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The Serine/Threonine Phosphatase PP5 Interacts with CDC16 and CDC27, Two Tetratricopeptide Repeat-containing Subunits of the Anaphase-promoting Complex*

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The evolutionarily conserved multisubunit complex known as the cyclosome or anaphase-promoting complex is involved in catalyzing the ubiquitination of diverse substrates in M phase, allowing their destruction by the 26 S proteasome and the completion of mitosis. Three of the eight subunits of the anaphase-promoting complex (CDC16, CDC23, and CDC27) have been shown to be phosphorylated in M phase, and their phosphorylation is required for the anaphase-promoting complex to be active as a ubiquitin ligase. Several subunits of the anaphase-promoting complex contain tetratricopeptide repeats, a protein motif involved in protein/protein interactions. PP5 is a serine/threonine phosphatase that also contains four copies of the tetratricopeptide repeats motif. Here we show by a combination of two-hybrid analysis and *in vitro* binding that PP5 interacts with CDC16 and CDC27, two subunits of the anaphase-promoting complex. Only the NH_2-terminal domain of PP5, containing all four tetratricopeptide repeats, is required for this physical interaction. Deletion analysis suggests that the site of binding to PP5 is localized to the COOH-terminal block of tetratricopeptide repeats in CDC16 and CDC27. In addition, indirect immunofluorescence showed that PP5 localizes to the mitotic spindle apparatus. The direct interaction of PP5 with CDC16 and CDC27, as well as its overlapping spindle localization in mitosis, suggests that PP5 may be involved in the regulation of the activity of the anaphase-promoting complex.

The sequential activation of cyclin-dependent kinases controls progression through the cell cycle (1). Cdc2 and its regulatory subunit cyclin B have been shown to drive the cell from G_2 into M phase, promoting the multiple events necessary for proper chromosome segregation between daughter cells (2). Along with kinase activation, proteolysis also plays a major role during the cell cycle (3–5; for review, see Ref. 6). The cyclosome, or anaphase-promoting complex (APC),[1] is a highly conserved multisubunit protein complex that recently has been shown to catalyze the ubiquitination of cyclin B, a necessary step to trigger its degradation by the 26 S proteasome pathway during telophase (6, 7). APC is required not only to exit M phase but also at an earlier stage to allow the initiation of anaphase. APC is activated at the metaphase-to-anaphase transition and apparently catalyzes the ubiquitination of several non-cyclin substrates; inhibition of APC leads to metaphase arrest (8, 9). In budding yeast it was demonstrated that PDST1, an inhibitor of chromosome segregation, was a good substrate for APC-dependent ubiquitination (10, 11). Destruction of this inhibitor is an obligatory step for anaphase to occur in yeast, and a similar requirement is likely in other organisms. Therefore, by targeting specific substrates for degradation at different stages of M phase, APC controls the progression and completion of mitosis (6). APC is also present at other stages of the cell cycle and may fulfill other crucial roles, particularly in coupling S and M phase, as suggested by genetic studies in yeast (12–15).

APC was recently purified from *Xenopus laevis* and is composed of eight subunits (16), two of which (CDC16 and CDC27) have also been characterized in yeast and mammals (9, 17–20). Four of the eight subunits of *X. laevis* APC contain tetratricopeptide repeats (TPR). This degenerate motif of 34 amino acids is widespread in evolution and is often found in tandem repeats. The TPR motif is thought to form amphipathic α-helices that mediate intra- and/or intermolecular protein/protein interactions (17, 21, 22). Indeed, in yeast it has been demonstrated that association between two APC subunits, CDC23 and CDC27, is dependent upon at least part of the TPR domain of each protein (17). Immunofluorescence studies in HeLa cells have shown that a portion of the cellular pool of CDC16 and CDC27 localizes to the spindle/centrosome throughout M phase, where it most likely acts as part of APC to catalyze the ubiquitination of diverse factors (9).

Recent evidence demonstrates that some subunits of *Xenopus* APC (CDC16, CDC23, and CDC27) become phosphorylated in M phase. Moreover, it was shown that only the phosphorylated form of APC was able to catalyze the ubiquitination of B-type cyclins and that this activity was abolished by phosphatase treatment of M phase-purified APC in a reconstituted system (16, 23). However, it remains to be discovered which kinases and phosphatases control these regulatory phosphorylations and dephosphorylations of APC.

PP5 is a recently discovered okadaic acid-sensitive, serine/threonine phosphatase whose substrates and biological functions are unknown and which is highly conserved throughout eukaryotes (24–26). The main structural distinction of PP5 compared with other serine/threonine phosphatases lies in the presence of a TPR domain, containing four repeats, located NH_2-terminal to the catalytic domain (24, 27). Several recent

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF018262 and AF018263.

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[1] The abbreviations used are: APC, anaphase-promoting complex; TPR, tetratricopeptide repeat(s); GST, glutathione *S*-transferase.

reports have shown that this TPR domain of PP5 is in fact important as an interface for protein/protein interactions. A direct interaction has been characterized between this TPR domain and the kinase-like domain of the atrial natriuretic peptide receptor (25). Additionally, a direct *in vivo* interaction between PP5 and the chaperone protein Hsp90 was shown to involve the TPR domain of PP5 (28, 29). Moreover, these studies indicate that PP5 is part of a large heterocomplex involved in the maturing process of steroid receptors. The presence of PP5 in this complex, which also includes Hsp90, the small acidic protein p23, and TPR-containing immunophilins, suggests that PP5 may be intimately involved in protein folding.

