The treatment of cells with histone deacetylase inhibitors (HDACi) was reported to reveal the acetylation of STAT1 at lysine 410 and lysine 413 (O. H. Krämer et al., Genes Dev. 20:473–485, 2006). STAT1 acetylation was proposed to regulate apoptosis by facilitating binding to NF-κB and to control immune responses by suppressing STAT1 tyrosine phosphorylation, suggesting that STAT1 acetylation is a central mechanism by which histone deacetylase inhibitors ameliorate inflammatory diseases (O. H. Krämer et al., Genes Dev. 23:223–235, 2009). Here, we show that the inhibition of deacetylases had no bearing on STAT1 acetylation and did not diminish STAT1 tyrosine phosphorylation. The glutamine mutation of the alleged acetylation sites, claimed to mimic acetylated STAT1, similarly did not diminish the tyrosine phosphorylation of STAT1 but precluded its DNA binding and nuclear import. The defective transcription activity of this mutant therefore cannot be attributed to STAT1 acetylation but rather to the inactivation of the STAT1 DNA binding domain and its nuclear import signal. Experiments with respective cDNAs provided by the authors of the studies mentioned above confirmed the results reported here, further questioning the validity of the previous data. We conclude that the effects and potential clinical benefits associated with histone deacetylase inhibition cannot be explained by promoting the acetylation of STAT1 at lysines 410 and 413.
cellular tyrosine phosphatases were inactivated by vanadate treatment (22). As both HDACi and vanadate are not STAT1-specific reagents, the authors produced a STAT1 mutant that mimicked the acetylated state, i.e., KK410/413QQ (25), to link HDACi-associated effects directly to the acetylation of STAT1 (22, 23). In perfect agreement with the results obtained with HDACi and wild-type STAT1, the acetylation-mimicking mutant was reported to remain unphosphorylated in IFN-treated cells, it did not accumulate in the nucleus or bind DNA, and hence it too was incapable of activating IFN target genes (22). Importantly, according to the authors, the glutamine mutations per se did not interfere with any of these STAT1 activities, as they reported that the treatment of cells with vanadate or small interfering RNA to inactivate tyrosine phosphatases completely rescued the phenotype of the acetylation-mimicking mutant KK410/413QQ. Remarkably, when tyrosine phosphatase activity was suppressed, the IFN stimulation of cells was shown to trigger Tyr701 phosphorylation of the mutant STAT1, which now accumulated in the nucleus and bound to GAS sites on DNA, culminating in the ability to activate IFN-responsive genes. Thus, based on this consistent data set the authors posited that the phosphorylation/dephosphorylation cycle of STAT1 and, hence, interferon signaling was controlled by the acetylation of residues 410 and 413 (22).

However, residues 410 and 413 are crucial for the NLS of activated STAT1 (29), a fact that received no attention in these studies. In agreement with general NLS requirements, lysines in these positions facilitate the association of activated STAT1 with importin-α, which is required for subsequent nuclear import (26, 29, 32). Thus, we wondered how the acetylation of NLS residues and the replacement of the lysines in positions 410 and 413 with glutamines to mimic acetylation affected STAT1 nucleocytoplasmic mobility and IFN signaling. Table 1 summarizes published results concerning the effects of Lys410/413 mutations on the tyrosine phosphorylation of STAT1, as well as DNA binding and the nuclear import of the activated molecule. As our results from the outset did not conform to the model proposed by Krämer et al., the key experiments published in references 22 and 23 were repeated to evaluate the biological significance of STAT1 acetylation. This exercise confirmed that the model presented by Krämer et al. in references 22 and 23 is incorrect. We show here that neither IFN nor HDACi triggered STAT1 acetylation. We show furthermore that the treatment of cells with HDACi did not interfere with the IFN-induced tyrosine phosphorylation of STAT1. In addition, the alleged acetylation-mimicking glutamine mutant of STAT1, i.e., KK410/413QQ, is demonstrated to be defective per se both in nuclear import and DNA binding. In further stark contrast to the previously published results (22, 23), phosphatase inhibition could not rescue the nuclear import and DNA binding deficiencies of acetylation-mimicking STAT1. Finally, we have repeated the previously published experiments with the original cDNAs encoding wild-type and acetylation-mimicking STAT1 that were provided by the authors of the published studies (22, 23). The experiments with these reagents did not reproduce the published results, whereas the contradictory findings reported here were fully confirmed. In sum, the claims that STAT1 acetylation controls immune responses by suppressing STAT1 tyrosine phosphorylation (22) and regulates apoptosis by facilitating STAT1 binding to NFκB (23) were not upheld by the results presented here.

