The upstream stimulatory factor USF1 is regulated by protein kinase CK2 phosphorylation

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ABSTRACT

The upstream stimulatory factors 1 (USF1) and 2 (USF2) are transcription factors which bind to E-box motifs of various promoters regulating a variety of different cellular processes. Only little is known about the regulation of USFs. Here, we identified protein kinase CK2 as an enzyme that phosphorylates USF1 but not USF2. Using deletion mutants and point mutants we were able to identify threonine 100 as the major phosphorylation site for CK2. It is well known that USF1 and USF2 form hetero-dimers. Binding studies revealed that the inhibition of CK2 kinase activity by a specific inhibitor enhanced binding of USF1 to USF2. Furthermore, transactivation studies showed that the inhibition of CK2 phosphorylation of USF1 stimulated transcription from the glucokinase promoter as well as the fatty acid synthetase promoter but not from the heme oxygenase-1 promoter. Thus, we have shown for the first time that CK2 phosphorylation of USF1 modulates two functionally important properties of USF1, namely hetero-dimerization and transactivation.

1. Introduction

The upstream stimulatory factors 1 (USF1) and 2 (USF2) participate in a large number of gene regulating networks affecting stress reactions, immune responses, the cell cycle, proliferation and metabolism [1]. USF1 and USF2 are encoded by different genes. They are ubiquitously expressed although the abundance of the USF proteins varies between different cell types [2,3]. The USF1 gene codes for a 43 kDa protein whereas USF2 codes for a 44 kDa protein. Both, USF1 and USF2 were first purified from HeLa cells [4,5]. In vivo the proteins exist mainly as USF1/USF2 hetero-dimers [2]. Experiments with USF knock-out mice revealed an asymmetrical regulation of the expression of the two isoforms, i.e. USF1+/− mice displayed an enhanced USF2 expression, whereas USF2+/− mice express less USF1 protein compared to wild-type mice [6]. Like many other transcription factors, USF1 is a phosphoprotein [7] where the degree of phosphorylation seems to vary between primary cells and cells in tissue culture. USF2 is also a phosphoprotein and in particular protein kinase A (PKA) and glycogen synthase kinase-3 (GSK3) are known to phosphorylate USF2 and to modulate its DNA binding activity [8].

The cellular processes regulated by USFs, like cell cycle, proliferation, carbohydrate metabolism, and embryonic development are also strongly regulated by protein kinase CK2 [9–13]. CK2 is a tetrameric enzyme consisting of two catalytic α- and α'-subunits and two non-catalytic β- and β'-subunits. Interestingly, the majority of CK2 targets are proteins involved in signalling, protein synthesis and transcriptional regulation [14–16]. According to the fact that we previously identified several transcription factors as interaction partners or as substrates for CK2, and taking into account known and overlapping functions between CK2 and USF proteins, we asked whether there is a direct or indirect functional link between USF proteins and CK2. In the present study, we demonstrate that in contrast to USF2, USF1 is phosphorylated by CK2 at threonine 100. As a consequence, this phosphorylation influences the interaction between USF1 and USF2 as well as the transcription factor activity of USF1.

2. Materials and methods

2.1. Chemicals and biological reagents

Tissue culture media were purchased from GIBCO. The foetal calf serum (FCS) was from PAA (Pasching, Austria) and [32P]γ-ATP and [32P]orthophosphate were purchased from Hartmann Analytic (Bruenschweig, Germany). The CK2 specific inhibitor CX-4945 was purchased from Selleckchem (Munich, Germany). CX-4945, 4,5,6,7-tetramobromobenzotriazole (TBB, VWR, Darmstadt, Germany) [17] and quinalizarin (LaboTest OHG, Niederschöna, Germany) [18] were dissolved in dimethyl sulfoxide (DMSO) to 10 mM stock solutions.
2.2. Cell lines

INS-1 cells [19] were cultured in RPMI1640 containing 10% foetal calf serum (FCS), 1% glutamine, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol. Cells were cultured at 37 °C and 5% CO2 in a humidified atmosphere in an incubator.

