A phosphorylation-acetylation switch regulates STAT1 signaling

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Cytokines such as interferons (IFNs) activate signal transducers and activators of transcription (STATs) via phosphorylation. Histone deacetylases (HDACs) and the histone acetyltransferase (HAT) CBP dynamically regulate STAT1 acetylation. Here we show that acetylation of STAT1 counteracts IFN-induced STAT1 phosphorylation, nuclear translocation, DNA binding, and target gene expression. Biochemical and genetic experiments altering the HAT/HDAC activity ratio and STAT1 mutants reveal that a phospho-acetyl switch regulates STAT1 signaling via CBP, HDAC3, and the T-cell protein tyrosine phosphatase (TCP45). Strikingly, inhibition of STAT1 signaling via CBP-mediated acetylation is distinct from the functions of this HAT in transcriptional activation. STAT1 acetylation induces binding of TCP45, which catalyzes dephosphorylation and latency of STAT1. Our results provide a deeper understanding of the modulation of STAT1 activity. These findings reveal a new layer of physiologically relevant STAT1 regulation and suggest that a previously unidentified balance between phosphorylation and acetylation affects cytokine signaling.

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The STAT signaling pathway is a paradigm for ligand-induced signaling from the cell surface to the nucleus. Cytokines and growth factors activate the transcription factor STAT1, which regulates the expression of physiologically important genes for cell growth, differentiation, apoptosis, and immune functions. Interferons (IFNs) are cytokines that induce dimerization of their cognate receptors leading to phosphorylation-dependent activation of the receptor-associated tyrosine kinases JAK1/2 and TYK2. These phosphorylate the C-terminal tyrosine residues Y⁷⁰¹ in STAT1 and Y⁶⁹⁰ in STAT2. Subsequently, STAT1 homodimers or STAT1/STAT2 heterodimers reciprocally interacting via their Src homology 2 (SH2) domains rapidly accumulate in the nucleus and induce STAT1 target genes (Ihle 2001; Platanius 2005; Stark 2007).

Receptor internalization, decreased kinase activity, and sumoylation of STAT1, SOCS, and PIAS proteins; as well as STAT1 dephosphorylation by phosphatases (PTPs)

followed by nuclear export counteract in vivo responses to IFN (Lim and Cao 2006; Kim and Lee 2007). The PTP T-cell protein tyrosine phosphatase (TCP45) dephosphorylates nuclear STAT1, which recycles STAT1 back to the cytoplasm (ten Hoeve et al. 2002). TCP45 additionally provides latency to previously activated STAT1 independent of the kinase and receptor status in vivo (Sakamoto et al. 2004b). However, the “timer” setting inactivation of phosphorylated nuclear STAT1 remains an enigma.

External as well as internal signals can induce the association of substrates with histone acetyltransferases (HATs) and histone deacetylases (HDACs) controlling the specificity and level of acetylation-dependent protein functions. Acetylation of histones mediated by HATs is a prerequisite for STAT1-dependent transcription (Paulson et al. 2002; Kouzarides 2007). Phosphorylated, nuclear STAT1 transiently binds the HAT CBP (Zhang et al. 1996), which can exist in a complex with the coactivator p/CIP. CBP cannot compensate a lack of p/CIP, which makes it difficult to define individual roles of these factors (Torchia et al. 1997). Recent data demonstrate that association of CBP with STAT1 and acetylation of histones at STAT1 target genes are temporally separated processes (Christova et al. 2007; Ramsauer et al. 2007).

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Strikingly, STAT1–CBP complex formation can even correlate with reduced histone acetylation (Nusinzon and Horvath 2003). Moreover, a STAT1 mutant that is unable to recruit CBP to chromatin still supports IFN-dependent transcription (Ramsauer et al. 2007). In agreement with such findings, IFN-dependent activation of STAT1 target genes requires GCN5 rather than CBP/p300 (Paulson et al. 2002).

Apart from tyrosine-phosphorylation, other functionally important post-translational modifications of STAT1 were identified (Lim and Cao 2006; Kim and Lee 2007). Recently, we described that CBP-mediated acetylation of STAT1 depends on the ϵ -amino group of lysine residues K⁴¹⁰ and K⁴¹³ (Krämer et al. 2006), which belong to the surface-exposed DNA-binding domain (DBD) common to all STATs. Dynamic acetylation of STAT1 is consistent with its interaction with both, HATs and HDACs. Remarkably, data from biochemical and genetic experiments show that HDAC activity is necessary for IFN-induced STAT1 activation (Nusinzon and Horvath 2003; Chang et al. 2004; Klampfer et al. 2004; Sakamoto et al. 2004a; Zupkovitz et al. 2006; Vlasáková et al. 2007). However, it is not clear whether HDACs and HDAC inhibitors (HDACis) affect STAT1 activity directly or by modulating other factors involved in STAT1 signaling.

Here we provide a mechanistic basis for the negative role of STAT1 acetylation in cytokine signaling. Following rapid activation of STAT1 via IFN, acetylation of STAT1 sets the timer for STAT1 inactivation via complex formation between acetylated STAT1 and the PTP TCP45. HDAC3 deacetylates STAT1, thus permitting phosphorylation and restimulation. Reversible, dynamic acetylation hence switches STAT1 between different functional modes. Our data provide novel insights into the covalent modification cycle, which limits the duration of the cytokine signal.

Results

Acetylation of STAT1 inhibits IFN-dependent STAT1 phosphorylation and nuclear translocation

IFNs can induce phosphorylation as well as acetylation of STAT1 in vitro and in vivo (Krämer et al. 2006; Hayashi et al. 2007; Tang et al. 2007). We examined the kinetics of STAT1 phosphorylation and acetylation in 293T cells treated with the type I interferon IFN α . Our data demonstrate that STAT1 acetylation follows STAT1 phosphorylation, and we found a correlation between STAT1 acetylation and dephosphorylation (Fig. 1A). Furthermore, using stringent immunoprecipitation (IP) conditions, we noted that acetylation and phosphorylation can transiently occur simultaneously on STAT1 (Fig. 1B). To evaluate the functional consequences of STAT1 acetylation, we analyzed whether a previous exposure to IFN α affects STAT1 phosphorylation. We found that, independent of ongoing protein synthesis or degradation and irrespective of the type of IFN and its cognate receptor, STAT1 could not be phosphorylated in re-exposed cells (Fig. 1C,D; Supplemental Fig. S1A), which confirms pre-

vious observations (Sakamoto et al. 2004a). If STAT1 acetylation contributes to this process, HDACis should equally prevent STAT1 phosphorylation. Indeed, consistent with a previous report (Klampfer et al. 2004), pretreatment with HDACis inhibited IFN α - or IFN γ -induced STAT1 phosphorylation in 293T, SK-Mel-37, and 2fTGH cells (Fig. 1E; data not shown). We could confirm such results in the presence of a caspase inhibitor or with MCF7 cells resistant to apoptosis induced by HDACis (Supplemental Fig. S1B,C; Krämer et al. 2008a). Thus, acetylation-dependent inactivation of STAT1 occurs independently of apoptosis. Furthermore, in agreement with the slow kinetics of HDACi-induced STAT1 acetylation (Krämer et al. 2006), coadministration of HDACi and IFN did not affect the extremely rapid IFN-induced phosphorylation of STAT1 (Fig. 1F), but accelerated its dephosphorylation. Delayed acetylation of STAT1 in the absence of IFN presumably stems from very low levels of nuclear STAT1 able to interact with CBP residing in the nucleus (Zhang et al. 1996; Meyer et al. 2003; Krämer et al. 2006). Consistently, short-term incubation with IFN α did not evoke STAT1 acetylation (Supplemental Fig. S1D). STAT1 acetylation correlates with its nuclear shuttling and time-delayed cytoplasmic accumulation of CBP (Krämer et al. 2006).

