The Scaffolding A/Tpd3 Subunit and High Phosphatase Activity Are Dispensable for Cdc55 Function in the \textit{Saccharomyces cerevisiae} Spindle Checkpoint and in Cytokinesis*

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Protein serine/threonine phosphatase 2A (PP2A) is a multifunctional enzyme whose trimeric form consists of a scaffolding A subunit, a catalytic C subunit, and one of several regulatory B subunits (B, B', and B''). The adenovirus E4orf4 protein associates with PP2A by directly binding the B or B'' subunits. An interaction with an active PP2A containing the B subunit, or its homologue in yeast, Cdc55, is required for E4orf4-induced apoptosis in mammalian cells and for induction of growth arrest in \textit{Saccharomyces cerevisiae}. In this work, Cdc55 was randomly mutagenized by low-fidelity PCR amplification, and Cdc55 mutants that lost the ability to transduce the E4orf4 toxic signal in yeast were selected. The mutations obtained by this protocol inhibited the association of Cdc55 with E4orf4, or with the PP2A-AC subunits, or both. Functional analysis revealed that a mutant that does not bind Tpd3, the yeast A subunit, as well as wild type Cdc55 in a \textit{tpd3A} background, can form a heterodimer with the catalytic subunit. This association requires C subunit carboxyl methylolation. The residual phosphatase activity associated with Cdc55 in the absence of Tpd3 is sufficient to maintain a partially active spindle checkpoint and to prevent cytokinesis defects.

PP2A\(^1\) is one of the major protein serine/threonine phosphatases in the cell, which plays a role in several cellular processes, including metabolism, transcription, RNA splicing, translation, cell cycle progression, morphogenesis, signal transduction, development, and transformation (1, 2).

Several reports indicate that the predominant form of PP2A in cells is a heterotrimer consisting of three subunits. Two of them, the 36-kDa catalytic C subunit and the 63-kDa scaffolding A subunit (PR65), form the core enzyme, and the regulatory B subunit binds the core enzyme to form the holoenzyme. The A and C subunits both exist as two isoforms (\(\alpha\) and \(\beta\)), which are closely related, whereas the B subunit is variable, and its multiple isoforms belong to at least three unrelated gene families, B/B55/PR55 (\(\alpha-\delta\) isoforms), B'/B56/PR61 (\(\alpha-\epsilon\) isoforms), and B'/PR72/PR130/PR59/PR48 (3). The core PP2A enzyme has also been shown to bind other cellular proteins, including striatin and SG2NA (4). Viral proteins, such as the SV40 small t antigen and the polyomavirus small and middle T antigens, can replace the cellular B subunits (5). The various cellular PP2A B subunits target the PP2A holoenzyme to different substrates and dictate its subcellular localization (reviewed in Ref. 3).

In \textit{Saccharomyces cerevisiae}, two closely related genes, PP2H1 and PP2H22, redundantly encode the major PP2A catalytic subunit (6). TPD3 encodes the only A subunit, and two distinct B subunits, encoded by CDC55 and RTS1, are highly homologous to mammalian B and B', respectively (7–9). Mutations of CDC55 are viable, but yield defects in cytokinesis and in the spindle checkpoint and result in abnormal cell morphology. Methylation of the C-terminal leucine residue of the PP2A catalytic subunit by a specific methyltransferase increases the affinity of this subunit for the regulatory subunits B/Cdc55 and B'/Rts1 and for Tpd3 both \textit{in vitro} and \textit{in vivo} (10–13). Yeast Cdc55 and mammalian PP2A-B55 subunits share an extensive homology (53% identity and 67% similarity) (7), and the middle third of both proteins shows 83% similarity. Members of the B third subunit family have been shown to contain four to seven WD repeats (14, 15).

Adenovirus E4orf4 protein is a multifunctional viral regulator. It down-regulates expression of genes that have been activated by E1A and cAMP (16, 17), induces hypophosphorylation of various viral and cellular proteins (16, 18), regulates alternative splicing of adenovirus mRNAs (18), and induces p53-independent apoptosis in transformed cells (19–21). E4orf4-induced apoptosis is specific to transformed cells (19–21). E4orf4 interacts with the PP2A holoenzyme through a direct association with the B0/B55 subunit (17), and can also bind PP2A complexes containing B'/B56 (23). We, and others, have recently shown that the interaction with PP2A complexes that include the B0/B55, but not the B'/B56 subunit, is required for induction of apoptosis by E4orf4 (22–24). Binding of the B subunit alone is not sufficient for induction of apoptosis, because an E4orf4 mutant, A3 (S95P), could bind the B subunit, whereas the A and C subunits were excluded from the complex, causing A3 to act as a dominant negative mutant (23). The presence of an active PP2A also contributes to other functions performed by the E4orf4 protein, including down-regulation of transcription (17, 25, 26) and alternative splicing (18).

