Interaction between Protein Phosphatase 5 and the A subunit of Protein Phosphatase 2A

EVIDENCE FOR A HETEROTRIMERIC FORM OF PROTEIN PHOSPHATASE 5*

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Members of the phosphoprotein phosphatase family of serine/threonine phosphatases are thought to exist in different native oligomeric complexes. Protein phosphatase 2A (PP2A) is composed of a catalytic subunit (PP2Ac) that complexes with an A subunit, which in turn also interacts with one of many B subunits that regulate substrate specificity and/or (sub)cellular localization of the enzyme. Another family member, protein phosphatase 5 (PP5), contains a tetratricopeptide repeat domain at its N terminus, which has been suggested to mediate interactions with other proteins. PP5 was not thought to interact with partners homologous to the A or B subunits that exist within PP2A. However, our results indicate that this may not be the case. A yeast two-hybrid screen revealed an interaction between PP5 and the A subunit of PP2A. This interaction was confirmed for endogenous proteins in vivo using immunoprecipitation analysis and for recombinant proteins by in vitro binding experiments. Our results also indicate that the tetratricopeptide repeat domain of PP5 is required and sufficient for this interaction. In addition, immunoprecipitated PP5 contains associated B subunits. Thus, our results suggest that PP5 can exist in a PP2A-like heterotrimeric form containing both A and B subunits.

The reversible phosphorylation of proteins, catalyzed by protein kinases and phosphatases, is a major mechanism for regulating many cellular processes, including intermediary metabolism, cell cycle progression, DNA replication, transcription, and protein translation (1–3). It is estimated that one-third of all cellular proteins undergo reversible phosphorylation, which cells utilize to regulate the functional properties of key regulatory proteins involved in specific pathways (4).

Based on homology of amino acid sequences and the similarity of three-dimensional structures, phosphoprotein phosphatases (PPPs)† are divided into three families designated PPP, PPM, and PTP (5, 6). The PPP and PPM families are comprised of phosphoserine- and phosphothreonine-specific enzymes, whereas the PTP family is comprised of phosphotyrosine-specific and/or dual specificity phosphatases. As suggested by the name, dual specificity phosphatases can dephosphorylate all three phosphoamino acids (7). Protein phosphatase 2A (PP2A) together with PP1, PP2B (calcineurin), PP4, PP5, PP6, and PP7 are classified in the PPP family (4).

PP5 exists as a heterotrameric component of a catalytic subunit (PP2Ac), an A subunit (also known as PR65), and a B subunit. There are three major families of B subunits, PR55/B, PR61/B′, PR72/B′, with multiple isoforms within each family (4–6, 8). The association of PP2Ac with different B subunits modulates the activity, substrate specificity, and/or (sub)cellular location of the holoenzymes, enabling them to control numerous cellular functions (3, 4, 9, 10).

Protein phosphatase 5 (PP5), another member of the PPP family of phosphatases, differs from the other serine/threonine phosphatases in that it contains regulatory and (sub)cellular targeting functions within a single polypeptide chain. Its C-terminal catalytic domain is related to those of PP1, PP2A, and PP2B, but its N-terminal domain consists of three tetratricopeptide repeats or TPRs that are missing from these other PPP family members (11, 12). TPR domains consist of a series of antiparallel amphipathic α helices that bundle together through hydrophobic interactions to form a cradle-shaped binding groove (13). Tetratricopeptide repeats are found not only in PP5, but also in a variety of unrelated proteins, and are thought to serve both targeting and regulatory functions. The TPR domain of PP5 interacts with a number of proteins, including the atrial natriuretic peptide receptor, Cdc 16p, and Cdc 27p (11, 14). PP5 also interacts with heat shock protein 90 in complexes with glucocorticoid receptors, and it has been demonstrated that overexpression of the TPR domain of PP5 blocks glucocorticoid-induced gene transcription (15, 16). Thus, PP5 has distinctive biochemical and biological properties because of its N-terminal TPR domain.

