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[17] Purification and Assay of the Budding Yeast Anaphase-Promoting Complex

By LORI A. PASSMORE, DAVID BARFORD, and J. WADE HARPER

Abstract

The anaphase-promoting complex (APC) is a central regulator of the eukaryotic cell cycle and functions as an E3 ubiquitin protein ligase to catalyze the ubiquitination of a number of cell cycle regulatory proteins. The APC contains at least 13 subunits in addition to two activator subunits, Cdc20 and Cdh1, that associate with the APC in a cell cycle-dependent manner. This chapter describes methods for preparation and assay of the APC from *Saccharomyces cerevisiae*. Highly active APC is purified from cells expressing Cdc16 fused with a tandem affinity purification (TAP) tag. Enzymatically active APC is achieved upon addition of recombinant Cdc20 or Cdh1 together with E1, Ubc4, ATP, and ubiquitin. Activity assays toward several endogenous substrates, including Clb2 and Pds1, are described. In addition, methods for observation of APC–coactivator and APC–substrate complexes by native gel electrophoresis are described.

Introduction

Progression through the mitotic phase of the cell cycle is regulated by ubiquitin-mediated proteolysis of regulatory proteins and by the periodic activity of the major mitotic kinase Cdk1/cyclin B. Ubiquitin-mediated proteolysis controls several transitions during mitosis, including chromosomal

METHODS IN ENZYMOLOGY, VOL. 398

0076-6879/05 $35.00
DOI: 10.1016/S0076-6879(05)98017-8

segregation, reorganization of the mitotic spindle, and exit from mitosis, in part through the ubiquitination of B-type cyclins. A major ubiquitin ligase controlling mitosis is the *a*naphase-*p*romoting *c*omplex, which is also referred to as the cyclosome (reviewed in Harper *et al.*, 2002; Peters, 2002). The APC is composed of at least 13 core subunits. Activation of the APC involves phosphorylation by cyclin-dependent kinases (Cdks) (Golan *et al.*, 2002; Kotani *et al.*, 1998; Kramer *et al.*, 2000; Rudner and Murray, 2000), as well as association with two coactivators, Cdc20 and Cdh1, which bind the APC in a cell cycle-dependent manner (Fang *et al.*, 1998; Kramer *et al.*, 2000; Visintin *et al.*, 1997; Zachariae *et al.*, 1998a). Substrate specificity is thought to be largely dictated by Cdc20 and Cdh1 (Burton and Solomon, 2001; Hendrickson *et al.*, 2001; Hilioti *et al.*, 2001; Pfleger and Kirschner, 2000; Pfleger *et al.*, 2001; Visintin *et al.*, 1997). In budding yeast, Cdc20 is largely responsible for ubiquitination of Pds1 at the metaphase–anaphase transition, whereas Cdh1 is largely responsible for ubiquitination of B-type cyclins, Cdc5, Hsl1, and Cdc20 itself (Harper *et al.*, 2002; Peters, 2002). There is some evidence that the coactivators may bind APC substrates directly (Burton and Solomon, 2001; Hilioti *et al.*, 2001; Pfleger and Kirschner, 2000; Pfleger *et al.*, 2001), with the assistance of the Doc1/Apc10 subunit (Passmore *et al.*, 2003). However, the precise function of the coactivators is unclear.

While much of our understanding of the components and function of the APC has derived from budding and fission yeast systems, only recently has budding yeast APC activity toward full-length natural substrates been reconstituted *in vitro*. This is in stark contrast to the situation with *Xenopus* and mammalian APC complexes, where activity assays against multiple substrates have been available for some time (Aristarkhov *et al.*, 1996; King *et al.*, 1995). This chapter describes methods for purification and assay of the budding yeast APC. In addition, it describes methods for visualization of APC complexes with both substrates and coactivator subunits using native gel analysis.

Overview of Assay Development

Enzymatic analysis of APC activity requires several components: (1) purified APC, (2) coactivator subunits Cdc20 or Cdh1, (3) E1 ubiquitin-activating enzyme, (4) an appropriate E2 ubiquitin-conjugating enzyme (UBC), and (5) appropriate substrates, in addition to ubiquitin and ATP. In the case of vertebrate APC, APC core complexes can be purified by conventional column chromatography to yield a preparation that is active in the presence of added Cdc20 or Cdh1. However, most frequently, APC core complexes are purified using a single-step immunopurification procedure involving anti-Cdc27 or anti-Cdc16 antibodies as an affinity reagent.

The ability to selectively tag yeast genes with epitope tags has made it possible to generate high-purity budding yeast APC using *t*andem *a*ffinity *p*urification (TAP) wherein the epitope tag is fused to the C terminus of Cdc16, a core APC subunit (Carroll and Morgan, 2002; Passmore *et al*., 2003; Zachariae *et al*., 1996). As described later, current procedures employ a TAP tag composed of the IgG-binding domain of protein A, one or more TEV protease cleavage sites, and a small peptide capable of binding to calmodulin in a calcium-dependent manner. Purification of Cdc16-TAP and its associated APC subunits involves (1) affinity chromatography on IgG Sepharose beads, (2) cleavage of bound fusion proteins with TEV protease, (3) capture of the Cdc16-calmodulin binding peptide by affinity chromatography on calmodulin beads in the presence of calcium, and (4) elution of the APC complex from the calmodulin beads in the presence of EGTA (Fig. 1B). Such preparations support the ubiquitination of full-length native yeast APC substrates (including Pds1, Hsl1, and Clb2), as well as an artificial substrate composed of residues 12–91 of sea urchin cyclin B, when combined with recombinant Cdc20 or Cdh1 as the activating subunit (Carroll and Morgan, 2002; Passmore *et al*., 2003). An alternative approach employing a single-step purification of Cdc16-HA using an anti-HA affinity resin has also been reported (Charles *et al*., 1998). The following section describes the purification and assay of budding yeast APC using the TAP-tag approach.