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We have recently demonstrated that E4orf4 induces PP2A-dependent growth arrest in \textit{S. cerevisiae}, by targeting PP2A to the anaphase-promoting complex/cyclosome (APC/cyclosome) and inhibiting the activity of the APC/C in mitosis (27). Our

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1 The abbreviations used are: PP2A, protein phosphatase 2A; E4orf4, early region 4 open reading frame 4; WT, wild type; APC/C, anaphase-promoting complex/cyclosome; HA, hemagglutinin.
results, together with previously reported work, further suggested that E4orf4 could be enhancing a physiological interaction of PP2A with the APC/C. Thus, the work in yeast indicated that E4orf4 can be used as a tool to further characterize PP2A function and regulation. We have also shown that the yeast system can be utilized to select for non-apoptotic mutants of E4orf4, which do not bind PP2A (28). These results indicate that a biological selection assay based on E4orf4 toxicity in yeast can identify mutations in the E4orf4-PP2A complex, which affect interactions within the complex.

In this work we designed a protocol to select for Cdc55 mutants that lost the ability to transduce the E4orf4 toxic signal. These mutants define residues in Cdc55 required for the interaction with the core enzyme and with E4orf4. Functional characterization of the mutants and the WT protein revealed that a low level of binding of the regulatory B/Cdc55 subunit to the catalytic C subunit is retained in the absence of the scaffolding A/Tpd3 subunit. The minimal PP2A activity associated with this complex is sufficient for maintaining a partially active spindle checkpoint and an apparently normal cytokinesis.

**Experimental Procedures**

**Yeast Strains and Media—**Yeast cells were grown either in YPD (1% yeast extract, 2% Bactopeptone, 0.015% l-tryptophan, 2% glucose) or in synthetic (SD) medium (29). Rapamycin was added to solid medium to 1 µM, as described by Voth et al. (31). Similar, a mutant cell was grown 1 day on synthetic medium with 2% raffinose at 30 °C, and then on synthetic medium with 2% raffinose, 2% glucose, and 1 µM rapamycin. Following growth, the cells were washed with water and resuspended in 2% glucose at 30 °C. The yeast strains used in this study are isogenic with strain W303, and include KY520 (cdc55Δ::URA3 marker) (27), YCp50-CDC55 (containing a URA3 marker) (30), pRS426-PM1, and pRS416-(HA)::PPH22 (12). Marker swap plasmid ura3::ADE2 was introduced into an ADE2 strain of wild type in pRS426 and pRS426-PM1, as described by Voth et al. (31). Similarly, a TRP1 marker was introduced into pRS416 and pRS416-(HA)::PPH22 instead of the URA3 marker, using the ura3::TRP1 marker swap plasmid.

**Plasmids—**Plasmids that have been previously described include p1414GALL-E4orf4 (containing a TRP1 marker) (27), YCP50-CDC55 (containing a URA3 marker) (30), pRS426-PM1, and pRS416-(HA)::PPH22 (12). Marker swap plasmid ura3::ADE2 was used to introduce an ADE2 instead of a URA3 marker in pRS426 and pRS426-PM1, as described by Voth et al. (31). Similarly, a TRP1 marker was introduced into pRS416 and pRS416-(HA)::PPH22 instead of the URA3 marker, using the ura3::TRP1 marker swap plasmid.

**Isolation of Plasmid DNA from Yeast—**The yeast cells containing Cdc55 open reading frame have been mutagenized at random by PCR amplification of CDC55 in the presence of 0.03 mM MnCl2, as previously described (22). The forward primer was CTC-GGAATCC. Plasmid YCp50-CDC55 has been digested with Sscl and MluI. The fragment containing the vector and sequences lying 916 bp upstream of the CDC55 coding frame and 330 bp downstream of it (defined as the linear vector) was gel-purified, as were the PCR products. The PCR products share homologous sequences with the vector, which include 120 bp upstream of the CDC55 open reading frame (positions −1036 to −916 relative to the first ATG) and 334 bp downstream of it (1910 to 2244). The linear vector and PCR products were transformed into KY520 yeast cells containing p1414GALL-E4orf4, where they underwent homologous recombination to produce a circular plasmid, as previously described (32). Selection for E4orf4-resistant clones was performed on galactose plates. Site-directed mutagenesis was performed using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer’s instructions.

**Isolation of Plasmid DNA from Yeast—**The yeast cells containing p1414GALL-E4orf4 and mutant YCP50-CDC55 were grown 1 day on medium containing tryptophan but lacking uracil, and then plated on plates lacking uracil and containing 2-amino-5-fluorobenzoic acid to select for plates that lack the plasmid encoding the TRP marker (33). Plasmid DNA was extracted from the clones obtained on 2-amino-5-fluorobenzoic acid as previously described (34).

**Immunoprecipitations and Phosphatase Assays—**For co-immunoprecipitations, yeast extracts were prepared by bead beating cells for 4 min at 4 °C in lysis buffer (250 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.5% Nonidet P-40, 0.1% Triton X-100, 50 mM NaF, 100 µM sodium vanadate, 1× complete protease inhibitor mixture (Roche Applied Science)). The lysates were spun to separate beads and debris from the clear lysate. The beads were washed twice more in the lysis buffer and immunoprecipitations were carried out in the same lysis buffer. Lysates and antibodies were rotated for 2 h at 4 °C. Protein A beads (Amersham Biosciences) were added and rotated for an additional hour. The beads were then washed three times in lysis buffer.

