Protein Phosphatase 2A Holoenzyme Assembly

IDENTIFICATION OF CONTACTS BETWEEN B-FAMILY REGULATORY AND SCAFFOLDING A SUBUNITS

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Protein serine/threonine phosphatase (PP) 2A is a ubiquitous enzyme with pleiotropic functions. Trimeric PP2A consists of a structural A subunit, a catalytic C subunit, and a variable regulatory subunit. Variable subunits (B, B’, and B’ families) dictate PP2A substrate specificity and subcellular localization. B-family subunits contain seven WD repeats predicted to fold into a β-propeller structure. We carried out mutagenesis of By to identify domains important for association with A and C subunits in vivo. Several internal deletions in By abolished coimmunoprecipitation of A and C subunits expressed in COS-M6 cells. In contrast, small N- and C-terminal By deletions had no effect on incorporation into the PP2A heterotrimer. Thus, holoenzyme association of B-family subunits requires multiple, precisely aligned contacts within a core β-propeller domain. Charge-reversal mutagenesis of By identified a cluster of conserved critical residues in By WD repeats 3 and 4. Acidic substitution of paired basic residues in By (RR165EE) abolished association with wild-type A and C subunits, while fostering incorporation of By into a PP2A heterotrimer containing an A subunit with an opposite charge-reversal mutation (EE100RR). Thus, binding of A and B subunits requires electrostatic interactions between conserved pairs of glutamates and arginines. By expressing complementary charge-reversal mutants in neuronal PC6-3 cells, we further show that holoenzyme incorporation protects By from rapid degradation by the ubiquitin/proteasome pathway.

The balance of protein kinase and phosphatase activities toward key proteins is central to many aspects of cellular physiology. Compared with kinases, protein phosphatases have received little attention, and appreciation that they may be just as precisely regulated as the enzymes whose action they oppose is relatively recent.

PP2A1 is one of the four major classes of serine/threonine phosphatases that also include PP1, PP2B (calcineurin), and PP2C. PP2A is highly conserved in eukaryotes (for recent reviews, see Refs. 1 and 2). It constitutes between 0.3% and 1% of total protein in mammalian cells (3, 4) and supplies the majority of soluble phosphatase activity toward phospho-serine and -threonine. PP2A is a holoenzyme of two or three subunits. A 36-kDa catalytic or C subunit complexes with a 65-kDa scaffolding A subunit to form the AC core enzyme; the core enzyme can bind a third, variable subunit to form the PP2A heterotrimer. In mammals, A and C subunits are each encoded by two highly similar genes (Aα/β and Cα/β), with Aα and Cα isoforms being more abundant. Regulatory subunits are encoded by three multigene families referred to as B, B’, and B’’. The B family (also known as PR55) consists of four genes, Bα, Bβ, Bγ, and Bδ, that give rise to proteins with molecular masses of 54–57 kDa (5–9). The B’ family (also referred to as B56 or PR61) consists of at least seven isoforms encoded by five genes (B‘α, B‘β, B‘γ, B‘δ, and B‘ε) (10–15), with molecular masses between 54 and 74 kDa. The four known members of the B’’ family are designated according to their masses as PR48 (16), PR59 (17), and PR72/130 (18). Several PP2A regulatory subunits show restricted tissue expression; for instance, Bβ and Bγ can only be detected in brain (6, 19). Proteins encoded by DNA tumor viruses, SV40 small t and polyoma virus small and middle T antigen, are a fourth group of proteins that bind to the PP2A core enzyme and subvert its activity as a suppressor of cellular transformation (20–22). The AC dimer has also been shown to interact with other proteins, including the WD repeat-containing proteins striatin and SG2NA (23).

Evidence is accumulating that regulatory subunits impart specific functions to PP2A holoenzymes (24, 25). For example, B-family regulatory subunits have been implicated in the regulation of cytoskeletal protein assembly (26–28), B’’ subunits participate in the developmental Wnt/β-catenin signal transduction cascade (29, 30), and B’ subunits may control the G1-S cell cycle transition (16, 17). Adenovirus type 5 appears to induce apoptosis by interaction of its E4orf4 protein with the Bα subunit of PP2A (31, 32). How regulatory subunits function to mediate the diverse physiological functions of PP2A is poorly understood. There is in vitro evidence that regulatory subunits affect enzymatic activity and substrate specificity of PP2A (33). Localization studies have suggested that regulatory subunits target PP2A holoenzymes to distinct subcellular compartments (11, 14, 19).