Construction of Yeast Strain Expressing Cdc16-TAP

We have used polymerase chain reaction (PCR)-based gene targeting with a modified version of the pFA6a-kanMX6 vector series (Fig. 1A) (Wach *et al*., 1998) to fuse the coding region for a C-terminal affinity purification tag onto the chromosomal copy of an APC gene (Passmore, 2003; Passmore *et al*., 2003). Because the gene is tagged at its 3′ end, the promoter region is not disrupted and expression of the fusion protein should be equivalent to that in wild-type yeast. This is important for the modification of APC subunits, as many of them are essential for growth and a change in their endogenous expression levels may be toxic. We use the protease-deficient yeast BJ2168 (*MAT***a** *leu2 trp1 ura3–52 pep4–3 prc1–407 prb1–1122 gal2*; ATCC 208277) (Jones, 1977; Zubenko *et al*., 1980) for gene targeting and protein purifications. The *CDC16* gene was tagged because, in previous studies, it had been tagged with at least nine Myc epitopes without affecting APC function (Zachariae *et al*., 1996) and an immunoprecipitation of APC from a *CDC16–9MYC* yeast strain had provided enough protein for analysis of its subunits by mass spectrometry (Zachariae *et al*., 1998b). These factors indicated that the C terminus of Cdc16p is

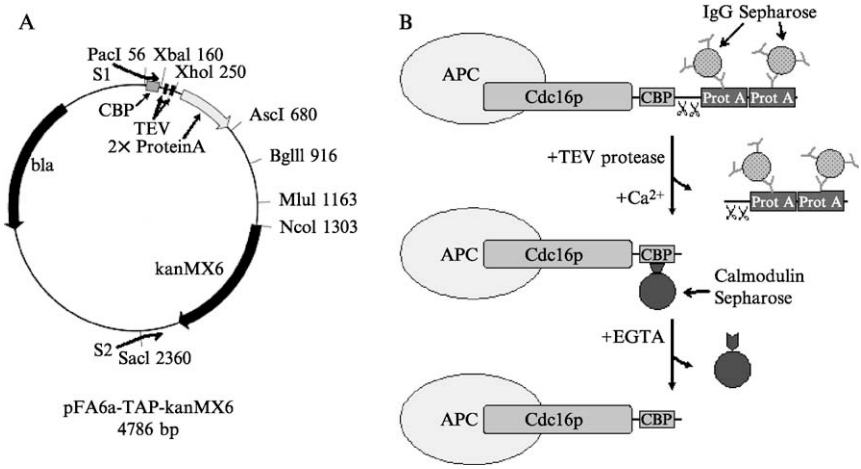


FIG. 1. Tandem affinity purification. (A) Schematic diagram of the pFA6a-TAP-kanMX6 vector. The positions of selected restriction enzyme cleavage sites are shown. The TAP tag was inserted into the *Pac*I/*Asc*I sites of the pFA6a vector and includes the coding region for the calmodulin-binding peptide (CBP), two TEV protease cleavage sites, and two protein A IgG-binding domains. This vector contains an *E. coli* origin of replication and an ampicillin resistance gene (bla) to allow propagation in bacteria and a kanamycin/G418 resistance gene (kanMX6) for selection in yeast. Oligonucleotides used for PCR-based gene targeting (S1 and S2) are shown schematically. (B) Overview of the TAP purification strategy. Clarified yeast lysates are loaded onto an IgG Sepharose column and washed thoroughly. The C-terminal protein A IgG-binding domains (Prot A) bind to the column and are cleaved off using TEV protease. The TEV eluate is supplemented with calcium and loaded onto a calmodulin Sepharose column. The calmodulin-binding peptide (CBP) binds to the column and the column is washed. The bound protein is eluted by the addition of EGTA, which chelates the calcium, thereby disrupting the interaction between calmodulin and the calmodulin-binding peptide.

solvent exposed and not required for APC function. We tried tagging *CDC16* with several different tags (Passmore, 2003) but the TAP tag (Puig *et al*., 2001; Rigaut *et al*., 1999) was the most efficient for APC purification.

The TAP tag was synthesized by recursive PCR with 14 overlapping oligonucleotides (Prodromou and Pearl, 1992) and the sequence was modified for optimal *S. cerevisiae* codon usage, avoiding repetitive DNA sequences (Passmore, 2003; Passmore *et al*., 2003). To increase the efficiency of TEV cleavage during purification, tandem TEV protease recognition sites were inserted within the tag. Thus, the TAP tag in pFA6a-TAP-kanMX6 contains an N-terminal calmodulin-binding peptide (originating from rabbit skeletal muscle myosin light chain kinase) (Blumenthal *et al*., 1985), followed by two TEV protease cleavage sites and, at its C terminus, two repeats of the *Staphylococcus aureus* protein A IgG-binding domain

(Fig. 1). The following protocol (adapted from Wach *et al*., 1998) can be used for PCR-based gene targeting and was used to create a yeast strain expressing Cdc16-TAP.

Buffers and Reagents

10× LiAc: 1 *M* lithium acetate (Sigma L–6883) adjusted to pH 7.5 with dilute acetic acid and filter sterilized
10× TE: 0.1 *M* Tris, pH 7.5, 10 m*M* EDTA, autoclaved
LiAc/TE: 1× LiAc, 1× TE, made fresh and filter sterilized
50% PEG: 50% (w/v) PEG 3350 (Sigma P–3640) in water (leave overnight on stirplate to allow the PEG to go into solution)
PEG/LiAc: 40% PEG 3350 (8 ml 50% PEG), 1× TE (1 ml 10× TE), 1× LiAc (1 ml 10× LiAc), prepared immediately prior to use and filter sterilized
Dimethyl sulfoxide (DMSO), Sigma D–8779
Salmon sperm DNA, Invitrogen 15632–011
YPD: For 1 liter, autoclave 20 g bacto-peptone and 10 g yeast extract in 950 ml water. Cool to approximately 60° and add sterile-filtered 2 *M* D-glucose to 0.11 *M*
G418/YPD plates: For 1 liter, autoclave 20 g bacto-peptone, 10 g yeast extract, and 20 g bacto-agar in 950 ml water. Cool to approximately 60° and add sterile-filtered 2 *M* D-glucose to 0.11 *M* and sterile-filtered G418 to 300 μg/ml (from a 25-mg/ml stock solution; Melford G0175)
IP buffer: 50 m*M* Tris-Cl, pH 8.0, 150 m*M* KCl, 10% (w/v) glycerol, 0.2 % (v/v) Triton X–100, and protease inhibitors.