**Immunoprecipitation phosphatase assays have been previously described (22). Briefly, immune complexes were prepared as described above and were washed twice more in PP2A buffer (50 mM imidazole, pH 7.2, 0.2 mM EDTA, 0.02% β-mercaptoethanol, 0.1% bovine serum albumin). The immune complexes were incubated with 50 nmol of peptide substrate RRA(pT)VA (35) in PP2A buffer for 60 min at 30 °C. The amount of free phosphate released was measured by a color reaction generated in the presence of molybdate and malachite green. Antibodies used in this work were: anti-E4orf4 (19), anti-Tpd3 (from J. R. Brouch), anti-Cdc55 antibodies, raised against a glutathione S-transferase-Cdc55 fusion protein, and anti-HA (Covance, Berkeley, CA).

**Results**

**Selection for Cdc55 Mutants That Cannot Mediate E4orf4 Toxicity in Yeast—**The protocol used to select for Cdc55 mutants that have lost the ability to transduce the E4orf4 toxic effect was based on the findings that E4orf4 inhibits growth of WT yeast but not of cdc55Δ cells, and that ectopic expression of a WT CDC55 gene in the cdc55Δ background restores E4orf4 toxicity (27, 36). It also relies on the finding that E4orf4-induced cell death requires an interaction between E4orf4 and an active PP2A, containing both catalytic and regulatory subunits (22, 29). The mutants selected by this protocol may be deficient in their ability to bind either E4orf4 or the PP2A-AC heterodimer. To obtain cdc55 mutants, the CDC55 gene was mutated by a low-fidelity PCR-based protocol, as described previously (22). The mutated PCR products and the linear vector described under “Experimental Procedures,” were transformed into cdc55Δ yeast cells containing p1414GALL-E4orf4. Homologous recombination in the transformed yeast cells produced circular plasmids containing the mutated PCR products (32) (Fig. 1). Transformants that grew on galactose (i.e. resistant to E4orf4) presumably contained a mutated Cdc55 protein that could not transduce the E4orf4 signal. Growth of the transformants on galactose was compared with that of cdc55Δ cells containing an empty vector or the vector expressing WT Cdc55. Only clones that grew as well as the vector-containing yeast were further analyzed. In the next stage, clones that became resistant to E4orf4 toxicity because of

**FIG. 1. A schematic representation of the generation of mutant Cdc55 plasmids.** The protocol for generation of mutant Cdc55 proteins is described in detail under “Experimental Procedures.”
because several of the mutants contained more than one mutation, a few substitutions were introduced as single mutations into the Cdc55 protein by site-directed mutagenesis. The additional mutants obtained by this procedure were tested for their ability to bind E4orf4 and TpD3, and the results are summarized in Fig. 2C and Table I.

**Binding of the Mutants to an Active PP2A—to determine whether the various mutants were associated with a functional PP2A-Table I.**

Functionality Analysis of Single Substitution Mutants—To test whether the various mutations affected Cdc55 functions, we examined their influence on two of the known phenotypes associated with CDC55. Cdc55 mutants demonstrate defective spindle checkpoint activity (37, 38), which could result from a diminished ability to promote dephosphorylation of Cdc28 (37), and from enhanced derepression of the APC/C activity in the yeast cells, leading to a less effective inactivation of the APC/C by the spindle checkpoint pathway (27). In addition, Cdc55 antagonizes the Tor signaling pathway, and such mutants exhibit rapamycin resistance as well as an enhanced sensitivity to benomyl (24). Strains defective in the spindle checkpoint defect caused by loss of CDC55, we examined their sensitivity to benomyl. Strains defective in the spindle checkpoint pathway were more sensitive to low levels of benomyl, a
PP2A Tpd3 Subunit Is Dispensable for Some Cdc55 Functions

Table I
Summary of Cdc55 mutations

| Clone number | Mutation | Cdc55 expression | Tpd3 binding | E4orf4 binding |
|--------------|----------|------------------|--------------|---------------|
| Type 1 clones: interact with Tpd3 at 50% or more of WT levels but bind E4orf4 with low efficiency |
| 1            | Q125R, I171F, I307S, K316R | +/−           | +/−          | −/+           |
| 68           | R206K, E290G, S309P, N372D | +/−           | +/−          | −/+           |
| 28           | E55G, S180G, I307T, Y344C | +           | +          | −          |
| 201          | D262N    | +/−           | +/−          | −/+           |
| 207          | Y344A    | +/−           | +/−          | −/+           |
| 81           | Y344C    | +/−           | +          | −          |
| 107          | V514D    | +/−           | +/−          | −/−          |
| Type 2 clones: interact with E4orf4 but not with Tpd3 |
| 126          | H100L, K118E | +          | −          | +          |
| K118E        | K118E    | +          | −          | +          |
| Type 3 clones: do not interact with Tpd3 or E4orf4 |
| 15           | T184I, M229T, R264G | +/−           | −          | −          |
| 73           | L102P, H333R, A471V | +/−           | −          | −          |
| 66           | Q125L, S508STOP | +          | −          | −          |
| 37           | F33S, V514D | +          | −          | −          |
| 19           | D262N    | +/−           | −          | −/−          |
| L102P        | L102P    | +/−           | −          | −/−          |
| 110          | S309P    | +/−           | −/−         | −          |
| 29           | S366P    | +/−           | −          | −          |
| Type 4 clones: enhanced Tpd3 binding |
| 38           | Q125R, L480Q | −/+          | +/−         | −/−          |
| 41           | T105A, S418G, M460L, N461D | −/+          | −/−         | −/−          |
| 87           | S440G    | −/+          | −/−         | −/−          |
| Type 5 clones: no effect on binding |
| 102          | T184A, I307A | +          | +          | +          |
| F33S         | F33S    | +          | +          | +/−         |
| Q125L        | Q125L   | +          | +          | +/−         |
| 44           | E290A   | +/−         | +/−         | −/+          |