To fully exclude nonspecific effects exerted by HDACi and to demonstrate the effect of STAT1 acetylation sites on phosphorylation, we transfected STAT1-null U3A cells and HeLa cells with vectors encoding wild-type or mutated STAT1. These mutants harbor lysine-to-glutamine (Q; STAT1^{K410,413Q}) or arginine (R; STAT1^{K410,413R}) exchanges in the potential STAT1 acetylation sites K⁴¹⁰ and K⁴¹³, which mimic the acetylated or nonacetylated state of STAT1, respectively (Krämer et al. 2006; Supplemental Fig. S1E). We found that upon stimulation with IFN α , STAT1^{K410,413R} became phosphorylated and accumulated in the nucleus just like wild-type STAT1. However, STAT1^{K410,413Q} remained unphosphorylated and did not undergo nuclear translocation (Fig. 1G,H; Supplemental Fig. S1F). Consistently, STAT1 and STAT1^{K410,413R}, although not STAT1^{K410,413Q}, bound Importin α 5 (Fig. 1I) mediating nuclear transport of phosphorylated STAT1 (Sekimoto et al. 1997; Melén et al. 2001).

STAT1 DNA-binding mutants do not accumulate in the nucleus because of their rapid export via a leptomycin B (LMB)-sensitive CRM1-dependent mechanism (Meyer et al. 2003). We tested the effect of LMB on nuclear accumulation of STAT1. Whereas wild-type STAT1 was retained in the nucleus after stimulation with IFN and LMB, no increase of STAT1^{K410,413Q} in the nucleus was detectable under such conditions (Supplemental Fig. S1G). Hence, upstream events such as decreased phosphorylation appear responsible for the latency of pseudo-acetylated STAT1. The resistance of acetylated STAT1 to IFN α is unlikely to be caused by decreased interactions with the IFN α receptor (IFNAR) or kinases, since wild-type STAT1 and STAT1^{K410,413Q} equally colocalized with these factors, and both coprecipitated with the IFNAR (Fig. 1J,K; Krämer et al. 2006).

Figure 1. Acetylation affects IFN α -induced phosphorylation and translocation of STAT1. (A) STAT1 was immunoprecipitated from lysates of 293T cells treated with IFN α for the time periods indicated. Acetylation, phosphorylation, and precipitation of STAT1 were analyzed by Western blot; (IP) immunoprecipitation; (pre) preimmune serum IP. STAT1 phosphorylation was analyzed with an antibody specifically recognizing STAT1 phosphorylated at Y⁷⁰¹ in this and all following experiments when indicated. (B) 293T cells were incubated with IFN α for 40 min (+). Anti-acetyllysine IPs formed under stringent conditions with RIPA buffer (1% SDS) were analyzed for the presence of pY⁷⁰¹-STAT1 by Western blot (IP with control IgG). (C) 293T cells were incubated with IFN α for 20 min or remained untreated (0). One-hundred-eighty minutes later, cells were restimulated for 20 min (180 + 20 min). Parallel cultures were kept naive and treated with IFN α for only 20 min. Cell lysates were probed for STAT1 phosphorylation and expression in Western blot. (D) As in C, except that the protein/RNA synthesis inhibitors Cycloheximide (CHX) and Actinomycin D (ActD), or the proteasomal inhibitor MG-132, had been added 1 h before initial IFN treatment. (E) 293T cells were incubated with HDACi [(V) VPA; (T) TSA] or left untreated (C). Twenty-four hours later, cells were stimulated with IFN α for 20 min (+). Lysates were analyzed as in C. (F) 293T cells were coincubated with IFN α and HDACi for up to 3 h. Lysates were analyzed as in C. (G) U3A cells were transfected with DNA for STAT1 (WT), lysine mutants K^{410,413Q} (QQ), K^{410,413R} (RR), or empty vector pcDNA3.1 (3.1); (2f) 2fTGH, STAT1-positive U3A parental cell line. Forty-eight hours later, IFN α was added to the cells for 1 h (+). The Western blot was probed as indicated. (H) Translocation of EGFP-STAT1 (WT) and indicated K^{410,413} mutants was assessed by live cell time-lapse fluorescence microscopy. Transfected U3A cells were treated with IFN α for 0–60 min. In this and all following microscopy experiments, the bar corresponds to 10 μ m if not stated otherwise. (I) Binding of STAT1 (WT/QQ/RR) to Importin α 5 was analyzed by GST pull-down with lysates of transfected U3A cells. Western blots were probed for STAT1 and GST-Importin α 5. Cells were treated for 1 h. Pull-down with GST alone did not precipitate STAT1 (not shown). (J) Confocal immunofluorescence microscopy shows colocalization of transfected wild-type (WT) and pseudo-acetylated STAT1 (QQ) with endogenous TYK2, JAK2, or IFNAR1/2 in U3A cells (numbers state “overlap equation” r_0). (K) Wild-type STAT1 and K^{410,413Q} were equally recovered in IFNAR2 IPs formed from lysates of transfected U3A cells. (L) U3A cells were transfected with plasmids for STAT1 (WT) or STAT1^{K410,413R} (RR). The time course of STAT1 phosphorylation and expression, and IFN-induced nuclear translocation of EGFP-tagged STAT1 (WT/RR) was analyzed 180 min after IFN stimulation. (M) U3A cells were transfected with DNA for STAT1 (WT) or STAT1^{K410,413R} (RR). Forty-eight hours later, IFN α was added for 3 h (+). Lysates were prepared under conditions disrupting protein–protein interactions (1% SDS). IPs formed with anti-STAT1 or anti-acetyllysine antibodies were probed for acetyllysine and STAT1, or STAT1, respectively. (N) GST pull-downs with the indicated fragments of CBP on lysates of U3A cells reconstituted with STAT1 or STAT1^{K410,413R} by Western blot against STAT1 or GST.