We have been characterizing an interaction between the A subunit of PP2A and a member of the heat shock transcription factor family, heat shock factor 2 (HSF2) (17, 18). Our data suggests that HSF2 competes with PP2Ac by binding to the same region of the A subunit and that HSF2 may represent a new type of PP2A regulatory protein. As a means to identify other potential regulators of PP2A that function by this mechanism, we screened a yeast two-hybrid library using the C-terminal region of the A subunit as a bait. One of the A subunit-interacting clones identified by this screen was a polypeptide encoding the TPR domain of PP5.

In this present study, we began to characterize the mechanistic and structural basis for the interaction between the PP2A A subunit and PP5. We confirmed that endogenous PP5

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† The abbreviations used are: PPP, phosphoprotein phosphatases; PP2A, protein phosphatase 2A; PP5, protein phosphatase 5; PP2Ac, catalytic subunit of PP2A; PR65, A subunit of PP2A; TPR, tetratricopeptide repeat; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; HSF2, heat shock factor 2; HEAT, huntingtin-elongation-A subunit-target of rapamycin.
and A subunit interact in vivo using immunoprecipitation analysis, as well as in vitro using recombinant proteins. Our results indicate that the tetratricopeptide repeat region of PP5 is sufficient for this interaction. We also found that the sequence surrounding the loop region of HEAT repeat 11 within the A subunit, which was previously shown to be important for binding to PP2Ac and HSF2, is also important for interaction with PP5 (18, 19). Furthermore, we found that PP5 associates with B subunits in vivo, indicating that PP5 exists in a heterotrimeric form reminiscent of the PP2A enzyme.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screening**—The bait construct containing PR65(205–589) (17) was made in pGAD-C1 using standard subcloning methodologies. PR65(205–589) fused to the GAL4 DNA-binding domain was introduced into yeast strain p69A-4A (20). The resulting strain was then transformed with a mouse whole embryo cDNA library (21). Screening was performed as described previously (17). In subsequent analysis, two-hybrid constructs for full-length PP5, TPR domain, and PP5 catalytic domain (lacking TPR domain) were made in pGBD-C2 using standard subcloning methodologies. PR65(205–589), the "bait" used to perform the yeast two-hybrid library screen, and/or pGBD-C2 (empty bait vector) and various PP5 target constructs made in pGAD-C2. The empty target vector pGAD-C2 was tested as a negative control.

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**Bait** | **Target** | **Interaction**
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pGBD-PR65(205–589) | pGAD-HSF2 | ++
pGBD-PR65(205–589) | pGAD-PP5 | ++
pGBD-PR65(205–589) | pGAD-TPR(1–19) | ++
pGBD-PR65(205–589) | pVP16-TPR(1–19) | +++
pGBD-PR65(205–589) | pGAD-Cat | =
pGBD-PR65(205–589) | pVP16 | =
pGBD-C2 | pGAD-HSF2 | =
pGBD-C2 | pGAD-PP5 | =
pGBD-C2 | pGAD-TPR(1–19) | =
pGBD-C2 | pVP16-TPR(1–19) | =
pGBD-C2 | pGAD-Cat | =
pGBD-C2 | pVP16 | =

**Fig. 1. Interaction between the A subunit of PP2A and PP5 in yeast two-hybrid assay.** A, depiction of full-length pGBD-PR65 and pGBD-PR65(205–589), the "bait" used to perform the yeast two-hybrid library screen. B, schematic representation of full-length PP5 and the region found to interact during the yeast two-hybrid library screen. C, schematic representation of full-length PP5 versus full-length PP2Ac. Areas highlighted include the catalytic domains, which are 40% identical and 60% similar in amino acid composition, and the TPR region of PP5.