Preparation of PCR Product

Oligonucleotide primers should be 5′ phosphorylated and PAGE purified. S1 oligonucleotides are designed to contain 40 bp of the 3′ end of gene to be tagged (up to but not including the stop codon) followed by the 24 bp pFA6a vector sequence, such that the gene is fused, in-frame, with the tag from the vector (Fig. 1A). The S2 oligonucleotide contains 40 bp complementary to the chromosomal region downstream of the targeted gene, followed by a 20 bp pFA6a vector sequence. To make the Cdc16-TAP strain, we used the following oligonucleotides (with vector-specific sequences in italics):

S1-TAP-CDC16: TAATGCCGACGATGATTTTGACGCAGATA TGGAACTGGAA *TCTCACGAAAAGAGAAGATGGAAG*

S2-CDC16: CTTTTACGTGTGGCTGCCTCTAAGAATTAAACT TCTTTTC*CATCG ATGAATTCGAGCTCG*

Perform a 200-μl PCR reaction using Expand high-fidelity polymerase (Roche Applied Science, 1732641), the S1 and S2 oligonucleotides, and the pFA6a-TAP-kanMX6 template. Use 20 cycles of PCR as follows: 95° 30 s, 54° 30 s, and 72° 3 min. Clean up the PCR products (~2.4 kb) using gel purification and the QIAEX II gel purification kit (Qiagen, 20021).

Transformation Protocol

1. Inoculate a 50-ml overnight culture in YPD using one large colony of BJ2168 yeast (less than 2 weeks old). Grow for approximately 24 h at 30°, 220 rpm.
2. Inoculate 300 ml fresh YPD with overnight culture to give an OD_{600nm} of 0.2 and grow at 30°, 220 rpm until the OD_{600nm} is 0.4–0.6.
3. Harvest cells in 50-ml Falcon tubes at 1000*g* for 5 min at room temperature.
4. Combining all pellets, wash once in 30 ml sterile water and once in 1.5 ml LiAc/TE.
5. Resuspend cells in 1.5 ml LiAc/TE and make competent by incubating at 30° for 30 min with gentle shaking (100 rpm).
6. To prepare the DNA for transformation, heat salmon sperm DNA to 100° for 10 min and then chill quickly on ice. For each transformation, mix 100 μg salmon sperm DNA with 1–4 μg of PCR product in a 1.5-ml tube. As a negative control, use TE buffer instead of PCR product.
7. Add 100 μl competent yeast to the DNA and mix well.
8. Add 0.6 ml PEG/LiAc solution to each tube and mix well by flicking the tube.
9. Incubate at 30° for 30 min with shaking (200 rpm).
10. Add 70 μl DMSO, mix by gentle inversion, heat shock for 15 min at 42°, and then chill on ice for 2 min.
11. Pellet cells at 6000*g* for 1 min, resuspend in 0.5 ml YPD, and recover by incubation at 30° and 200 rpm for 2 h.
12. Finally, pellet cells at 6000*g* for 1 min, resuspend in 0.6 ml YPD, and plate onto six G418/YPD plates (100 μl/plate) using glass beads to spread the cells. Grow inverted plates for 3 days at 30°.
13. After 3 days, the transformed yeast grow out as large colonies on a thin lawn of background colonies. There should be no colonies on the negative control plates. Streak out several of the large colonies onto fresh G418/YPD plates to obtain single colonies.

Verification of Integration

Integration of the purification tag at the 3′ end of the endogenous *CDC16* gene should be verified by colony PCR as described (Wach *et al.*, 1998). Colony PCR confirms that the integration has occurred at the correct locus and that the yeast strain is haploid for the targeted gene. Briefly, design four primers so that a PCR product from one set of primers (V1 and K2) spans the 5′ integration site and a PCR product from a second set of primers (K3 and V4) spans the 3′ integration site (V1 and V4 are gene specific; K2 and K3 are vector specific). The presence of correctly sized PCR products indicates a positive transformant. PCR reactions are performed in tubes containing a small quantity of yeast microwaved on high power for 1 min (Wach *et al.*, 1998). For the CDC16-TAP strain, the following oligonucleotides were used:

V1-CDC16: GCACAAATCAT TGTACCTAAAGCC
V4-CDC16: GGAACCTTGAACTTGAACAGCG
K2-kanMX6: CGGATGTGATGTGAGAACTGTATCCTAGC
K3-kanMX6: GCTAGGATACAGT TCTCACATCACATCCG

The PCR products should be 1.6 kb with V1-CDC16 and K2-kanMX6; 1.4 kb with K3-kanMX6 and V4-CDC16; and 3.1 kb with V1-CDC16 and V4-CDC16 (0.7 kb for a wild-type strain).

A small proportion of clones that have integrated tags as determined by colony PCR do not express functional tags, perhaps due to frameshifts or other mutations. Therefore, small-scale immunoprecipitations should also be performed for several clones to test for the presence of functional tags. Inoculate 200 ml YPD with 5 ml of a saturated overnight culture and grow at 30° with shaking (200 rpm) until the $OD_{600\ nm}$ is 0.6–1.0. (Use untagged BJ2168 yeast for a negative control.) Harvest cells by centrifugation at 3000*g* for 12 min at 4°. Rinse pellets once with cold water, flash freeze in a dry ice/ethanol bath, and store at −80°. For immunoprecipitations, add an equal volume of acid-washed glass beads (425–600 μm; Sigma) to frozen cell pellets and IP buffer to cover the glass beads (~0.4 ml). Lyse yeast by vortexing on maximum speed at 4° for 30 s, followed by a 30-s incubation on ice, for a total of 8 min of vortexing. Transfer the lysate, without glass beads, into 1.5-ml tubes and centrifuge at 20,000*g* for 20 min at 4°. Transfer the supernatant to a new tube and estimate the protein concentration using the Bio-Rad protein assay.

Equilibrate 20 μl of IgG Sepharose (Amersham Biosciences 17–0969–01) in IP buffer. Add equal amounts of protein extract to the resin and incubate at 4° on a pinwheel rotor for 1 h. Wash IgG Sepharose three times with IP buffer. To elute protein, add 40 μl 2× SDS–PAGE sample buffer to each sample and vortex. Boil samples for 10 min, vortex, and load

directly onto an 8% SDS–PAGE large gel. Western blot with anti-Protein A antibody (SPA–27, Sigma P–2921; 1:2400 dilution). Cdc16-TAP should be visible as a 118-kDa band (99 kDa after TEV cleavage).