The crystal structure of the scaffolding Aα subunit of PP2A has been solved (34), confirming a previous model based on secondary structure prediction and mutagenesis studies (35). The A subunit is a hook-shaped protein made up almost entirely of 15 imperfect repeats, each about 40 amino acids long. Each of these HEAT repeats (named after proteins that contain them: huntingtin, elongation factor, A subunit, and TOR kinase) consists of two antiparallel, amphipathic α-helices. Loops between the two helices (intrarepeat loops) form a continuous...
ridge along the inside of the hook, providing interaction surfaces for catalytic and regulatory subunits. Regulatory subunits and viral antigens bind to the 10 N-terminal repeats, whereas the catalytic subunit binds via repeats 11–15 (35, 36).

The PP2A C subunit is thought to have a roughly globular structure similar to that of the related PPI C subunit (37, 38).

Knowing how regulatory subunits fold and interact with the core PP2A dimer is crucial for our understanding of the diverse roles of PP2A in cells. Here, we carry out deletion and site-directed mutagenesis in combination with structure modeling to identify domains and amino acids important for holoenzyme association of B-family regulatory subunits. By complementary charge-reversal mutagenesis, we show that adjacent arginines in By critically interact with adjacent glutamates in the Aα subunit.

**EXPERIMENTAL PROCEDURES**

**Structure Modeling**—An amino acid alignment of B-family regulatory subunits from different phyla was submitted to the 3D-PSSM protein fold recognition web server (39), which generated a first-round model based on the structure of the Gβ1 subunit of heterotrimeric G proteins (40). This model was globally and locally optimized for bond lengths, angles, and torsions of side chains using the steepest decent algorithm of the Swiss PDB Viewer software (41). In addition, breaks in the backbone were ligated with a cutoff value of 3.0 Å, and hydrogen atoms were added to the model. Ribbon diagrams and surface representations of the optimized B subunit model were rendered, annotated, and analyzed using Rasmol and Swiss PDB Viewer software.

**RESULTS AND DISCUSSION**

**Structure Prediction of PP2A B-family Regulatory Subunits**—Mammalian B-family regulatory PP2A subunits (Bo-β) display high degrees of sequence conservation (>80% amino acid identity). Secondary structure prediction suggests that B-family regulatory subunits are almost entirely composed of β-sheets and turns, whereas the B′ and B′′ subunits are mostly α-helical. Thus, PP2A regulatory subunit families have distinct primary and secondary structures. As has been noted previously (44), B-family regulatory subunits contain several degenerate WD repeats (four to seven, depending on the isoform and motif search threshold). WD (also called WD40 or Gβ) repeats are loosely defined, ~40-amino acid sequence motifs that often end with the tryptophan-aspartate (WD) dipeptide (45). The amino acid sequence of By, a representative member of the B subunit family, aligned by WD repeat motifs is shown in Fig. 1A. Seven degenerate WD repeats are separated by regions of 13–46 residues in length (c-d loops).

The two WD repeat-containing proteins whose three-dimensional structure has been solved to date are the Gβ1 subunit of heterotrimeric G proteins (40) and the p40 subunit of the arp2/3 actin filament branching complex (p40-ARC; Ref. 46). Both proteins fold into a seven-bladed β-propeller, a toroid structure in which seven twisted, antiparallel β-sheets are radially arranged around a common center. Each WD repeat contributes the outer (d) β-strand of one propeller blade and the inner three β-strands (a–c) of the next propeller blade (Fig. 1B). This phase-shift of sequence and structural motifs allows for closure of the torus by a “velcro” mechanism (45). Sequences preceding the first WD repeat and trailing the last repeat may protrude from the core toroid (Fig. 1B).

Three web-based threading protein fold prediction algorithms (3D-PSSM (39), FUGUE (47), and 123D (genomic.sanger.ac.uk/123D/123D.html)) identified Gβ1 as the closest structural homolog of PP2A B-family regulatory subunits, despite low sequence similarity (~15% identity). The structure of B-family regulatory subunits was modeled based on the Gβ1 crystal structure (see “Experimental Procedures”). A ribbon diagram of this model shows the seven-bladed β-propeller fold characteristic of WD repeat-containing proteins (Fig. 1C). Because PP2A B-family regulatory subunits are larger than Gβ1, portions of the larger loops connecting WD repeats are missing from the model.