2fTGH, STAT1-positive U3A parental cell line. Forty-eight hours later, IFN α was added to the cells for 1 h (+). The Western blot was probed as indicated. (H) Translocation of EGFP-STAT1 (WT) and indicated K^{410,413} mutants was assessed by live cell time-lapse fluorescence microscopy. Transfected U3A cells were treated with IFN α for 0–60 min. In this and all following microscopy experiments, the bar corresponds to 10 μ m if not stated otherwise. (I) Binding of STAT1 (WT/QQ/RR) to Importin α 5 was analyzed by GST pull-down with lysates of transfected U3A cells. Western blots were probed for STAT1 and GST-Importin α 5. Cells were treated for 1 h. Pull-down with GST alone did not precipitate STAT1 (not shown). (J) Confocal immunofluorescence microscopy shows colocalization of transfected wild-type (WT) and pseudo-acetylated STAT1 (QQ) with endogenous TYK2, JAK2, or IFNAR1/2 in U3A cells (numbers state “overlap equation” r_0). (K) Wild-type STAT1 and K^{410,413Q} were equally recovered in IFNAR2 IPs formed from lysates of transfected U3A cells. (L) U3A cells were transfected with plasmids for STAT1 (WT) or STAT1^{K410,413R} (RR). The time course of STAT1 phosphorylation and expression, and IFN-induced nuclear translocation of EGFP-tagged STAT1 (WT/RR) was analyzed 180 min after IFN stimulation. (M) U3A cells were transfected with DNA for STAT1 (WT) or STAT1^{K410,413R} (RR). Forty-eight hours later, IFN α was added for 3 h (+). Lysates were prepared under conditions disrupting protein–protein interactions (1% SDS). IPs formed with anti-STAT1 or anti-acetyllysine antibodies were probed for acetyllysine and STAT1, or STAT1, respectively. (N) GST pull-downs with the indicated fragments of CBP on lysates of U3A cells reconstituted with STAT1 or STAT1^{K410,413R} by Western blot against STAT1 or GST.

Further evidence that acetylation allows dephosphorylation of STAT1 is provided by STAT1^{K410,413R} exhibiting delayed deactivation kinetics with prolonged phosphorylation and nuclear localization after IFN stimulation (Fig. 1L). Additionally, IFN-induced phosphorylation of STAT1^{K410,413R} was insensitive to a previous exposure to

IFN as well as to HDACi (data not shown). IP of STAT1 or STAT1^{K410,413R} from reconstituted U3A cells followed by anti-acetyllysine Western blot, as well as the reverse experiment revealed that IFN α -induced acetylation of STAT1 is dependent on K⁴¹⁰ and K⁴¹³ (Fig. 1M). Since different interaction of wild-type STAT1 and STAT1^{K410,413R}

with CBP could prevent acetylation of STAT1^{K410,413R}, we performed a pull-down experiment to demonstrate direct interactions of these proteins (Zhang et al. 1996). Both STAT1 variants equally bound to fragments of CBP containing the two reported STAT1–CBP contact regions (Fig. 1N). These findings strongly indicate that acetylation of STAT1 (likely at lysines K⁴¹⁰ and/or K⁴¹³) critically counteracts cytokine-induced STAT1 activation.

Acetylation of STAT1 inhibits IFN-induced STAT1-dependent gene expression

HDACs have been reported to counteract STAT1 signaling (Nusinzon and Horvath 2003; Chang et al. 2004; Klampfer et al. 2004; Sakamoto et al. 2004a; Vlasáková et al. 2007). To gain insights into the underlying molecular mechanism, we transfected U3A cells with vectors

for STAT1 or K⁴¹⁰, K⁴¹³ mutants and a GAS-luciferase reporter plasmid harboring promoter elements of IFN α -responsive STAT1 target genes subject to induction by an activated STAT1 homodimer (Torchia et al. 1997; Platanias 2005). Wild-type STAT1 potently activated this reporter after IFN α stimulation. STAT1^{K410,413R} activated IFN-dependent transcription even more robustly, whereas STAT1^{K410,413Q} failed to induce reporter activity (Fig. 2A).

By quantitative real-time PCR, we determined the expression of the IFN α -induced, endogenous STAT1 target genes *isg15* and *ubch8* in U3A cells stably transfected with vectors for STAT1. ISG15 and UBC8 play important roles in the immune response and in several cancers (Dao and Zhang 2005; Krämer et al. 2008b; Okumura et al. 2008), and these genes are induced by an activated STAT1/STAT2 homodimer binding to an ISRE sequence

Figure 2. Lysines 410 and 413 of STAT1 control IFN-induced transcription. (A) A luciferase assay with a GAS-Luc construct quantifies transcriptional activity of the indicated STAT1 variants in U3A cells. IFN α -induced (24 h) reporter activation by wild-type STAT1 is set as 100%. Lysates were analyzed for STAT1 expression; (WT) wild-type; (QQ) STAT1^{K410,413Q}; (RR) STAT1^{K410,413R}; (3.1) empty vector pcDNA3.1. In this and all following luciferase experiments, luciferase activity is normalized to β -Gal activity. (B) IFN α -induced (8 h) expression of the indicated STAT1 target genes was analyzed by quantitative RT-PCR with RNAs from G418-resistant U3A cells stably expressing indicated STAT1 constructs or GFP. Fold induction relates to corresponding untreated cells. The Western blot verifies equal STAT1 expression. (C) Lysates from U3A cells transfected and stimulated as in A were analyzed for UBC8 expression. (D) ABCD assay showing STAT1–DNA complex formation. U3A cells were transfected as in A and treated with IFN α for 1 h; (GRE) control oligonucleotide. The Western blot was probed as indicated. (E) Translocation of EGFP-STAT1^{K410Q} or EGFP-STAT1^{K413Q} was assessed in U3A cells by live cell time-lapse fluorescence microscopy. Cells were treated with IFN α (0–60 min). (F) STAT1 (WT), STAT1^{K410Q}, or STAT1^{K413Q} was expressed in U3A cells. Cells were stimulated with IFN for 1 h (+). (Top panel) STAT1 phosphorylation and expression were determined by Western blot. (Bottom panel) Binding to Importin α 5 was analyzed by GST pull-down and Western blot. (G) Luciferase assay results (GAS-Luc) obtained with U3A cells transfected with STAT1 harboring individual or combined K to Q and/or R exchanges of K⁴¹⁰/K⁴¹³. (H) Expression of UBC8 in U3A cells transfected with STAT1 constructs stated, was analyzed as in C. (I) An ABCD assay shows the DNA binding of wild-type STAT1 (WT), STAT1^{K410Q}, and STAT1^{K413Q} after IFN stimulation.

(Nyman et al. 2000; Pfeffer et al. 2004). IFN α strongly enhanced the expression of both genes in STAT1-positive cells. STAT1^{K410,413R} induced *isg15* and *ubch8* even more potently than wild-type STAT1, while STAT1^{K410,413Q} was unable to mediate significant induction of these genes (Fig. 2B). Western blot analyses showed that this also translates into corresponding UBCH8 protein levels in U3A cells (Fig. 2C).

Next, we assessed STAT1–DNA complex formation with a GAS consensus oligonucleotide (Meyer et al. 2003). Both STAT1 and STAT1^{K410,413R} bound this DNA element upon IFN stimulation (Fig. 2D; Supplemental Fig. S1H). Consistent with all our observations that STAT1^{K410,413Q} is resistant to IFN α , this protein was not recovered with the GAS sequence. To dissect potential site-specific effects, we used STAT1 mutants harboring single K-to-Q exchanges (Supplemental Fig. S1E). STAT1^{K410R} and STAT1^{K413R} were responsive to IFN like wild-type STAT1 (data not shown). In contrast, amino acid exchanges mimicking acetylation of K⁴¹⁰/K⁴¹³ (STAT1^{K410Q}, STAT1^{K413Q}) rendered these mutants refractory to IFN α . Furthermore, STAT1 with combined K-to-Q and K-to-R mutations demonstrated that a single acetylated K⁴¹⁰/K⁴¹³ moiety already precludes STAT1 activation (Fig. 2E–I).