Purification of Yeast APC$^{CDC16\text{-}TAP}$

The TAP purification protocol (Passmore *et al.*, 2003) was modified from Rigaut *et al.* (1999) and is outlined in Fig. 1B. We routinely perform the entire purification in 1 day over ~12 h. However, the purification can be stopped before TEV cleavage and stored overnight at 4°.

Yeast Cultures

To prepare yeast pellets, use a saturated overnight culture, grown from one or two fresh *CDC16-TAP* yeast colonies, to inoculate 10–20 liters of YPD (0.5–2.0 ml overnight culture per liter YPD in a 2-liter flask). Grow yeast cultures overnight with shaking (200 rpm) at 30° to an $OD_{600\ nm}$ of approximately 1.0. The cells must be harvested before they reach stationary phase, as the major yeast proteases become more active, the cell wall becomes thicker, and protein synthesis decreases at stationary phase (Jones, 2002; Werner-Washburne *et al.*, 1993). Harvest the yeast at 3000*g* for 12 min at 4°, rinse once in cold water (to remove extracellular proteases), flash freeze in a dry ice/ethanol bath or liquid nitrogen, and store at –80°.

Buffers and Reagents

Buffers used for large-scale purifications are in italic*s* and are listed in Table I. All purification steps should be performed at 4° unless otherwise indicated. It is important to prechill buffers to 4° before adjusting the pH due to the temperature dependence of Tris buffers. To avoid problems with pH fluctuations, HEPES buffer can be used instead of Tris.

To prepare IgG Sepharose FF (Amersham Biosciences 17–0969–01), pack the resin into a Vantage L column (Amicon) using 0.125 ml resin for every 10 g of yeast pellet. An empty PD10 column (Amersham Biosciences), or similar gravity flow column, may also be used. However, we have observed that lower yields are obtained using gravity flow columns. Wash the column with five bed volumes of *TST* (Table I). To remove unbound IgG, wash with three bed volumes each of *0.5 M HAc, TST,* and *0.5 M HAc*. Wash the column thoroughly with *TST* until the pH returns to 7.6 (monitor the pH using pH strips). Chill it to 4° and equilibrate with *IgG buffer 1* just before use. To prepare calmodulin Sepharose 4B (Amersham

TABLE I
BUFFERS FOR APC PURIFICATION

Buffer name	Composition
TST	50 m*M* Tris-Cl, pH 7.6, 150 m*M* NaCl, 0.05% (v/v) Tween 20
0.5 *M* HAc	0.5 *M* acetic acid, adjusted to pH 3.4 with NH_4CH_3COOH
Lysis buffer	50 m*M* Tris-Cl, pH 8.0, 150 m*M* KCl, 10% (w/v) glycerol
DNase I	1 mg/ml DNase I (Roche 104159) in water
Igepal CA-630	10% (v/v) Igepal CA-630 (Sigma I-3021) in water
IgG buffer 1	50 m*M* Tris-Cl, pH 8.0, 150 m*M* KCl, 10% (w/v) glycerol, 0.5 m*M* EDTA, 2 m*M* EGTA, 0.1% (v/v) Igepal CA-630
IgG buffer 2	50 m*M* Tris-Cl, pH 8.0, 150 m*M* KCl, 10 % (w/v) glycerol, 0.5 m*M* EDTA, 1 m*M* DTT, 0.1% (v/v) Igepal CA-630
CaM wash buffer	10 m*M* Tris-Cl, pH 8.0, 150 m*M* NaCl, 10% (w/v) glycerol, 3 m*M* DTT, 1 m*M* Mg-acetate, 2 m*M* $CaCl_2$, 0.1% (v/v) Igepal CA-630
CaM elution buffer	10 m*M* Tris-Cl, pH 8.0, 150 m*M* NaCl, 10% (w/v) glycerol, 3 m*M* DTT, 1 m*M* Mg-acetate, 2 m*M* EGTA, 0.1% (v/v) Igepal CA-630
Protease inhibitors	Complete EDTA-free protease inhibitor cocktail tablets (Roche Applied Science 1873580). Add one tablet for every 100 ml buffer
Phosphatase inhibitors	50 m*M* NaF, 25 m*M* β-glycerophosphate, 1 m*M* Na-orthovanadate

Biosciences 17–0529–01), pack 0.1 ml resin per 10 g yeast pellet into a Bio-Rad polyprep column or empty PD10 column. Wash the column with 10 bed volumes of water and then with 20 bed volumes of *CaM wash buffer* and chill to 4°.

Preparation of Yeast Extracts

1. Thaw cell pellets from 10 to 20 liters yeast culture at room temperature and resuspend in an equal volume of *lysis buffer* with *protease inhibitors* and *phosphatase inhibitors*. Add 1/1000 volume *DNase I*.

2. Lyse cells by passing through an Emusiflex C5 homogenizer (Avestin, Canada) on ice, four times at 20,000 psi. The Emulsiflex C5 homogenizer uses a high-pressure pump to push a continuous flow of cell paste through an adjustable homogenizing valve. Cells are subjected to a shear force due to the rapid change in pressure at the homogenizing valve as well as a physical force due to impact with the homogenizing valve, resulting in cell lysis. We have also successfully used a French press to lyse the cells.

3. Clear lysate by centrifuging in a JA20 Beckman rotor at 19,000 rpm (45,000*g*) for 30 min at 4°. (The lysate remains quite turbid after centrifugation.)

4. Add EDTA to a final concentration of 0.5 m*M* (from 0.5 *M* EDTA stock) and *Igepal CA–630* (a nonionic detergent) to a final concentration of 0.1% (v/v).

Purification on IgG Sepharose

1. Load the cleared lysate onto the IgG Sepharose Vantage column at 0.5–1.0 ml/min using a P1 pump (or slowly pass the lysate over the column, trying not to disturb the bed of resin, if using a gravity flow column).

2. Wash the IgG column with 60 bed volumes of *IgG buffer 1* with *protease inhibitors* and *phosphatase inhibitors*, followed by 30 bed volumes of *IgG buffer 2* with *phosphatase inhibitors*. The EGTA in *IgG buffer 1* chelates calcium to release any calmodulin that may be bound to the calmodulin-binding peptide. We have observed that if EGTA is omitted from *IgG buffer 1*, only ~60% of the APC binds to the calmodulin column. This suggests that endogenous yeast calmodulin binds to the calmodulin-binding peptide of the tagged APC subunit during purification and blocks binding to calmodulin resin. (The purification may be stopped here and stored overnight if necessary.)