**MATERIALS AND METHODS**

**Cell culture, cDNAs, and reagents.** Human HeLa S3, U3A, and 293T cells were grown at 37°C in a humidified 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS; Sigma) and antibiotics. Mammalian expression plasmids for wild-type (pSTAT1-GFP) and mutant KK410/413EE (EE) STAT1 fused to green fluorescent protein (GFP) have been described already (29); STAT1 was C-terminally Flag tagged. The site-directed mutagenesis of pSTAT1-GFP (QuickChange; Stratagene) generated mutants KK410/413QQ (QQ) and KK410/413RR (RR). Mutations were verified by DNA sequencing. Transient transfections were carried out using Lipo-fectamine Plus (Invitrogen) as described previously (3). Unless stated otherwise, the treatment of cells was with human IFN-α (100 U/ml; Calbiochem), human IFN-γ (20 ng/ml; Calbiochem), 1.5 mM VPA (Sigma), 30 nM TSA (Sigma), and 1 mM pervanadate. Mammalian expression constructs published by Krämer et al. (22) encoding the wild type and QQ mutant of STAT1 were requested from the authors in February 2010, and dried cDNA was obtained on 14 October 2010. DNA was extracted with Tris-EDTA (TE) and was used to transform DH5α bacteria. Single bacterial colonies were amplified, and plasmid DNA was purified using the Qiagen Maxiprep protocol. The resulting cDNAs were of comparable quality, as judged by readings of the optical density at 260 and 280 nm, which were 1.92 for STAT1-QQ and 1.87 for STAT1-WT, respectively. Clones of both plasmids were partially sequenced in house using the Big Dye sequencing protocol (Applied Biosystems), followed by capillary electrophoresis using a commercial source (Gene Services Nottingham).

**Quantitative Western blotting and immunoprecipitation.** Cell extractions were performed in whole-cell extraction buffer (50 mM Tris-HCl, pH 7.4, 7.5 mM NaCl, 1 mM sodium pervanadate [VO43–], 30 mM NaF, 10 mM glycerol phosphate, 1 mM EDTA, 2 mM EGTA, 10% [vol/vol] glycerol, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], and Complete [Roche] protease inhibitors) for 30 min on ice. Soluble components were separated by centrifugation at 14,000 × g for 15 min at 4°C and used for immunoprecipitation (30) and Western blotting. Western blotting entailed the labeling of primary antibodies with IRdye800-conjugated secondary antibodies. Control readings were obtained with diluted cell extracts to confirm that signal intensities were within the linear range. Where the presence of multiple proteins/protein modifications was examined, this was done consecutively for each antibody using the same blot. Bound antibodies were stripped off nitrocellulose membranes by incubation for 30 min twice in 1 mM Na2CO3, 0.1% (wt/vol) Tween-20, 0.1% (wt/vol) sodium deoxycholate, 1% (wt/vol) SDS at 55°C for 30 min, followed by three washes with Tris-buffered saline (TBS) and one wash with TBS with 0.1% (wt/vol) Tween-20.

