Regulation of Protein Phosphatase 2A Activity by Heat Shock Transcription Factor 2

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Heat shock transcription factor (HSF) mediates the stress-induced expression of heat shock protein genes (hsp). However, HSF is required for normal cell function even in the absence of stress and is important for cell cycle progression, but the mechanism that mediates these effects of HSF is unknown. Here, it is shown that a member of the HSF family, HSF2, interacts with the PR65 (A) subunit of protein phosphatase 2A (PP2A). HSF2 binding to PR65 blocks its interaction with the catalytic subunit, due to competition between HSF2 and catalytic subunit for the same binding site in PR65. In addition, overexpression of HSF2 stimulates PP2A activity in cells, indicating the relevance of HSF2 as a regulator of PP2A in vivo. These results identify HSF2 as a dual function protein, capable of regulating both hsp expression and PP2A activity. This could function as a mechanism by which hsp expression is integrated with the control of cell division or other PP2A-regulated pathways.

HSF is a transcription factor that has a well-characterized function in up-regulating the expression of hsp genes following exposure of cells to stress conditions, mediated by its binding to heat shock elements in the promoters of these genes (1–6). However, a number of results indicate that stress-induced hsp expression is not the only function of HSF and in fact suggest that HSF is important for the normal growth and development of cells. For example, the HSF gene is essential for viability of yeast even under normal, nonstress conditions (7–9), and deletion of the HSF gene in Drosophila melanogaster results in defects in oogenesis and early larval development, with evidence indicating that these defects are not due to altered basal hsp expression but rather to some other as yet unknown target or function of HSF (10). Further, a yeast cell cycle mutant blocked in G2 was identified that maps to the HSF gene, indicating that some function of HSF is important for progression through the cell cycle (11).

Protein phosphatase 2A (PP2A) is involved in regulating a number of important cellular processes including intermediary metabolism, signal transduction, and cell cycle progression by dephosphorylating and thereby modulating the activity of proteins that control these processes (12–16). PP2A is composed of a core heterodimer containing a protein called PR65 (A subunit) and a catalytic subunit. This core heterodimer associates with a large number of different B-type variable subunits to form mixed populations of PP2A heterotrimers (holoenzyme) in cells (17–24).

To gain insight into the functions of HSF in normal cellular growth and development described above, we utilized the yeast two-hybrid approach to identify cellular proteins that interact with HSFs. We found that a member of the HSF family, HSF2, interacts specifically with the PR65 subunit of PP2A. Deletion mapping demonstrated that a region in PR65 previously shown to be involved in interacting with the catalytic subunit is also required for binding to HSF2, suggesting that HSF2 directly competes with catalytic subunit for binding to PR65. This was confirmed by our subsequent binding experiments. We also show that overexpression of HSF2 leads to increased PP2A activity in cells, indicating the relevance of HSF2 as a regulator of PP2A in vivo. We hypothesize that the regulation of PP2A activity by HSF2 functions as a mechanism for cross-talk between the hsp expression pathway and PP2A-regulated pathways in the cell, including regulation of cell division.

EXPERIMENTAL PROCEDURES

Plasmids—Bait constructs containing full-length mouse HSF1 and HSF2 were made in pGBD-C1 using standard subcloning methodologies. 3′ truncation mutants of pGBD-HSF2 and pGAD-PR65 were made by performing restriction digestion of selected unique sites, filling in with T4 DNA polymerase, and then ligating to delete sequences from the 3′ end. The sites in HSF2 utilized were SphI (pGBD-HSF2 (1–168)), T7hII (pGBD-HSF2 (1–281)), BglII (pGBD-HSF2 (1–387)), and BamHI (pGBD-HSF2 (1–473)), whereas the sites in PR65 used were BglII (pGAD-PR65 (1–325)) and Bsa36I (pGAD-PR65 (1–378)). Yeast Two-Hybrid Screening—Bait constructs containing full-length mouse HSF1 and HSF2 were made in pGBD-C1 and used to screen a human brain cDNA library in yeast strain pJ69–4A, as described previously (25, 26). Library plasmids that were scored positive by their ability to confer growth on plates lacking adenine were isolated and confirmed by back-transformation into pJ69–4A with the bait constructs.

β-Galactosidase Assay—For analysis of β-galactosidase activity in yeast harboring two-hybrid constructs, yeast extracts were incubated in 50 mM NaHPO₄-Na₂HPO₄ (pH 7.0), 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol. After addition of 1 mM/ml of o-nitrophenyl-β-d-galactoside substrate, samples were incubated at 30 °C for 5 min, and then the A₄₂₀₅ was measured.