3. Carefully remove resin from the IgG column and transfer it into round-bottomed 2-ml tubes. For every 100 μl of IgG Sepharose, add 100 μl *IgG buffer 2* and approximately 10 units (or 15 μg) TEV protease (Invitrogen, 10127–017). Shake the cleavage reactions at 16°, 100 rpm for 2 h.

4. After cleavage, the APC should be present in the supernatant. Spin down the resin (1000*g*, 1 min, 4°). Remove the supernatant and wash the resin twice with a small volume of *IgG buffer 2* (approx. 0.3 bed volumes each wash). Pool the TEV supernatant and washes. In our hands, approximately 95% of Cdc16-TAP is cleaved and eluted with TEV protease, as shown by immunoblotting for the protein A tag.

5. Spin the supernatant at 10,000*g* for 1 min to ensure all IgG beads are removed.

Purification on Calmodulin Resin

1. To the supernatant from the TEV cleavage reaction, add 1 *M* $CaCl_2$ to a final concentration of 5 m*M*. Save 35 μl for SDS–PAGE analysis.
2. Incubate the TEV supernatant with calmodulin Sepharose in a sealed column on rollers for 1 h at 4°. The calmodulin-binding

peptide of the TAP tag will bind to calmodulin in the presence of calcium.

3. After binding, allow the column to drain by gravity (save 35 μl of this flow through for SDS–PAGE analysis). Wash with 70 bed volumes *CaM wash buffer* by carefully pipetting several milliliters of buffer onto the resin at a time, trying to maintain an even bed of resin.
4. Elute protein from the calmodulin Sepharose in *CaM elution buffer* in at least 15 fractions of one bed volume each. Be careful not to disturb the bed of resin when pipetting elution buffer. The APC usually elutes in fractions 2–6.

Analysis and Concentration

5. Analyze samples by SDS–PAGE on an 18 × 16-cm 8% SDS–polyacrylamide gel and silver stain using the method of Ansorge (1985). Generally, 35 μl of the TEV eluate or 100 μl of each calmodulin elution fraction should be loaded onto the gel. We can resolve all of the APC subunits (except Apc4 and Apc5, which run at the same position, Fig. 2) using a modified gel composition of

Resolving gel: 0.75 *M* Tris, pH 9.2, 8% 37.5:1 acrylamide:bis-acrylamide, 0.1% (w/v) SDS

Stacking gel: 0.25 *M* Tris, pH 6.8, 5.1% 37.5:1 acrylamide:bis-acrylamide, 0.1% (w/v) SDS

Running buffer: 25 m*M* Tris, 192 m*M* glycine, pH 8.3, 0.1% SDS

6. Pool the calmodulin elution fractions containing the APC (usually fractions 2–6 contain most of the APC).
7. Concentrate the pooled fractions using YM–50 Centricons or YM–50 Microcons (Millipore). Estimate the protein concentration using the Bio-Rad protein assay and a bovine serum albumin calibration standard made up in *CaM elution buffer*. For *in vitro* assays, a concentration of 0.05–0.1 mg/ml is sufficient.
8. Aliquot the concentrated protein, flash freeze in dry ice/ethanol or liquid nitrogen, and store at –80°. Purified APC loses activity after multiple freeze–thaws.

Comments

A silver-stained gel of a typical APC purification is shown in Fig. 2. After the first two steps of the TAP purification (purification on an IgG Sepharose column and elution with TEV protease), a series of bands

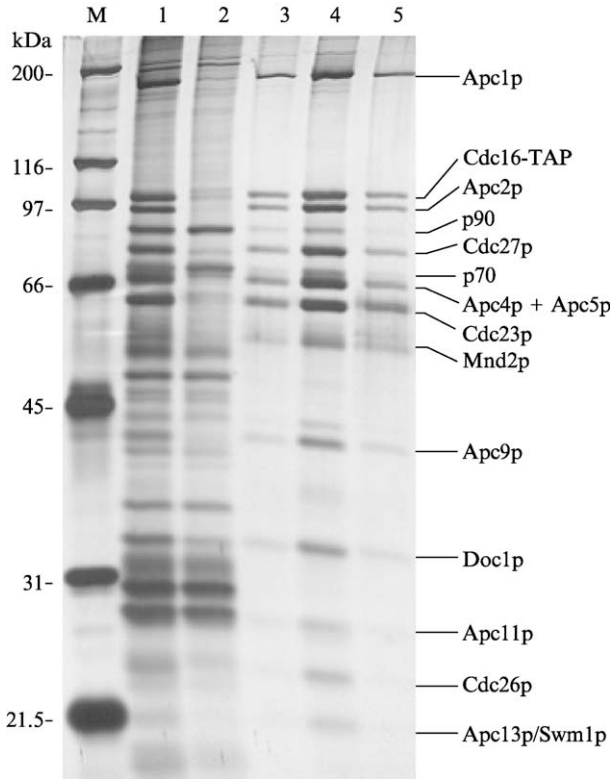


FIG. 2. TAP purification of endogenous APC from *S. cerevisiae*. Analysis of APC purification from *CDC16-TAP* yeast by silver-stained SDS–PAGE. Cdc16-TAP binds to IgG Sepharose and is eluted by cleavage with TEV protease (eluate, lane 1). The eluate is bound to calmodulin Sepharose (flow through, lane 2), washed, and eluted with EGTA (elution fractions, lanes 3–5). Proteins identified by MALDI-TOF mass spectrometry are labeled. p90 is a Cdc16p degradation product, and p70 is the Hsp70 family heat shock protein Ssa2. The diffuse band at 55–60 kDa contains keratins, which originate in contaminated SDS stock solutions. M, molecular weight markers. Reproduced from Passmore *et al.* (2003) with permission.

corresponding to the correct molecular weights for APC subunits is visible (Fig. 2, lane 1). After subsequent purification on calmodulin resin, the APC is ~95% pure (Fig. 2, lanes 3–5) with a yield of ~10–50 μg APC from 10 liters of yeast culture. In contrast, a negative control purification from untagged BJ2168 yeast yields no major purified proteins (not shown). We have confirmed the identities of all of the 13 APC subunits using MALDI-TOF mass spectrometry (Passmore *et al.*, 2003). We also identified two