The results are the average of three binding experiments as described in the legend to Fig. 2. The levels of expression and binding are: +, 67–100% of WT levels; +/−, 33–67% of WT levels; −/+, 15–37% of WT levels; −, less than 15% WT levels.

microtubule-depolymerizing agent, probably because of an increased proportion of cells that go through mitosis in the absence of an intact spindle (40, 41). To simplify analysis, each class of cdc55 mutations to be examined was represented only by mutants with single amino acid substitutions. We expected that mutants, which were dramatically reduced in their ability to bind an active PP2A, would lose their resistance to sublethal concentrations of benomyl, whereas mutants associating with WT PP2A levels would retain benomyl resistance. Surprisingly, three mutants that associated with 15% or less of WT PP2A activity were almost as resistant to benomyl as WT Cdc55. One mutant, D262N, which was associated with WT levels of PP2A activity, was not as resistant to benomyl as the WT yeast (Fig. 4).

The macrolide drug rapamycin inhibits yeast growth by inhibiting essential activities of Tor kinase that are mediated at least partially through Tap42. cdc55Δ strains are resistant to rapamycin, because Cdc55 is required to reverse Tor-mediated phosphorylation of Tap42, and in its absence even low levels of Tor kinase activity are sufficient for cell growth (39). The various single substitution mutants were grown in the presence of 100 nM rapamycin, a concentration to which the WT strain is sensitive, but on which the cdc55Δ strain can grow. As shown in Fig. 4 mutants Y344A and Y344C that bind WT levels of phosphatase activity are as sensitive to rapamycin as the WT strain. Mutant D282N is rapamycin-resistant despite its ability to associate with WT levels of PP2A activity. Mutants that were associated with low levels of PP2A activity were more resistant to rapamycin than WT yeast. However, K118E was less resistant than other mutants retaining low level association with the active phosphatase, such as L102P and S309P.

The finding that mutant D282N, which binds WT PP2A activity levels, behaves like a cdc55Δ mutant may be explained by suggesting that this mutant cannot maintain an active conformation of the phosphatase toward substrates that are larger than the peptide used in the dephosphorylation assays. Alternatively, it may be unable to interact with physiological substrates of the enzyme. However, the finding that mutants K118E and S309P, which do not bind Tpd3 (Fig. 2) and are associated with a low phosphatase activity (15% of WT levels, Fig. 3), behave like WT Cdc55 when grown on benomyl, is much harder to explain. These results suggest that the scaffolding A/Tpd3 subunit and a high phosphatase activity may be dispensable for the activity of Cdc55 in the spindle checkpoint.

Cdc55 Maintains a Partially Active Spindle Checkpoint in the Absence of Tpd3—To test whether WT Cdc55 can maintain an active spindle checkpoint in the absence of Tpd3, we examined the viability of a tpd3Δ strain in the presence of nocodazole, another microtubule-depolymerizing agent, and compared it to the viability of WT and cdc55Δ yeast cells. Because the various strains differ greatly in their growth properties, we could not directly compare their growth on benomyl. Cells were arrested at G1 by treatment with α-factor and then released from the G1 block in the presence of nocodazole. Samples were removed at various times after release and analyzed for viability by a colony formation assay. Cells with an intact spindle checkpoint arrest in mitosis and retain viability, whereas cells defective in the spindle checkpoint proceed through mitosis in the absence of a spindle and lose viability (12). As shown in Fig. 5, WT yeast cells retained viability up to 4 h in nocodazole, whereas viability of cdc55Δ cells declined to 4% of their viability in the absence of nocodazole. Viability of tpd3Δ yeast cells remained at an intermediate level of 50%. Thus, Tpd3 is dispensable for Cdc55 function in the spindle checkpoint.

Cdc55 Can Bind the PP2A Catalytic Subunit in the Absence of Tpd3—Retention of the spindle checkpoint function by Cdc55 proteins that are unable to bind Tpd3 raises the question of whether Cdc55 can bind the PP2A catalytic subunit in the absence of Tpd3. It has been previously reported that cross-linking of purified PP2A complexes in vitro led to the detection...
of BC heterodimers (42). However, the existence of such heterodimers in vivo has never been reported. To determine whether a WT Cdc55 protein is capable of binding the PP2A catalytic subunit in the absence of Tpd3, the binding of Cdc55 to HA-Pph22 was compared in WT and tpd3Δ backgrounds. Cell extracts prepared from WT, cdc55Δ, and tpd3Δ yeast cells expressing HA-Pph22 were subjected to immunoprecipitation with anti-Cdc55 antibodies. The presence of HA-Pph22, Cdc55, and Tpd3 in the immune complexes was measured by an enzymatic reaction, using a phosphorylated peptide as a substrate. The level of phosphate release was measured by a colorimetric reaction, and relative phosphatase activity was determined as a percentage of WT Cdc55-associated activity. These experiments were repeated three times and error bars denote the S.D.
absence of Tpd3 at higher than background levels, although binding is greatly diminished compared with Cdc55-HA-Pph22 association in the presence of Tpd3.