Moreover, in 293T cells, phosphorylation of endogenous STAT1 is suppressed by STAT1^{K410,413Q} *in trans* (Fig. 3A). U3A cells restored with STAT1 and STAT1^{K410,413Q} recapitulate this finding, as the latter prevents phosphorylation of the wild type (Fig. 3B). Consistent with these data, STAT1^{K410,413Q}, STAT1^{K410Q}, STAT1^{K413Q}, or HDACi treatment inhibited nuclear signaling and DNA binding of endogenous STAT1 (Fig. 3C–G; data not shown). Our findings indicate that acetylated STAT1 inhibits activation of nonacetylated STAT1 *in trans*.

Increasing evidence indicates that acetylation negatively affects IFN-induced STAT signaling (Nusinzon and Horvath 2003; Chang et al. 2004; Klampfer et al. 2004; Sakamoto et al. 2004a; Zupkovitz et al. 2006; Vlasáková et al. 2007). Therefore, we asked if our mutant mimicking nonacetylated STAT1 (Fig. 1M) is resistant to HDACi-induced inactivation. We reconstituted U3A cells with wild-type STAT1 and STAT1^{K410,413R} and treated these cells with IFN α and VPA. As expected, signaling by wild-type STAT1 was inhibited by acetylation. Expression of ISG15 was inhibited more strongly than UBCH8, which likely results from a complex mechanism by which HDACi induce expression of UBCH8, but not of ISG15 (Krämer et al. 2003; data not shown). In sharp contrast, signaling by STAT1^{K410,413R} was significantly induced upon inhibition of HDACs (Fig. 3H). These data demonstrate that acetylation per se can promote IFN-induced signaling, whereas acetylation of STAT1 counteracts this process.

Independent of stimulation with IFN, STAT1 dimerizes with other STAT1 or STAT2 molecules (Gupta et al. 1996; Stancato et al. 1996; Braunstein et al. 2003; Mao et al. 2005; Mertens et al. 2006). The *trans*-dominant-negative effect of STAT1^{K410,413Q} (Fig. 3A–G) suggests its dimerization with wild-type STAT1. Co-IP analyses, indeed,

demonstrated that HA-tagged STAT1, STAT1^{K410,413Q}, and STAT1^{K410,413R} interacted equally well with Flag-tagged STAT1 and with endogenous STAT2, independent of K-to-Q mutations in the STAT1 DBD (Fig. 3I–K). Besides being congruent with the observation that HDACi do not affect STAT1 dimerization (Nusinzon and Horvath 2003), our data indicate that the unresponsiveness of acetylated STAT1 and STAT1^{K410,413Q} to IFN is not just due to a defect in dimerization.

Considering these results and colocalization of STAT1^{K410,413Q} with STAT2 shown by confocal microscopy (Fig. 3L), we examined whether STAT1^{K410,413Q} inhibits STAT2 phosphorylation *in trans*. IFN α still induced STAT2 phosphorylation in the presence of STAT1^{K410,413Q} (Fig. 3M), demonstrating that mimicking acetylation of STAT1 at these lysine residues inactivates specifically STAT1.

A recent report shows acetylation of STAT3 at K⁶⁸⁵ (Yuan et al. 2005) corresponding to K⁶⁷⁹ in STAT1 (Supplemental Fig. S1I). Acetylation of STAT1 at this site in principle could impair STAT1 signaling. However, we observed no differences in phosphorylation levels of wild-type STAT1 and STAT1^{K679Q} (Fig. 3N). Hence, mimicking acetylation of STAT1 at K⁴¹⁰ and K⁴¹³ very specifically counteracts STAT1 signaling.

STAT1 activity is regulated by CBP and GCN5

Previously, we found that CBP is the HAT responsible for acetylation of STAT1, and that CBP translocates to the cytoplasm in response to IFN- α or HDACi (Krämer et al. 2006). The slow kinetics of HDACi-induced, compared with IFN-induced, acetylation of STAT1 correlates with delayed cytosolic appearance of CBP (data not shown). Since CBP specifically acetylates STAT1 (Krämer et al. 2006; Tang et al. 2007), we asked whether this HAT contributes to the termination of STAT1 signaling. Positive effects of cytosolic CBP/p300 on STAT signaling at the level of the IFNAR were observed in overexpression systems (Tang et al. 2007). This study, however, did not analyze the effect of STAT1 acetylation on IFN-induced signaling, and numerous reports clearly demonstrate that HDAC activity is required for IFN-induced STAT1 activation (Nusinzon and Horvath 2003; Chang et al. 2004; Klampfer et al. 2004; Sakamoto et al. 2004a; Zupkovitz et al. 2006; Vlasáková et al. 2007). Because unphysiological overexpression of the global regulator CBP cannot distinguish between artificially increased basal expression and IFN-induced expression of STAT1 target genes (O'Shea et al. 2005; Lim and Cao 2006), we used RNAi to analyze the role of CBP in STAT1 signaling induced by the physiological stimulus IFN. We generated 293T cells stably expressing shRNAs targeting CBP. Our data demonstrate that silencing of CBP significantly attenuated STAT1 dephosphorylation (Fig. 4A). Accordingly, reporter gene assays and analyses of endogenous ISG15 and UBCH8 levels showed enhanced IFN α -triggered STAT1-dependent transcription in cells with reduced CBP expression (Fig. 4B–D). These data are consistent with specific acetylation of STAT1 by CBP and enhanced IFN-induced signaling via nonacetylatable STAT1 (Figs. 1M, 2A–C, 3H).

Figure 3. Phosphorylation and DNA binding of STAT1 are regulated by acetylation. (A) 293T cells were transfected with vectors for HA-STAT1^{K410,413Q} (QQ) or pcDNA3.1. Cells were treated for 20 min with IFN α (+). STAT1 phosphorylation and expression were analyzed by Western blot. (B) STAT1 phosphorylation was assessed in U3A cells transfected with STAT1^{K410,413Q} (QQ; 1 μ g) and wild-type STAT1 (0.5 μ g). Cells were treated for 20 min (+). (C) Luciferase assay with a GAS-Luc reporter shows the transcriptional activity of endogenous STAT1 in 293T cells in the presence of increasing amounts of QQ or pcDNA3.1 (3.1; set as 100% for 24 h of IFN stimulation). The Western blot shows augmenting QQ expression. (D) Same as in C except that an ISRE-Luc reporter was used. (E) Same as in A except that cells were treated for 24 h and probed for UBCH8. (F) The ABCD assay shows the DNA binding of endogenous STAT1 from 293T cells in the presence of increasing amounts of HA-STAT1^{K410,413Q} (QQ; +, IFN α for 1 h). (G) 293T cells were left untreated (C) or incubated with VPA (V; 24 h). Equally treated parallel cultures were exposed to IFN α for 1 h. Lysates were analyzed by ABCD assay. (H) U3A cells were transfected with STAT1 (WT or RR). The induction of IFN α -induced (8 h) expression of the indicated STAT1 target genes in the absence (IFN α ; individually set as 100% for each STAT1 variant) or presence of VPA (1.5 mM) during IFN treatment (IFN α + V) or after a 24-h preincubation (V/IFN α) was analyzed by quantitative RT-PCR (note the different scales). (I) 293T cells were transfected with HA-tagged STAT1 (WT), STAT1^{K410,413Q} (QQ), or STAT1^{K410,413R} (RR) and Flag-STAT1. STAT1 homodimerization was assessed by IP against HA, followed by anti-Flag blot (pre, control IgG IP). (J) U3A cells were transfected with STAT1 (WT, QQ, or RR). IPs were done with anti-HA or irrelevant IgG (pre) and probed for STAT1 and endogenous STAT2. (K) U3A cells were transfected with STAT1 (GFP-WT and HA-WT, or GFP-QQ and HA-QQ). IPs were done with an anti-GFP antibody or irrelevant IgG (pre) and probed for STAT1 variants and endogenous STAT2. (L) Confocal immunofluorescence microscopy shows colocalization of wild-type (WT) and pseudo-acetylated STAT1 (QQ) with endogenous STAT2 in U3A cells (numbers state "overlap equation" r_0). (M) U3A cells were transfected with the indicated plasmids for STAT1 (WT/QQ/RR) or pcDNA3.1. STAT2 phosphorylation and expression were analyzed by Western blot (+, IFN α for 20 min). (N) STAT1^{K679Q} phosphorylation was compared with phosphorylation of wild-type STAT1 and STAT1^{K410,413Q} (QQ) in transfected U3A cells treated with IFN α for 20 min (+).