2.3. Plasmids

The cDNA of human USF1 was amplified by PCR introducing EcoR1 and Xho1 restriction sites at the 5′- and 3′-end, respectively. The PCR product was inserted into the plasmid pGEX-5X-1 (GE Healthcare, Freiburg, Germany) in frame with the GST-coding sequence. The USF1 deletion mutants ranging from codon 1–99, 1–163, 1–194, 11–104, 164–194, 104–163 and 167–220 were also produced by PCR simultaneously introducing BamH1 and EcoR1 sites for the insertion into the pGEX-5X-1 vector. Phospho-deficient mutants of USF1 were created based on the pGEX-5X-1-USF1wt plasmid using the QuikChange mutagenesis kit from Stratagene and the following mutagenesis primers:

5′ TCC AGG GTG CTT TCG CCA GTG ATG ATG CAG3′
5′ CTG CAT CAT CAG TGG CGA AAG CAC CCT GGA3′

5′ TTG ACA CGG A GG G GC A GC T GC T GA G AC G CA3′
5′ TGC GTC TCA GCA GCT GCC CCC TCC GTG TCA A3′

For eukaryotic expression USF1 was cloned into the EcoR1 and Xba1 sites of p3x FLAG-Myc-CMV24 thus generating a fusion construct with an N-terminal FLAG-tag and a C-terminal myc-tag. The cDNA of human USF2 was cloned into the Xho1/HindIII sites of pcDNA 3.1 (Invitrogen, Darmstadt, Germany) to generate pcDNA–USF2. The plasmid was used for the T7-polymerase dependent in vitro translation of USF2 by a reticulocyte lysate. The pancreas specific glucokinase reporter construct GK1448-luc (rGCK) contains the rat promoter sequence from nt −990 to +14 [20]. The sequence was produced by PCR from rat genomic DNA simultaneously introducing Kpn1 and Nco1 sites and cloned into the pGL3 luciferase reporter vector (Promega GmbH, Mannheim, Germany). The rat fatty acid synthetase (rFAS) luciferase reporter is from Addgene (#8890). The heme oxygenase-1 reporter (hHO-1) contains the −4.5 kb HO-1 promoter fragment which was a kind gift of Dr. Anupam Agarwal, University of Florida, Gainesville [21]. The dominant negative USF–mutant (CMV566 A-USF) was from Addgene (#33360).

2.4. Antibodies

Goat anti-mouse IgG (No. 115-035-146) and goat anti-rabbit IgG (No. 111-035-144) were purchased from Dianova (Hamburg, Germany). The anti-USF1 antibody is a rabbit polyclonal IgG directed against the C-terminus of USF1 (c-20, sc-229), the anti-USF2 antibody is a rabbit polyclonal IgG directed against the C-terminus of USF2 (c-20, sc862). Both antibodies, the mouse monoclonal anti-USF1 (sc-390027) which is directed against the N-terminus of USF1 ranging from amino acids 75–160, and the rabbit HA-probe antibody (Y-11, sc-805) were purchased from Santa Cruz (Biotechnology Inc., Heidelberg, Germany). The mouse monoclonal antibody FLAG M2 (F1804) and the mouse monoclonal antibody α-tubulin (clone DM1A) were from Sigma-Aldrich (Munich, Germany).

Fig. 1. Phosphorylation of USF1 and USF2 by the CK2 holoenzyme. The recombinant full-length GST-USF1 and GST-USF2 were incubated with recombinant CK2 holoenzyme in the presence of [32P]γ-ATP for 30 min at 37 °C. CK2 was loaded as a control (lane C) to show the autophosphorylation. The samples were separated in a 12.5% SDS-polyacrylamide gel and the radioactivity was detected by autoradiography (A). Molecular weight markers are indicated on the left.

Fig. 2. In vivo phosphorylation of USF1. INS-1 cells were treated with 10 μM of the CK2 inhibitor CX-4945 or equivalent volumes of DMSO for 24 h and then in vivo labelled with [32P]orthophosphate for 4 h. USF1 was immunoprecipitated from the cell extract and analysed on a 10% SDS polyacrylamide gel and transferred to a PVDF membrane. Phosphate-labelled proteins were identified by autoradiography; the Western blot analysis was subsequently performed with an USF1 specific antibody. The phosphate incorporation rate [%] was calculated after normalization to the amount of USF1 in the Western blot analysis.
2.5. In vitro translation of USF2

Human USF2 was in vitro translated from a pcDNA3.1 construct in the presence of \[^{35}S\]methionine according to the manufacturer's recommendations (TNT T7 coupled reticulocyte lysate system, Promega GmbH, Mannheim, Germany).