**TABLE 1. Functional characterization of wild-type STAT1 and STAT1 variants mutated in positions 410 and 413**

<table>
<thead>
<tr>
<th>STAT1 construct</th>
<th>pTyr701</th>
<th>DNA binding</th>
<th>pTyr701 STAT1 nuclear import</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>RR</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>QQ</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EE</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>AA</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

a +, functionality; −, loss of functionality.
in stripping buffer (25 mM glycine, 2% SDS, pH 2) at 65°C. Primary antibodies were purchased from Cell Signaling (anti-pTyr701 STAT1, order no. 9171 and anti-acetylated lysine, order no. 9441 [formerly sold by NEB]), Santa Cruz (anti-STAT1, C24, E23, anti-simian virus 40 [SV40] T antigen [Pab 108], order no. sc-148), Abcam (anti-acetyl-histone 3 and H3[lys9]), and Sigma (anti-FLAG and anti-β-actin).

Immunoﬂuorescence microscopy. Cells were ﬁxed with methanol at −20°C, washed in phosphate-buffered saline (PBS), and blocked with 20% (vol/vol) FCS–PBS. Samples were incubated overnight with either anti-STAT1 antibody (C24; dilution, 1:6,000) or anti-pTyr701 STAT1 antibody diluted 1:250 in 20% (vol/vol) FCS–PBS. After three washes with PBS, the cells were incubated with appropriate species-speciﬁc afﬁnity-puriﬁed Cy3-conjugated immunoglobulins (Jackson Research) in 20% (vol/vol) FCS–PBS. A Leica DMLB microscope was used as described previously (3).

EMSA. For electrophoretic mobility shift assay (EMSA), U3A cells (1.8 × 10⁵) were transiently transfected. Thirty-six hours later, the cells were left untreated or were treated as described in the ﬁgure legends. After two washes with ice-cold PBS, the cells were incubated on ice for 5 min in 70 μl cytoplasmic extraction buffer (10 mM KCl, 1 mM EDTA, 20 mM HEPES-NaOH, pH 7.4, 0.1 mM sodium pervanadate, 1.5 mM MgCl₂, 10% [vol/vol] glycerol, 1 mM DTT, Complete protease inhibitors, 0.2% [vol/vol] NP-40, and 0.1 mM PMSF), followed by centrifugation for 20 s at 4°C and 14,000 × g. The supernatant (cytoplasmic extract) was isolated and kept on ice. The pellet was resuspended in 70 μl nuclear extraction buffer (420 mM KCl, 1 mM EDTA, 20 mM HEPES-NaOH, pH 7.4, 0.1 mM pervanadate, 1.5 mM MgCl₂, 20% [vol/vol] glycerol, 1 mM DTT, Complete protease inhibitors, and 0.1 mM PMSF), and incubated for 30 min on ice. After centrifugation for 15 min at 4°C and 14,000 × g, the nuclear extract was isolated and combined with the corresponding cytoplasmic extract. A Bradford assay (Bio-Rad) was used to conﬁrm equal protein concentrations for the different extracts. Five micrograms of protein was incubated in a 14-μl reaction mix (containing 1 mg/ml bovine serum albumin (BSA), 1 mM DTT, 2 μg poly(dI-dC), 20 mM HEPES-NaOH, pH 7.9, 40 mM KCl, 1 mM MgCl₂, 0.4 mM EDTA, 0.1 mM EGTA, 4% [vol/vol] Ficoll, and 1 mM double-stranded [γ-³²P]CTP-labeled DNA probe) for 20 min at room temperature. The DNA probe (5'-TGATTTCCCGGAAATGACGGGC-3' with 5'-TGAG-3' overhangs at the 5' end of both strands) harbored a single GAS site (underlined) from the IRF-1 gene promoter (35). For the immune detection of DNA-bound STAT1 (supershift), anti-STAT1 antibody (C24) was added to the reaction mix (1 μl). DNA-protein complexes were resolved at 4°C and 400 V on 4.7% nondenaturing polyacrylamide gels using Tris-borate-EDTA buffer (140 mM Tris-HCl, pH 8.0, 22 mM boric acid, and 2 mM EDTA). After a running time of 90 min, gels were dried; radioactivity was detected and quantiﬁed by phosphorimaging (FLA-3000; Fujifilm).

