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Molecular Basis of Competition between HSF2 and Catalytic Subunit for Binding to the PR65/A Subunit of PP2A

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We recently identified the existence of a novel interaction between heat shock transcription factor 2 (HSF2) and the PR65/A subunit of protein phosphatase 2A (PP2A) and showed that HSF2 is able to compete with the PP2A catalytic subunit for binding to PR65. To elucidate the mechanistic basis of this competition between HSF2 and catalytic subunit at the molecular level we have sought to characterize sequences within PR65 that are important for interaction with HSF2. The results identify the intra-repeat loop within HEAT repeat 11 of PR65 as critical for interaction with HSF2. Analysis of point mutants within this loop region of PR65 identify lysine 416 as a residue critical for interaction with HSF2. Interestingly, this same lysine residue of PR65 is important for its binding to catalytic subunit. These results suggest that HSF2's ability to interfere with catalytic subunit binding to PR65 is due to competition between HSF2 and catalytic subunit for at least one amino acid residue of PR65, lysine 416. These data support the hypothesis that HSF2 represents a new type of PP2A regulatory protein.

PP2A plays a critical role in regulating a number of cellular processes including intermediary metabolism, signal transduction, and cell cycle progression by dephosphorylating and thereby modulating the activity of proteins that control these processes (1–7). For example, PP2A dephosphorylates and inactivates MEK1, ERK1, and ERK2 to down-regulate growth stimulatory pathways and prevent uncontrolled growth (8, 9). This enzyme is composed of a core heterodimer containing a protein called PR65 (also called A subunit) and the catalytic subunit, which associate with a large number of different B-subunits to form the mixed population of PP2A heterotrimers (holoenzyme) found in cells. The PR65 protein, whose structure has recently been solved, contains 15 repeats of a structural domain called the HEAT motif which span the entire PR65 polypeptide (10). The B-subunits bind the N-terminal region of PR65 containing HEAT repeats 1–10 while the catalytic subunit interacts with the C-terminal region of PR65 containing HEAT repeats 11–15 (11–14). Association of these B-subunits regulates PP2A function, with the nature of the regulation dependent on the identity of the B-subunit which is associated. For example, some B-subunits alter substrate preference of the enzyme while others target the enzyme to specific cellular locations (15–22).

We have recently described a novel interaction that occurs between heat shock transcription factor 2 (HSF2) and the PR65 subunit of PP2A (23). We also showed that HSF2 is able to block the interaction between PR65 and the catalytic subunit of PP2A. Initial mapping data suggested that this is due to competition between HSF2 and catalytic subunit for binding to the same region of the PR65 protein. Based on these results we hypothesize that HSF2 represents a new type of PP2A-regulatory protein, distinguished from the B-type subunits by the ability to bind the C-terminal region of PR65 and block its interaction with catalytic subunit.

The goal of the experiments in this paper was to define the region of PR65 which is important for interaction with HSF2, and to determine the molecular basis for the competition between HSF2 and catalytic subunit for binding to PR65. The results reveal that sequences in PR65 between amino acids 205–419 are important for interaction with HSF2. Near the

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Abbreviations used: HSF2, heat shock transcription factor 2; PP2A, protein phosphatase 2A; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.

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C-terminal boundary of this region we have identified the lysine residue at position 416 of PR65 as critical for interaction with HSF2. Interestingly, this same residue has previously been shown to be important for catalytic subunit binding to PR65, which we have also confirmed in this study (14). Thus, these results identify lysine 416 of PR65 as important for the binding of both HSF2 and catalytic subunit, suggesting that the ability of HSF2 to interfere with catalytic subunit binding to PR65 is due to competition between HSF2 and catalytic subunit for at least one critical contact residue within PR65.

MATERIALS AND METHODS

Exonuclease III and site-directed mutagenesis. A series of C-terminal exonuclease III mutants of PR65 were made by cutting pACT-PR65 (23) with *Aat*II and *Xho*I restriction endonucleases, and then following the manufacturer's instructions for the Stratagene *Exo*III-Mung Bean Nuclease kit. Single amino acid mutants of PR65 were made using the Stratagene Quikchange kit, employing the following mutagenic primers (bold indicates altered nucleotides, and only top strand (sense) primer is indicated). All of the mutations were confirmed by DNA sequencing: PR65 (E413A), 5′ GTGGAGCTGGCTG**C**GGACGCCAAGTGG 3′; PR65 (D414A), 5′ GAGCTGGCTGAGG**CG**GCCAAGTGGCGG 3′; PR65 (A415Y), 5′ CTGGCTGAGGAC**TA**CAAGTGGCGGGTG 3′; PR65 (K416A), 5′ GCTGAGGACGCC**GC**GTGGCGGGTGCGG 3′; PR65 (W417A), 5′ GAGGACGCCAAG**GC**GCGGGTGCGGCTG 3′; PR65 (R418A), 5′ GACGCCAAGTGG**GC**GGTGCGGCTGGCC 3′.

