Identification of the PP2A-interacting region of heat shock transcription factor 2

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Abstract Previous work in our laboratory demonstrated the existence of an association between heat shock transcription factor 2 (HSF2) and the serine/threonine phosphatase 2A, which is mediated by interaction between HSF2 and the A subunit (also called PR65) of this protein phosphatase. In light of the importance of HSF2-PP2A association for HSF2 cellular function, in this study, we have sought to dissect the sequences within HSF2 that are important for interaction with the A subunit of PP2A. The results of these experiments indicate that the HSF2 region comprising amino acids 343–363 is important for A subunit interaction. This region includes part of the C-terminal leucine zipper motif of HSF2 called heptad repeat C (HR-C). The results of transfection/immunoprecipitation experiments also show that deletion of the 6 amino acids from 343 to 348 from HSF2 (HSF2 (Δ 343–348)), is sufficient to prevent HSF2 from interacting with PP2A. These data provide insight into a new functional domain of HSF2, the PP2A A subunit-interacting region.

INTRODUCTION

Heat shock factor 2, or HSF2, is a member of the heat shock factor family of DNA-binding proteins. It is related to the HSF1 protein, which is responsible for turning on transcription of the *hsp70i* and other *hsp* genes in response to stress, and can interact with the same DNA sequence (heat shock element [HSE]) as HSF1, but its function had largely remained elusive (Sarge et al 1991, 1993; Schuetz et al 1991; Pirkkala et al 2001; Voellmy 2004). However, we recently obtained evidence that at least 1 function of HSF2 is to mediate an intriguing property of the *hsp70i* promoter called bookmarking (Xing et al 2005).

Bookmarking refers to an unusual lack of compaction that is exhibited by the promoters of the *hsp70* and other genes during mitosis, in contrast to the more compacted state of the bulk of the genomic DNA (Martinez-Balbas et al 1995; Michelotti et al 1997; John and Workman 1998; Sarge and Park-Sarge 2005). For example, multiple DNase I hypersensitive sites are found between –300 and the +1 transcription start site of the *hsp70i* promoter in mi-

totic chromatin, indicating that this region is not compacted and is accessible to proteins (Martinez-Balbas et al 1995). Preventing compaction of the *hsp70* promoter is important for the ability of cells to express this gene in early G1, thereby allowing them to survive stress if it occurs during this part of the cell cycle (Sarge and Park-Sarge 2005; Xing et al 2005).

HSF2 plays a central role in bookmarking the *hsp70* promoter during mitosis by recruiting the serine/threonine phosphatase PP2A to this promoter and also interacting with the CAP-G subunit of the condensin complex to promote dephosphorylation/inactivation of the condensin complex, thereby preventing compaction of this specific region of chromosomal DNA in mitotic cells (Xing et al 2005). HSF2 associates with PP2A by interacting with the A subunit of this protein phosphatase (Hong and Sarge 1999; Hong et al 2000). Our previous work involving yeast 2-hybrid analysis of a series of relatively large C-terminal truncation mutants of the HSF2 protein indicated that the amino acid sequence of HSF2 important for interaction with the A subunit of PP2A was somewhere in the region between amino acids 281 and 387 (Hong and Sarge 1999). In light of the importance of the interaction between HSF2 and PP2A for HSF2 function in cells and to gain insight into the structure-function basis

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of this interaction, we sought in this study to more precisely identify the amino acid sequence within HSF2 that is important for interaction with the A subunit of PP2A. The results show that the HSF2 region between amino acids 343 and 363 is important for interaction with the PP2A A subunit. This region includes part of the C-terminal leucine zipper motif of HSF2 called heptad repeat C (HR-C). The results also demonstrate that deletion of the 6 amino acids from 343 to 348 from HSF2 (HSF2 [Δ 343–348]), is sufficient to prevent HSF2 from being able to interact with PP2A. The results of this study have now identified the location of a new functional domain of HSF2, the PP2A A subunit-interacting region.

METHODS

Yeast 2-hybrid analysis

Yeast strain pJ694A was cotransformed with pGAD-A subunit (plasmid with Gal4 activation domain fused to the A subunit of PP2A) and either pGBD-HSF2 full-length (1–517), pGBD-HSF2 (169–517), pGBD-HSF2 (230–517), pGBD-HSF2 (310–517), pGBD-HSF2 (357–517), or pGBD-HSF2 (387–517) and then streaked on –TL, –TLH, or –TLA plates as previously described (Hong and Sarge 1999). The GBD plasmids express the HSF2 sequences fused to the Gal4 binding domain. The ability of each protein pair to interact was determined by scoring growth on the selective plates lacking adenine or histidine. The exonuclease III truncation mutagenesis of HSF2 was performed with a Stratagene Exo III–Mung bean nuclease kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions, following the strategy described previously (Hong et al 2000). For assay of interaction strength with β -galactosidase activity, yeast extracts were incubated with Z Buffer (60 mM Na₂HPO₄ and 40 mM NaH₂PO₄ [pH 7.0], 10 mM KCl, 1 mM MgSO₄, 50 mM β -mercaptoethanol). After addition of 4 mg/mL O-nitrophenyl- β -D-galactoside substrate, samples were incubated at 30°C for 30 minutes, then the optical density at 420 nm (OD₄₂₀) was measured (Xing et al 2005).