RESULTS AND DISCUSSION

Our analyses of the effects of lysine acetylation on the function of STAT1 started with an attempt to conﬁrm STAT1 acetylation. The previously published experiments were done with several different cell lines; however, crucial experiments were performed with 293T cells, which express SV40 large T antigen (22, 23). Because SV40 large T antigen binds to cellular factors, including the transcriptional coactivators p300 and CBP (2), and CBP is responsible for the acetylation of STAT1 according to the published results (22, 23), we first conﬁrmed by Western blotting that our 293T cells, but not primary and spontaneously immortalized mouse embryonic ﬁbroblasts (MEF), expressed SV40 large T antigen. Figure 2A shows that this was indeed the case. We then stimulated 293T cells with IFN-α for 3 h as described by Krämer et al. (see Fig. 1A of reference 22), followed by the immunoprecipitation of the endogenous STAT1 from whole-cell extracts and the probing of the immunoprecipitates with antiacetylysine-specific antibody. Additionally, 293T cells were treated with 1.5 mM VPA for 24 h, either alone or in conjunction with subsequent IFN-α for 3 h, as this was described to boost the acetylation of STAT1 (see Fig. 6A of reference 23). However, despite repeated attempts, in our hands neither approach gave a signal for acetylated STAT1 (Fig. 2B). Human ﬁbrosarcoma 2TGH cells are another cell line that was reported to harbor acetylated STAT1 upon stimulation with IFN-α (see Fig. 6F of reference 23). We thus used 2TGH-derived U3A cells (31), which lack STAT1 expression, and reconstituted them with Flag-tagged STAT1. However, immunoprecipitated Flag-tagged STAT1 did not show reactivity with acetyllysine antibody irrespective of IFN-α.
Moreover, treatment with deacetylase inhibitors was shown to preserve (12), lysine acetylation is efficiently stabilized by HDACi, sodium butyrate (NaBu) (Fig. 3D). Even at more than 10-fold higher than published HDACi concentrations, STAT1 acetylation fails to detect fraction of acetylated STAT1 is an unlikely explanation for the lack of antiaacetyllysine immunoreactivity.

To our knowledge, STAT1 acetylation has been observed in two other instances, but the data quality was not definitive (16, 40), and according to those studies there was no impairment of STAT1 phosphorylation associated with acetylation. A third study on STAT1 acetylation reported a lack of immunodetectable STAT1 lysine acetylation in B-cell lines irrespective of treatment with IFN-α or TSA (27). Our failure to detect acetylated STAT1 is unlikely to be explained by antibody differences, as preparations of the commercially available acetyllysine antibody were used in the present and the earlier studies (22, 23). Another factor relevant for successful detection is the abundance of acetylated STAT1 in cells, which to our knowledge has not been quantified. However, Krämer et al. report that the target of acetylation is phosphorylated STAT1 (22, 23), which makes up 30 to 40% of STAT1 in interferon-stimulated cells (43). According to reference 22, acetylation is required to recruit phosphatase TC45 to the activated STAT1. If we assume that at least one monomer of each phosphodimer needs to be acetylated for efficient phosphatase recruitment, it is safe to presume that in IFN-stimulated cells about 10 to 20% of STAT1 molecules are acetylated at steady state. Contrary to labile postranslational modifications, like the SUMO conjugation of STAT1, which is difficult to preserve (12), lysine acetylation is efficiently stabilized by deacetylase inhibitors according to the published experiments (22, 23). Moreover, treatment with deacetylase inhibitors was reported to completely suppress the IFN-induced phosphorylation of STAT1 (see Fig. 1E in reference 22), suggesting that the quantitative acetylation of STAT1 is readily achieved under these conditions. Thus, it is reasonable to assume that a large fraction, probably in excess of 30 to 40% of the cellular STAT1 content, should have been acetylated in our experiments. Therefore, a very small modified and, hence, difficult-to-detect fraction of acetylated STAT1 is an unlikely explanation for the lack of antiaacetyllysine immunoreactivity.