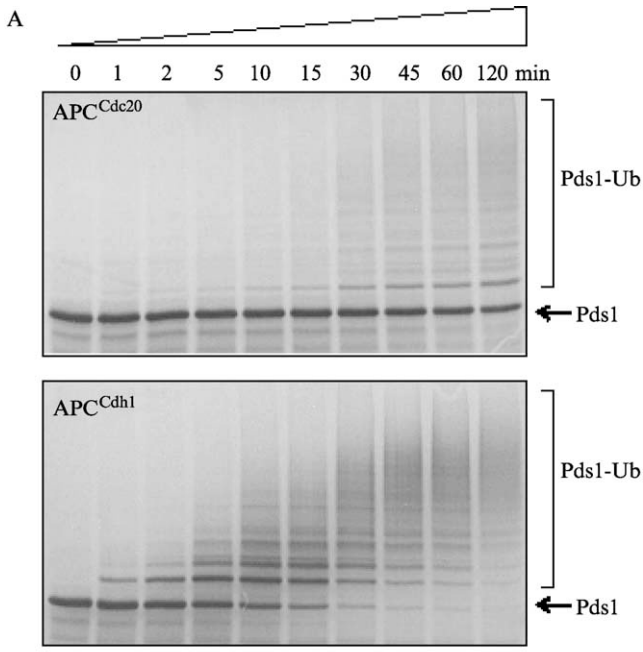
contaminating proteins: p90 is a 90-kDa degradation product of Cdc16 whereas p70 is the Hsp70 family heat shock protein Ssa2. Hsp70 binds to extended hydrophobic polypeptide segments (Bukau and Horwich, 1998), but its presence does not necessarily indicate unfolding or disassembly of the APC because it is often a contaminant in protein purifications. Ssa2 is a highly expressed protein (Garrels *et al*., 1997) and it had a high frequency of occurrence in genome-wide mass spectrometric studies in yeast, being present in up to 54% of all protein purifications, as well as in mock purifications from untagged strains (Gavin *et al*., 2002; Ho *et al*., 2002). The relative intensities of p90 and p70 on SDS–PAGE decrease after concentration of the APC, probably because they are not integral components of the APC and they pass through the concentrator membrane.

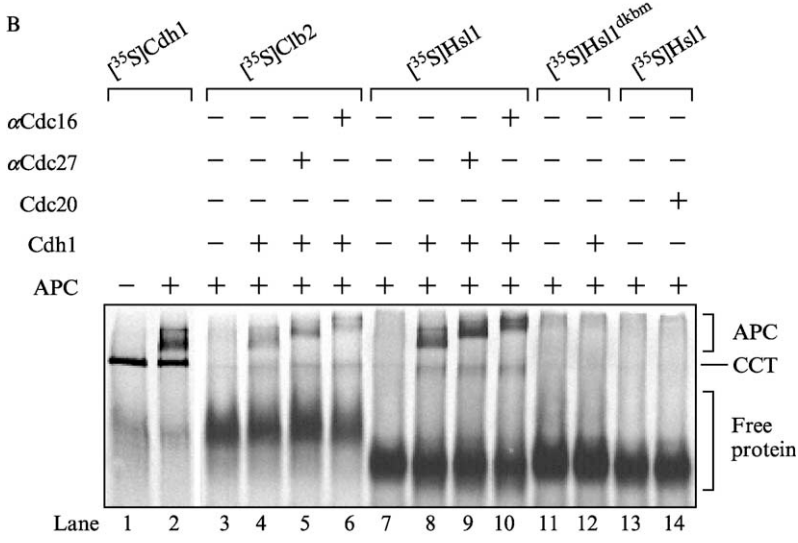
We have examined the stability of the APC in response to pH change (Passmore, 2003). These studies showed that APC is highly sensitive to pH less than 7.5 and that low pH may cause the APC to precipitate or dissociate. The APC is stable at higher pH and it should be purified in a buffer with a pH of 8.0. Because the pH of Tris buffers is temperature dependent, one must ensure that all buffers are kept at 4° and that APC is stored on ice. (As mentioned earlier, we have also used HEPES buffer to avoid this problem.)

The TAP tag was designed specifically to allow for the efficient recovery of proteins present in low quantities (Rigaut *et al*., 1999). Its success is probably dependent on several factors. First, both the protein A–IgG and calmodulin-binding peptide (CBP)–calmodulin interactions are high affinity. For the CBP–calmodulin interaction, the K_d is less than 10 n*M* (Blumenthal *et al*., 1985). The affinity of the protein A–IgG interaction varies with the type of IgG and the pH but it should bind tightly under the conditions used here (human IgG and pH 8.0; Harlow and Lane, 1999). Second, these protein–protein interactions are very specific, unlike those found in many other affinity/epitope tags. Finally, the elution methods (TEV cleavage from IgG Sepharose and EGTA elution from calmodulin Sepharose) are specific and gentle, preventing dissociation of the protein complex.

Assay of APC Using Native Substrates

As an E3 ubiquitin ligase, the *in vivo* role of the APC is to catalyze the conjugation of polyubiquitin chains onto specific substrate proteins. This activity is regulated both spatially and temporally. We have developed an *in vitro* ubiquitination assay to study APC activity against endogenous yeast targets and therefore in a physiologically relevant context (Passmore,


A
0 1 2 5 10 15 30 45 60 120 min
APC^Cdc20
Pds1-Ub
Pds1
APC^Cdh1
Pds1-Ub
Pds1


B
[35S]Cdh1
[35S]Clb2
[35S]Hsl1
[35S]Hsl1dkbm
[35S]Hsl1
αCdc16 − − − + − − − + − − − −
αCdc27 − − + − − − + − − − − −
Cdc20 − − − − − − − − − − − +
Cdh1 − + + + − + + + − + − −
APC − + + + + + + + + + + +
APC
CCT
Free protein
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14

2003; Passmore *et al.*, 2003). Purified APC is incubated with yeast E2 (Ubc4p), ubiquitin, ATP, and ^{35}S-labeled substrate, and the entire reaction mixture is analyzed by SDS–PAGE followed by autoradiography. Using this assay, purified yeast APC is active as an E3 ubiquitin ligase toward specific yeast substrates, including Pds1, Clb2, and Hsl1, but it is not active toward, nonphysiological substrates such as Cln1 (Fig. 3A) (Passmore *et al.*, 2003). The activity of the APC is dependent on the presence of either the Cdc20 or the Cdh1 coactivator. It catalyzes the formation of polyubiquitin chains onto substrate, resulting in high molecular weight polyubiquitinated products. Thus, the *in vitro* assay reflects the *in vivo* function of the APC.