Ppm1 Is Required for the Spindle Checkpoint Function of Cdc55 and for the Tpd3-independent Interaction of Cdc55 with the PP2A Catalytic Subunit—It has been well documented that carboxyl methylation of the PP2A catalytic subunits, including yeast Pph21 and Pph22, is required for their efficient interaction with the A/Tpd3 and B/Cdc55 subunits. Deletion of the S. cerevisiae PPM1 gene, encoding a methyltransferase that modifies the PP2A catalytic subunits, leads to reduced binding of Cdc55 and Tpd3 to PP2A-C and increases benomyl sensitivity of yeast cells (12, 13). To determine whether Ppm1 is required for the Tpd3-independent checkpoint activity of Cdc55, we tested the benomyl sensitivity of two Cdc55 mutants that cannot bind Tpd3 (126 and K118E) in the ppm1Δ genetic background. As demonstrated in Fig. 7A, cdc55Δ/ppm1Δ yeast cells expressing Ppm1 and the K118E and 126 mutations were as resistant as 25 μg/ml benomyl as were cells expressing Ppm1 with WT Cdc55. However, resistance was completely lost when the mutants were expressed in the absence of Ppm1. Both mutants behaved like a cdc55Δ mutant on rapamycin. These results suggest that Ppm1 is required for the Tpd3-independent checkpoint activity of Cdc55.

To determine whether C subunit methylation was required for the Tpd3-independent association of Cdc55 with Pph22, WT Cdc55 and mutant 126 were introduced into a cdc55Δ/ppm1Δ yeast strain expressing HA-Pph22 together with an empty vector or the vector expressing Ppm1. Fig. 7B demonstrates that binding of both WT and mutant Cdc55 proteins to HA-Pph22 was reduced in the ppm1Δ genetic background, as compared with their binding in yeast cells expressing Ppm1.

These results are consistent with the conclusion that mutant 126 retains resistance to sublethal benomyl levels by binding low levels of the catalytic subunit in the absence of Tpd3. In contrast with their activity in the spindle checkpoint, the low phosphatase levels associated with the mutants appear not to be sufficient for the role of Cdc55 in inhibition of TOR-mediated signaling.

Defects in Tpd3 Binding Are Not Associated with Morphological Defects of cdc55 Mutations—cdc55Δ cells show defects in cytokinesis, often yielding multinucleate elongated cells that possess pronounced constrictions along their lengths (7). To investigate whether Cdc55 mutants that are unable to bind Tpd3 show this morphological defect, exponentially growing cultures of cdc55Δ cells expressing WT Cdc55, the K118E and 126 mutants, or no Cdc55 proteins were harvested and cells were visualized by light microscopy. As seen in Fig. 8, the mutant cells are not associated with the cdc55 defect, and have
FIG. 8. Tpd3 binding is not required for Cdc55 function in cytokinesis. Samples of exponentially growing cultures of cdc55Δ yeast cells transformed with the indicated Cdc55-expressing plasmids or an empty vector were subjected to light microscopy. A magnification of 200-fold is shown.

a WT-like appearance. Thus, regulation of cytokinesis can be maintained by Cdc55 proteins that are unable to bind Tpd3.

Based on our results we conclude that Cdc55 can interact with the PP2A catalytic subunit in the absence of Tpd3, albeit at low levels. The residual phosphatase activity associated with Cdc55 under these conditions is sufficient to maintain some of the Cdc55 functions.

DISCUSSION

In this work we used a selection protocol based on PP2A-dependent E4orf4-induced growth arrest in S. cerevisiae to find biologically interesting Cdc55 mutants. The characterization of these mutants revealed the surprising and novel finding that Cdc55 functions.

Types of Mutants Identified by the Selection Protocol—E4orf4 induces PP2A-B/Cdc55 subunit-dependent growth arrest and cell death in both yeast and mammalian cells (22–24, 27, 36). Furthermore, E4orf4 must interact with an active PP2A enzyme to induce cell death (22, 23). Thus selection of Cdc55 mutants that are unable to transduce the E4orf4-induced Cdc55-dependent growth arrest in yeast was expected to identify residues in the yeast Cdc55 subunit involved in its interaction with the PP2A core heterodimer and with E4orf4, as well as mutations that inhibit expression or destabilize the Cdc55 protein. The second type of mutations has not been further studied in this work.

Cdc55 mutations obtained by the approach described here can be divided into 4 classes. Type 1 mutations reduce E4orf4 binding to Cdc55, but do not interfere dramatically with Tpd3 binding. Type 2 mutations affect Tpd3, but not E4orf4 binding. Type 3 mutations interfere with both E4orf4 and Tpd3 binding, and type 4 mutations bind enhanced levels of Tpd3 compared with their expression levels. Because many of the mutants obtained in the screen contained more than one amino acid substitution, single substitutions were introduced by site-directed mutagenesis to determine more precisely which residues were responsible for disrupting the Cdc55 interactions.