GCN5 catalyzes the acetylation of histones, but not of STAT1 (Paulson et al. 2002; Kouzarides 2007; Tang et al. 2007). We therefore analyzed the function of this HAT in IFN-induced signaling via an siRNA approach. This experiment showed that ablation of GCN5 decreased the induction of the GAS-reporter and of endogenous UBCH8 (Fig. 4E,F). Degradation or nuclear export of GCN5 cannot, however, explain why HDACi or pre-treatment with IFN α inhibit STAT1 signaling, as GCN5 remained stable and nuclear under these conditions

(Supplemental Fig. S2A). Our data demonstrate opposing effects of CBP and GCN5 on IFN-dependent STAT1 signaling. In contrast to GCN5, CBP can negatively regulate STAT1 activity in this context.

HDAC3 catalyzes STAT1 deacetylation

VPA, which selectively inhibits the class I HDACs HDAC1, HDAC2, HDAC3, and HDAC8, induces STAT1 acetylation (Göttlicher et al. 2001; Krämer et al. 2006). These HDACs are therefore likely candidate deacetylases

Figure 4. HATs and HDACs control STAT1 acetylation and phosphorylation. (A) 293T cells stably expressing shRNAs against CBP (shRNA CBP) or a nontargeting control vector (shRNA Ctl) were treated with IFN α . STAT1 phosphorylation, expression, and efficiency of the CBP knockdown were analyzed by Western blot. (B) Luciferase assay with the GAS-Luc construct in these cells. IFN α was added for 6–24 h (values for shRNA Ctl cells induced with IFN α for 24 h are set as 100%). (C) Real-time PCR was performed to analyze *ubch8* expression in 293T cells harboring shRNA Ctl or shRNA CBP. Cells were treated for 8 h with IFN α . (D) Western blot shows induction of UBCH8 and ISG15 in 293T cells bearing shRNA Ctl or shRNA CBP after addition of IFN α for 24 h. (E) Activation of the GAS-Luc reporter after knockdown of GCN5 by siRNAs (ctl, nontargeting siRNA) in 293T cells treated with IFN α (24 h). (F) UBCH8 induction in cells treated as in D. The Western blot was probed as indicated. (G) The Western blot demonstrates the interaction of endogenous STAT1 with endogenous HDACs, which were immunoprecipitated from 293T cell lysates. Efficient HDAC depletion was confirmed by Western blot (not shown). (H) Localization of HDAC1 and HDAC3 in

cytosolic (Cyt) and nuclear fractions (Nuc) prepared from 293T cells. (I) STAT1 acetylation in 293T cells overexpressing CBP and HDAC3 was analyzed by STAT1-IP and anti-acetyllysine Western blot. (J) Same as in I with CBP or siRNAs for HDAC3. (K) STAT1 phosphorylation was analyzed in 293T cells transfected with siRNA against HDAC3 or nontargeting control siRNA. Cells were IFN-treated for 20 min. (L) Same as in E, with siRNAs against HDAC3.

for STAT1. In co-IP experiments with endogenous proteins from 293T cells, STAT1 was recovered in a complex with HDAC1 and HDAC3 (Fig. 4G; Krämer et al. 2006). In contrast to HDAC1, HDAC3 localizes to both, nucleus and cytoplasm, and interacts with STAT1 in both cellular compartments (Fig. 4H; Chang et al. 2004; data not shown). Thus, HDAC3 is the most likely STAT1 deacetylase in the cytosol initiating STAT1 signaling.

Cotransfection experiments confirmed that HDAC3 strongly counteracts CBP-mediated STAT1 acetylation (Fig. 4I). Likewise, siRNA-induced ablation of HDAC3, similar to CBP overexpression, promoted STAT1 acetylation (Fig. 4J). Remarkably, knocking down HDAC3 equally translated into reduced STAT1 phosphorylation and attenuated IFN-dependent transcriptional activation (Fig. 4K,L). Hence, HDAC3 antagonizes STAT1 acetylation, which allows IFN-triggered STAT1 signaling. These observations provide an explanation why cytokine-induced STAT1 target gene expression requires HDAC activity. Our data further reveal that CBP and HDAC3 are the enzymes antagonistically regulating acetylation and ultimately signaling of STAT1.

PTP-dependent inactivation of acetylated STAT1

Activation by kinase-mediated phosphorylation and attenuation by PTP-mediated dephosphorylation are hallmarks of STAT1 signaling. We noted significant

differences in the phosphorylation of STAT1 and STAT1^{K410,413Q} in vitro and in cells. Although TYK2 catalyzed phosphorylation of both proteins in vitro (Fig. 5A), only wild-type STAT1 was phosphorylated in TYK2-transfected U3A cells (Fig. 5B). Notably, application of the general PTP inhibitor vanadate together with IFN α not only maintained phosphorylation of endogenous STAT1 in 293T cells (Supplemental Fig. S2B), but also permitted phosphorylation and nuclear translocation of STAT1^{K410,413Q} in U3A and 293T cells (Fig. 5C,D; Supplemental Fig. S2C). In response to this treatment, STAT1^{K410,413Q} even induced endogenous UBCH8 and bound cognate DNA (Fig. 5E,F; Supplemental Fig. S2D). Hence, PTPs antagonize kinase activity and appear to critically mediate STAT1's acetylation-dependent repression.

Since acetylated STAT1 forms a cytosolic complex with NF κ B p65 in vitro and in vivo (Krämer et al. 2006; Hayashi et al. 2007), NF κ B and I κ Bs could attenuate STAT1^{K410,413Q}. However, STAT1^{K410,413Q} remained unresponsive to IFN upon inactivation of p65 by siRNAs or attenuation of I κ Bs by TNF α (Fig. 5D,E; Supplemental Fig. S3A). These results, along with the observation that STAT1^{K410,413Q} and STAT1 interacted equally well with PIAS1, SOCS1, and SUMO1 (Supplemental Fig. S3B), disfavor alternative PTP-independent regulatory mechanisms.

