Mutually exclusive STAT1 modifications identified by Ubc9/substrate dimerization-dependent SUMOylation

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ABSTRACT

Post-translational modifications control the physiological activity of the signal transducer and activator of transcription STAT1. While phosphorylation at tyrosine Y701 is a prerequisite for STAT1 dimerization, its SUMOylation represses the transcriptional activity. Recently, we have demonstrated that SUMOylation at lysine K703 inhibits the phosphorylation of nearby localized Y701 of STAT1. Here, we analysed the influence of phosphorylation of Y701 on SUMOylation of K703 in vivo. For that reason, an Ubc9/substrate dimerization-dependent SUMOylation (USDDS) system was developed, which consists of fusions of the SUMOylation substrate and of the SUMO-conjugating enzyme Ubc9 to the chemically activatable heterodimerization domains FKBP and FRB, respectively. When FKBP fusion proteins of STAT1, p53, CRSP9, FOS, CSNK2B, HES1, TCF21 and MYF6 are coexpressed with Ubc9-FRB, treatment of HEK293 cells with the rapamycin-related dimerizer compound AP21967 induces SUMOylation of these proteins in vivo. For STAT1-FKBP and p53-FKBP we show that this SUMOylation takes place at their specific SUMOylation sites in vivo. Using USDDS, we then demonstrate that STAT1 phosphorylation at Y701 induced by interferon-β treatment inhibits SUMOylation of K703 in vivo. Thus, pY701 and SUMO-K703 of STAT1 represent mutually exclusive modifications, which prevent signal integration at this molecule and probably ensure the existence of differentially modified subpopulations of STAT1 necessary for its regulated nuclear cytoplasmic activation/inactivation cycle.

INTRODUCTION

Functions of proteins are often controlled by post-translational modifications, such as phosphorylation, myristoylation, acetylation, ubiquitination and SUMOylation. These modifications can be constitutively or regulated and often prime or hinder further modifications (1). Protein SUMOylation is a reversible conjugation process with strong similarity to ubiquitination where the SUMO protein is attached in a process of three enzymatic steps via an isopeptide bond to the ε-amino group of a lysine residue of the substrate protein. In a fourth step, SUMOylation can be released by SUMO-specific proteases (2). SUMOylation is involved in the regulation of several proteins and, consequently, potentially interferes with other regulatory protein modifications. Interestingly, some transcription factors, such as HSF1, GATA-1 and MEF2A, are regulated by phosphorylation-dependent SUMOylation (3–5), while MEF2D, HIC1, NF-IL-6 and SP-3 show an interplay between SUMOylation and acetylation (6–9). Furthermore, SUMOylation competes with IκBα ubiquitination (10) and, as we have demonstrated recently using Ubc9 fusion-directed SUMOylation (UFDS), SUMOylation inhibits STAT1 phosphorylation at Y701 (11).

The in vivo analysis of the interplay between different protein modifications mentioned above is often hampered by the low level of the specific modifications in the cell and by the lacking possibilities to manipulate a specific protein modification independently of other modifications. To increase the low level of SUMOylation in vivo, we have developed Ubc9 fusion-directed SUMOylation (UFDS) (11). However, because of the static fusion of Ubc9 to the substrate protein of interest, this method is not suited to study the kinetics of SUMOylation or the sequential SUMOylation after different preceding modification events. To overcome these limitations, we now introduce the Ubc9/substrate...
dimerization-dependent SUMOylation (USDDS) system. Instead of a static fusion, this system makes use of a chemically inducible interaction between Ubc9 and the substrate of interest allowing substrate-directed SUMOylation in vivo at a controlled time point. USDDS now enables us to study induced SUMOylation in dependence on other pre-existing modifications. Here, we demonstrate USDDS with eight substrate proteins and used USDDS to analyse the effect of STAT1 phosphorylation at Y701 on its SUMOylation at K703.