Yeast two-hybrid and β-galactosidase assay. The ability of proteins to interact in the yeast two-hybrid system was tested by cotransfection of bait constructs (in pGBD) and target constructs (in pGAD) into yeast strain pJ69-4A, and then examining for growth on plates lacking adenine, following established protocols (23, 24). For analysis of β-galactosidase activity in yeast harboring two-hybrid constructs, yeast extracts were incubated in 50 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0), 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol. After addition of 1mg/ml of *o*-nitrophenyl-β-D-galactoside (ONPG) substrate, samples were incubated at 30°C for 5 min and then the OD at 420 nm was measured.

Expression of recombinant proteins and GST-pull-down assay. Full-length wild-type GST-PR65, 6 × His-HSF2, and 6 × His-catalytic subunit were previously described (23). GST fusion proteins of different PR65 point mutants were generated by subcloning the appropriate portion of the coding regions of the PR65 point mutants (made as described above) into pGEX2T. The different constructs were expressed in bacteria and then purified according to the manufacturer's instructions. For the *in vitro* binding assay, GST-PR65 or GST-PR65 K416A mutant were bound to glutathione-agarose beads, incubated with 6× His-HSF2 or 6× His-catalytic subunit 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 4× with binding buffer, bound HSF2 or catalytic subunit was analyzed by SDS–PAGE and Western blot using HSF2 and Cα antibodies (25; Upstate Biotechnology).

RESULTS

To dissect the sequences in PR65 important for HSF2 interaction, we made N-terminal deletion constructs (Fig. 1A) and C-terminal exonuclease III deletion constructs (Fig. 1B) and tested the ability of these truncation mutants to interact with full-length HSF2 in the yeast two-hybrid assay. As shown in Fig. 1A, a mutant containing PR65 amino acids 205–589 is able to interact with HSF2 in this assay, but a mutant containing amino acids 327–589 is not, suggesting that the N-terminal boundary of the sequences important for HSF2 interaction exists somewhere between amino acids 205 and 327. With regard to the C-terminal boundary of these sequences, Fig. 1B shows that C-terminal exonuclease III truncation mutants of PR65 ending with amino acid 419 or later are able to interact with full-length HSF2 in the two-hybrid assay. However, further truncation of PR65 to amino acid 415 results in a significant loss of interaction, which decreases further upon truncation to amino acid 413. The PR65 mutant ending at amino acid 408 is unable to interact with HSF2 in this assay. These results suggest that the C-terminal boundary of the sequences required for HSF2 interaction is near amino acids 415–419 of PR65. As summarized in Fig. 1C, the results of these two experiments suggest that sequences in PR65 between amino acids 205–419 are important for interaction with HSF2.

The position of this C-terminal boundary within PR65 was particularly interesting with respect to our hypotheses because this region contains a lysine residue (K416) previously shown to be important for PR65 interaction with catalytic subunit (14). Based on this result, we hypothesized that the competition between HSF2 and catalytic subunit for binding to PR65 could be due to the possibility that both of these proteins require one or more identical amino acid residues in PR65 for binding.

To test this hypothesis we used site-directed mutagenesis to make a number of single amino acid substitutions in the region of PR65 near lysine 416 and then determined the effects of these mutations on interaction with HSF2 in the two-hybrid assay. The mutations made were all alanine substitutions except for alanine 415, which was changed to tyrosine to determine the effect of substitution of a bulky side chain for the small alanine side chain. The results of this experiment, shown in Fig. 2, support our hypothesis by revealing that mutation of lysine 416 of PR65 to alanine results in a 90% reduction in β-galactosidase activity relative to wild-type PR65. Interestingly, while four of the other mutants we made in this region of PR65 (E413A, D414A, A415Y, and W417A) showed similar β-galactosidase activity with HSF2 as wild-type PR65, the construct containing a mutation of arginine 418 to alanine consistently exhibited at least a 40% increase in β-galactosidase activity. Possible interpretations of this result with respect to the HSF2:PR65 interaction are presented in the Discussion.