**Fig. 2. Specificity of interaction between the A subunit and PP5.** Test of yeast two-hybrid interaction between pGBD-PR65(205–589), the bait used to perform the yeast two-hybrid library screen, and/or pGBD-C2 (empty bait vector) and various PP5 target constructs made in pGAD-C2. The empty target vector pGAD-C2 was tested as a negative control.

**Immunoprecipitation (IP) analysis of the A subunit-PP5 interaction.** A, PP5-containing complexes were immunoprecipitated using anti-PP5 monoclonal antibodies, and then immunoprecipitated proteins were subject to Western blot using anti-A subunit polyclonal antibodies. B, A subunit antibodies were used to perform two rounds of immunoprecipitation of HeLa cell extracts, followed by Western blot analysis of the two pellets (P1 and P2) and remaining supernatant (S) using anti-PP5 monoclonal antibodies. The immunoprecipitation was carried out from 1794 μg of initial cell extract, 20 μg of the extract was loaded in the lane marked WCE, and 1718 μg of protein remained in the supernatant after the two sequential immunoprecipitations. The lower panel is a Western blot of these samples probed with anti-A subunit antibodies to determine efficiency of immunoprecipitation. C, same as in B except that PP5 antibodies were used for two sequential immunoprecipitations, and Western blot was probed using anti-A subunit antibodies. The immunoprecipitation was carried out from 1794 μg of initial cell extract, 20 μg of the extract was loaded in the lane marked WCE, and 1681 μg of protein remained in the supernatant after the two sequential immunoprecipitations. The lower panel is a Western blot of these samples probed with anti-PP5 antibodies to determine the efficiency of immunoprecipitation.
acid substitutions within the A subunit (PR65) protein were made using the Stratagene QuikChange Mutagenesis Kit. Using the following mutagenic primers (bold indicates the altered nucleotides, and only the sense primer is indicated). All of the mutations were confirmed by DNA sequencing: PR65 (E413A), 5'-GTGGAACCTGCTCAGGACGCCAGAAGTGGA-3'; PR65 (D414A), 5'-GAGCTGTGAGGGCCACATTGCGG-G-3'; PR65 (A415Y), 5'-CTGCTGTAAGACTCAAAATGCGGCTGT-3'; PR65 (K416A), 5'-GCTGAGGACCGCCTGCGTGGCGGTCG-3'; PR65 (R418A), 5'-GACGCCAGTTGGGCGTGGCTGCCGC-3'; PR65 (L421A), 5'-TGCGGCGTGCGCGCGCCACATCTGAG-3'.

For analysis of β-galactosidase activity in yeast harboring two-hybrid constructs, yeast extracts were incubated with Z Buffer (60 mM NaHPO4 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 ~30–45 min and then the Amax was measured.

RESULTS

To identify potential PP2A regulatory proteins, a mouse whole embryo cDNA library was screened using a C-terminal portion of the A subunit of PP2A (PR65, 205–589) as the bait (Fig. 1A) (20–22). This truncated form of the A subunit does not contain the region responsible for binding the B subunits, and thus we were able to focus on identifying proteins that bind to the C-terminal region of the A subunit. One of the positives identified was a clone containing an N-terminal portion of PP5 comprising amino acids 7–139, which will hereafter be referred to as TPR7–139 (Fig. 1B) (12). Interestingly, this portion of PP5 contains most of the TPR domain of this protein. The domain organization of PP5 relative to PP2Ac, which lacks these N-terminal TPR repeats, is shown in Fig. 1C.

To further characterize this interaction, a full-length clone of PP5, as well as the N-terminal TPR domain (comprising amino acids 1–159), the original PP5 partial clone (TPR7–139), and the C-terminal catalytic domain of PP5 (Cat), were tested for their ability to interact with PR65, 205–589) using the yeast two-hybrid assay. Yeast transformed with the A subunit bait PR65, 205–589) + PP5 as well as those transformed with PR65, 205–589) + TPR7–139 and PR65, 205–589) + TPR7–139 all grew on selective media, indicating the existence of an interaction (Fig. 2). Yeast transformed with the A subunit bait PR65, 205–589) + Cat, PR65, 205–589) + empty target vectors pGAD-C2 or pVP16, as well as those transformed with the empty bait vector (pGBD) and the aforementioned target constructs, all failed to grow on selective media (Fig. 2). These results support the hypothesis that the TPR domain of PP5 interacts with the A subunit of PP2A.