MATERIALS AND METHODS

Plasmids

We amplified the cDNA encoding for the FKBP domain from pc4EN-F1E and the FRB (T2098L) domain from pc4-RHE (ARGENT Regulated Heterodimerization Kit) by PCR using the primers FKBP-EcoRI (5′-GGCGGAATTCATCAGAGGATGTCAGTGGAACACCATC-3′) and FKBP-XbaI (5′-GGCGCTCTAGATTTAGAAGCTC-3′) or the primers FRB-EcoRI (5′-GGCGGAATTCATCAGAGGATGTCAGTGGAACACCATC-3′) and FRB-XbaI (5′-GGCGCTC TAGATTCACTAGTCTTTGAGATTCGTCGGAACA CATGATA-3′) and cloned it into the EcoRI and XbaI sites of pcDNA3 (Invitrogen) to obtain the pcDNA3-MCS-FKBP/FRB expression vectors. We have taken the cDNA-encoding human STAT1α from the pcDNA3-STAT1-Ubc9 plasmid (11) by BamHI/EcoRI digestion, and cloned it into the BamHI and EcoRI sites of pcDNA3-MCS-FKBP/FRB to generate the mammalian STAT1-FKBP/FRB expression vectors. Dependent on an EcoRI site in the coding sequence, seven C-terminal amino acids of the human STAT1α in the STAT1-FKBP/FRB fusion proteins are missing. We then have taken the cDNA encoding for human p53 from the plasmid pcDNA3-p53-Ubc9 by BamHI/EcoRI digestion, and cloned it into the BamHI and EcoRI sites of pcDNA3-MCS-FKBP/FRB to generate the mammalian STAT1-FKBP/FRB expression vectors. For generation of pcDNA3-MCS-FKBP2. The ORF-FKBP fusion protein expression vectors were obtained by recombination of the above described destination vector with the ORF (Table 1) harbouring entry plasmids using the Gateway recombination system (Invitrogen).

Transfection, cell lysis and western blotting

HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium with high glucose, complemented with 10% fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. We performed transfection of 50–80% confluent HEK293 cells in 12-well plates using the polyethylenimine transfection reagent according to Christina Ehrhardt et al. (13). We grew the transfectants for 24 h, then lysed them in 150 μl of gel loading buffer [160 mM Tris–HCL, pH 6.8, 4% (w/v) sodium dodecyl sulphate (SDS), 20% (v/v) glycerol, 0.5% β-mercaptoethanol (v/v), 0.008% (w/v) bromophenol blue] and incubated them for 10 min at 95°C. For western blot analysis, we separated the proteins by SDS–PAGE, blotted the proteins on a PVDF membrane and detected them with specific primary antibodies [α-SUMO1 (Cell Signaling), α-STAT1 (Cell Signaling), α-p53 (1C12, Cell Signaling), α-SUMO1 (Cell Signaling), α-FKB1 (1-026A, Affinity Bioreagents)], a horseradish peroxidase-conjugated secondary antibody, the Immobilon™ Western (Millipore) and the LAS-3000 imaging system (Fuji). For the interferon-stimulation experiments, we grew the transfectants for 24 h and then either stimulated them with 1000 U/ml of interferon-β or left them unstimulated for 1 h. Then the cells were incubated further 0, 1, 2 or 4 h in the medium with the interferon-β without or after adding AP21967 to a final concentration of 1 μM. Then we lysed the cells and analysed the proteins by western blotting.

RESULTS

AP21967-dependent binding of Ubc9 to various substrate proteins induces their SUMOylation

Post-translational protein modifications can act separately, together or even counteract each other to integrate
transfected cells with the dimerizer AP21967 leads to a strongly enhanced SUMOylation of STAT1-FRB and p53-FRB already after 1 h, which reaches saturation after 2–4 h (Figure 2A and B). The estimated stoichiometry of the SUMOylation of STAT1 and p53 in USDDS is similar to that of UFDS. However, USDDS clearly functions in an inducible manner. We also tested STAT1-FKBP, p53-FKBP and FKBP fusions of the proteins listed in Table 1 in combination with Ubc9-FRB. Again, we found an even slightly stronger, AP21967-induced SUMOylation of the STAT1- and p53-FKBP fusion proteins (Figure 2C and D). Furthermore, we found AP21967-induced SUMOylation of the CRSP9-FKBP, FOS-FKBP, TCF21-FKBP, CSNK2B-FKBP, MYF6-FKBP and HES1-FKBP (Figure 2E–J). Overall, the results by the USDDS system summarized in Table 1 resemble the data obtained using the UFDS system. However, there are clear differences in SUMOylatability at least for HES1 or MYF6, which are SUMOylated in USDDS only. These differences could result from structural constrains of the static UFDS system, which are not present in the more flexible USDDS approach.