Type 1 mutations interfering with E4orf4 binding to Cdc55, but not with Tpd3 binding, include single substitutions in several of the seven WD repeats present in the Cdc55 protein (WD4 (D262N), WD6 (Y344C, Y344A), and WD7 (V514D)). Not surprisingly, the results indicate that E4orf4 and Tpd3 binding require different Cdc55 residues, facilitating their simultaneous binding to Cdc55. Interestingly, mutation Y344C was identified in the screen in two independently obtained clones: clone 28 (E85G, S180G, I307T, Y344C) and clone 81 (Y344C).

The selection protocol described here yielded only one type 2 mutation that interfered with Tpd3, but not with E4orf4 binding (clone 126). A single substitution mutant (K118E), which was derived from mutant 126 and shares similar properties with it, contains a charge-reversal mutation in the inter-repeat loop between WD2 and WD3. Similar charge reversal mutations in WD3, WD4, and the inter-repeat loop between them have been previously described to affect the interaction between Bγ and the AC subunits of mammalian PP2A (14).

Several mutations belong to class 3 and interfere with both E4orf4 and Tpd3 binding. Some of these mutants have two substitutions, both of which contribute to the altered interactions. For example, clone 37 contains two substitutions, F33S and V514D. F33S reduces mildly the interactions with Tpd3 and E4orf4. V514D reduces Tpd3 binding to intermediate levels, and reduces significantly E4orf4 binding. The combined effect of both substitutions leads to complete inhibition of binding to both Cdc55 binding partners. In addition to mutants containing substitutions that additively affect Cdc55 interactions, we also found single substitutions that affected binding to both proteins. These include L102P, D262G, S309P, and S368P. These substitutions introduce into the polypeptide amino acids, such as glycine and proline, that may contribute to more dramatic changes in local conformation, thus altering surfaces involved in binding both Tpd3 and E4orf4. Indeed, mutation D262N reduces mostly E4orf4 binding, and affects Tpd3 binding only mildly, whereas substitution D262G completely inhibits both interactions. These results suggest that D262 may be directly involved in E4orf4 binding, and that adjacent residues, affected only by a more drastic change in local conformation induced by the glycine substitution, could be required for the interaction with Tpd3.

The fourth class of mutants includes three mutants that are expressed weakly, but appear to bind relatively enhanced levels of Tpd3. These mutants contain substitutions in several residues that are not conserved between yeast and mammalian cells, and are located toward the C terminus of Cdc55. Thus some mutations in Cdc55 may lead to changes in the protein that allow a higher affinity of interaction with PP2A-AC. The fact that evolution did not favor such alterations suggests that some feature of PP2A control could involve quick association and disassociation of different regulatory subunits and the core enzyme. Indeed, it has been shown that methylation of the PP2A-C subunit, which is required for association of the B and B′ subunits with the core enzyme (11–13, 43), is altered during the cell cycle (44). These findings indicate that the B subunit content of the heterotrimeric enzyme may be subject to cell cycle control. In another example, the composition of PP2A heterotrimers was transiently altered during the initial stage of retinoic acid-induced granulocytic differentiation (45).

The physical interaction between Cdc55 and Tpd3, detected by Western blots, and the interaction between Cdc55 and an active PP2A-AC core enzyme, measured by immunoprecipitation phosphatase assays, are generally in agreement (Figs. 2 and 3). Mutations inhibiting the interaction with Tpd3, or
reducing significantly Cdc55 expression led to significantly reduced levels of Cdc55 association with an active PP2A. Mutations that affect E4orf4 binding, but not Tpd3 binding, or those that reduce binding to Tpd3 only mildly, do not significantly affect the interaction with an active PP2A. However, two mutants, clones 41 and 68, which bind Tpd3 efficiently (more than 50% of WT levels), showed a more significant inhibition of association with an active PP2A (15% of WT levels). These mutants may have lost the ability to interact with the C subunit, and as a result may be unable to form a stable and functional heterotrimeric complex. In contrast, mutation E290A appears to be consistently associated with a 2-fold higher PP2A activity compared with WT Cdc55, although it binds normal levels of Tpd3. This mutation possibly enhances the interaction of Cdc55 with the C subunit.

All of the single substituted amino acids that affect E4orf4 or Tpd3 binding are identical or similar to the corresponding amino acids in the mammalian Bα subunit. Indeed, a representative mutation from each class of mutations was introduced into the mammalian Bα subunit and was shown to confer binding properties similar to those demonstrated in yeast (results not shown).

It has been suggested that monomeric subunits of PP2A, found outside the heterotrimer, are unstable in both Drosophila (46, 47) and mammalian cells (14). However, our results indicate that in yeast cells this is not always the case, because several Cdc55 mutants that were unable to integrate into the PP2A heterotrimer were expressed at WT levels (for example, clones 37, 66, 126, and K118E). These results are in agreement with previous reports showing that when individual subunits cannot participate in PP2A heterotrimer formation in S. cerevisiae their expression levels remain unaltered (12, 13, 48, 49).