To determine whether endogenous PP5 and A subunit proteins interact, we immunoprecipitated PP5 from extracts of HeLa cells followed by Western blot using anti-A subunit antibodies. As a negative control we also subjected these extracts to immunoprecipitation using nonspecific mouse IgG antibodies and probed for A subunit. The results indicate that PP5 is found in a complex with A subunit in vivo (Fig. 3A), while little or no A subunit was immunoprecipitated by the negative control IgG antibodies. To determine the proportion of total endogenous cellular PP5 and A subunit that are associated, we subjected extracts of HeLa cells to sequential immunoprecipitations. To measure the efficiency of immunoprecipitation we also subjected each of these samples to Western blot using the antibody used to immunoprecipitate. The results of the A subunit immunoprecipitation (Western probed for PP5) indicate that a significant proportion of cellular PP5 does appear to immunoprecipitate with A subunit in the two sequential immunoprecipitations (Pellet 1 (P1) and Pellet 2 (P2)) (Fig. 3B). A fraction of PP5 does appear in the supernatant (lane marked “S”), but so does a fraction of the cellular PR65 (see lower panel), indicating that our immunoprecipitation was not able to

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**Plasmids**—A bait construct containing full-length human A subunit (PR65) was made in pGBD-C1 using standard subcloning methodologies. 5’ and 3’ truncation mutants were made by performing restriction digestion of selected unique sites, filling in with Klenow, and then ligating to delete the sequences. The sites utilized were BgII (pGBD-PR65, 205–225), BsrGI (pGBD-PR65, 205–267), BgIII (pGBD-PR65, 205–293). 3’ truncation mutants of human PR65 (205–589) were generated using exonuclease III deletion as described previously (18).

**Site-directed Mutagenesis and β-Galactosidase Assay**—Single amino acid substitutions within the A subunit (PR65) protein were made using the Stratagene QuikChange Mutagenesis Kit. Using the following mutagenic primers (bold indicates the altered nucleotides, and only the sense primer is indicated). All of the mutations were confirmed by DNA sequencing: PR65 (E413A), 5’-GTGGAACCTGCTCAGGACGCCAGAAGTGGA-3’; PR65 (D414A), 5’-GAGCTGTGAGGGCCACATTGCGG-G-3’; PR65 (A415Y), 5’-CTGCTGTAAGACTCAAAATGCGGCTGT-3’; PR65 (K416A), 5’-GCTGAGGACCGCCTGCGTGGCGGTCG-3’; PR65 (R418A), 5’-GACGCCAGTTGGGCGTGGCTGCCGC-3’; PR65 (L421A), 5’-TGCGGCGTGCGCGCGCCACATCTGAG-3’.

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**FIG. 5.** Deletion mapping of regions required for the A subunit-PP5 interaction. A, N- and C-terminal deletion mutants of the A subunit were constructed and tested for their ability to interact with TPR\(_{7-139}\) in the yeast two-hybrid assay. B, depiction of the region within the A subunit required for interaction with PP5.

pull down all of this protein, possibly due at least in part to the association of PR65 with cellular substructures such as microtubules. Thus it is unclear whether this fraction of PP5 remaining in the supernatant is complexed with a subunit or not.

The reverse experiment, in which PP5 antibodies were used in two sequential immunoprecipitations followed by Western blot using A subunit antibodies, reveals that while some of the cellular A subunit clearly immunoprecipitates with PP5 (most in the first immunoprecipitation (IP)-Pellet 1 (P1)) a substantial proportion remains in the supernatant and thus appears not to be associated with PP5 (Fig. 3C). This is expected as a large proportion of cellular A subunit is associated with PP2A catalytic subunit and thus would not be immunoprecipitated by PP5 antibodies.