**Deletion Mutagenesis**—To define regions and residues in B-family regulatory subunits critical for association with the AC core dimer, we carried out deletion and site-directed mutagenesis of the By coding sequence. Mutant B by cDNAs carrying an N-terminal FLAG epitope tag were transiently expressed in COS-M6 cells in combination with the scaffolding Aα subunit tagged with a C-terminal EE epitope (42). FLAG-By was immunoprecipitated and washed extensively, and in vivo incorporation into the PP2A heterotrimer was assayed by blotting By immunoprecipitates for transfected Aα and endogenous C subunits. The ability of By mutants to associate with the core enzyme was quantified by densitometry as the ratio of C to By subunit bands in the same lane. In general, mutating By af-
fected A and C subunit binding to similar degrees, supporting the notion that regulatory subunits interact with a structural unit of A and C subunits. A schematic diagram of the B deletion and truncation mutants is shown in Fig. 2A.

B subunit family members differ considerably in their first 20–30 residues. Deletion of the variable 20 N-terminal amino acids of B (1–20) had little effect on binding to the A and C subunit (Fig. 2B), consistent with a role of these residues in mediating isoform-specific functions. This deletion extends into the predicted first (d) -strand of WD repeat 1, which, according to the crystal structures of Gβ1 and p40-ARC, is critical for closure of the -propeller core by interacting with the c-strand of WD repeat 7 (see Fig. 1B). It is conceivable that the FLAG epitope tag can substitute for the 5 residues deleted from WD repeat 1; alternatively, the boundaries of this structural motif in B may require revision.

At the C terminus, truncating the 8 amino acids that follow the last WD repeat in B (440–447) had no effect on holoenzyme association. Extending the truncation by just 6 amino acids (434–447) to include the predicted c-strand of WD repeat 7 caused an almost complete loss of A and C subunit binding. Thus, residues 434–439 are required for holoenzyme association, presumably because they interact with N-terminal residues to maintain the toroid structure of B.

Four internal deletions throughout the B protein ranging from 12 to 32 residues in length completely abrogated coimmunoprecipitation of A and C subunits (3–7% of wild-type); only the 381–401 deletion displayed close to wild-type binding activity (Fig. 2B). Three of these critical deletions (128–156, 259–270, and 370–401) are predicted to affect surface-exposed loops connecting WD repeats, whereas Δ26–38 deletes a portion of WD repeat 1 predicted to be buried in the protein. The apparent intolerance of the B core (residues 21–439) to small deletions suggests that the interaction of B-family subunits with the AC dimer requires precise alignment of multiple interacting residues.

Charge-reversal Mutagenesis—Site-directed mutagenesis was carried out to delineate specific sites of holoenzyme interaction. All B residues that were mutated are perfectly conserved in other mammalian B-family isoforms and their orthologs in worms, fruit flies, and yeast. Carrying out similar mutagenesis experiments with the A subunit, we had previously identified charged residues in HEAT repeats 3 (Glu100...
and Glu\(^{101}\) and 5 (Arg\(^{183}\)) important for binding to regulatory subunits and viral tumor antigens (42). Hence, we focused the By mutagenesis on charge-reversal of basic and acidic residues with the goal of identifying electrostatic interactions with the Aα subunit.

Three acidic-to-basic mutations of conserved residues in the N-terminal third of By (E66R, EE89RR, and D112K) had no effect on holoenzyme association (Fig. 3). In contrast, four By mutants in WD repeat 3 (RR165EE, D184R, E186R, and DD192RR) and two mutants in the loop connecting WD repeat 3 and WD repeat 4 (D212K and IK213EE) displayed severely reduced binding to A and C subunits (between 2% and 12% of wild-type). Mapping to WD repeat 4, By mutant ED219RR incorporated into the PP2A holoenzyme normally, whereas E223R was defective. Because By Δ259–270 was binding-incompetent (Fig. 2B), we tested the effect of mutating all acidic residues in this region. By-D259R did not bind to the AC dimer, whereas By EE266RR, E269R, and D270R had little or no effect on the ability of By to associate with A and C subunits. Lastly, the E343R mutation in WD repeat 6 had an intermediate effect on the ability of By to incorporate into the PP2A heterotrimer (40% residual binding of the C subunit; Fig. 3).

