threonine, Tyr: tyrosine, UV: ultraviolet light

Key Words: Stat5a, Stat5b, Serine Phosphorylation, Transformation, Serine-Threonine Kinase, Review

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