Expression of Recombinant Proteins and GST Pull-down Assay—Full-length HSF1, HSF2, PR65, and catalytic subunits were subcloned into pQE30 (for His fusion proteins) or pGEX2T (for GST fusion proteins), expressed in bacteria, and then purified according to the manufacturer’s instructions. For the in vitro binding assay, GST-HSF1 or GST-HSF2 were bound to glutathione-agarose beads, incubated with His₄-PR65 for 1 h at 4 °C in binding buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 0.1% Triton X-100, 14 mM β-mercaptoethanol). After washing four times with binding buffer, bound PR65 was analyzed by SDS-PAGE and Western blot using anti-PR65 monoclonal antibodies (27). For competition binding experiments, GST-PR65 was bound to glutathione-agarose, washed, and then incubated in binding buffer with 1 μg of His₄-catalytic subunit in the absence or presence of increasing amounts (1 and 2 μg) of His₄-HSF2 or His₄-HSF1 protein at 4 °C for 60 min. The beads were then washed four times with binding buffer, and
HSF2 Interaction with PR65

the binding of catalytic subunit was determined by Western blots using anti-catalytic subunit polyclonal antibody (28, 29).

Immunoprecipitation Analysis—Extracts of human K562 erythroleukemia cells made in Buffer C (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) were incubated with 4 μl of either HSF1 or HSF2 polyclonal antibodies for 1 h at 4°C with gentle inversion mixing. Protein G-agarose was added and incubated for 3 h at 4°C with gentle mixing. After collecting by centrifugation, the complexes were washed three times with Buffer C, and the bound proteins then analyzed by SDS-PAGE and Western blot using anti-PR65 antibodies (27). Levels of HSF1 and HSF2 in the extracts were determined by Western blot using HSF1 and HSF2 polyclonal antibodies (30). The experiments comparing levels of PR65 in pellets versus supernatants of immunoprecipitations using HSF2 and Ca⁺⁺ polyclonal antibodies were performed similarly except that TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween 20) was used instead of Buffer C and that two sequential rounds of immunoprecipitation were done. Supernatants of the immunoprecipitations were collected by trichloroacetic acid precipitation, and then both pellet (pellets 1 and 2) and supernatant proteins were analyzed by Western blots using PR65 antibodies.

HSF2 Transient Transfection and PP2A Assay—NIH 3T3 cells were mock transfected or transfected with β-actin parental plasmid or β-actin-HSF2 using LipofectAMINE Plus (Life Technologies, Inc.) following the manufacturer’s protocol, and then extracts prepared from these cells were assayed for PP2A activity by the method of Cohen et al. (31) using 32P-labeled phosphorylase a as substrate. The assay was performed in the presence and absence of 3 nM okadaic acid, and PP2A activity was determined by subtraction of the activity obtained in 3 nM okadaic acid from the total phosphatase activity (measured in absence of okadaic acid).

RESULTS

To identify HSF-interacting proteins, a human brain yeast two-hybrid library was screened using full-length mouse HSF1 or HSF2 as bait (25, 26, 32, 33). One of the positives identified using the HSF2 bait was the protein PR65, which is a subunit of PP2A (34, 35). A full-length clone of the PR65 protein was then tested for its ability to interact with HSF2 in the yeast two-hybrid system (36). Yeast transformed with the HSF2 bait plasmid and the full-length PR65 plasmid (HSF2 + PR65) or with the HSF2 bait plasmid and the original PR65 partial clone (HSF2 + ΔPR65) are able to grow on media lacking adenine, providing genetic evidence of an interaction between these proteins (Fig. 1A). Yeast transformed with the HSF2 plasmid and activation domain pACT2 plasmid, HSF2 plasmid alone, or PR65 plasmid and Gal4-binding domain pGPD plasmid are unable to grow on selective media (Fig. 1A). PR65 interacts with a catalytic subunit protein (Ca⁺⁺) to form the core complex of PP2A (12–16), and therefore we also tested whether HSF2 interacts with the Ca⁺⁺ protein. Yeast transformed with the HSF2 bait plasmid and a plasmid containing full-length Ca⁺⁺ (37) were not able to grow on selective media, indicating that these two proteins do not interact (Fig. 1A).

To quantitate the interaction between HSF2 and PR65 in the yeast two-hybrid system, we measured β-galactosidase activity in extracts of yeast transformed with the same plasmid combinations tested above. The results of this analysis confirm the ability of HSF2 to interact with both full-length PR65 and the truncated PR65 clone originally isolated from the library (ΔPR65) but not with the catalytic subunit of PP2A (Ca⁺⁺) (Fig. 1B). HSF2 shares several regions of homology with its related family member HSF1 (25, 38, 39). However, yeast transformed with an HSF1 bait plasmid and the full-length PR65 plasmid are not able to grow on selective media, indicating that these two proteins do not interact and thus that the PR65 interaction is specific to HSF2 (data not shown).

To provide additional evidence of the interaction between HSF2 and PR65, we tested the ability of recombinant proteins to interact in vitro using a GST pull-down assay. GST-HSF1 and GST-HSF2 were bound to glutathione-agarose, incubated with recombinant PR65, and washed extensively, and then PR65 binding was measured by Western blot analysis of proteins bound to the resin using PR65 antibodies. The results of this experiment demonstrate that recombinant PR65 interacts in vitro with GST-HSF2 but not GST-HSF1, providing additional evidence of the HSF2-PR65 interaction as well as its specificity (Fig. 2A). To obtain evidence of interaction between endogenous HSF2 and PR65 expressed in cells, we also performed immunoprecipitation analysis. As shown in Fig. 2B, PR65 is immunoprecipitated with HSF2 antibodies but not HSF1 antibodies, indicating that endogenous HSF2 and PR65 proteins expressed in cells interact and that this interaction is specific to HSF2.