2.6. Purification of recombinant proteins

A colony of transformed Escherichia coli BL21(DE3) was grown in LB medium supplemented with 60 μg/ml ampicillin at 37 °C overnight. One ml of this pre-culture was used to inoculate 250 ml LB medium supplemented with 60 μg/ml ampicillin and the bacteria were grown with continuous shaking at 30 °C. At an OD\(_{600}\) nm of 0.4−0.5, 0.1 mM IPTG (Carl Roth, Karlsruhe, Germany) was added and the bacteria were grown at 30 °C for a further 4 h. Bacteria were harvested by centrifugation at 2600 × g and 4 °C for 10 min. The bacterial pellet was resuspended in 10 ml buffer (20 mM Tris–HCl, pH 8.0, 25 mM NaH\(_2\)PO\(_4\), 25 mM Na\(_2\)HPO\(_4\) supplemented with protease inhibitor (Complete®, EDTA-free, Roche Diagnostics, Mannheim, Germany)). Cells were lysed on ice by stirring for 30 min with 10 mg of lysozyme, followed by the addition of 300 mM NaCl and subsequent sonication. Then, 1 ml of 10% Triton X-100 was added and the suspension was stirred on ice for 1 h. After centrifugation at 20,000 × g and 4 °C for 10 min, the supernatant was subjected to affinity purification with 300 μl glutathione sepharose beads (GE Healthcare, Freiburg, Germany). The cell lysate was incubated with the beads by shaking in 300 μl elution buffer (50 mM Tris–HCl, pH 8.5, 20 mM reduced glutathione (Sigma Aldrich, Munich, Germany)) at 6 °C for 1 h. The eluted proteins were dialysed in 10 mM Tris–HCl, pH 7.5, 10% glycerol at 4 °C overnight. The concentration of the proteins was determined by Bradford assay (BioRad, Munich, Germany). Size and purity of the proteins were analysed by Coomassie blue staining and Western blotting.

2.7. Pull-down assay

The pull-down assay was essentially done as described by Sun et al. [22]. Purified GST- or GST-tagged proteins (20 μg) were immobilized on GSH-sepharose and equilibrated with PBS-T binding buffer (PBS, pH 7.4, 1% Tween 20). Immobilized proteins were incubated with USF2 product from the in vitro translation reaction. After washing with cold PBS-T, bound proteins were eluted with SDS sample buffer (65 mM Tris–HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) and analysed by SDS polyacrylamide gel electrophoresis, followed by protein staining with Coomassie blue and autoradiography.

2.8. In vitro phosphorylation of USF proteins with protein kinase CK2

Recombinant GST-tagged USF1 proteins were mixed with equal amounts of CK2 holoenzyme in a volume of 20 μl of kinase buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl\(_2\), 50 μM ATP, 1 mM DTT). To start the reaction, we added 2 μCi \[^{32}P\]γATP and incubated the samples for 30 min at 37 °C. The reaction was stopped by adding 10 μl of 3x concentrated SDS sample buffer. Finally, samples were

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**Fig. 3.** Phosphorylation of different USF1 proteins by the CK2 holoenzyme. (A) shows the complete sequence of the human USF1 protein with putative CK2 sites in bold. The recombinant GST-USF1 fragments as shown in (B) were incubated with CK2 holoenzyme in the presence of \[^{32}P\]γATP for 30 min at 37 °C. The samples were separated in a 12.5% SDS polyacrylamide gel and subjected to autoradiography (C). In the lower part (D) the Coomassie blue staining of the gel is shown. The putative CK2 phosphorylation sites of USF1, T100 and T110 were mutated to alanine, and the corresponding cDNAs were expressed as GST-tagged fusion proteins, either as single or double mutant. Proteins were phosphorylated by CK2, separated in a 12.5% SDS polyacrylamide gel and incorporated radioactivity was detected by autoradiography (E). Equal amounts of proteins were used as shown in the Coomassie blue stained gel (F). Molecular weight markers are indicated on the left.
For the luciferase reporter assay, INS-1 cells were seeded into a 6-well plate in a total volume of 2 ml/well of cell culture medium and cultured overnight. Cells were then transfected with 4 μg plasmid DNA by using Turbofect® Transfection Reagent (Thermo Scientific, St. Leon-Rot, Germany). 24 h after transfection cells were treated with the CK2 inhibitors. We used CX-4945 in a final concentration of 10 μM, TBB and quinalizarin were used in a final concentration of 50 μM over a period of 24 h.