In an effort to determine whether PP5 may be involved in regulating the activity of APC, we have examined its physical interaction with two subunits of APC (CDC16 and CDC27) which are phosphorylated during M phase. We show that PP5 is in fact able to associate with CDC16 and CDC27 through their respective TPR domains. Furthermore, epitope-tagged PP5 can localize to the mitotic spindle where CDC16 and CDC27 have been shown to be present (9). These results suggest that PP5 may be involved in the regulation of APC activity.

EXPERIMENTAL PROCEDURES

Constructs—All constructs were made using standard techniques. Each construct was sequenced to verify the correct frame as well as the proper sequence of any linker introduced during the cloning procedure. The two-hybrid plasmids (pBTM116, pVP16, and pLexA-lamin) were generous gifts from S. Hollenberg and J. A. Cooper (Fred Hutchinson Cancer Research Center) (30, 31). To facilitate the construction of the multiple two-hybrid constructs used in this report, the polylinker region of the pBTM116 and the pVP16 plasmids was remodeled to make three different versions of pBTM116 or pVP16 (A, B, and C), differing in-frame at the *Bam*HI site and containing an additional *Not*I site. The LexA-lamin fusion (human lamin C amino acids 66–230 in pBTM116) used as a negative control has been described elsewhere (30). PEX5 fused to VP16 was a generous gift from S. Subramani (University of California, San Diego) and was used as an unrelated TPR-containing protein control for the two-hybrid experiments. All CDC16 and CDC27 constructs were derived from the human full-length CDC16 and CDC27 cDNAs in pBluescript, pSTU65, and pSTU16, respectively, and were generous gifts from P. Hieter (Johns Hopkins University School of Medicine).

Xenopus and Mouse PP5 cDNAs—A full-length mouse PP5 cDNA was isolated from a macrophage λ-Zap library (kindly provided by Dr. Chris Glass, University of California, San Diego), using a probe encoding part of the NH_2-terminal TPR domain of PP5, and both strands of the longest clone were completely sequenced. The PP5 full-length cDNA subcloned in pBluescript SK (pMW0551/2 and 069) was used to generate all mouse PP5-derived constructs of this study. A version of mouse PP5 with a COOH-terminal VSV-G-tag (32), designated PP5-tag, was engineered by ligating a pair of complementary oligonucleotides between a *Pst*I site in the COOH terminus of PP5 and a *Bam*HI in pMW0551/2 resulting in pMW059. These oligonucleotides reconstitute the COOH terminus of PP5 and extend it with the VSV-G-tag and a stop codon. The resulting PP5-tag was then subcloned into a pCDNA3 vector in the *Xba*I and *Eco*RI sites, creating pVO120. A nearly full-length cDNA encoding *Xenopus* PP5 was isolated from an oocyte cDNA library with a mouse PP5 probe (TPR domain only) using low stringency hybridization conditions. The longest *Xenopus* PP5 cDNA was fully sequenced on both strands.

Glutathione S-Transferase (GST) Fusion Constructs—To make GST fusion constructs, the *Bam*HI/*Eco*RI fragment of CDC27 (amino acids 295–823) was cut out from LexA-CDC27(295–823) and ligated into pGEX-KG to generate GST-CDC27(295–823). Similarly, the CDC27 *Bgl*II/*Eco*RI fragment (amino acids 537–823) was cloned into pGEX-3X to generate GST-CDC27(537–823).

Two-hybrid Interactions—The two-hybrid interaction assays were done according to previously published protocols using the *Saccharomyces cerevisiae* strain L40 (30, 31). As an initial step, each LexA fusion was tested against VP16 alone to determine whether it could activate the transcription of the reporter genes *HIS3* and *LACZ*, in which case it was eliminated from further study. Similarly, each VP16 fusion was assayed with a LexA-lamin fusion control. Plasmids encoding a fusion between the DNA binding domain LexA and the construct of interest were cotransformed in the L40 strain with a plasmid encoding a fusion between the activation domain VP16 and a second protein of interest. After growth of the double transformants on selective media (−Trp, −Leu plates), several individual transformants were tested for their ability to activate the transcription of the two integrated reporter genes *HIS3* and *LACZ*. An interaction was scored positive based on the ability to activate transcription of the *HIS3* reporter gene, based on whether yeast colonies were able to grow on −His plates after 3–5 days at 30 °C. The ability to activate the *LACZ* reporter was also checked by a filter assay as described (30).

Expression of PP5-tag from Baculovirus-infected Sf9 Cells—VSV-G-tagged PP5 (PP5-tag) from pVO120 (*Bam*HI/*Cla*I) was inserted into SLP10 baculovirus vector, and recombinant full-length PP5-tag was expressed in Sf9 cells using baculovirus as described (33). To check for expression of PP5-tag, aliquots of PP5-tag Sf9 extracts and SLP10 Sf9 extracts (mock) were analyzed by immunoblotting using the P5D4 antibody. A band of 58 kDa corresponding to PP5-tag was detected in the PP5-tag baculovirus-infected cells but not in the mock-infected cells. These PP5-tag extracts were then used for *in vitro* binding assays.

In Vitro Binding—The synthesis of GST-CDC27(295–823) and GST-CDC27(537–823) in Y1090 cells was induced for 4 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The bacterial pellet was resuspended in 18 ml of TEDG buffer (50 mM Tris, pH 7.5, 0.4 M NaCl, 1.5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin). GST fusion proteins were incubated with glutathione-agarose beads (Sigma) at 4 °C, and washed once in binding buffer (8 mM Tris, pH 7.5, 120 mM KCl, 8% glycerol, 4 mM dithiothreitol, 0.1% Nonidet P-40). Beads were then incubated with 5 μl of unfractionated whole cell extracts of Sf9 cells expressing PP5-tag in 400 μl of binding buffer containing 1% milk. After 2 h of rotating at 4 °C, the binding reactions were washed three times in the same binding buffer with no milk. Beads were finally resuspended in sample buffer, boiled for 5 min, and the products of the binding reaction were resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transfer to nitrocellulose and blocking (in 5% milk, 150 mM NaCl, 50 mM Tris, pH 7.5, 0.02% Tween 20), the membrane was immunoblotted with an anti-VSV-G-tag antibody (P5D4, 1/1,000 dilution), then with anti-mouse horseradish peroxidase-conjugated secondary antibody (1/3,000 dilution), and detected by ECL treatment (Amersham). The membrane was reprobed with anti-CDC27 antibody (9) (1/2,000 dilution) followed by anti-rabbit horseradish peroxidase (1/4,000 dilution) to check for the presence of the expected GST-CDC27 fusions.