In agreement with these data, STAT1 acetylated via HDACi treatment was phosphorylated and bound to

DNA after treatment with IFN α and vanadate (Fig. 5G). Likewise, combined application of these stimuli induced phosphorylation of STAT1^{K410Q} and STAT1^{K413Q} in U3A cells. Both proteins could then bind GAS DNA as well as GCN5 and induce transcriptional activation (Fig. 5H–J).

Our data and those from Melén et al. (2001) show that local charge alterations within the STAT1 DBD do not prevent STAT1 signaling per se. Furthermore, STAT1 acetylation site mutants as well as STAT1 acetylated via HDACi are structurally intact and in principle permissive for phosphorylation. Consistent with this, acetylation of STAT1 does not compromise its intracellular localization in the uninduced state (Figs. 1H,J, 3L; Krämer et al. 2006). Acetylation of STAT1, rather, represents a PTP-dependent refractory state of STAT1.

TCP45 binds to acetylated STAT1

We next investigated which PTPs are relevant for the inactivation of STAT1 following acetylation. SHP2 (SH2-containing PTP) and TCP45 are PTPs negatively regulating STAT1 signaling (ten Hoeve et al. 2002; Wu et al. 2002). To clarify their role in the STAT1 phospho-acetyl switch, we generated 293T cells in which PTP levels are stably suppressed by shRNAs. Reporter assays with these cells confirmed that SHP2 and, even more potently, TCP45 suppress endogenous STAT1 signaling (Fig. 6A). IFN α -induced reporter gene activation was furthermore unaffected by vanadate treatment in cells bearing shRNAs against SHP2 and TCP45 (data not shown), which supports the view that they are the key PTPs for STAT1.

Since, similar to vanadate treatment, the down-regulation of TCP45 by shRNA rescued STAT1 signaling in the presence of STAT1^{K410,413Q} (Fig. 6B), we focused on this PTP. If TCP45 prevents IFN α -induced phosphorylation of acetylated STAT1, HDACis should not inhibit STAT1 activation in 293T cells with reduced TCP45 levels. Indeed, STAT1 phosphorylation and DNA recognition were unaffected by HDACis in such cells (Fig. 6C). Likewise, IFN α induced phosphorylation and strong nuclear accumulation of wild-type STAT1 and STAT1^{K410,413Q} in TCP45-depleted U3A cells (Fig. 6D; Supplemental Fig. S3C). In U3A shTCP45 cells, STAT1^{K410,413Q}-activated transcription of endogenous STAT1 target genes (Fig.

Figure 5. PTPs inactivate acetylated STAT1. (A) STAT1 (WT) and STAT1^{K410,413Q} (QQ) are phosphorylated by TYK2 in vitro. The Western blot was probed for phosphorylated STAT1 and total STAT1. (B) Transfected TYK2 (50 ng) phosphorylates STAT1 (WT) but not STAT1^{K410,413Q} (QQ) in U3A cells. Western blotting was done as in A. (C) U3A cells were transfected with STAT1, STAT1^{K410,413Q}, or pcDNA3.1. Sodium vanadate (VO $_4^{3-}$) had been added for 30 min, and then IFN α was added for 20 min. Western blotting was done as in A. (D) U3A cells were transfected with EGFP-STAT1^{K410,413Q} and analyzed by live cell time-lapse immunofluorescence. VO $_4^{3-}$ or TNF α and IFN α were added at the time points indicated. (E) UBCH8 induction was analyzed by Western blot in U3A cells transfected with wild-type STAT1 or STAT1^{K410,413Q}. Cells were exposed to VO $_4^{3-}$ or TNF α and IFN α for 24 h. (F) Lysates from U3A cells transfected with STAT1 or STAT1^{K410,413Q} were subjected to ABCD assay for phospho-STAT1–DNA binding. Cells were incubated with VO $_4^{3-}$ for 30 min followed by IFN α for 1 h; (GRE) control oligonucleotide. (G) 293T cells were incubated with VPA (V; 24 h), then with VO $_4^{3-}$ for 30 min, followed by IFN α for 1 h. Lysates were analyzed for STAT1 phosphorylation and DNA binding by Western blot and ABCD assay. (H) U3A cells transfected with STAT1 (WT), STAT1^{K410Q} (410Q), or STAT1^{K413Q} (413Q) were analyzed for STAT1 phosphorylation and subjected to ABCD assay analyzing STAT1 binding to DNA and to GCN5. Cells were incubated as in F; (3.1) empty vector pcDNA3.1. (I) U3A cells were transfected as in H. Luciferase assay was performed to analyze STAT1-dependent gene induction. The Western blot shows equal expression of STAT1 variants. (J) Western blot assessing UBCH8 induction after 24 h of IFN α plus vanadate treatment in U3A cells transfected as in I.

6E). The knockdown of this PTP equally rendered STAT1^{K410Q} and STAT1^{K413Q} responsive to IFN α (Fig. 6F), which triggered transcriptional activation of STAT1 target genes (Fig. 6G,H).

Importantly, co-IP analyses showed that STAT1 mutants mimicking acetylation of K^{410,413} efficiently bound TCP45 (Fig. 7A). This finding provides a rationale for the unresponsiveness of previously activated, acetylated STAT1 to IFN. Therefore, we studied by immunofluorescence whether IFN α not only induces acetylation of STAT1, but also affects the localization of endogenous TCP45 in intact cells. Indeed, periods of IFN α treatment inducing STAT1 acetylation (Fig. 1A,M) caused colocalization of TCP45 and STAT1, together with cytoplasmic translocation of this PTP (Fig. 7B; Supplemental Fig. S3D). Co-IP analyses further confirmed that STAT1 acetylation via IFN α induces formation of a complex containing STAT1 and TCP45 (Figs. 1A, 7C).

Further analyses showed that STAT1 phosphorylation peaks at ~20 min and starts to cease at ~40 min of IFN- α treatment. Consistent with the reported nuclear dephosphorylation of STAT1 (Haspel et al. 1996; Haspel and Darnell 1999; ten Hoeve et al. 2002), LMB did not alter this kinetic (Fig. 7D). We performed IP experiments with timed cytosolic and nuclear fractions to analyze the interaction of acetylated STAT1 and TCP45. Data obtained with this approach demonstrate that beginning dephosphorylation of STAT1 correlates with acetylated STAT1 binding to TCP45 in the nucleus. Additionally, there is a time-dependent accumulation of cytosolic TCP45 in a complex with acetylated STAT1 (Fig. 7E).

Finally, we asked for how long acetylation of STAT1 inhibits IFN-induced signaling. For this purpose, we pulsed cells with IFN and chased them to study their ability to reinduce STAT1 phosphorylation. After a chase time of 2 h, we could clearly detect phosphorylated STAT1 (Fig. 7F). Remarkably, the return of STAT1 to restimulation capacity was associated with decreased STAT1 acetylation, loss of CBP binding, and reassociation with HDAC3 (Fig. 7G).

Our interaction studies and functional analyses demonstrate the dynamic regulation of STAT1 via CBP, HDAC3, and TCP45. Hence, these factors are key elements of a phospho-acetyl switch regulating STAT1 activity (Fig. 7H).