We then compared the effect of these point mutations in PR65 on its interaction with catalytic subunit in the two hybrid assay. As shown in Fig. 3, the results

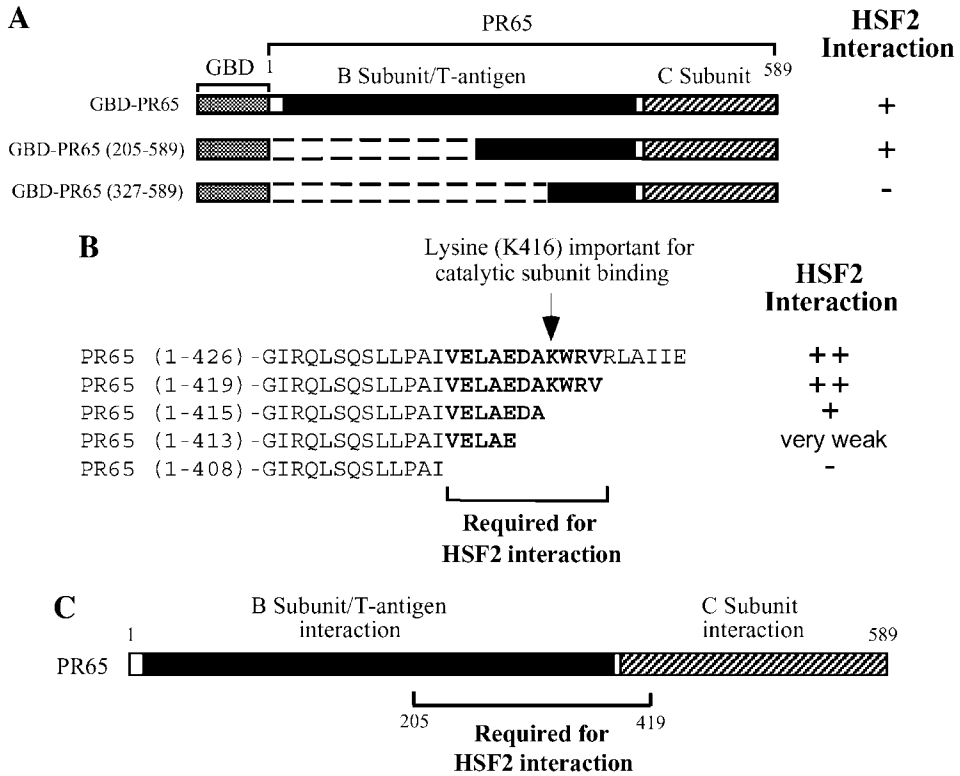

FIG. 1. Analysis of interaction between HSF2 and PR65 N-terminal truncation mutants (A) and C-terminal exonuclease III mutants (B) by yeast two-hybrid assay. PR65 mutant constructs were tested for ability to confer growth of yeast on media lacking adenine in combination with a plasmid containing full-length wild-type HSF2. (C) indicates position of sequences in PR65 indicated by our results to be important for interaction with HSF2.

confirm the importance of lysine 416 of PR65 for interaction with catalytic subunit. Mutation of this residue to alanine results in an 80% reduction in β-galactosidase relative to wild-type PR65. However, our results also identify two other amino acids in this region of PR65 whose mutation alters interaction with catalytic subunit. Mutations of aspartic acid 414 (D414A) or alanine 415 (A415Y) reduce β-galactosidase activity by 85 and 25%, respectively, relative to wild-type PR65.

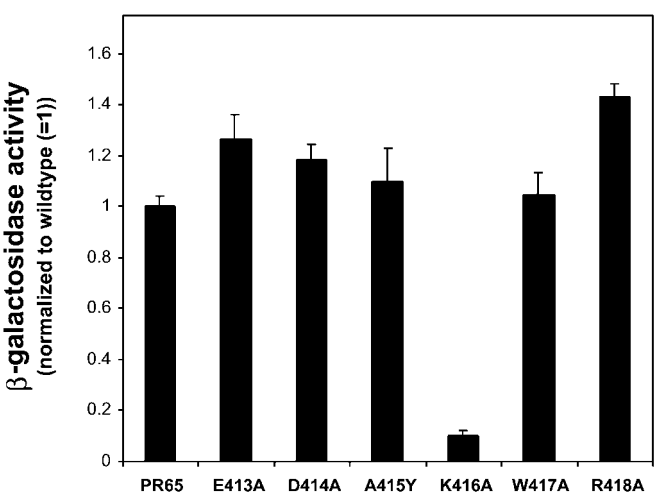

FIG. 2. Analysis of effect of PR65 point mutants on interaction with HSF2. The indicated point mutants of PR65 were transformed into yeast with full-length HSF2 and then β-galactosidase activity was measured in yeast extracts. Values are normalized to wild-type PR65 (=1).