Indirect Immunofluorescence—COS-1 cells were transfected as described (34) by calcium phosphate precipitation. After 20 h, cells were refed and grown an additional 20–24 h. Cells were fixed using fresh 3% paraformaldehyde in phosphate-buffered saline for 10 min, permeabilized for 30 min in phosphate-buffered saline containing 0.1% Triton X-100, 0.2 M glycine, and 2.5% fetal bovine serum. Coverslips were incubated with the primary antibody (P5D4, 1/1,000 dilution), directed against the VSV-G-tag, then with a fluorescein-conjugated secondary antibody (goat anti-mouse antibody, 1/750 dilution). When double labeling was performed, cells previously treated as above were then incubated with rat anti-tubulin antibody (1/500 dilution) and Texas red anti-rat antibody (Biomeda Corp., 1/500 dilution). Coverslips were mounted on slides with 100 mM Tris, pH 8.0, 90% glycerol, 1 mg/ml phenylenediamine, containing Hoechst dye 33342 (1 μg/ml) to detect DNA.

RESULTS

PP5 Is a Highly Conserved Serine/Threonine Phosphatase Containing Four TPR—PP5 is a serine/threonine phosphatase that contains an NH_2-terminal TPR domain with four repeats (Fig. 1*A*). The sequence of human and rat PP5 has been reported elsewhere (24, 25, 35). As part of this study, we isolated and sequenced cDNAs encoding PP5 from mouse and *X. laevis* cDNA libraries. Fig. 1*B* presents an alignment of PP5 protein sequences, including human (24, 26), rat (25, 35), mouse, and *Xenopus* (this study; GenBank accession numbers AF018262 and AF018263, respectively). The high degree of homology observed among the PP5 sequences from four vertebrates demonstrates that this phosphatase is highly conserved, not only in the COOH-terminal phosphatase domain but also in the NH_2-terminal TPR domain thought to be responsible for determin-