PP2A Can Function as a Complex Consisting of Cdc55 and the Catalytic Subunit—When the mutants obtained in the E4orf4-based selection process were tested to find if they retained Cdc55 functions, it appeared that mutants deficient for Tpd3 binding could still maintain an intact spindle checkpoint, as analyzed by their ability to grow on sublethal concentrations of benomyl (Figs. 4 and 7). These mutants could also undergo apparently normal cytokinesis (Fig. 8). Furthermore, WT Cdc55 also retained partial checkpoint activity in the absence of Tpd3 (Fig. 5 and Ref. 38). Two possibilities may theoretically be compatible with these findings. Cdc55 may bind the catalytic subunit in the absence of the α/Tpd3 subunit and direct the PP2A C-Cdc55 heterodimer to dephosphorylate spindle checkpoint and cytokinesis-related substrates. Alternatively, Cdc55 may have PP2A-independent activities. Our results support the suggestion that Cdc55 and the C subunit form a heterodimer. Both mutant 126 and WT Cdc55 could bind low levels of Pph22 without binding Tpd3. The association of mutant 126 with Pph22 was inhibited when the methyltransferase PPM1 gene was deleted (Fig. 7B). It has been previously reported that Ppm1 is responsible for PP2A-C methylation, which is required for association of Tpd3 and regulatory subunits B/Cdc55 and B/Rts1 with the catalytic subunit (12, 13). It now appears that PP2A-C subunit methylation also affects its binding to Cdc55 in the absence of Tpd3. Furthermore, loss of Cdc55-C dimer formation in the ppm1Δ background was accompanied by loss of growth on benomyl. Although we cannot rule out the possibility that methylation is also involved in an as yet unidentified PP2A-independent function of Cdc55, our results are consistent with the simpler model suggesting that the spindle checkpoint is maintained by the Cdc55-C subunit dimer. Although it has been previously reported that B-C complexes could be found when purified PP2A complexes were subjected to cross-linking (42), to our knowledge this is the first time that an A-subunit-independent association between B and C subunits was demonstrated in vivo, and was assigned biological activities.

It has been previously reported that in S. cerevisiae, Tpd3 cellular concentration is limiting for both catalytic and regulatory Rts1 subunit binding (49). However, Cdc55 levels were shown to be much lower than levels of all other subunits. During normal mitosis or when the spindle is damaged by drugs, it may be essential that Cdc55 target PP2A activity to spindle checkpoint components fast, to prevent premature cell cycle progression. Binding to the non-limiting PP2A-C subunit directly may provide a more efficient solution to such emergencies than competition for PP2A AC heterodimers. Several Cdc55 mutants, which associated with low levels of phosphatase activity (less than 15% of WT levels: K118E, 126, L102P, and S309P), retained WT Cdc55 activity in the spindle checkpoint but could not antagonize Tor signaling as well as WT Cdc55 (Figs. 4 and 7). Mutants K118E and 126 were also shown to support an apparently normal cytokinesis (Fig. 8). These results may suggest that lower levels of Cdc55-associated phosphatase activity are required for the Cdc55 functions in the spindle checkpoint and in cytokinesis than are required to inhibit TOR signaling. Low levels of Cdc55-C heterodimer formation also appear not to be sufficient for E4orf4-mediated signaling, because mutant 126, which does not bind Tpd3, was obtained by virtue of conferring resistance to E4orf4, and a tpd3Δ strain was reported to be resistant to E4orf4 (27).

In summary, study of the interaction between the viral regulator E4orf4 and PP2A, a key cellular protein, provided novel insights into the structure and function of PP2A. Furthermore, the set of cdc55 mutants obtained here will be useful in the future when structural data on Cdc55 becomes available, and can be correlated with these mutants.