Discussion

The molecular mechanisms responsible for STAT acetylation are under intense investigation, and IFN-dependent signaling via STAT1 is a paradigm for a pathway requiring deacetylase activity. We analyzed STAT1 signaling in vitro and in cells using a wide variety of conditions and methods. Our data indicate that a functional phospho-acetyl switch, regulated by an acetylation/deacetylation balance, modulates STAT1 signaling.

We initially reported that acetylated STAT1 inhibits NF κ B (Krämer et al. 2006). Now, we demonstrate that acetylation of STAT1 counteracts its own activity. Our

findings provide a mechanistic basis for the suspected negative role of acetylation on IFN-dependent signaling (Nusinzon and Horvath 2003; Chang et al. 2004; Klampfer et al. 2004; Sakamoto et al. 2004a; Zupkovitz et al. 2006; Vlasáková et al. 2007).

Both phosphorylation and acetylation are versatile modulators of protein functions and interactions (Schreiber and Bernstein 2002). Cross-talk between serine phosphorylation and lysine methylation has been described for the kinetochore protein DAM1 and the transcription factor p53 (Fischle et al. 2003; Zhang and Dent 2005). Our data identify STAT1 as a signaling molecule subject to an unexpected cross-regulation between phosphorylation and lysine acetylation. IFN-induced phosphorylation promotes nuclear translocation of STAT1, which enables it to interact with the acetyltransferase CBP. Acetylation of STAT1 by CBP correlates with the formation of a STAT1–TCP45 complex, dephosphorylation, and latency of STAT1. We conclude that the highly active PTP TCP45 acts as a “transmission control protein” docking to and inhibiting previously activated STAT1. STAT1 acetylation hence conveys information terminating stimulation and regulating restimulation.

Extensive conformational changes are necessary for STAT1 dephosphorylation. The “pocket” residues Q³⁴⁰, Q⁴⁰⁸, and G³⁸⁴ are required for spatial reorientation of a parallel to an anti-parallel STAT1 dimer presenting the pY⁷⁰¹ to TCP45 (Zhong et al. 2005; Mertens et al. 2006). It appears feasible that after initial activation, and once acetylated in the nucleus by CBP, STAT1 is stabilized in its anti-parallel structure providing access for TCP45. Since HDACs inhibit IFN-induced phosphorylation and promote dephosphorylation of STAT1, acetylation of lysines K⁴¹⁰ and K⁴¹³ may facilitate both, disengagement of STAT1 from DNA and presentation to TCP45 (Meyer et al. 2003; Mao et al. 2005; Mertens et al. 2006). Although K⁴¹⁰ and K⁴¹³ provide contacts of STAT1 with the DNA backbone (Chen et al. 1998; Melén et al. 2001), these lysines do not contribute to specific STAT1–DNA contacts, and 12 additional amino acid side chains connect STAT1 with DNA (Chen et al. 1998; Melén et al. 2001). In agreement with this, phosphorylated STAT1^{K410,413Q} can bind DNA, albeit slightly weaker than the wild type. Consistent with the observation that these residues do not belong to the “pocket” residues required for N-terminal dimerization, mutagenesis of K⁴¹⁰, K⁴¹³ does not affect STAT1 dimerization. Furthermore, the activity of STAT1^{K410,413Q} under conditions in which TCP45 is blocked either by shRNA or vanadate strongly indicates that this protein is structurally intact.

Acetylation of STAT1 within its surface-exposed DBD determines functionally relevant interactions of STAT1 with other regulators of signaling. Since HATs interact with all STATs, acetylation and deacetylation might regulate each of them. Nevertheless, assessing the specific consequences of acetylation requires individual analyses. In the case of STAT1, acetylation clearly acts inhibitorily. In contrast, acetylation seems to affect STAT3 dimerization, and it remains to be determined

Figure 6. TCP45 inactivates acetylated STAT1. (A) Luciferase assay with a GAS-Luc construct in 293T cells stably expressing shRNAs against SHP2 and TCP45. IFN α -induced (24 h) shRNA Ctl cells are set as 100%. The Western blot shows efficient phosphatase depletion and UBCH8 induction with shorter and longer (*) film exposure. (B) Luciferase assay was performed as in A in the presence of STAT1^{K410,413Q} (QQ). (C) 293T cells expressing TCP45 shRNA were treated with HDACis [(V) VPA; (T) TSA; (B) sodium butyrate; (C) not treated with HDACi] and 24 h later with IFN α for 20 min (+). STAT1 phosphorylation and expression were analyzed. (Right panel) Binding of phosphorylated STAT1 to the GAS oligonucleotide was assessed by ABCD assay and Western blotting; (GRE) control oligonucleotide. (D) U3A cells stably expressing STAT1 (WT) or STAT1^{K410,413Q} (QQ) were transfected with TCP45 shRNA for 48 h and treated with IFN α for 1 h (+), or (–) left untreated, and analyzed for nuclear translocation of STAT1 by immunofluorescence microscopy. (Right panel) Equally transfected U3A cells were analyzed for phosphorylation and expression of STAT1. (E) U3A cells transfected with STAT1^{K410,413Q} and TCP45 shRNA. IFN α was added for 24 h (+). Expression of STAT1 and its target genes UBCH8 and ISG15, and shRNA efficiency were analyzed by Western blot. (F) U3A cells transfected with TCP45 shRNA and wild-type STAT1 (WT), STAT1^{K410Q}, STAT1^{K413Q}, or pcDNA3.1 were treated with IFN α (1 h). (Top panel) STAT1 phosphorylation, expression, and shRNA efficiency were analyzed by Western blotting. (Bottom panel) Binding of STAT1 to GAS-DNA was analyzed via ABCD assay (cf. Fig. 2E–I). (G) U3A cells transfected as in F were analyzed for GAS-Luc activation (induction by wild-type STAT1 set as 100%). Cells were incubated with IFN α for 24 h. (H) Same as in E, analysis of STAT1 (WT), STAT1^{K410Q}, and STAT1^{K413Q}.

how STAT3 signaling is terminated (Yuan et al. 2005). Acetylation of STAT3 residues corresponding to K⁴¹⁰ and K⁴¹³ in STAT1 can be ruled out, as these residues are arginines in STAT3 (Supplemental Fig. S3E), which could well explain why HDACis do not inhibit STAT3. Moreover, the fact that STAT1 acetylation does not suppress STAT2 function is consistent with the observation that STAT2 is inactivated neither by TCP45 nor by HDACis (Sakamoto et al. 2004b; Vlasáková et al. 2007).

Equal to phosphorylation, acetylation does not necessarily enhance gene activation, and the physiological consequences of histone versus nonhistone protein modification by HATs and HDACs are clearly different (Schreiber and Bernstein 2002; Yang 2004). Reminiscent of data collected for STAT2 (Paulson et al. 2002), our results support that GCN5 is required for IFN-induced STAT1/STAT2 signaling. In contrast to CBP, GCN5 catalyzes the acetylation of histones, but not of STAT1 (Paulson et al. 2002; Tang et al. 2007). In agreement with this finding, HDACis promote IFN-induced signaling, but only if IFN-dependent gene induction is evoked by a non-acetyltable STAT1 molecule. CBP and GCN5 even show

opposing effects, as CBP-mediated acetylation of STAT1 within its DBD is dominant over STAT1 activation. Our results provide a rationale for the negative role of STAT1 acetylation on IFN signaling. They elucidate a novel, specific role of CBP, which is increasingly appreciated as a nonhistone protein acetyltransferase and a negative regulator of gene expression (Munshi et al. 1998; Zhang and Dent 2005). In agreement with several studies (Nusinzon and Horvath 2003; Chang et al. 2004; Klampfer et al. 2004; Sakamoto et al. 2004a; Zupkovitz et al. 2006; Vlasáková et al. 2007), our data demonstrate that CBP induces STAT1 acetylation and suppresses IFN-dependent activation. Therefore, acetylation of STAT1 via CBP dominantly restricts the duration of IFN signaling in human cells responsive to this cytokine.