Fluorescence microscopy

Immunoprecipitation analysis was performed as follows according to our established procedures (Goodson et al 2001). HeLa cells grown on coverslips in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and 50 μ g/mL gentamicin were transfected with expression plasmids for GFP-HSF2, GFP-HSF2 (Δ 343–348), GFP-HSF2 (Δ 343–363), or GFP alone with Effectene (Qiagen) as the transfection reagent. After incubation at 37°C for 30 minutes, the medium was removed and the coverslips washed with 1 $\times$ phosphate-buffered saline

(PBS) for 5 minutes. A solution containing 3.7% paraformaldehyde in 1 $\times$ PBS was added, and after 20 minutes incubation at room temperature, coverslips were washed with 1 $\times$ PBS for 5 minutes. Coverslips were incubated for 5 minutes with 50 ng/mL 4',6-diamidino-2-phenylindole in PBS to allow visualization of DNA. Coverslips were then washed briefly 3 times in distilled water and mounted onto a slide spotted with 15 μ L of Vectashield (Vector Laboratories). Excess fluid was wicked from the coverslip, and the edges of the coverslip were sealed with fingernail polish. The fluorescence was then visualized with a Nikon fluorescence microscope, and pictures were taken with a Nikon Spotcam digital imaging camera.

Immunoprecipitation analysis

Immunoprecipitation analysis was performed as follows according to our established procedures (Xing et al 2005). HeLa cells that were transfected as described above with expression plasmids for GFP-HSF2, GFP-HSF2 (Δ 343–348), or GFP-HSF2 (Δ 343–363) were washed 3 times with ice-cold PBS, followed by lysis in 5 volumes of lysis buffer (25 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid [pH 7.9], 10% glycerol, 100 mM KCl, 0.1 mM ethylenediaminetetraacetic acid, 12.5 mM MgCl₂, 0.5% NP-40). Lysates were cleared by centrifugation at 13 000 rpm for 10 minutes at 4°C. Soluble lysates were then pre-cleared by incubation with nonspecific (control) rabbit immunoglobulin G (IgG) and protein G-sepharose beads for 1 hour at 4°C with gentle rotation. Precleared extracts were then incubated with anti-GFP antibody (Clontech) or species-matched nonspecific control IgG and 25 μ L of Protein G Sepharose (Amersham) for 1 hour at 4°C with rotation. After washing beads 3 times for 5 minutes each at 4°C in the lysis buffer (described above), bound proteins were released by boiling in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample dye and then subjected to SDS-PAGE and Western blot analysis with antibody raised against the catalytic subunit of PP2A (Upstate Biotech.).

RESULTS

Our previous study of yeast 2-hybrid analysis of a series of relatively large C-terminal truncation mutants of the HSF2 protein suggested that the region from amino acids 281–387 appeared to be important for interaction with the A subunit of PP2A (Hong and Sarge 1999). However, in that previous study, we had not examined the interaction between N-terminal truncation mutants of HSF2 and the A subunit in the yeast 2-hybrid assay. Therefore, in one experiment of this study, we sought to obtain information about the N-terminal boundary of sequences in HSF2 important for interaction with the A subunit by testing the

Fig 1. Yeast 2-hybrid analysis of interaction between the PP2A A subunit and an N-terminal truncation series of HSF2. Yeast strain pJ694A cotransformed with pGAD-A subunit and either pGBD-HSF2 full-length (1–517), pGBD-HSF2 (169–517), pGBD-HSF2 (230–517), pGBD-HSF2 (310–517), pGBD-HSF2 (357–517), or pGBD-HSF2 (387–517) were streaked on –TL, –TLH, or –TLA plates. Relative growth of each pairing on the selective plates was then scored. pGAD contains the sequences fused to the Gal4 Activation Domain, and pGBD the sequences fused to the Gal4 Binding Domain. In the schematic, the locations of the HSF2 DNA binding domain (DBD), heptad repeat A/B (HR-A/B, which is the trimerization domain of the protein), and the heptad repeat C (HR-C) are shown.

interactions in the yeast 2-hybrid assay between the A subunit and different N-terminal truncation mutants of HSF2. As shown in Figure 1, N-terminal mutants that begin with amino acids 169, 230, or 310 exhibit interaction with the A subunit that is similar to that of full-length HSF2. Further N-terminal truncation to amino acid 357 (HSF2 [357–517]), however, resulted in a slightly weaker interaction. Truncation to amino acid 387 of HSF2 (HSF2 [387–517]) was associated with loss of the interaction with the A subunit. These results suggest that sequences between amino acids 310 and 387 of HSF2 are important for its interaction with the A subunit of PP2A.