24 h after treatment cells were collected by lysing in 1× lysis buffer (LB, Promega, Mannheim, Germany) and measured with the Luciferase Reporter Assay System (Promega) following the manufacturer’s recommendations.

For Western blot analysis, cell lysates were centrifuged at 13,000 ×g to remove cell debris. The protein content was determined with BioRad reagent dye (BioRad, Munich, Germany). Protein extracts were immediately used for Western blot analysis or stored at −20 °C.

For in vivo [32P]orthophosphate labelling, INS-1 cells were seeded on a 10 cm cell culture plate in a total volume of 5 ml/plate of culture medium. After a period of 24 h cells were washed three times with phosphate-free DMEM and incubated in phosphate-free DMEM with supplements for 2 h. After this time, cells were labelled with 100 μCi [32P]orthophosphate/dish for a period of 4 h.

Cells were extracted with RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecylsulfate (SDS)), supplemented with protease inhibitor complete® and PhosSTOP (Roche Diagnostics, Mannheim, Germany) for 20 min on ice. After lysis cell debris was removed by centrifugation at 13,000 ×g for 30 min at 4 °C. The protein content was measured with the BioRad reagent dye (BioRad, Munich, Germany). 1.2 mg of cell extract was pre-cleared twice with protein G sepharose beads (GE Healthcare, Freiburg, Germany), blocked for 1 h with 10% bovine serum albumin (BSA) (PAA Laboratories GmbH, Pasching, Austria) over a period of 1 h. The supernatant was incubated with rabbit polyclonal antibody USF1 (sc-229) for 2 h at 4 °C. Beads were washed seven times with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4). Bound proteins were eluted with 30 μl SDS sample buffer (130 mM Tris–HCl, pH 6.8, 0.02% bromophenol blue (w/v), 10% β-mercaptoethanol, 20% glycerol (v/v), and 4% SDS) by incubating at 95 °C for 5 min. Proteins were separated in a 10% SDS polyacrylamide gel, transferred to a PVDF-membrane and phosphorylation was visualized by autoradiography. Subsequently, the blot was incubated with the polyclonal antibody USF1 (sc-229) as a control for protein loading. Protein bands were visualized by the ECL Lumilight system of Roche Diagnostics (Mannheim, Germany).

2.11. Duolink® in situ proximity ligation assay

For the detection of the interaction between USF1 and USF2, we used the Duolink® in situ proximity ligation assay (PLA, Sigma-Aldrich, USA) according to the manufacturer’s protocol with INS-1 cells. Proteins were detected with mouse monoclonal antibody against USF1 (sc-390027) or rabbit polyclonal antibody against USF2 (sc-862). The detection of the interaction signals was carried out by red fluorescence microscope (Zeiss, Jena, Germany). The quantification of PLA dots was performed by counting single dots in 50 cells of different areas on the cover slips.

2.12. Statistical analysis

Microsoft Excel 2007 software was used to analyse the data. Results of luciferase reporter assays were expressed as mean ± standard deviation (SD) of at least three independent experiments. Statistical analysis of the data was performed using the Student’s t-test (two-tailed, paired). Statistical significant differences were shown as follows: **p < 0.01 or *p < 0.05.
3. Results

3.1. USF1 but not USF2 is a direct CK2 substrate

Although it was shown that both USF1 and USF2 are phospho-proteins and that protein kinase CK2 contributes considerably to the whole human phospho-proteome, so far nothing is known whether both USFs are substrates for protein kinase CK2.