**USDDS targets the specific SUMOylation sites**

To prove that USDDS displays specificity for the in vivo SUMOylation sites of p53 and STAT1, we coexpressed the mutant proteins p53K386R-FRB and STAT1K703R-FRB with Ubc9-FKBP and EGFP-SUMO1. In HEK293 cells, we could not identify any SUMOylation of STAT1K703R-FRB (Figure 3A) but only weak second site SUMOylation of p53K386R-FRB (Figure 3B) which was also detected with the UFDS system (11). Obviously, the induced heterodimerization of the Ubc9 fusion protein with the substrate fusion proteins leads preferentially to a modification at their specific SUMOylation sites. This let us suppose that USDDS is a useful tool to analyse the dynamic interplay between SUMOylation and other protein modifications.

**USDDS demonstrates that pY701 excludes K703 SUMOylation of STAT1**

It has been shown that interferon-γ stimulation of STAT1-transfected COS7 cells, a treatment which also increases STAT1 phosphorylation (20), leads to an enhanced SUMOylation of STAT1 at K703. Using UFDS, we have already demonstrated that STAT1 SUMOylation at K703 inhibits Y701 phosphorylation (11), but it was not possible to study the opposite effect by UFDS (data not shown), possibly due to the static SUMOylation of STAT1 in this experimental setting. Because of that, we now used USDDS to study the effect of STAT1 phosphorylation at Y701 in K703 SUMOylation in vivo (Figure 4). Therefore, we first coexpressed STAT1-FRB, Ubc9-FKBP and EGFP-SUMO1 in HEK293 cells. After 24 h, we stimulated the cells with interferon-β for 1 h to induce STAT1 phosphorylation and subsequently incubated the cells with 1 μM AP21967.
Figure 2. AP21967-induced in vivo SUMOylation of STAT1 and p53. (A) STAT1-FRB and EGFP-SUMO1 or (B) p53-FRB and EGFP-SUMO1 were cotransfected into HEK293 cells either alone (−) or together with Ubc9-FKBP (+). (C) STAT1-FKBp and EGFP-SUMO1 or (D) p53-FKBp and EGFP-SUMO1 or (E) CRSP9-FKBp and EGFP-SUMO1 or (F) TCF21-FKBp and EGFP-SUMO1 or (G) FOS-FKBp and EGFP-SUMO1 or (H) CSNK2B-FKBp and EGFP-SUMO1 or (I) MYF6-FKBp and EGFP-SUMO1 or (J) HES1-FKBp and EGFP-SUMO1 were cotransfected into HEK293 cells either alone (−) or together with Ubc9-FRB (+). After 24 h, the cells were stimulated with the AP21967 (1 μM) for the indicated times. Fusion proteins in the extracts of the transfectants were detected by western blot using (A and C) a STAT1 antibody (α-STAT1), (B and D) a p53 antibody (α-p53) or (E–J) a FKBP antibody (α-FKBP). E-S1-STAT1-FRB(FKBP) = STAT1-FRB(FKBP) fusion protein conjugated with coexpressed EGFP-SUMO1; E-S1-p53-FRB(FKBP) = p53-FRB(FKBP) conjugated with coexpressed EGFP-SUMO1; E-S1-CRSP9-FKBp = CRSP9-FKBp conjugated with coexpressed EGFP-SUMO1; E-S1-TCF21-FKBp = TCF21-FKBp conjugated with coexpressed EGFP-SUMO1; E-S1-FOS-FKBp = FOS-FKBp conjugated with coexpressed EGFP-SUMO1; E-S1-CSNK2B-FKBp = CSNK2B-FKBp conjugated with coexpressed EGFP-SUMO1; E-S1-MYF6-FKBp = MYF6-FKBp conjugated with coexpressed EGFP-SUMO1; E-S1-HES1-FKBp = HES1-FKBp conjugated with coexpressed EGFP-SUMO1; and E = EGFP-Tag.
to induce STAT1 SUMOylation by the heterodimerization of STAT1-FRB with Ubc9-FKBP. The transfectants were lysed after different incubation times and the proteins were analysed by a pY701-STAT1 specific antibody that recognizes the tyrosine 701 phosphorylation also in SUMOylated STAT1 (11) (Figure 4A). It can be seen that interferon-β stimulation induced Y701 phosphorylation of endogenous STAT1 and of the STAT1-FRB fusion protein. However, pY701 could not be detected in the gel region where SUMOylated STAT1-FRB migrates, neither after 1 h nor 2 h or 4 h of interferon-β stimulation. A subsequent western blot using a STAT1 specific antibody clearly detects SUMOylated STAT1-FRB after 1, 2 and 4 h incubation with AP21967. Hence, STAT1 phosphorylation at Y701 excludes K703 SUMOylation.