Buffers and Reagents

Plasmids: All plasmids are constructed using *S. cerevisiae* open reading frames (ORFs). APC substrates (Pds1, Clb2, and Hsl1) and coactivators (Cdc20 and Cdh1) are cloned into vectors containing a T7 promoter (either pET or pRSET) so they can be produced by *in vitro* transcription/translation (IVT). All plasmids for IVT should be highly purified, e.g., using the HiSpeed plasmid midi kit (Qiagen 12643).

20× ubiquitination buffer: 800 m*M* Tris, pH 7.5, 200 m*M* $MgCl_2$, 12 m*M* dithiothreitol (DTT). Aliquot and store at –20°.

0.1 *M* ATP (Amersham Biosciences 27-2056-01): Aliquot and store at –20°. Avoid multiple freeze–thaws.

5 mg/ml ubiquitin: Dissolve ubiquitin (Affiniti, UW8795) to 5 mg/ml in 10 m*M* Tris, pH 7.0. Aliquot and store at –20°.

E2: His_6-Ubc4p (E2) can be overexpressed and purified from *Escherichia coli* using standard procedures. Dilute purified Ubc4 to 500 ng/μl in 10 m*M* Tris, pH 7.5, 150 m*M* NaCl, and 2 m*M* DTT. Aliquot and store at –80°.

FIG. 3. *In vitro* APC assays. (A) Time courses of the activities of APC with Cdc20 (APC^{Cdc20}) and APC with Cdh1 (APC^{Cdh1}) toward the substrate Pds1. APC was incubated with E2 (Ubc4), ubiquitin, ATP, ^{35}S-labeled substrate, and one of the coactivators, Cdc20 or Cdh1. Samples were taken at the indicated time points and added to SDS–PAGE loading buffer. The addition of polyubiquitin chains onto ^{35}S-labeled Pds1 results in the appearance of high molecular weight smears correlating with the disappearance of unmodified Pds1 from the bottom of the gel. (B) Analysis of APC–coactivator and APC–substrate interactions. In the first two lanes,^{35}S-Cdh1 was incubated with and without APC. In the remaining lanes, ^{35}S-labeled Clb2, 6His-$Hsl1^{667–872}$, or 6His-Hsl1 containing D- and KEN-box mutations (6His-$Hsl1^{667–872\ dkbm}$) were mixed with APC in the presence or absence of Cdh1 or Cdc20. All samples were run on a native gel and analyzed by autoradiography. Antibodies to Cdc27 or Cdc16, which retard the migration of the APC, were added to some samples. Reproduced from Passmore *et al.* (2003) with permission.

1 μg/μl ubiquitin aldehyde: Dissolve ubiquitin aldehyde (Affiniti, UW8450) to 1 μg/μl in 10 m*M* Tris, pH 7.0. Aliquot and store at –20°.
200 μ*M* LLnL: Dissolve LLnL (*N*-acetyl-Leu-Leu-Norleu-al; Sigma A6185) to 20 m*M* in DMSO and then dilute to 200 μ*M* with water. Aliquot and store at –20°.
APC buffer: 10 m*M* Tris, pH 8.0, 150 m*M* NaCl, 10% (w/v) glycerol, 1 m*M* Mg-acetate, 0.01% (v/v) Igepal CA-630, 2 m*M* EGTA, and 3 m*M* DTT. Aliquot and store at –20°.
4× SDS–PAGE loading buffer: 0.2 *M* Tris-Cl, pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, and bromphenol blue to color.

In Vitro *Transcription/Translation*

Coactivators and substrates are obtained using a rabbit reticulocyte-coupled *in vitro* transcription/translation (IVT) system, as these proteins are difficult to purify from overexpression systems. The incorporation of [^{35}S]methionine into the IVT system also provides a convenient and sensitive mechanism to label substrates for direct visualization on autoradiograms. A problem with using IVT-produced proteins is that other proteins from the IVT mixture might affect the assay. In these ubiquitylation assays, proteins independent of Cdc20 or Cdh1 are not activating the APC because the addition of reticulocyte lysate alone does not stimulate APC activity (Passmore *et al.*, 2003).

Prepare substrates and coactivators by IVT using the TNT T7 Quick coupled *in vitro* transcription/translation kit (Promega L1170). The TNT T7 Quick IVT system contains T7 RNA polymerase, nucleotides, salts, and ribonuclease inhibitor for *in vitro* transcription from a T7 promoter, as well as a rabbit reticulocyte lysate solution and amino acids to allow *in vitro* translation. Therefore, when a plasmid containing a gene under the control of a T7 promoter is incubated in the TNT solution, the gene product is expressed and may undergo posttranslational processing (e.g., chaperone-mediated folding). The IVT reactions should be performed fresh for each assay, as freeze/thaw cycles inactivate Cdc20 and Cdh1. For ubiquitination assays, synthesize substrates using [^{35}S]methionine and coactivators using unlabeled methionine. For [^{35}S]methionine IVT reactions, use 40 μl TNT T7 Quick Master Mix, 1 μg plasmid DNA, 4 μl [^{35}S]methionine (Redivue L-[^{35}S]methionine, Amersham AG1594), and water to 50 μl. For IVT reactions with unlabeled methionine, use 1 μl 1 m*M* methionine instead of [^{35}S]methionine. Incubate the reactions at 30° for 90 min. [^{35}S] Methionine-labeled reactions can be checked by running 3 μl on SDS–PAGE (see later).