The results of these PR65 point mutant analyses, which are summarized in Fig. 4, indicate that lysine 416 of PR65 is very important for the binding of both HSF2 and catalytic subunit. However, they also suggest that there are differences in how these two proteins interact with PR65, as shown by the differential effects of mutations in other nearby residues on PR65 interaction with HSF2 vs. catalytic subunit.

To obtain independent confirmation of these results concerning the importance of lysine 416 for interaction with both HSF2 and catalytic subunit, we also compared the binding of HSF2 and catalytic subunit to wild-type PR65 vs. mutant PR65 (K416A) using recombinant proteins in an *in vitro* binding assay. The results, shown in Fig. 5, support this finding by demonstrating that GST-PR65 containing the K416A mutation exhibits reduced binding to both HSF2 and catalytic subunit relative to wild-type GST-PR65.

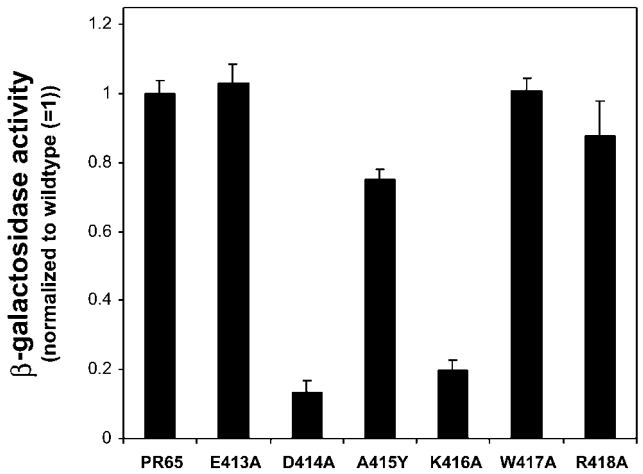

FIG. 3. Analysis of effect of PR65 point mutants on interaction with catalytic subunit. The indicated point mutants of PR65 were transformed into yeast with full-length catalytic subunit and then β-galactosidase activity was measured in yeast extracts. Values are normalized to wild-type PR65 (=1).

Quantitation of this experiment reveals that HSF2 and catalytic subunit binding to the K416A mutant is decreased by 60 and 85%, respectively, compared to wild-type PR65.

DISCUSSION

One of the key findings of this study is that lysine 416 of PR65, which had previously been implicated as important for catalytic subunit binding, is also critical for interaction of this protein with HSF2 (14). This result provides an explanation, at the molecular level, for the ability of HSF2 to compete with catalytic subunit for binding to PR65 (23). It suggests that this competition is more than just a matter of steric interference, and that these two proteins actually share at least one critical contact residue in PR65.

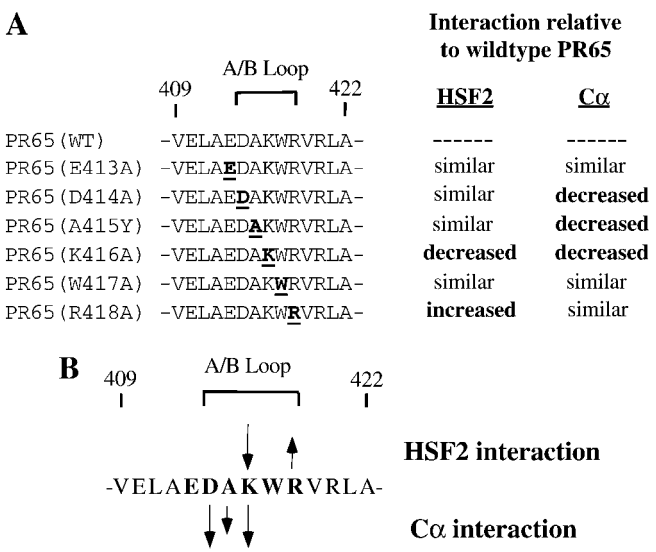

FIG. 4. Summary of analysis of PR65 amino acid residues important for interaction with HSF2 or catalytic subunit. (A) shows the mutants that were tested and the results for each in the interaction assay relative to wild-type PR65, while the cartoon in (B) depicts the magnitude and direction of the change (down and up arrows indicate decreased and increased interaction, respectively) only for those mutants which exhibited a difference relative to wild-type PR65.