**Y701 phosphorylation and K703 SUMOylation are mutually exclusive**

The results obtained by UFDS (11) and USDDS (above) let us suppose that Y701 phosphorylation and K703 SUMOylation are mutually exclusive modifications. To further characterize the dynamic interplay between Y701 phosphorylation and K703 SUMOylation of STAT1 by USDDS in *vivo*, we applied USDDS to analyse the influence of K703 SUMOylation on Y701 phosphorylation of both STAT1-FRB and STAT1-FKBP (Figure 4B and C) and extended to analysis of the influence of Y701 phosphorylation on K703 SUMOylation on STAT1-FKBP (Figure 4C). Therefore, we transfected HEK293 cells with the respective expression plasmids and stimulated the transfectants first with interferon-β and then with AP21967, or vice versa. Western blot analysis of the transfectants for the STAT1-FRB (Figure 4B) or STAT1-FKBP (Figure 4C) revealed that no double modification of STAT1 by SUMOylation and Y701 phosphorylation is detectable under any of the stimulation scenarios although single Y701 phosphorylation or SUMOylation are clearly detectable. Hence, STAT1 is phosphorylated at Y701 or SUMOylated at K703 but cannot carry both modifications at the same time. A closer inspection of the blots also shows a weak decrease in the pY701 signal of STAT1-FRB and STAT1-FKBP when the cells were stimulated first with interferon-β and then with AP21967 (Figure 4B, P-STAT1-FRB; Figure 4C, P-STAT1-FKBP). This is possibly due to the kinetics of the interferon-β stimulation in HEK293 where the induced tyrosine 701 phosphorylation declines after about 2 h of stimulation. Furthermore, SUMOylation of STAT1-FRB (FKBP) could reduce the amount of protein that can be re-phosphorylated at Y701. Apart from this, we cannot completely rule out that there is also an effect of AP21967 on dephosphorylation or degradation of pY701-STAT1-FRB. However, USDDS clearly reveals that STAT1 SUMOylation at K703 and STAT1 phosphorylation at Y701 are mutually exclusive while interferon-β stimulation does not inhibit overall SUMOylation in the cell (Figure 4).

**DISCUSSION**

In the control of protein function, post-translational protein modifications can act separately, together or even counteract each other to integrate extracellular signals and to ensure a specific function of a protein at the appropriate localization within the cell (1). Here, we have characterized the dynamic interplay between Y701 phosphorylation and K703 SUMOylation of STAT1 by USDDS. The results obtained together with the finding...
that SUMOylation of STAT1 at K703 inhibits the phosphorylation at Y701 (11) support a model where phosphorylation of tyrosine 701 and SUMOylation of lysine 703 of STAT1 represent mutually exclusive modifications which prevent signal integration at these molecule and probably ensure the existence of differentially modified subpopulations of STAT1, pY701-STAT1 and SUMO-K703-STAT1, necessary for its regulated nuclear cytoplasmic activation/inactivation cycle (Figure 4D).

In the upper blot, the open arrow head indicates the positions of the E-S1-STAT1-FRB or -FKBP that are not decorated by the pY701-STAT1 antibody (α-pY701-STAT1).
Our results are partially confirmed by recent data that show a reduced SUMOylation of a STAT1 pY701-peptide in vitro (24) and are apparently in contrast to the described enhancement of STAT1 SUMOylation by interferon-γ stimulated phosphorylation of STAT1. However, very recently an interferon-γ induced STAT1 phosphorylation of S727 by the MKK6/p38 pathway has been described, which enhances SUMOylation at K703 (25). Hence, the intriguing possibility exists that, depending on the phosphorylated site, STAT1 K703 SUMOylation can be stimulated or inhibited offering the possibility for a complex regulation.

The analysis of the interplay of different modifications at one protein will be one of the difficult tasks to be solved in future description of signalling processes. We have developed the USDDS system that combines the effective and specific SUMOylation of UFDS with an inducible heterodimerization that makes it possible to reach controlled SUMOylation of a substrate protein at any time point within a sequential scenario of modification events. Although we can not exclude some forced artificial SUMOylation by the USDDS, we have demonstrated that this is a unique method for studying the kinetics and the dynamic interplay of protein SUMOylation with other post-translational modifications.

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