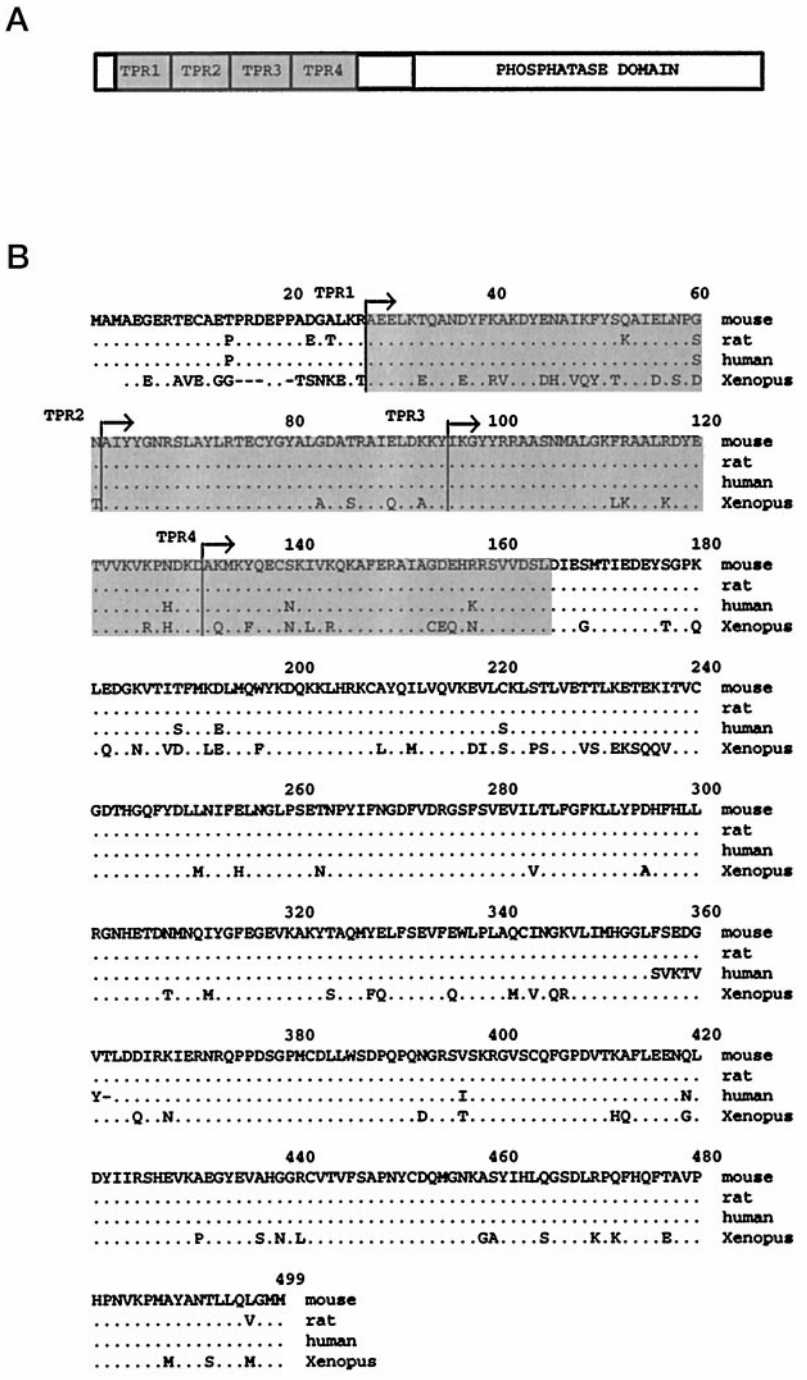

FIG. 1. **Protein structure and sequence comparison of the serine/threonine phosphatase PP5 from vertebrate species.** *Panel A*, schematic representation of PP5 with the four NH_2-terminal TPR and the COOH-terminal phosphatase domain indicated. *Panel B*, alignment of PP5 amino acid sequences from mouse and *Xenopus* (this study) and rat and human (24–26, 35). Amino acid changes compared with the mouse sequence are indicated by *capital letters*, amino acid identities by *dots*, and deleted residues by *dashes*.

ing the specificity of interacting proteins.

Two-hybrid Interaction between PP5 and Two Subunits of APC (CDC16 and CDC27)—To investigate whether PP5 may interact with some TPR proteins belonging to APC, fusion proteins were constructed using the two-hybrid system vectors pBTM116 and pVP16. Fig. 2 summarizes the constructs used in this study for PP5, CDC16, and CDC27. Each full-length or deletion construct was fused independently either to the LexA DNA binding domain (in pBTM116), or to the VP16 activation domain (in pVP16). Interactions were tested by cotransformation in the *S. cerevisiae* strain L40, containing the two reporter genes, *HIS3* and *LACZ*, which are under the control of upstream LexA operating sequences.

PP5 Interacts with CDC16 and CDC27 through Its Amino-terminal TPR Domain—As shown in Fig. 3, full-length PP5(1–499) strongly interacts with CDC16(2–619), CDC27(2–823), and CDC27(237–823) fusions. The TPR domain alone, PP5(4–165), was sufficient for strong interaction with CDC16 and CDC27, demonstrating that the phosphatase domain of PP5 is not required for this association. In general, these interactions were observed regardless of whether the TPR domain of PP5 was assayed as a fusion with the VP16 activation domain or as a fusion with the LexA DNA binding domain (Fig. 3 and Tables I and II). Other investigators recently demonstrated by coimmunoprecipitation that CDC16 interacts with CDC27 in yeast (17). We exploited this reported interaction as a positive control and demonstrated that human CDC16(2–619) interacts with human CDC27(2–823) (Fig. 3 and Table III). Furthermore, deleting the three NH_2-terminal TPR of CDC16 (CDC16(142–619)) did not affect its binding to CDC27(2–823) (Table III). In contrast, deleting the NH_2-terminal 294 amino acids of CDC27 abrogates the interaction with CDC16(142–619), suggesting

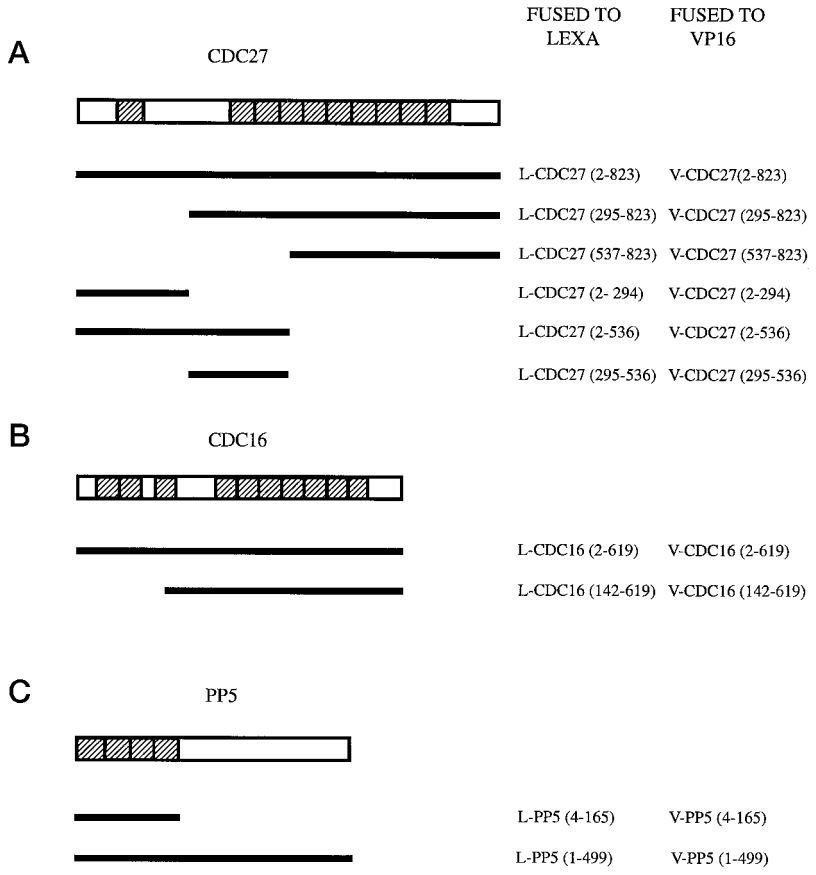

FIG. 2. **CDC27, CDC16, and PP5 constructs.** The human CDC27 and CDC16 and the mouse PP5 cDNAs were used to derive all constructs. *Hatched boxes* represent the position of a TPR motif. Both CDC27 and CDC16 contain 10 TPR. The phosphatase PP5 has only four TPR. The amino acid limits of the different deletions fused to LexA and VP16 in the two-hybrid vectors pBTM116 and pVP16 are shown in *parentheses* after the name of the construct. For instance, L-CDC27(2–823) denotes a fusion between the DNA binding domain LexA and the CDC27 protein from amino acid 2 to amino acid 823. V-CDC27(2–823) denotes the same CDC27 construct fused to the VP16 activation domain. The other CDC27, CDC16, and PP5 constructs were named accordingly.