The results of the deletion and site-directed mutagenesis experiments are summarized in Fig. 3B. By mutants were classified as critical or noncritical depending on the amount of coimmunoprecipitated C subunit (<15% and ≥40% of wild-type, respectively). Most critical amino acid substitutions cluster in the middle of the molecule (165–259) encompassing WD repeats 3 and 4. Li and Virshup (48) have recently reported that two fragments of B'α can bind to the Aα subunit in glutathione S-transferase pull-down assays. Intriguingly, the corresponding regions in Bo and B'PR72 also interacted with Aα, even though PP2A regulatory subunit families display little primary amino acid similarity and are classified into different structural families according to protein fold prediction algorithms (39, 47). The N-terminal “A subunit binding domain” defined by Li and Virshup corresponds to By residues 172–270, a region that we show here contains many residues necessary for holoenzyme association in vivo. The C-terminal A subunit binding domain encompasses By residues 302–360. We mutated E343 in this region, which, according to Li and Virshup’s domain alignment (48), is invariant in B, B', B'' subunits, and we observed an intermediate effect on PP2A holoenzyme formation.

Identification of Interacting Residues—We speculated that evolutionarily conserved and surface-exposed, charged residues of By interact with residues of opposite charge in Aα that

![Fig. 3. Identification of By residues important for holoenzyme association. A. wild-type (w.t.) FLAG-tagged By, the indicated site-directed mutants, or empty vector was expressed in COS-6 cells and tested for association with transfected Aα (EE-tagged) and endogenous C subunits by coimmunoprecipitation (coIP). The percentage binding of the C subunit was quantified as described in the Fig. 2 legend and is shown as the average of two to five experiments. B. summary of By deletion and site-directed mutagenesis results. Critical mutations displaying <15% wild-type C subunit binding activity are indicated on the top of the domain diagram; noncritical mutations (≥40% wild-type binding) are indicated on the bottom of the domain diagram.](image1)

![Fig. 4. Identification of interacting residues in By and Aα. Wild-type (w.t.) or mutant FLAG-tagged By and EE-tagged Aα subunits were coexpressed in the indicated combinations in COS-M6 cells and tested for association by FLAG immunoprecipitation, followed by immunoblotting for PP2A subunits.](image2)

![Fig. 5. Ubiquitination and proteasome-mediated degradation of monomeric By subunits in PC6-3 cells. A. the indicated combinations of wild-type (w.t.) and mutant By and Aα subunits (ER, EE100RR) were transiently expressed in PC6-3 cells, and total lysates were immunoblotted for By (FLAG tag) and the endogenous C subunit. B. indicated mutations of calcium/calmodulin-dependent protein kinase II (CaMKII) were expressed in PC6-3 cells and treated for 2 h before lysis with or without (−) or with (+) the proteasome inhibitor MG-132 (50 μM). Total lysates were immunoblotted for the indicated proteins. C. FLAG-By D212K was transfected and immunoprecipitated from PC6-3 cells treated for 6 h in the absence or presence of 50 μM MG-132. Aliquots of immunoprecipitates were blotted for the FLAG tag (left) and ubiquitin (right). To account for increased levels of By after MG-132 treatment, twice the volume of the control immunoprecipitate was analyzed. The distributions of high molecular weight FLAG tag and ubiquitin immunoreactivities do not correspond well because of the disproportion of ubiquitin and FLAG epitopes in larger By species.](image3)
we previously identified as critical for regulatory subunit association (Glu<sup>100</sup>, Glu<sup>101</sup>, and Arg<sup>183</sup>; Ref. 42). Consequently, we coexpressed charge-reversal mutants of the Aα subunit (EE100RR and R183E) with all opposite charge-reversal mutants of the Bγ subunit and tested for complementation, i.e. restoration of holoenzyme assembly by coimmunoprecipitation. We were unable to show association of any of the acidic-to-basic mutants of Bγ with the basic-to-acidic mutant Aα R183E (data not shown). There are three potential reasons for this: 1) we may have not mutated the interacting residue in Bγ, 2) Aα or Bγ mutations, while potentially affecting interacting residues, may introduce structural changes that misalign other important amino acids, and 3) Aα R183 may not interact directly with regulatory subunits.

However, when we paired Aα EE100RR with Bγ RR165EE, we observed binding that was comparable to wild-type subunits (Fig. 4). By RR165EE was unable to coimmunoprecipitate another binding-defective, acidic-to-basic Aα mutant, DW139RR (data not shown), demonstrating that the observed complementation is not a consequence of altering the overall charge of the proteins. Thus, PP2A holoenzyme association is critically dependent on electrostatic interactions between adjacent glutamates in the A subunit (Glu<sup>100</sup> and Glu<sup>101</sup> in Aα) and adjacent arginines in B-family regulatory subunits (Arg<sup>165</sup> and Arg<sup>166</sup> in Bγ). Because the EE100RR mutation in Aα interferes with binding of all regulatory subunit families (42), it is likely that B’ and B” subunits also interact via basic residues. We reversed the charge of a pair of conserved lysine residues in Bε that is in a position similar to By Arg<sup>165</sup> and Arg<sup>166</sup> (Bε KK173DD), but this mutation did not disrupt PP2A holoenzyme assembly (data not shown). Additional studies are necessary to identify points of contact between Aα and B’/B” subunits.