We therefore focused on the reported consequences of STAT1 acetylation, namely, the inhibition of IFN-α- and IFN-γ-induced STAT1 Tyr701 phosphorylation. It was reported that a 24-h pretreatment of 293T cells with HDACi VPA (1.5 mM) or TSA (30 nM) precludes the subsequent 20-min IFN-α-induced tyrosine phosphorylation of STAT1 (see Fig. 1E in reference 22). To attempt to reproduce this experiment, 293T cells were left untreated or were treated with HDACi VPA (1.5 mM) or TSA (30 nM) for 24 h, followed by stimulation with IFN-α for 20 min as described above. IFN treatment alone caused the expected Tyr701 phosphorylation of STAT1 (Fig. 3A). However, in stark contrast to the published results, pretreatment with HDACi TSA (30 mM) or VPA (1.5 mM) left the IFN-α-induced tyrosine phosphorylation of STAT1 undiminished (Fig. 3A). Three quantitative Western blotting experiments generated no statistically significant differences regarding IFN-α-induced Tyr701 phosphorylation between untreated and HDACi-treated cells (Fig. 3A). We additionally studied the effects on STAT1 phosphorylation of increasing concentrations of VPA (Fig. 3B), TSA (Fig. 3C), or another HDACi, sodium butyrate (NaBu) (Fig. 3D). Even at more than 10-fold higher than published HDACi concentrations, STAT1 was strongly phosphorylated in response to IFN-γ, not at all resembling the complete loss of IFN-α- or γ-induced Tyr701 phosphorylation reported in Fig. 1E and data not shown of reference 22. We thus inferred that low HDACi concentrations...
did not alter the activation of STAT1. This conclusion agrees with numerous previous studies, which reported the unchanged or even prolonged tyrosine phosphorylation of STAT1 at low HDACi concentrations similar to those used here (5, 33, 36).

We next studied the IFN-induced activation of the acetylation-mimicking mutant of STAT1, KK410/413QQ (termed QQ). This mutant was reported to remain unphosphorylated in U3A cells after 1 h of IFN-α (A) or even prolonged tyrosine phosphorylation of STAT1 at low HDACi concentrations similar to those used here (5, 33, 36).

We then examined the DNA binding of the acetylation-mimicking STAT1 mutant KK410/413QQ, which was shown to bind to GAS sites (see Fig. 5F in reference 22). However, to reveal the mutant’s DNA binding activity, previously cells needed to be treated with a phosphatase inhibitor, e.g., vanadate (22). According to the authors, this treatment was required to overcome the excessive dephosphorylation caused by the phosphatase recruitment of the pseudoacetylated STAT1. The QQ mutant therefore bound to DNA in the presence of a tyrosine phosphatase inhibitor (see Fig. 5F, S1H, and S2D in reference 22). According to the published data, these results establish that acetylation-mimicking STAT1 KK410/413QQ, if phosphorylated, is capable of binding to GAS-containing DNA. The dependence of DNA binding and transcriptional activity on phosphatase inhibition (as shown in Fig. 5E in reference 22) is of pivotal importance for the proposed phosphorylation-acetylation switch model. For one thing, it confirms the acetylation-mimicking activity of the QQ mutant. More crucially, it represents the cornerstone of the proposed model on which the authors rest their central conclusion that acetylation inhibits STAT1 signaling by facilitating the recruitment of phosphatase activity to the activated transcription factor (Table 1).