A very attractive hypothesis is that STAT1 acetylation acts as a “memory mark” designating previously activated STAT1. Within a “STAT1 modification code,” TCP45 apparently reads out acetylation of this transcription factor. Feed-forward mechanisms for STAT1 acetylation promoting association with TCP45 might be established by the CBP bromodomain recognizing

Figure 7. Acetylation of STAT1 recruits TCP45 terminating the IFN signal. (A) U3A cells were transfected with TCP45 substrate trapping mutants TCP45^{C216S/D182A}. Anti-TCP45 precipitates were analyzed for co-IP of STAT1 or its K⁴¹⁰/K⁴¹³ acetylation site mutants (QQ/410Q/413Q; [pre] preimmune IP) and efficient precipitation of TCP45. (B) Immunofluorescence analysis of HeLa cells analyzing dynamic localization of endogenous STAT1 and endogenous TCP45 after IFN α stimulation. Bar, 50 μ m. (C) TCP45^{C216S/D182A} IPs from 293T cells treated with IFN α as in B were subjected to Western blot against STAT1 and TCP45. (D) 293T cells were stimulated with IFN α for 0–60 min. STAT1 phosphorylation and STAT1 expression, in the absence or presence of LMB, were monitored by Western blotting against STAT1, acetyl-lysine, and TCP45. (E) TCP45^{C216S/D182A} IPs from cytosolic and nuclear extracts from 293T cells treated with IFN α for the time periods indicated were subjected to Western blotting against STAT1, acetyl-lysine, and TCP45. (F) 293T cells were incubated with IFN α for 8 h (Pulse). After removal of IFN α , cells were retreated with IFN α for 20 min (+) or not restimulated (–) at 1-h intervals. The presence of phosphorylated STAT1, STAT1, CBP, and HDAC3 was determined by Western blot. (G) STAT1 IPs were done from the same lysates as in F. STAT1 acetylation and precipitation, and binding of CBP and HDAC3 to STAT1 was determined 1–3 h after removal of IFN α (Chase). (H) Model illustrating the dynamic modification of STAT1. A phospho-acetyl switch inhibits STAT1 upon its acetylation-dependent recruitment of TCP45 following activation by IFN. STAT1 homodimers serve as the example.

N^c-acetylated proteins, the IFN-induced cytosolic translocation of CBP, and the reduced interaction of acetylated STAT1 with HDACs (Yang 2004; Krämer et al. 2006; Tang et al. 2007). In addition, dephosphorylated anti-parallel STAT1 dimers remain intact and exit to the cytoplasm, tethered via their N-terminal domains (Mertens et al. 2006). Upon nuclear export, dimers or even oligomers of STAT1 (Gupta et al. 1996; Stancato et al. 1996; Vinkemeier and Meyer 2005; Mertens et al. 2006) within a complex containing even a limited number of acetylated STAT1 molecules, bound to CBP and TCP45, can counteract activation of the STAT1 pool. Via such a mechanism, unnecessary activation, energy-consuming degradation, and a potential loss of cytoplasmic functions of STAT1 is prevented (Krämer et al. 2006; Lim and Cao 2006; Kim and Lee 2007). Termination of STAT1 acetylation, via dissociation of CBP and association of HDAC3, in turn shifts the balance to permissiveness for restimulation. Deacetylation of STAT1 by HDACs may provide a molecular switch to restore inducible STAT1 in the cytoplasm, which would close the cycle of STAT1 activation and inactivation (Fig. 7H). Such precise and dynamic acetylation/deacetylation-depen-

dent regulatory circuits may have evolved to adjust cytokine-induced gene expression rapidly and economically in vivo.

In addition, both transcription factors, STAT1 as well as NF κ B, induce inflammatory mediators contributing to inflammatory diseases, such as rheumatoid arthritis or Crohn's disease, STAT1 acetylation may hence be a central mechanism by which HDACs ameliorate pathological settings with repeatedly released large amounts of proinflammatory cytokines (Blanchard and Chipoy 2005).

Materials and methods

Cell lines, transfections, microscopy, drugs, and chemicals

Cells were maintained as described (Krämer et al. 2003, 2006). Transfections were done with PEI (Sigma, for 293T) or Lipofectamine (Invitrogen; as recommended). Unless stated otherwise, 1 μ g DNA/12-well or 5 μ g DNA/10-cm plate were transfected, and cells were harvested 48 h later. Stable cell lines were generated with G418 (500 μ g/mL) or Puromycin (2 μ g/mL). Immunofluorescence staining and image analysis of cells were performed as in Krämer et al. (2006).

Colocalization coefficients represent “overlap equation” (Krämer et al. 2008a):

$$r_0 = \frac{\sum (R_i G_i)}{\sqrt{\sum R_i^2 \sum G_i^2}}$$

Drugs and chemicals were purchased as in Krämer et al. (2006, 2008a,b). If stated, cells were incubated with 10^3 U of IFN α , 50 ng/mL TNF α , 10 ng/mL LMB, 10 μ g/mL Cycloheximide, 1 μ g/mL Actinomycin D, 20 μ M MG-132, or HDACi [(VPA) 1.5 mM VPA; (TSA) 30 nM TSA; sodium butyrate: 1.5 mM] for time periods indicated in the figure legends. Vanadate was used at concentrations of 0.1 mM for 24-h incubations and at 1 mM for shorter treatments.

Preparation of cell lysates, IP, pull-down, immunoblotting and ABCD (avidin-biotin-coupled DNA) assay

These techniques were carried out as described (Sekimoto et al. 1997; Krämer et al. 2003, 2006, 2008b). Inputs represent 10% of lysates used.

Luciferase reporter assays and quantitative PCR

In all luciferase assays (Göttlicher et al. 2001; Krämer et al. 2006), 24 h after transfection, 10^3 U of IFN α was added for 6–24 h. Assays were performed in triplicate and normalized to β -galactosidase activity. The data shown are representative for at least two independent experiments. For detailed transfection schemes, see the Supplemental Material.

Total RNA was isolated using Illustra RNeasy spin (GE Healthcare). cDNAs were synthesized with the RT-System (Promega). cDNAs were subjected to one-step real-time PCR in an IQ5 cycler (Bio-Rad) using SYBR Green (Abgene) and fluorescein (annealing temperature: 63°C). Data are $\Delta\Delta$ – Ct values (unstimulated/stimulated samples) normalized to 18S rRNA (Krämer et al. 2008b).

Antibodies, sequences for quantitative PCR and gene silencing, and in vitro phosphorylation assay

Details are provided in the Supplemental Material.

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