As a complementary approach we also tested the ability of recombinant PP5 and A subunit proteins to interact in vitro using a GST pull-down assay. GST or GST-PR65\(_{205-589}\) were bound to glutathione-agarose, incubated with purified recombinant 6xHis-TPR\(_{1-159}\) or 6xHis-PP5, and washed extensively. The binding of 6xHis-PP5 or 6xHis-TPR\(_{1-159}\) was then measured by Western blot analysis using either PP5 antibodies or 6xHis antibodies, respectively. The results of this experiment demonstrate that both PP5 (Fig. 4A) and its TPR domain (Fig. 4B) interact in vitro with recombinant A subunit, but not with GST alone. Similar interactions with PP5 and TPR domain were observed using GST fusion proteins containing full-length A subunit (data not shown). These data provide additional evidence of the PP5-A subunit interaction and support the hypothesis that the TPR domain of PP5 is the region responsible for interacting with the A subunit.

To better understand the interaction between PP5 and the A subunit, we determined the regions within the A subunit polypeptide that are important for this interaction by constructing 3' truncation mutants of full-length A subunit and testing their ability to interact with TPR\(_{7-139}\) in the yeast two-hybrid system. The results demonstrate that 3' truncation mutants of the A subunit that terminate at amino acids 378 and 325 are unable to interact with TPR\(_{7-139}\) (Fig. 5A). We also tested 3' truncation mutants of the portion of the A subunit (PR65\(_{205-589}\)) used as bait during the initial library screen. We found that 3' truncation mutants that terminate at amino acid 426 were able to interact with TPR\(_{7-139}\) but that further deletion to amino acid 408 rendered this protein unable to interact (Fig. 5A). Finally, we tested a 5' truncation mutant of full-length A subunit that begins at amino acid 325 and found that it too was unable to interact with TPR\(_{7-139}\) (Fig. 5A). These results suggest that the region within the A subunit between amino acids 205 and 426 is important for interaction with the TPR domain of PP5 (Fig. 5B).

As indicated in Fig. 5B, amino acids 205–426 of the A subunit overlap the region of this protein previously shown to be involved in interacting with PP2Ac (amino acids 408–426) (19, 23). Interestingly, the region of overlap contains a loop region in HEAT repeat 11 of the A subunit that we and others have demonstrated to be important for interaction with PP2Ac (18, 19). This prompted us to speculate that amino acids within this loop region of the A subunit may also be important for interaction with the TPR domain of PP5. To test this hypothesis we analyzed point mutants within this region for their ability to interact with the TPR domain of PP5 in the yeast two-hybrid assay, as measured by β-galactosidase activity in yeast extracts. The mutations made were all alanine substitutions except for alanine 415, which was changed to tyrosine to determine the effect of substituting a bulky side chain for the small alanine side chain. As shown in Fig. 6A, mutations of aspartic acid 414 (D414A) and leucine 421 (L421A) resulted in a small reduction in β-galactosidase activity relative to wild type A subunit, suggesting that these residues could be involved in the interaction with the TPR domain of PP5. Somewhat to our surprise, mutation of four other residues led to either a moderate (E413A) or large (K416A, W417A, R418A) increase in β-galactosidase activity. We postulate that the side chains of these particular residues in this region of the A subunit are deleterious to its interaction with the TPR domain, perhaps because of steric or ionic repulsions between them and groups in the TPR domain that are then relieved when these side chains are changed to that of alanine. The A415Y mutation resulted in only a small increase in β-galactosidase activity. To
rule out the possibility that significant differences in expression of any of the mutants in yeast could account for differences in \( \beta \)-galactosidase activity we observed, we performed Western blot of yeast containing each of the A subunit constructs with A subunit antibodies and did not observe any large differences in levels that could account for the results (Fig. 6B), which are summarized in Fig. 6C.