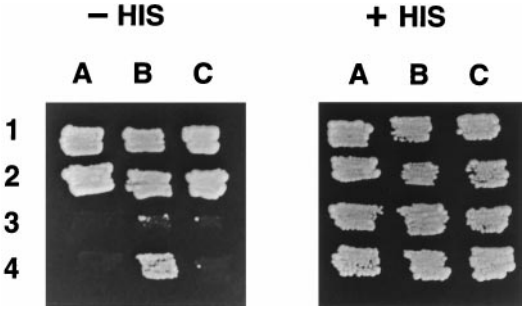

FIG. 3. **Two-hybrid interactions between PP5, CDC16, and CDC27.** The *S. cerevisiae* strain L40 was cotransformed with a combination of LexA fusions and VP16 fusions. *Left*, the yeast strain expressing two interacting hybrid proteins grows on −His plates. *Right*, all cotransformed strains grow in the presence of His. *1A*, L-CDC27(295–823) with V-PP5(4–165); *1B*, L-PP5(4–165) with V-CDC27(2–823); *1C*, L-PP5(4–165) with V-CDC27(295–823); *2A*, L-CDC16(2–619) with V-PP5(4–165); *2B*, L-PP5(1–499) with V-CDC27(2–823); *2C*, L-PP5(1–499) with V-CDC27(295–823); *3A*, L-CDC27(295–536) with V-PP5(4–165); *3B*, L-lamin with V-CDC27(2–823); *3C*, L-PP5(4–165) with V-PEX5; *4A*, L-lamin with V-PP5(4–165); *4B*, L-CDC16(2–619) with V-CDC27(2–823); *4C*, L-CDC27(2–823) with V-PEX5.

TABLE I
PP5/CDC27 two-hybrid interactions

LexA fusions	VP16 fusions	Interaction
L-PP5(4–165)	V-CDC27(2–823)	+
L-PP5(4–165)	V-CDC27(295–823)	+
L-PP5(4–165)	V-CDC27(537–823)	−
L-PP5(4–165)	V-CDC27(2–294)	−
L-PP5(4–165)	V-CDC27(2–536)	−
L-PP5(4–165)	V-CDC27(295–536)	−
L-PP5(1–499)	V-CDC27(2–823)	+
L-PP5(1–499)	V-CDC27(295–823)	+
L-PP5(1–499)	V-CDC27(537–823)	−
L-PP5(1–499)	V-CDC27(2–294)	−
L-PP5(1–499)	V-CDC27(2–536)	−
L-CDC27(295–536)	V-PP5(4–165)	−
L-CDC27(295–823)	V-PP5(4–165)	+
L-CDC27(295–823)	V-PP5(1–499)	+

TABLE II
PP5/CDC16 two-hybrid interactions

LexA fusions	VP16 fusions	Interaction
L-PP5(4–165)	V-CDC16(2–619)	−
L-PP5(4–165)	V-CDC16(142–619)	+
L-PP5(1–499)	V-CDC16(2–619)	−
L-PP5(1–499)	V-CDC16(142–619)	+
L-CDC16(2–619)	V-PP5(4–165)	+
L-CDC16(2–619)	V-PP5(1–499)	+
L-CDC16(142–619)	V-PP5(1–165)	+
L-CDC16(142–619)	V-PP5(1–499)	+

that the NH_2-terminal region of CDC27 (containing a single TPR) is important for binding to CDC16.

PP5, CDC16, and CDC27 Are Unable to Bind to PEX5, Another TPR-containing Protein—To confirm the specificity of the PP5 interaction with CDC16 and CDC27, we examined the PP5 TPR domain for its ability to interact with another member of the TPR family, the peroxisomal receptor protein PEX5, which contains eight TPR (36, 37). When assayed in the two-hybrid system as LexA fusion proteins, the TPR domain of PP5 was unable to interact with PEX5 fused to the VP16 activation domain (Fig. 3 and Table IV). Similarly, there was no interaction between different CDC16 or CDC27 LexA fusions and VP16-PEX5 (Fig. 3 and Table IV). These results suggest that PP5, CDC16, and CDC27 do not bind indiscriminately to all TPR-containing proteins and that the interactions of PP5 with the two subunits of APC examined here represent specific protein/protein interactions.

Identification of the Regions of CDC16 and CDC27 Involved in the Binding of the PP5 TPR Domain—A series of constructs

TABLE III
CDC16/CDC27 two-hybrid interactions

LexA fusions	VP16 fusions	Interaction
L-CDC16(2–619)	V-CDC27(2–823)	+
L-CDC16(142–619)	V-CDC27(2–823)	+
L-CDC16(142–619)	V-CDC27(295–823)	–
L-CDC16(142–619)	V-CDC27(537–823)	–
L-CDC16(142–619)	V-CDC27(2–294)	–
L-CDC16(142–619)	V-CDC27(2–536)	–
L-CDC16(2–619)	V-CDC16(2–619)	–
L-CDC16(142–619)	V-CDC16(142–619)	–
L-CDC27(295–823)	V-CDC16(2–619)	–
L-CDC27(295–823)	V-CDC27(2–823)	–
L-CDC27(295–823)	V-CDC27(295–823)	–
L-CDC27(295–823)	V-CDC27(537–823)	–
L-CDC27(295–823)	V-CDC16(142–619)	–