Monomeric By Is Degraded by the Ubiquitin/Proteasome Pathway—All By mutants could be expressed to similar, high levels in COS-M6 cells, a cell line that supports plasmid replication due to expression of the SV40 large T antigen. Studying the effects of By mutants in the neuronal PC6-3 subline of PC12 cells (49), in which much lower levels of expression can be achieved, we noticed that holoenzyme formation-defective mutants could be expressed to at most 10% of wild-type By levels. This is shown for two mutants in Fig. 5A. Importantly, expression levels of By RR165EE, but not D212K, could be rescued by coexpression of the complementary Aα EE100RR mutant, indicating that low expression is a consequence of failure to incorporate into the PP2A holoenzyme. To investigate the mechanism of this effect, PC6-3 cells were treated with the proteasome inhibitor MG-132 for 2 h before immunoblotting. Proteasome inhibition resulted in a massive increase of Bγ protein levels but had no effect on levels of another transfected protein (calcium/calmodulin-dependent protein kinase IIα) or the endogenous PP2A C subunit (Fig. 5B). MG-132 treatment led to the accumulation of higher molecular weight species of By D212K that were immunoreactive for ubiquitin (Fig. 5C).

Similar results were obtained with the RR165EE mutant and wild-type Bγ.

It was previously shown that transfected B’ subunits quantitatively incorporate into the PP2A holoenzyme (11) and that ablation of PP2A A or C subunits by RNA interference decreases the stability of regulatory subunits in <i>Drosophila</i> Schneider cells (25). The present results provide further evidence that PP2A subunit expression levels are stringently controlled in cells and suggest ubiquitination and proteasome-mediated degradation as a mechanism for rapid removal of monomeric regulatory subunits.

Structure modeling and site-directed mutagenesis support the model of PP2A holoenzyme structure shown in Fig. 6. B-family regulatory subunits adopt a β-propeller fold that is found in other proteins engaged in multiple protein-protein

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**Fig. 6. Model of the PP2A holoenzyme.** A space-filling representation of the structure of the Aα subunit (34) is arranged with model structures of the C and Bγ subunits (based on the PP1 catalytic subunit (38) and Gβ1 (40), respectively; see the text). Residues whose mutation disrupts subunit association (critical) are indicated in black; interacting residues are colored green. Arg<sup>183</sup> (R183) and Trp<sup>257</sup> (W257) are highlighted as representative of several critical residues in HEAT repeats 5 and 7 of the Aα subunit (42). Some critical Bγ residues are not shown because they are either absent from the model (Lys<sup>214</sup> and Asp<sup>259</sup>) or are buried (Asp<sup>192</sup>).
interactions (40, 46). By mutational complementation, we identified electrostatic interactions between two conserved arginines in the outer (d) strand of WD repeat 3 of B and two glutamates in the intrarepeat loop of HEAT repeat 3 of A. Previous domain mapping and site-directed mutagenesis of the A subunit (35, 36, 42) and the present data argue for multiple additional contacts between regulatory and scaffolding units. Also, regulatory subunit binding to the AC dimer is expendable for intersubunit interactions. Instead, N-terminal residues is the C subunit (50–53). Critical amino acids in B are located C-terminal of the A subunit-contacting residues Arg<sup>165</sup> and Arg<sup>166</sup> and cluster in WD repeats 3 and 4 and the intervening loop. We propose that this region forms extensive contacts with the intrarepeat loops of HEAT repeats 4–7 of the A subunit, where many residues important for regulatory subunit binding are localized (42). Consistent with possible isoform-specific functions, we find that the divergent N-terminal tail of B is expendable for intersubunit interactions. Instead, N-terminal residues of B-family regulatory subunits may determine the subcellular localization of PP2A holoenzymes by interacting with specific anchoring proteins (19). Compatible with this view, the B/N terminus faces away from the A subunit in our PP2A holoenzyme model. This report addresses the structural basis of PP2A holoenzyme function but requires ultimate verification and refinement by other methods such as crystallography.

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