In the previous study from our laboratory (Hong and Sarge 1999), yeast 2-hybrid assays of a series of C-terminal truncation mutants demonstrated that a fragment of HSF2 containing amino acids 1–387 is able to interact with the A subunit of PP2A, but that a fragment containing HSF2 amino acids 1–281 is not (Hong and Sarge 1999). To more precisely define the C-terminal boundary of sequences in HSF2 important for interaction with the A subunit, we made an exonuclease III C-terminal truncation series of the HSF2 protein and then tested the ability of the different truncation mutants to interact with the

A subunit in the yeast 2-hybrid assay. The results, shown in Figure 2, agree with our previous data (Hong and Sarge 1999) by showing that C-terminal truncation mutants of the HSF2 protein ending at amino acid 387 or later are able to interact strongly with the A subunit of PP2A. Further truncation of HSF2 to amino acid 363 (HSF2 [1–363]) did not significantly alter the interaction. However, truncation to amino acid 354 (HSF2 [1–354]) results in a significant decrease in interaction. HSF2 (1–348) displays interaction with the A subunit that is similar to that of HSF2 (1–354), but loss of the next 6 amino acids (HSF2 [1–342]) appears to result in loss of interaction. The results in Figure 2 suggest that the region of HSF2 between amino acids 343 and 363 is important for interaction with the A subunit of PP2A, which is consistent with the results in Figure 1. The location of this region relative to the other functional domains of the HSF2 protein is shown in Figure 3A. To test the importance of sequences in this region of HSF2 for interaction with the A subunit, we made 2 yeast 2-hybrid bait constructs that deleted different amounts of this region of HSF2. In one of these HSF2 deletion mutants we removed amino acids 343–348 of HSF2 (GFP-HSF2 [Δ343–348]),

	Heptad Repeat C	A subunit Interaction
HSF2 (1–387)–LTSEDPVTMDSILNDNINLLGKVELLDYLSIDCSLEDFQAMLSGRQFSIDPD		+++
HSF2 (1–363)–LTSEDPVTMDSILNDNINLLGKVELLDYL		+++
HSF2 (1–354)–LTSEDPVTMDSILNDNINLL		+
HSF2 (1–348)–LTSEDPVTMDSILN		+
HSF2 (1–342)–LTSEDPVTM		-
HSF2 (1–339)–LTSEDP		-

Fig 2. Yeast 2-hybrid analysis of interaction between the PP2A A subunit and an exonuclease III-generated C-terminal truncation series of HSF2. Yeast strain pJ694A cotransformed with pGAD-A subunit and either pGBD-HSF2 full-length (1–517), pGBD-HSF2 (1–363), pGBD-HSF2 (1–354), pGBD-HSF2 (1–348), pGBD-HSF2 (1–343), or pGBD-HSF2 (1–339) were streaked on –TL, –TLH, or –TLA plates. Relative growth of each pairing on the selective plates was then scored.

Fig 3. (A) Schematic depicting the HSF2 region indicated to be important for interaction with the A subunit of PP2A relative to other HSF2 functional domains. The locations of the HSF2 DNA binding domain (DBD), heptad repeat A/B (HR-A/B, which is the trimerization domain of the protein), and the heptad repeat C (HR-C) are shown. Closed and open diamonds in the sequence portion shown indicate conserved hydrophobic amino acid residues that form the 2 registers of the leucine zipper. (B) Yeast strain pJ694A was cotransformed with pGAD-A subunit and either pGBD-HSF2 wild type (1–517), pGBD-HSF2 (Δ 343–348), pGBD-HSF2 (Δ 343–363), or pGBD, and then interaction strength was tested by performing a β -galactosidase activity assay on extracts of yeast containing each plasmid pair.

whereas in the other, we made a slightly larger deletion of amino acids 343–363 HSF2 (GFP-HSF2 [Δ 343–363]), that removed the entire putative A subunit-interacting region. As shown in Figure 3B, these constructs both exhibited decreased interaction with the A subunit of PP2A in the yeast 2-hybrid assay, with the larger deletion (Δ 343–363) showing slightly lower interaction than the smaller deletion (Δ 343–348). These results support the importance of this region of HSF2 for interaction with the A subunit of PP2A.