TABLE IV
Control two-hybrid interactions

LexA fusions	VP16 fusions	Interaction
L-PP5(4–165)	VP16	–
L-PP5(1–499)	VP16	–
L-CDC27(295–823)	VP16	–
L-CDC27(295–536)	VP16	–
L-CDC16(2–619)	VP16	–
L-LAMIN	VP16	–
L-LAMIN	V-PP5(4–165)	–
L-LAMIN	V-PP5(1–499)	–
L-LAMIN	V-CDC27(2–823)	–
L-LAMIN	V-CDC27(295–823)	–
L-LAMIN	V-CDC27(537–823)	–
L-LAMIN	V-CDC27(2–294)	–
L-LAMIN	V-CDC27(2–536)	–
L-LAMIN	V-CDC27(295–536)	–
L-LAMIN	V-CDC16(2–619)	–
L-LAMIN	V-CDC16(142–619)	–
L-LAMIN	V-PEX5	–
L-PP5(4–165)	V-PEX5	–
L-PP5(1–499)	V-PEX5	–
L-CDC27(295–823)	V-PEX5	–
L-CDC27(295–536)	V-PEX5	–
L-CDC16(2–619)	V-PEX5	–
L-CDC16(142–619)	V-PEX5	–

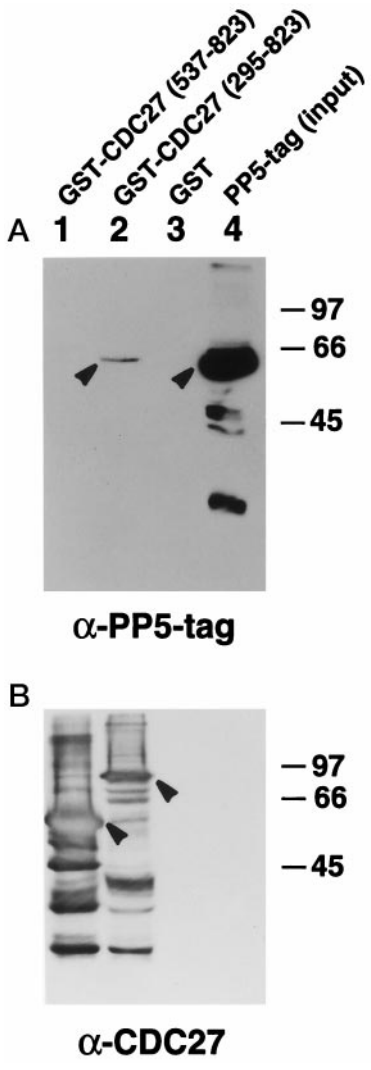

FIG. 4. ***In vitro* binding of PP5 with GST-CDC27(295–823) but not GST-CDC27(537–823).** GST, GST-CDC27(295–823), and GST-CDC27(537–823) were expressed in *Escherichia coli*. Equivalent amounts of proteins were bound on GSH-agarose beads, and the washed beads were incubated with extracts of Sf9 cells expressing PP5-tag. After washings, bound proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by an immunoblotting with a monoclonal antibody directed against the VSV-G-tag (*panel A*). The membrane was then reprobed with an anti-CDC27 antibody (*panel B*). The *arrows* in *panel A* denote PP5-tag bound to GST-CDC27(295–823) (*lane 2*) and PP5-tag present in the Sf9 cell extracts (*lane 4*). In *panel B*, the *left arrow* denotes the GST-CDC27-(537–823), and the *right arrow* shows the GST-CDC27(295–823).

was made to delineate further the region of interaction between PP5 and CDC16 and CDC27. A deletion of the three NH_2-terminal TPR of CDC16 (CDC16(142–619)) did not affect its interaction with PP5, indicating that this region is not required for binding (Table II). Similarly, we observed that deletion of the NH_2-terminal 294 residues of CDC27 (CDC27(295–823)), which includes the first of 10 TPR, had no impact on its interaction with PP5 (Table I). However, further subdivision of the nine remaining TPR abolished any interaction of the CDC27 fusions (537–823, 2–294, 2–536, 295–536) with PP5, demonstrating that the integrity of at least part of the nine repeats present at the COOH terminus of CDC27 is important for binding with PP5 (Fig. 3 and Table I). This result also confirms that the interaction between CDC27 and PP5 is dependent on the presence of the TPR in each protein. This, together with the lack of interaction observed with PEX5, reinforces the conclusion that the TPR domain of PP5 does not bind nonspecifically to TPR-containing domains in general.

In Vitro Binding between GST-CDC27 and PP5-tag—To confirm our two-hybrid analysis, GST-fusion proteins of CDC27 and CDC16 were made and tested in a binding experiment with a VSV-G epitope-tagged derivative of PP5 expressed in baculovirus-infected insect cells. Immunoblot analysis of the binding reaction using an antiserum against the VSV-G-tag (32) indicates that PP5 is able to interact with a GST-CDC27 fusion protein containing the nine COOH-terminal TPR of CDC27 (Fig. 4*A*, *lane 2*) but not with GST alone (Fig. 4*A*, *lane 3*). Consistent with the two-hybrid analysis, there was no detectable binding of PP5 with a GST-CDC27 fusion protein containing only five COOH-terminal TPR (Fig. 4*A*, *lane 1*). The membrane was reprobed with an anti-CDC27 antibody to confirm the presence of the GST-CDC27 fusion proteins in the binding assay (Fig. 4*B*). These *in vitro* binding results confirm that PP5 binds to CDC27 in the same region defined by the two-hybrid experiment. However, we were unable to see significant binding of PP5-tag to GST-CDC16 fusion proteins, suggesting that the optimal binding conditions were different for CDC16 and CDC27 in this *in vitro* binding assay or, alternatively, that the interaction between PP5 and CDC16 requires an additional factor (data not shown).