Before we report the results of experiments evaluating the validity of this set of published data, we first need to comment on details of the methodology that was used to demonstrate STAT1 DNA binding in the published study (22). To evaluate
the sequence-specific DNA binding of a protein, the sequence of the DNA must be known. “GAS consensus oligonucleotides” and “GAS sequences” are mentioned numerous times in reference 22, yet the only precise information about the actual GAS sequence that was used can be found in the legend to Fig. S1H, where it is stated, “A pulldown assay with a biotinylated GAS oligonucleotide (ABCD-assay) was used to detect STAT1-DNA binding. The oligonucleotide represents two palindromic TCC half-sites, contacted by the DNA binding domains of an activated STAT1 dimer.” This statement does not fully clarify the issue, since GAS sites are nonameric (TTCN TNGAA) and thus are not represented by two palindromic TCC half sites (9). Nonetheless, this and other statements suggest that the authors wanted to study the DNA binding of dimers of activated STAT1. However, instead of treating cells with IFN-γ, which is the most potent and specific STAT1-activating stimulus, IFN-α was used to assess DNA binding. IFN-α stimulation gives rise predominantly to STAT1/STAT2 heterodimers that bind together with another protein, IRF-9, to an unrelated DNA site (9, 15), such that relatively few STAT1 dimers capable of GAS binding remain. These incomplete descriptions and the very difficult-to-interpret study design precluded the exact repetition of the experiments as published. We decided therefore to follow established procedures that generate unambiguous results to examine the DNA binding of dimers of wild-type and mutant STAT1, respectively. This included the use of electrophoretic mobility shift assay (EMSA) rather than precipitation methods like the ABCD assay, as the latter uses denaturing conditions to resolve DNA binding complexes, thus destroying information on the size and composition of the DNA-associated protein complexes. This limitation makes precipitation assays less useful for studying sequence-specific DNA binding proteins. As the probe, we used radiolabeled DNA containing a single high-affinity STAT1 binding site, the GAS element of the IRF-1 promoter (35). Also, cells were treated with IFN-γ to generate large quantities of activated STAT1 dimers, which facilitates analyses of GAS-specific DNA binding activity.

Using whole-cell extracts from U3A cells transiently reconstituted with STAT1 variant proteins as described previously (22), we observed a single protein-DNA complex for wild-type STAT1 (Fig. 4C, lanes 1 and 2) that was reactive with STAT1 antibody (lane 10), indicating that the DNA binding complex contained STAT1 as expected. We then tested the DNA binding of the acetylation-mimicking mutant KK410/413QQ. Although abundant activated STAT1 was present in the extracts (Fig. 4B), there was no binding of STAT1 QQ to GAS-containing DNA (lanes 3 and 4). The identical negative outcome was seen with mutant KK410/413EE (lanes 5 and 6), which we had shown previously to dimerize yet to be defective in DNA binding (29), while the arginine mutant KK410/413RR retained binding to the GAS site (lanes 7 and 8). These results demonstrated that positive charges in positions 410 and 413 are indispensable for the binding of STAT1 to its recognition site on DNA.