The PP2A heterotrimer contains a B subunit that associates with the N-terminal domain of the A subunit. Our finding that PP5 interacts with the A subunit prompted us to examine whether this complex also contains a B subunit. To test this hypothesis we immunoprecipitated PP5 complexes from extracts of HeLa cells and probed Western blots of the immunoprecipitate with antibodies that recognize different B subunits. As shown in Fig. 7A, we found that a member of the B' family, PR72, immunoprecipitates with PP5. A control IgG does not immunoprecipitate this protein, indicating specificity. We were unable to detect any B subunit isomers from the PR55/B or PR56/B' family (data not shown). Sequential immunoprecipitation analysis of this association of B' with PP5 revealed that, similar to what was observed for the proportion of cellular A subunit associated with PP5 and consistent with our expectation (see Fig. 3C), while we can clearly detect a fraction of B' associated with PP5 (Pellet 1 (P1)) there remains a large fraction of B' not associated with PP5 (supernatant (S)) (Fig. 7B), even though the immunoprecipitation appears to have efficiently pulled down the B' protein in the extracts (lower panel). Again, this is consistent with the expectation that a substantial portion of B' is found in PP2A heterotrimer in the cell and is not associated with this complex. It is also possible that other B subunits besides those we tested are associated in some A subunit-PP5 complexes. However, our results do suggest that

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**Fig. 6. Analysis of interaction between the A subunit and PP5 using point mutants.** A, the indicated A subunit point mutants in the pGAD target vector were transformed into yeast with pGBD-TPR(7–139), and then \( \beta \)-galactosidase activity was measured in yeast extracts. Values are normalized to wild type (wt) A subunit (=1). B, the levels of wild type and point mutant A subunit proteins in the yeast were determined by subjecting extracts of yeast transformed with the different A subunit constructs listed in A to SDS-PAGE and Western blot using anti-A subunit antibodies. C, summary indicating amino acids important for interaction with PP5.
the “PP5-A subunit heterodimer” associates with at least one B subunit in vivo to form novel heterotrimERIC complexes containing PP5, A subunit, and a B subunit.

DISCUSSION

Based on our results, we propose that PP5 can exist in a native complex in vivo with the A subunit of PP2A and a B subunit, like that of the PP2A heterotrimer (Fig. 8). Our data also suggest that PP5 binds to the A subunit via its N-terminal TPR domain, although it is possible that contacts between the catalytic domain of PP5 and the A subunit may also contribute to this interaction.

Eukaryotic cells express a large number of different protein kinases. However, the number of genes encoding protein phosphatases is limited and does not appear to equal the diversity of the protein kinases. Furthermore, it has been estimated that approximately one-third of all cellular proteins undergo reversible phosphorylation. A major question, then, is how such a limited number of dephosphorylating enzymes can counterbalance the action of so many protein kinases. The idea has developed that proteins that regulate protein phosphatases are key players in determining the properties of the enzyme. Our data may help answer this question as it suggests a “modular” design of phosphatases in the PPP family such that one catalytic subunit can be replaced by another. This could represent one mechanism for generating diversity of function among this family of protein phosphatases.

Our data also illustrate the fact that TPR domains and HEAT domains can interact. These two domains, both of which are involved in protein-protein interactions, are structurally similar in that they are both composed of antiparallel amphipathic α helices (24). However, TPR motifs generate extended surface grooves, while surface grooves are not a feature of tandem HEAT repeats (24–26). Instead, HEAT repeats generate a ridge-like motif, which creates an interaction interface (24, 25). Therefore, TPR and HEAT motifs illustrate how a common structural repeat unit can assemble related motifs to create different structures.

In summary, our findings indicate a relationship between the native complexes formed by PP5 and PP2A in cells. Further studies to characterize this relationship should increase understanding of how phosphatase functions are regulated within the cell.

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