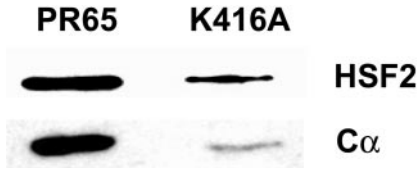

FIG. 5. *In vitro* binding analysis of 6 × His-HSF2 and 6 × His-catalytic subunit with wild-type GST-PR65 vs. GST-PR65 (K416A). GST-PR65 and GST-PR65 (K416A) were bound to glutathione agarose and then incubated with purified 6 × His-HSF2 or 6 × His-catalytic subunit. After extensive washing, the amount of HSF2 or catalytic subunit bound was determined by Western blot analysis.

Our results also suggest that although both HSF2 and catalytic subunit appear to require lysine 416 of PR65 for interaction, there are differences in how these two proteins interface with PR65. This interpretation is based on our finding that mutation of two residues near lysine 416, aspartic acid 414 and alanine 415, affects interaction of catalytic subunit but has no apparent effect on HSF2 binding. Both of these residues are exposed on the protein surface and thus are potentially available for interaction. The mutation we made at alanine 415 of PR65 is a substitution of a bulky tyrosine side chain for the relatively compact methyl group of alanine, and thus we hypothesize that this mutation may reduce catalytic subunit binding by interjecting this large side chain into the interface between these two proteins, creating steric interference.

This same logic could also explain why HSF2 interaction with PR65 actually increases when arginine 418 is mutated to alanine. We postulate that the relatively large, charged side chain of this arginine could be interfering with HSF2 binding due to either steric interference or charge repulsion with some group in the PR65-binding interface of HSF2. This interference could be relieved by changing arginine 418 to the much smaller, uncharged side chain of alanine. Interestingly, it has recently been shown that a mutation which changes this arginine residue to tryptophan (R418W) is found in a human melanoma cell line, suggesting this mutation could be tumorigenic (26). We hypothesize that this could be mediated by effects of this substitution to decrease interaction of PR65 with catalytic subunit due to replacement of the polar arginine side chain with the bulky, largely hydrophobic side chain of tryptophan, potentially leading to altered PP2A function and disregulated cell division.

Another interesting point worth discussing further is our data suggesting that aspartic acid 414 of PR65 is important for interaction with catalytic subunit. This aspartic acid occupies a position that is conserved among most of the HEAT repeats of PR65 (27). The crystal structure of PR65 reveals that in most of the HEAT repeats that have an aspartic acid at this position the carboxyl-group in the side chain of this residue is involved in a charge-charge interaction with conserved arginine residues (lysine in some repeats) in the preceding HEAT repeat (10). These inter-repeat interactions play an important role in stabilizing the PR65 structure, explaining the conservation at these positions in PR65. However, what is interesting is that the crystal structure of PR65 suggests that the aspartic acid at position 414 in HEAT repeat 11, the one suggested by our studies to be important for catalytic subunit binding, does not participate in this interaction with the arginine residue of the preceding HEAT repeat (arginine 381) (10). Measurement of the closest distance between the carboxyl oxygen atoms and nitrogen atoms in the side chains of these two residues (based on crystal structure coordinates) reveals that they are approximately 7 angstroms apart. This distance is beyond that typically expected for a strong interaction of this type. The corresponding aspartic acid residues in HEAT repeats 5–10, as well as HEAT repeat 12, do appear to form the interaction with arginine or lysine residues in the preceding HEAT repeat. One interpretation of this data, which would be consistent with the results of our mutation analysis, is that aspartic acid 414 does not participate in this inter-repeat charge-charge interaction with arginine 381 because it is required for interaction with chemical groups within the catalytic subunit. Indeed, this feature of aspartic acid 414 could serve to distinguish HEAT repeat 11 from many of the other HEAT repeats of PR65, and thereby perhaps help target catalytic subunit binding to this region of the protein.

In summary, the data presented in this paper suggest the underlying mechanistic basis by which HSF2 competes with catalytic subunit for binding to PR65 and support the hypothesis that HSF2 represents a new type of PP2A regulatory protein. Future studies will seek to further characterize the structure of the HSF2:PR65 interaction, and attempt to identify other potential members of this class of regulatory protein.

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