Lysines 410 and 413 are part of the nuclear import signal for activated STAT1 (29). However, little is known about how the acetylation of lysine residues or their charge-neutralizing mutation affects NLS activity. It was reported for STAT1 that the charge-neutralizing mutation of lysines 410 and 413 to glutamine does not per se impair the nuclear import of the phosphorylated protein (see Fig. 5D in reference 22). We therefore transiently reconstituted U3A cells with wild-type or mutant STAT1 and used immunofluorescence microscopy to observe the activation and intracellular distribution of STAT1 in response to IFN-α stimulation. Wild-type STAT1 showed the well-known IFN-α-induced activation and concomitant nuclear accumulation (42) (Fig. 5A). The acetylation-mimicking QQ mutant showed similarly strong IFN-α-induced activation, substantiating our Western blotting results but again contradicting the published experiments (see Fig. 1G in reference 22). Importantly, however, the phosphorylated mutant did not accumulate in the nucleus but rather stayed in the cytoplasm (Fig. 5A), in further contradiction of the published results (see Fig. 5C and D in reference 22). The glutamate mutation of residues 410 and 413 also precluded nuclear accumulation, and the activated protein stayed in the cytoplasm, whereas the arginine mutation of these positions did not impair STAT1 nuclear accumulation (Fig. 5A). We additionally tested whether treatment with vanadate could overcome the nuclear import defect of the glutamine mutants of STAT1, as shown in Fig. 5D of the earlier study (22). However, this was not the case, as the phosphorylated glutamine as well as the glutamate mutants remained in the cytoplasm (Fig. 5A). Control experiments verified the tyrosine phosphatase-inhibiting activity of the vanadate preparation used here. Vanadate measurably prolonged the IFN-α-induced nuclear accumulation of wild-type STAT1 (Fig. 5B), which is explained by the inability of tyrosine-phosphorylated STAT1 to return to the cytoplasm (30). When this experiment was performed in HeLa cells using IFN-γ stimulation, the same outcome was observed, namely, strong phosphorylation yet defective nuclear import of the acetylation-mimicking STAT1 QQ mutant, while the phosphorylated arginine mutant retained nuclear import (Fig. 5C). Interestingly, in the presence of H₂O₂, which was used in this experiment in conjunction with pervanadate to inhibit tyrosine phosphatase activity, phosphorylated STAT1 formed paracrystals. This phenomenon is explained by the concomitant H₂O₂-dependent inactivation of STAT1 SUMO conjugation (12). Of note, the QQ mutant formed paracrystals in the cytoplasm, providing corroborating evidence for the efficient phosphorylation and loss of nuclear import. We thus concluded that positive charges in positions 410 and 413 are required both for sequence-specific DNA binding and the nuclear import of activated STAT1. These results agree with data independently obtained for STAT1 (28), Max transcription factor (14), and the Rothmund-Thomson-syndrome gene product RECOL4 (11), whose nuclear import is precluded by charge-neutralizing replacements with glutamine or alanine of lysine residues required for NLS functioning.

Given the complete lack of agreement with the reported results, we requested the original mammalian expression vectors encoding wild-type STAT1 and the acetylation-mimicking mutant that were used in the published studies (22, 23). DNA sequencing of ~100 bp upstream and ~800 bp downstream of the STAT1 coding sequence revealed no differences between the two constructs (data not shown), and they matched the sequence of pcDNA3.1 TOPO vector (Invitrogen), thus confirming the published information (23). The wild-type STAT1-encoding sequence was identical to the GenBank entry
NM_007315 for human STAT1. The cDNA of STAT1 acetylation-mimicking mutant QQ showed the expected (single-base) changes of codons 410 and 413 resulting in mutations to glutamine (see Fig. S1 in the supplemental material). We found three additional single-base changes affecting codons 636, 740, and 742, one of which resulted in the mutation of the STAT1 protein at Lys636→Arg (Fig. 6A; also see Fig. S1 in the supplemental material). Residue 636 is situated in the SH2 domain and is part of a conserved antiparallel β-sheet that constitutes the core of this domain (6), but subsequent experiments showed that the protein mutated in position 636 was expressed in mammalian cells, indicating that this amino acid change did not alter protein structure. U3A cells then were transfected with these constructs to determine tyrosine phosphorylation and the nuclear translocation of the published proteins. As shown in Fig. 6B, quantitative Western blotting demonstrated that wild-type STAT1 and the acetylation mutant QQ both were equally well tyrosine phosphorylated, with no statistically significant differences between them. Similarly, immunofluorescence microscopy confirmed both the undiminished tyrosine phosphorylation of acetylation-mimicking STAT1 (determined by GFP epifluorescence) and the localization of phosphorylated STAT1 (pSTAT1), determined by labeling with anti-pTyr701-STAT1 antibody. Nuclei were labeled with Hoechst dye. Note the appearance of STAT1 paracrystals in the presence of H2O2. Bars, 10 μm.