In order to find out whether USF1 and/or USF2 can be direct substrates for CK2, we performed kinase assays where bacterially expressed and purified USF proteins were incubated with [32P]γ-ATP and the CK2 holoenzyme. Phosphorylated proteins were analysed on a 12.5% SDS polyacrylamide gel followed by Coomassie blue staining and autoradiography. As shown in Fig. 1A we detected a phosphorylated protein for GST-USF1 but not for GST-USF2. A control lane (C) demonstrates autophosphorylation of CK2. The Coomassie blue stained gel in Fig. 1B shows equal amounts of loaded GST-USFs after the phosphorylation reaction. Together, these data show that USF1 but not USF2 is a substrate for CK2.

Next, we aimed to show the impact of the USF1 phosphorylation by CK2 in vivo. To do so, we labelled INS-1 cells with [32P]orthophosphate either in the absence or in the presence of the CK2 specific inhibitor CX-4945 [23] and analysed USF1 phosphorylation after immunoprecipitation followed by Western blot analysis and autoradiography. As shown in Fig. 2, a phosphorylated protein band for USF1 was detected with the expected molecular weight. Importantly, the CK2 inhibitor CX-4945 reduced phosphorylation of USF1 by about 23%. Thus, these results indicate that USF1 is a CK2 substrate under in vivo conditions.

3.2. Threonine 100 is the major CK2 site within USF1

In contrast to many other protein kinases, CK2 phosphorylates serine or threonine residues in an acidic environment [24]. To identify the amino acid residues within USF1 targeted by CK2, we started an in silico analysis for a CK2 S/T-x-x-D/E/pY sequence [25] representing a CK2 phosphorylation site. We detected seven putative CK2 phosphorylation sites on the polypeptide chain of USF1 (Fig. 3A) but none on the polypeptide chain of USF2.

In order to map the CK2 phosphorylation site(s) on the polypeptide chain of USF1 we used different GST-tagged fragments of USF1 harbouring amino acids 1–99, 1–163, 167–220, 11–104, 169–194, 195–310, 1–194 and 104–194 (Fig. 3B) and performed kinase assays with [32P]γATP and the CK2 holoenzyme. As shown in Fig. 3C USF1 fragments ranging from amino acid 1–163, 11–104 and 1–194 were strongly phosphorylated. Fig. 3D shows the corresponding Coomassie blue staining of the gel demonstrating that we have used comparable amounts of proteins for the phosphorylation reaction. The phosphorylation of these various fragments allowed us to narrow down the CK2

Fig. 5. Duolink® in situ proximity ligation assay (PLA) of USF1 and USF2 in INS-1 cells. (A) INS-1 cells were incubated with 10 μM of the CK2 inhibitor CX-4945 or an equal amount of the solvent control for 24 h. Cells were subjected to Duolink® in situ proximity ligation assay using antibodies against USF1 and USF2. For negative control, a single staining with the USF1 and USF2 antibodies as well as the PLA probes was performed. Immunofluorescence was analysed using a Zeiss Axioskop fluorescence microscope (Zeiss, Jena, Germany). (B) Single dots were counted in all images, spots in 50 cells of the solvent control set 100% and the spots in the treated cells analogously analysed. Statistical analysis was performed by using Students t-test. * significant difference p < 0.05, ** significant difference p < 0.01.
phosphorylation sites to amino acids threonine 100 and threonine 110. In the next step we mutated either threonine 100 or threonine 110 or both to alanine which cannot be phosphorylated by CK2. These mutant proteins were bacterially expressed, purified and then phosphorylated as described above and analysed on a 12.5% SDS polyacrylamide gel followed by an autoradiography. Fig. 3E shows a strong reduction in the phosphorylation of the threonine USF1T100A mutant whereas there was only a slight reduction in the phosphorylation of the USF1T110A mutant. The double mutant USF1T100A/T110A showed the same reduction in phosphorylation as the USF1T100A mutant. Thus, we conclude from these results that threonine 100 is the major phosphorylation site for CK2 although we cannot totally exclude an additional other minor phosphorylation site.