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REFERENCES
1. Mumby, M. C., and Walter, G. (1993) Physiol. Rev. 73, 673–699
2. Fuerst, S., and Hennings, B. A. (1995) Biochim. J. 311, 17–29
3. Sontag, E. (2001) Cell Signal. 13, 7–16
4. Moreno, C. S., Park, S., Nelson, K., Ashby, D., Hubalek, F., Lane, W. S., and Pallas, D. C. (2000) J. Biol. Chem. 275, 5257–5263
5. Mumby, M. (1995) Semin. Cancer Biol. 6, 229–237
6. Sennod, A. A., Cohen, P. T., and Stark, M. J. (1990) EMBO J. 9, 4339–4346
7. Heslop, S., Kuan, J. P., and Perona, R. A. (1991) Mol. Cell. Biol. 11, 5767–5780
8. van Zyl, W., Huang, W., Sennod, A. A., Stark, M., Camier, S., Werner, M., Marek, C., Sentenac, A., and Broach, J. R. (1992) Mol. Cell. Biol. 12, 4946–4959
9. Shu, Y., Yang, H., Hallberg, E., and Hallberg, R. (1997) Mol. Cell. Biol. 17, 3242–3253
10. Bryant, J. C., Westphal, R. S., and Wadzinski, B. E. (1999) Biochem. J. 339, 241–246
11. Tolskyy, T., Lee, J., Vafai, S., and Stock, J. B. (2000) EMBO J. 19, 5682–5691
12. Wu, J., Tolskyy, T., Lee, J., Boyd, K., Stock, J. B., and Broach, J. R. (2000) EMBO J. 19, 5672–5681
13. Wei, H., Ashby, D. G., Moreno, C. S., Gries, E., Yeong, F. M., Corbett, A. H., and Pallas, D. C. (2001) J. Biol. Chem. 276, 1570–1577
14. Trach, S., Ruediger, B., Walter, G., Daga, R. K., Barwacz, C. A., and Cribbs, J. T. (2002) J. Biol. Chem. 277, 20750–20755
15. Griswold-Prenner, I., Kamibayashi, C., Marouka, E. M., Mumby, M. C., and Derynck, R. (1999) Mol. Cell. Biol. 19, 5637–5645
16. Muller, U., Kleinberger, T., and Shenk, T. (1992) J. Virol. 66, 5867–5878
17. Kleinberger, T., and Shenk, T. (1993) J. Virol. 67, 7556–7560
18. Kanopka, A., Muhlemann, O., Petersen-Mahrt, S., Estmer, C., Ohrmalm, C., and Akusjarvi, G. (1998) J. Virol. 72, 5755–5764
19. Marcellus, R. C., Lavoie, J. N., Boivin, D., Shore, G. C., and Akusjarvi, G. (1998) J. Virol. 72, 2975–2982
20. Lavoie, J. N., Nguyen, M., Marcellus, R. C., Branton, P. E., and Shore, G. C. (1998) J Cell Biol. 140, 637–645
21. Marcellus, R. C., Lavoie, J. N., Boivin, D., Shore, G. C., Ketner, G., and Branton, P. E. (1998) J. Virol. 72, 7144–7153
22. Shtrichman, R., Kleinberger, T., and Akusjarvi, G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10880–10885
23. Shtrichman, R., Kleinberger, T., and Akusjarvi, G. (1998) Oncogene 19, 3757–3765
24. Marcellus, R. C., Chan, H., Paquette, D., Thirlwell, S., Boivin, D., and Branton, P. E. (2000) J. Virol. 74, 7669–7677
25. Bondesson, M., Ohman, K., Mannervik, M., Fan, S., and Akusjarvi, G. (1996) *J. Virol.* **70**, 3844–3851

26. Whalen, S. G., Marcelhus, R. C., Whalen, A., Ahn, N. G., Ricciardi, R. P., and Branton, P. E. (1997) *J. Virol.* **71**, 3545–3553

27. Kernitzer, D., Sharf, R., and Kleinberger, T. (2001) *J. Cell Biol.* **154**, 331–344

28. Afifi, R., Sharf, R., Shtrichman, R., and Kleinberger, T. (2001) *J. Virol.* **75**, 4444–4447

29. Sherman, F., Fink, G. R., and Hicks, J. B. (1982) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

30. Nickels, J. T., and Broach, J. R. (1996) *Genes Dev.* **10**, 382–394

31. Voth, W. P., Jiang, Y. W., and Stillman, D. J. (2003) *Yeast* **20**, 985–993

32. Evans, D. R. H., Myles, T. M., Hofsteenge, J., and Hemmings, B. A. (1999) *J. Biol. Chem.* **274**, 24038–24046

33. Toyn, J. H., Gunyuzlu, P. L., White, W. H., Thompson, L. A., and Hollis, G. F. (2000) *Yeast* **16**, 553–560

34. Arensma, A. (2002) *Methods Enzymol.* **332**, 260–270

35. Deana, A., and Pinna, L. A. (1988) *Biochim. Biophys. Acta* **968**, 179–185

36. Roopchand, D. E., Lee, J. M., Shahinian, S., Paquette, D., Bussey, H., and Branton, P. E. (2001) *Oncogene* **20**, 5278–5290

37. Minshall, J., Straight, A., Rudner, A. D., Dernburg, A. F., Belmont, A., and Murray, A. W. (1996) *Curr. Biol.* **6**, 1609–1620

38. Wang, Y., and Burke, D. J. (1997) *Mol. Cell. Biol.* **17**, 620–626

39. Jiang, Y., and Broach, J. R. (1999) *EMBO J.* **18**, 2782–2792

40. Li, R., and Murray, A. W. (1991) *Cell* **66**, 519–531

41. Hoyt, M. A., Totis, L., and Roberts, B. T. (1991) *Cell* **66**, 507–517

42. Kamibayashi, C., Likteig, R. L., Estes, R., Walter, G., and Mumby, M. C. (1992) *J. Biol. Chem.* **267**, 21864–21872

43. Yu, X. X., Du, X., Moreno, C. S., Green, R. E., Ogris, E., Feng, Q., Chou, L., McQuoid, M. J., and Pallas, D. C. (2001) *Mol. Biol. Cell* **12**, 185–199

44. Turowski, P., Fernandez, A., Favre, B., Lamb, N. J., and Hemmings, B. A. (1995) *J. Cell Biol.* **129**, 387–410

45. Li, X., Scuderi, A., Letsou, A., and Virshup, D. M. (2002) *Mol. Cell. Biol.* **22**, 3674–3684

46. Silverstein, A. M., Barrow, C. A., Davis, A. J., and Mumby, M. C. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 4221–4226

47. Gentry, M. S., and Hallberg, R. L. (2002) *Mol. Biol. Cell* **13**, 3477–3492
The Scaffolding A/Tpd3 Subunit and High Phosphatase Activity Are Dispensable for Cdc55 Function in the Saccharomyces cerevisiae Spindle Checkpoint and in Cytokinesis

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