Next, we extended our immunoprecipitation analysis to determine what proportion of the PR65 protein in the cell is associated with HSF2, by comparing the amount of PR65 that is immunoprecipitated with HSF2 antibodies through two sequential immunoprecipitations (pellets 1 and 2) versus the amount that remains in the supernatant of the immunoprecipitation. For comparison, we also determined the relative proportion of PR65 that is immunoprecipitated with anti-Ca⁺⁺ antibodies. The results (Fig. 2C) demonstrate that a significant proportion of cellular PR65 is immunoprecipitated with HSF2 antibodies.

To further characterize the HSF2-PR65 interaction, we examined the regions of the HSF2 and PR65 polypeptides, which are important for this interaction. We constructed 3’ truncation mutants of each protein and tested their ability to interact with the full-length partner in the yeast two-hybrid system. The results demonstrate that 3’ truncation mutants of the HSF2 protein that terminate at amino acids 473 or 387 are able to
interact with PR65, but further deletion of HSF2 protein to amino acid 281 results in a loss of interaction with PR65 (Fig. 3A). For PR65, 3' truncation of the C-terminal region to amino acid 378 resulted in a loss of interaction with HSF2 (Fig. 3B). These results suggest that the regions between amino acids 281 and 387 of HSF2 and between amino acids 378 and 589 (wild type C terminus) of PR65 are required for interaction.

As indicated in Fig. 3B, a region of PR65 identified by our deletion analysis to be required for interaction with HSF2 (amino acids 378–589) overlaps the region of this protein that was previously shown to be involved in interacting with the catalytic subunit (28, 29, 40). This suggested the interesting possibility that HSF2 could compete with catalytic subunit for binding to PR65. To test this prediction, we measured the binding of catalytic subunit to immobilized GST-PR65 in the absence or presence of HSF2 protein. As shown in Fig. 4, the addition of HSF2 to the binding reaction decreased binding of the catalytic subunit to PR65, whereas the addition of HSF1 protein had no effect. Quantitation indicated that the two amounts of HSF2 tested (1 and 2 μg) decreased catalytic subunit binding to PR65 by 24% and 81.8%, respectively. These results are consistent with the results of our interaction domain mapping analysis above and indicate that HSF2 could function to sequester PR65 from catalytic subunit by competitive interaction.
The overexpression of HSF2 leads to a significant increase in PP2A activity. To test this hypothesis, we determined whether overexpression of HSF2 protein in cells alters PP2A activity. NIH 3T3 cells were transfected with an HSF2 expression plasmid or the parental expression construct, and then PP2A activity in extracts of these cells was measured by in vitro dephosphorylation assay using phosphorylase α as substrate. The results show that overexpression of HSF2 leads to a significant increase in PP2A activity (2.7-fold) in cells (Fig. 5).

**DISCUSSION**

HSF2 has previously been characterized as a transcriptional regulator of hsp gene expression, particularly in cells undergoing differentiation and development (44–47). However, our results now suggest that in addition to its role as a transcription factor, HSF2 also has a novel and unexpected function as a regulator of PP2A activity. One possible hypothesis is that these dual functions of the HSF2 protein evolved to provide a mechanism for cross-talk between regulation of hsp expression and PP2A-regulated pathways, particularly pathways involved in control of cell division. This cross-talk could function to coordinate hsp expression with the regulation of cell division or cell differentiation, to ensure the presence of sufficient levels of molecular chaperones to meet the needs of dividing or differentiating cells. However, we also recognize the alternative possibility that the PP2A-regulating function of HSF2 may be completely independent of its function in regulating hsp expression. Indeed, previous results have indicated an apparent lack of correlation between HSF2 and hsp expression during mouse development, leading to speculation that HSF2 may perform other functions important for developing/differentiating cells (48). Similar conclusions were drawn from studies in Drosophila, which showed that deletion of the HSF gene causes developmental defects without apparent effects on hsp expression (10).

Our proposed model for HSF2 regulation of PP2A is shown in Fig. 6. In this model, we hypothesize that HSF2 binding to PR65 prevents it from interacting with catalytic subunit due to competition between HSF2 and catalytic subunit for the same PR65 but in the context of associated catalytic subunit (17–24). The biological significance of our hypothesized function of HSF2 in regulating the availability of PR65 for binding to catalytic subunit is clearly demonstrated by results showing that mutations in PR65 that disrupt its ability to interact with catalytic subunit are associated with human lung and colon cancers (49).

In summary, the results of our study extend the function of HSF2 beyond its classical role as a regulator of hsp expression to include a new role as a modulator of cellular PP2A activity. Thus, fully understanding HSF2 activity in cells will require us to elucidate both of these functional roles and their relationship to each other, which could provide insight into the mechanisms of cross-talk that link important signaling pathways in cells.