3.3. Phosphorylation of USF1 by CK2 affects dimerization with USF2

USF1 and USF2 form heterologous dimers in the cell in order to function as transcription factors. In the next step we asked whether the CK2 phosphorylation might influence the dimerization of USF1 with USF2. For these experiments we used GST-USF1 either in the wild-type form (USF1wt) as well as the USF1T100A or USF1T110A mutant, which were incubated with in vitro translated [35S]methionine labelled USF2 protein. As shown in Fig. 4A we found a protein band for USF2 in the control cells set to 100%. Statistical analysis was performed by using Students t-test. * significant difference p < 0.05, ** significant difference p < 0.01. The corresponding Western blot analysis of the FLAG-tagged USF1 is shown below the graph. α-Tubulin served as a loading control.

To further substantiate these findings we used the proximity ligation assay (PLA) which is a very appropriate and convenient method for the detection of protein–protein interactions in cells [26] in particular with regard to the location and quantification of the interaction. When INS-1 cells were analysed with the PLA method we could detect a positive reaction for the interaction of USF1 and USF2 exclusively in the nucleus (Fig. 5A). The quantification of the spots revealed an about 1.6-fold
induction of the interaction after inhibition of CK2 with CX-4945 (Fig. 5B). Thus, these results together with the pull-down assay demonstrate that the phosphorylation of USF1 by CK2 modulates its association with USF2.

3.4. Phosphorylation of USF1 by CK2 at threonine 100 affects transcriptional activity of USF

Since USF1 is known to act as a transcription factor [3], we next analysed whether the phosphorylation of USF1 by CK2 might influence its transcription factor activity. The glucokinase promoter is known to be regulated by USF1 [27] and therefore this promoter construct was used for our studies. We transfected the luciferase reporter construct with the glucokinase promoter GK1448-luc (rGCK) together with either an empty vector or a vector construct with a coding sequence for FLAG-USF1 and measured the luciferase activity. As shown in Fig. 6A in the presence of the FLAG-USF1 protein there was a clear increase in the luciferase activity compared to the control with the empty vector. To confirm that the increase in reporter activity is exclusively due to USF1 we repeated the experiment with a dominant negative mutant of USF (AUSF), which is capable of hetero-dimerizing with endogenous USF but not of binding to DNA [28]. As expected the dominant negative USF mutant was unable to turn on the glucokinase promoter (AUSF).

The co-expression of USF1WT together with a dominant negative USF mutant reduced the transcription of the glucokinase promoter to basal level (USF1/AUSF). These results demonstrate that the glucokinase promoter is suitable for the analysis of the transactivation function of USF1.

To further analyse the impact of CK2 on the phosphorylation of USF1 we performed the glucokinase reporter studies also in the presence of several known CK2 inhibitors, namely CX-4945, TBB, and quinalizarin. When using these three different inhibitors they indeed reduced CK2 activity to about 40–50% as assessed by a phosphorylation assay with the CK2 specific peptide with the sequence RRRDDSDDDD [29].
of USF1 multimers. Here, we show that CK2 phosphorylation of USF1 reduces the interaction of USF1 with USF2 in vitro and in vivo.

Over the last ten years a number of transcription factors have been detected, which are phosphorylated by CK2 and where this phosphorylation modulates transcriptional activity. CK2 phosphorylation enhances transcriptional activity of ATF4 [15], Nrf2 [39], HIF1α [40], AP-2α [41], UBF [42] and FoxM1c [43] and reduces transcriptional activity of PDX-1 [16] and CHOP [44] just to mention but a few. In the case of ATF4, there was a first indication that the effect of the CK2 phosphorylation of the transcription factor may vary depending on the type of promoter [15,45]. Here, we have shown that CK2 phosphorylation of USF1 led to a down-regulation of the transcription factor activity at least with regard to the glucokinase promoter and FAS promoter, whereas the HO-1 promoter is not affected. These results support the previous observation with ATF4 and support the idea about a promoter specific regulation. In summary, the present study has shown that protein kinase CK2 specifically targets USF1 by phosphorylation and that CK2 phosphorylation influences the transcription factor activity and the interaction of USF1 with USF2.

5. Conclusion

For the first time, we provided data that USF1 is a substrate for protein kinase CK2. We identified threonine 100 as the major phosphorylation site for USF1. The phosphorylation of threonine 100 regulated the interaction of USF1 with USF2 and the transcriptional activity of USF1 in a promoter specific manner.

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