FIG. 5. Acetylation-mimicking STAT1 mutant is phosphorylated but not imported in the nucleus. (A) U3A cells were transiently reconstituted with GFP-tagged wild-type STAT1 (WT), mutant KK410/413QQ (QQ), mutant KK410/413EE (EE), or mutant KK410/413QRR (RR). After 48 h the cells were treated with 1,000 U/ml IFN-α for the indicated times. Where indicated (VO43−), cells were pretreated for 45 min with 1 mM pervanadate before the addition of IFN-α for another 60 min. Shown are immunofluorescence images depicting the localization of STAT1 (determined by GFP epifluorescence) and the localization of phosphorylated STAT1 (pSTAT1), determined by labeling with anti-pTyr701-STAT1 antibody. (B) U3A cells were transfected with GFP-tagged wild-type STAT1. Forty-eight hours later, cells were treated for 0 to 6 h with 1,000 U/ml IFN-α alone or together with 1 mM pervanadate (+VO43−). Shown are GFP epifluorescence micrographs that depict the distribution of STAT1. (C) HeLa cells were transiently transfected with GFP-tagged wild-type STAT1 (WT) or mutant QQ or RR as indicated. After 36 h the cells were left untreated or were treated for 60 min with IFN-γ (20 ng/ml). Where indicated (VO43−/H2O2), cells were pretreated for 45 min with 0.8 mM pervanadate and 0.2 mM H2O2 prior to the addition of IFN-γ. Shown are immunofluorescence images depicting the localization of STAT1 (determined by GFP epifluorescence) and the localization of phosphorylated STAT1 (pSTAT1), determined by labeling with anti-pTyr701-STAT1 antibody. Nuclei were labeled with Hoechst dye.
here conclusively exclude the possibility that STAT1 signaling is regulated by a phosphorylation-acetylation switch as proposed by Krämer et al. (22). The proposed switch mechanism is based on the experimental demonstration (22) that the treatment of cells with HDACi prevents the IFN-induced phosphorylation of STAT1, and that the resulting inhibition of STAT1 signaling can be overcome by the inactivation of cellular phosphatases. We disprove this claim by showing that the treatment of cells with HDACi results in the undiminished phosphorylation of STAT1. Validation of the model proposed in reference 22 furthermore hinges on the use of a single reagent, namely, the supposed acetylation-mimicking glutamine 410/413 mutant of STAT1. It was reported that glutamine mutations of residues 410 and 413 prevented IFN-induced phosphorylation of STAT1, and that the resulting inhibition of STAT1 signaling could be overcome by the inactivation of cellular phosphatases (22). We show here that the IFN-induced phosphorylation of the glutamine mutant both are inactivated, which prevents this protein from functioning as a signal transducer and activator of transcription, i.e., STAT1. The invalidated so-called acetylation-mimicking glutamine mutant is pivotal for Krämer et al. to demonstrate that acetylated STAT1 controls both IFN signaling and cell death. At present, the claims put forth in references 22 and 23 were found to be without experimental justification, as are attempts to link the protective effects of histone deacetylase inhibitors against cancer and inflammation to the Lys410/Lys413 acetylation of STAT1 (4, 10, 17, 18, 34, 44). As reported here, despite of our best attempts to reproduce exactly the experiments of references 22 and 23, we found no experimental confirmation of the results presented there. We are unable to provide a scientifically acceptable explanation for the complete dichotomy of our findings from those reported by Krämer et al.

ACKNOWLEDGMENTS

We note that the publisher of references 22 and 23 was first notified of the results reported here in November 2009. This work was supported by Deutsche Forschungsgemeinschaft (KFO192) grants VI 218/4 and VI 218/2 (to U.V.).
REFERENCES