Localization of PP5 to the Mitotic Spindle—Colocalization immunofluorescence studies with tubulin have shown that CDC16 and CDC27 are present on the mitotic spindle apparatus from prophase to cytokinesis (9). Because PP5 can bind directly to these two subunits of APC, we wanted to determine

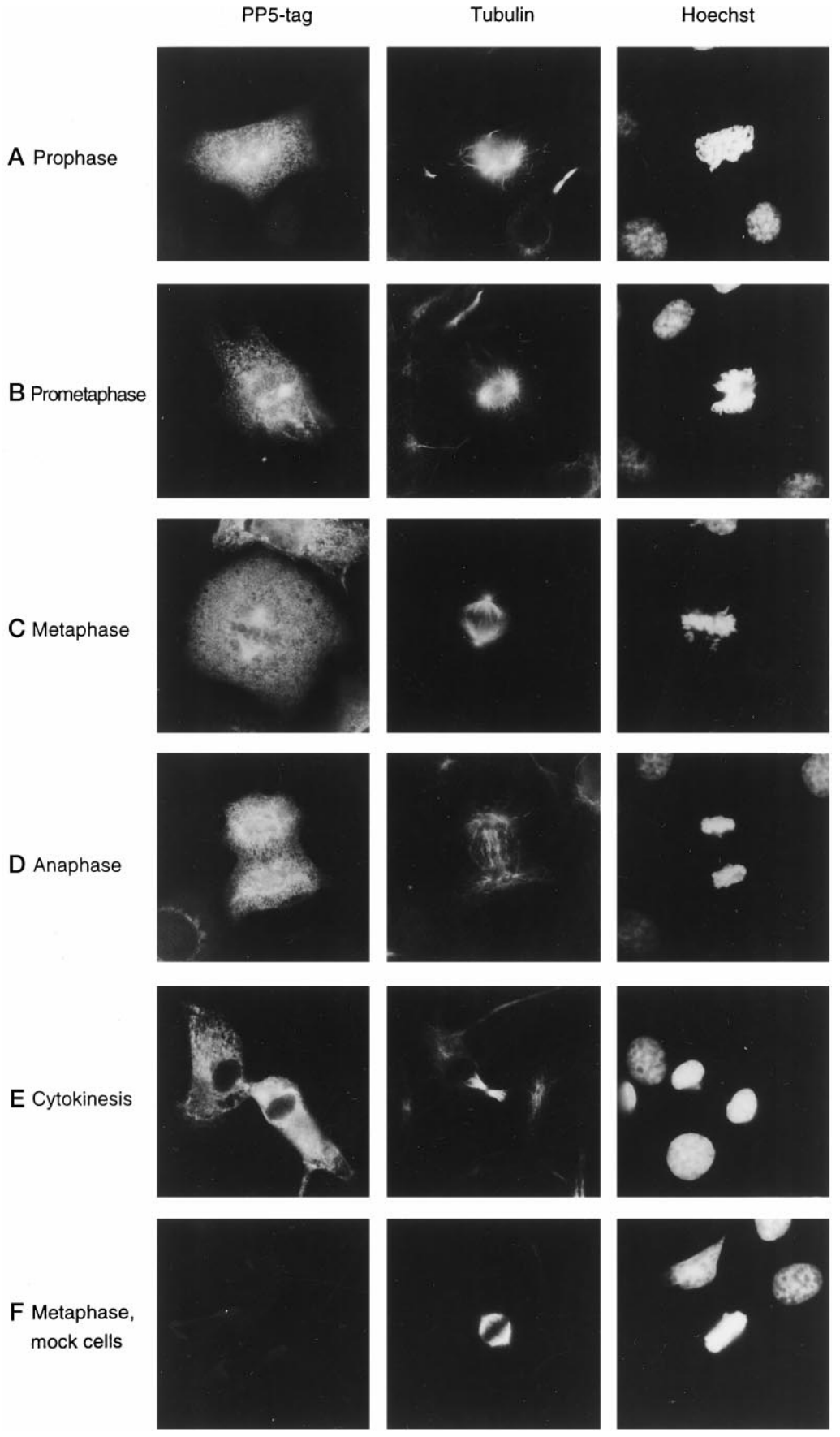

FIG. 5. **Immunolocalization of PP5-tag at different mitotic stages.** PP5-tag (*panels A–E*) and mock- (*panel F*) transfected COS-1 cells grown on coverslips were subjected to indirect immunofluorescence. Cells were triple stained with anti-PP5-tag antibody (P5D4, *left panels*), anti-α-tubulin (*middle panels*), and Hoechst dye (*right panels*). Fluorescein-conjugated anti-mouse and Texas red-conjugated antisera were used to detect PP5-tag and α-tubulin, respectively.