Finally, to allow us to perform a functional test of the importance of the HSF2 region between amino acids 343 and 363 for interaction with the A subunit of PP2A in mammalian cells, we made GFP-fusion mammalian expression constructs of HSF2 that contained the same 2 deletions, (Δ 343–348) and (Δ 343–363) and that were tested in the yeast 2-hybrid constructs (Figure 3B). Our goal

was to transfect these 2 HSF2 deletion constructs into HeLa cells and determine whether the removal of these amino acid sequences affects their ability to interact with PP2A compared with wild-type HSF2. The results shown in Figure 4A indicate that both the GFP-HSF2 (Δ 343–348) and the GFP-HSF2 (Δ 343–363) deletion mutant proteins display a subcellular distribution that is similar to that of wild-type HSF2, with some detectable in the cytoplasm but more being localized to the nucleus of the transfected cells. Consistent with the results of a previous study in our laboratory, some of the GFP-HSF2 signal in the nucleus is present in brightly staining structures, which our past data indicate are promyelocytic leukemia (PML) nuclear bodies (Goodson et al 2001).

Next, to compare the ability of these different protein constructs to interact with PP2A, we immunoprecipitated extracts of cells transfected with each construct with anti-

Fig 4. Amino acids 343–348 in the C-terminal domain of HSF2 are required for its interaction with protein phosphatase 2A in human cells in vivo. (A) Expression plasmids containing wild-type GFP-HSF2, GFP-HSF2 (Δ343–348), GFP-HSF2 (Δ343–363), or GFP alone were transfected into HeLa cells, which were then analyzed by fluorescence microscopy, with 4',6-diamidino-2-phenylindole staining used to visualize DNA. (B) Extracts of HeLa cells transfected with GFP-HSF2, GFP-HSF2 (Δ343–348), or GFP-HSF2 (Δ343–363) were subjected to immunoprecipitation with anti-GFP antibodies followed by Western blot of the immunoprecipitates with antibodies against the PP2A catalytic subunit. Lysates were subjected to anti-GFP Western blot to show similar expression levels of the different GFP-HSF2 fusion proteins, and to Western blot with antibodies against β-actin as a loading control.

GFP antibodies (with nonspecific species-matched IgGs as negative control) and then probed a Western blot of the immunoprecipitates with antibodies against the PP2A catalytic subunit. We chose to probe for the catalytic subunit because we wanted to examine the interaction between the functional intact PP2A enzyme and the wild type vs deletion mutant HSF2 proteins. Also, previous data indicate that levels of the A subunit of PP2A are limiting with respect to the catalytic subunit, indicating that there is likely to be little if any free A subunit in the cell (Gentry and Hallberg 2002). The results of this experiment show that wild-type HSF2 coimmunoprecipitates with PP2A catalytic subunit, but that neither the GFP-HSF2 (Δ343–348) nor the GFP-HSF2 (Δ343–363) deletion mutant proteins were able to do so (Fig 4B). This suggests that even deletion of the 6 amino acids 343–348 is sufficient to prevent HSF2 from interacting with PP2A when expressed in cells.

DISCUSSION

Our previous work indicated that the region between amino acids 281 and 387 of HSF2 is required for its interaction with the A subunit of PP2A (Hong and Sarge 1999). The results of this study have now dissected this interaction domain to amino acids 344–363. The results also indicate that deletion of the 6-amino acid region between 343 and 348 of HSF2 is sufficient to prevent interaction of this protein with PP2A, identifying this amino acid sequence as a region that is very important for the

interaction. The effect of this small deletion in preventing HSF2 interaction with PP2A does not appear to be due to any gross difference in the expression or stability between the different HSF2 constructs because the anti-GFP Western blot data shows that the levels of the 2 HSF2 deletion mutant proteins are similar to that of wild-type HSF2. The data in Figure 4A also indicate that the effect can't be ascribed to differences in subcellular localization because the patterns of the deletion mutant and wild-type HSF2 are very similar.

However, although our data do show that deletion of amino acids 343–348 of HSF2 results in loss of its interaction with PP2A, it is important to note that our results also suggest that the first part of the HR-C leucine zipper motif appears to contribute to this interaction as well. This leucine zipper motif is conserved in other members of the HSF family, including HSF1 (Rabindran et al 1991; Sarge et al 1991). Thus, it is conceivable that the corresponding sequences in these other HSFs could also be involved in interactions with PP2A and, if so, that this could be important for the function or regulation of these factors. These and other questions related to the association between PP2A and HSFs will be the subjects of future investigations.

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