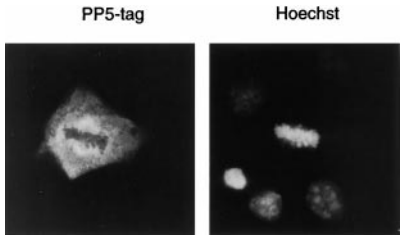

FIG. 6. **Single label immunolocalization of PP5-tag.** PP5-tag-transfected COS-1 cells were subjected to immunofluorescence as in Fig. 5 but with no tubulin staining.

whether PP5 can also localize to the spindle. Indeed, we observed significant PP5 localization to the spindle (Fig. 5*C*) in mitotic cells. Mock-transfected control cells exhibited no staining of the mitotic apparatus (Fig. 5*F*). Double label immunofluorescence with an anti-tubulin antibody confirmed that PP5 colocalized with the duplicated centrosomes in prophase (Fig. 5*A*) and with some, if not all, of the fibers forming the spindle in prometaphase and metaphase cells (Fig. 5, *B* and *C*). However, the staining of PP5 was more diffuse than that of tubulin, suggesting similar but not identical localization (Fig. 5*C*). In contrast to tubulin, there was no obvious localization of PP5 to the aster fibers (Fig. 5*C*). Single label immunofluorescence confirmed PP5 staining of the mitotic spindle (Fig. 6), ruling out the possibility that the observed colocalization with tubulin was caused by cross-reactivity between the secondary antibodies or bleed-through between the fluorescein and Texas red channels. In anaphase, no obvious staining of PP5 was observed on the spindle (Fig. 5*D*), suggesting the dissociation of PP5 from the mitotic spindle at the completion of metaphase. Finally, clear midbody localization of PP5 was not detected during cytokinesis (Fig. 5*E*), in contrast to what was observed for CDC16 and CDC27 (9). Taken together, these immunofluorescence data show that PP5 localizes to the mitotic spindle, at least through metaphase.

DISCUSSION

PP5 Interacts with CDC16 and CDC27—PP5 binds to some proteins through its NH_2-terminal TPR-containing domain, including the chaperone Hsp90 (28). As proposed by others (28, 29, 38), the TPR domain of PP5 therefore appears to be responsible for its specificity in binding putative substrate proteins. We have examined the ability of PP5 to interact with two different subunits of APC, CDC16 and CDC27, both of which contain TPR. A combination of two-hybrid analysis and *in vitro* studies allows us to conclude that PP5 interacts with CDC16 and CDC27 through its NH_2-terminal TPR domain. By using deletion constructs of the CDC16 and CDC27 proteins, we were able to delineate the site of interaction with PP5. We demonstrated that the TPR of CDC27 were required for binding with PP5. An *in vitro* binding assay between PP5 and GST-CDC27 further confirmed our two-hybrid analysis. Taken together, the binding data suggest that the TPR domain of PP5 is able to interact specifically with the TPR-containing COOH-terminal region of these two subunits of APC.

PP5 Localizes to the Mitotic Spindle—Transfection of an epitope-tagged derivative of PP5 in COS-1 cells allowed us to detect PP5 localized to the mitotic spindle. Although Chen *et al.* (24) did not observe identical localization of endogenous PP5, this could be explained by the low abundance of PP5 or its inaccessibility to antibodies. Using the epitope-tagged derivatives described in this study, our immunolocalization data clearly suggest that PP5 localizes to the mitotic spindle and may thus play an important role in M phase. Because our binding data also demonstrate an interaction between PP5 and two TPR-containing subunits of APC which are associated with the spindle/centrosome throughout mitosis (9), we conclude that the spindle localization of PP5 reflects a biologically significant function.

Putative Roles of the PP5/CDC16 and PP5/CDC27 Interactions—CDC16 and CDC27, along with at least one other subunit of APC, CDC23, become phosphorylated at the metaphase/anaphase transition, and these regulatory phosphorylations have been shown to be necessary for the ubiquitination of B-type cyclins (16). Therefore, one possible role of PP5 would be to maintain a dephosphorylated, inactivated state of APC just prior to anaphase. An okadaic acid-sensitive phosphatase has indeed been shown to control the ubiquitination of B-type cyclins, possibly through APC dephosphorylation; however, the phosphatase responsible for this activity has not been identified (23).

During anaphase and telophase when APC is activated as part of a ubiquitin ligase complex, PP5 may not remain associated with APC. Indeed, we could not detect any obvious localization of PP5 on the spindle/centrosome from anaphase to cytokinesis, indicating that PP5 may dissociate from the mitotic apparatus at the metaphase/anaphase transition, at which time APC becomes fully activated and remains present on the spindle. Although it is unknown whether APC subunits are still phosphorylated in G_1, APC remains active for a portion of G_1 prior to inactivation of its ubiquitin ligase activity (39). It would therefore be interesting to determine whether PP5 plays a role in this regulatory event.

Despite numerous attempts, we were unable to observe an *in vivo* interaction between subunits of APC and PP5. Using extracts from interphase cells or from cells arrested in metaphase by taxol treatment, there was no detectable coimmunoprecipitation of transfected PP5-tag and endogenous CDC27 or CDC16. Similarly, Chen *et al.* (40) were unable to detect an *in vivo* interaction between CDC27 and unphosphorylated Rb, although they characterized this interaction thoroughly using a combination of *in vitro* binding assays and two-hybrid interactions. This apparent lack of *in vivo* interaction, however, may be caused by the technical problem that APC, containing CDC16 and CDC27, is a very large multisubunit complex of more than 1,000 kDa, which may compromise efficient coimmunoprecipitation.

In summary, our results demonstrate interactions between PP5 and both CDC16 and CDC27, together with a similar spindle localization for these proteins during M phase. Alternatively, the localization of PP5 on the spindle may reflect another role independent of the regulation of APC activity. For instance, many distinct kinases are involved in mitosis and exhibit spindle localization, and, conceivably, the role of PP5 may be to regulate one or more of these activities. Indeed, we have observed that PP5 is able to interact with cdc2 and mos, two kinases that exhibit spindle localization during mitosis (41–43). Therefore, the interaction between PP5 and subunits of APC may simply serve to anchor PP5 to the spindle, facilitating its interaction with such kinases or other unidentified targets.

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The Serine/Threonine Phosphatase PP5 Interacts with CDC16 and CDC27, Two Tetratricopeptide Repeat-containing Subunits of the Anaphase-promoting Complex

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