Ubiquitination Assays

Ubiquitination assays are performed in 10-μl reaction volumes containing
0.50 μl 20× ubiquitination buffer
0.27 μl 0.1 *M* ATP
1.33 μl 5 mg/ml ubiquitin
2.00 μl purified E2 (yeast Ubc4, 500 ng/μl)
0.20 μl 1 mg/ml ubiquitin aldehyde
0.10 μl 200 μ*M* LLnL
1.00 μl ^{35}S-labeled substrate (produced in IVT)
0.67 μl purified APC (~50 ng/μl)
2.00 μl unlabeled Cdc20 or Cdh1 (produced in IVT)
1.93 μl H_2O

Prepare a reaction mixture on ice containing the ubiquitination buffer, ATP, ubiquitin, E2, ubiquitin aldehyde, LLnL, and water and then add substrate, APC, and coactivator as required. Incubate the reaction at room temperature for 45 min and stop it by adding 4 μl of 4× SDS–PAGE loading buffer. For negative controls, substitute APC with APC buffer, or coactivator with mock IVT reaction (i.e., IVT without plasmid). To control for substrate specificity, D- and/or KEN-box substrate mutants or nonphysiological substrates may be used.

Ubiquitin aldehyde is a specific inhibitor of ubiquitin C-terminal hydrolases (Hershko and Rose, 1987; Melandri *et al*., 1996; Pickart and Rose, 1986) and is included to prevent the hydrolysis of polyubiquitin chains. The proteasome inhibitor LLnL (Orlowski *et al*., 1993; Vinitsky *et al*., 1992) is included to prevent degradation of the polyubiquitinated proteins. The addition of exogenous E1 does not enhance the ubiquitination reaction, as E1 is abundant in reticulocyte lysate (Ciechanover *et al*., 1982; Haas and Bright, 1988). The optimal amounts of Ubc4 and APC should be determined by titration, as different preparations will have different activities.

SDS–PAGE and Autoradiography

For SDS–PAGE, standard gel compositions should be used (Sambrook and Russell, 2001) with the Bio-Rad Mini-PROTEAN 3 system (or similar). For visualizing ubiquitinated products, 8% minigels (8 × 7.3-cm gel plates) with 15-well combs provide good resolution. Run the entire reaction mixture (14 μl) on the gel at 140 V until the dye front reaches the bottom. Stain the gel with Coomassie blue (50% methanol, 10% acetic acid), destain (20% methanol, 10% acetic acid), dry on Whatman paper at 80° for 1 h, and expose to BioMax MR–1 film (Kodak).

In the presence of E1, E2, ubiquitin, ATP, and ^{35}S-labeled substrate, active APC conjugates ubiquitin proteins onto the substrate to form a polyubiquitin chain. As successive ubiquitins are added onto the substrate, its molecular mass increases by 8.6-kDa increments (the molecular mass of ubiquitin). On the autoradiogram, a ladder of ^{35}S-labeled substrate appears, increasing in average molecular weight as the reaction proceeds (Fig. 3A). Whereas other assays have only detected the conjugation of monoubiquitin onto one to three substrate lysines, this assay shows polyubiquitin chain formation. The APC with Cdh1 (APC^{Cdh1}) is more active than the APC with Cdc20 (APC^{Cdc20}) perhaps due to more efficient processing of Cdh1 relative to Cdc20 in the reticulocyte lysate (Passmore *et al.*, 2003).

Visualization of APC–Coactivator and APC–Substrate Complexes by Native Gel Electrophoresis

Because the core APC is active in ubiquitination reactions, it must interact with coactivators, E2, and substrates. To examine these interactions further, we have used an APC-binding assay (Passmore *et al.*, 2003). Traditional binding assays on columns could not be used, as IVT-produced Cdc20 and Cdh1 bind nonspecifically to affinity resins such as calmodulin Sepharose and Ni-NTA agarose. Native gels separate proteins based on their size, shape, and charge and therefore can be used to investigate protein–protein interactions that alter one or more of these parameters. A native gel system had been used previously to examine the interactions of the chaperonin containing TCP1 (CCT) with its substrates, one of which is Cdh1 (E. A. McCormack and K. R. Willison, unpublished results) (Liou and Willison, 1997; Valpuesta *et al.*, 2002). A modified version of this native gel assay using purified APC and proteins produced using IVT can be used to examine APC interactions (Passmore *et al.*, 2003).

APC–Coactivator Interactions

To examine APC–coactivator interactions, ^{35}S-labeled Cdh1 produced using IVT is mixed with purified APC. Interactions with APC can be monitored by changes in the mobility of the labeled coactivator. ^{35}S-labeled Cdh1 migrates as two species on a native gel (Fig. 3B, lane 1). A diffuse, faster migrating band probably represents free Cdh1, whereas a slower, more discrete band corresponds with the migration of CCT. This CCT comes from the rabbit reticulocyte lysate and is required for proper Cdh1 processing (Camasses *et al.*, 2003). (CCT is a eukaryotic chaperonin that binds to specific substrates and assists in their folding.) Upon addition

of APC, two additional bands appear (Fig. 3B, lane2). These bands migrate very slowly in native gels and represent APC–coactivator complexes, as they undergo a band shift upon the addition of antibodies directed against APC subunits (Passmore *et al.*, 2003). In addition, they correspond with the migration position of purified APC (Passmore *et al.*, 2003).

Binding reactions are performed in 14 μl with the following compositions:

2 μl purified APC (~50 ng) or *CaM elution buffer* (Table I) for negative control

2 μl ^{35}S-labeled Cdh1 (produced in IVT as described earlier)

0.7 μl 100 m*M* $CaCl_2$

$CaCl_2$ is required for proper migration on the native gels (E. A. McCormack, unpublished results). Adjust the volume to 14 μl with binding buffer (10 m*M* Tris-Cl, pH 8.0, 150 m*M* NaCl, 3 m*M* DTT, 1 m*M* Mg-acetate, 2 m*M* EGTA). Incubate samples at room temperature for 15 min, add 1.5 μl native gel sample buffer (see later), and load the entire reaction onto a 5.25% native gel.

We have observed that lower amounts of Cdc20 bind to the APC than Cdh1 in the native gel assay (Passmore *et al.*, 2003). Instead, most Cdc20 remains bound to CCT, suggesting that yeast Cdc20 is processed poorly by rabbit CCT. This Cdc20 interacts poorly with the APC and explains why APC^{Cdc20} has reduced ubiquitin ligase activity compared to APC^{Cdh1} (Fig. 3A). This may be due to species-specific differences between yeast Cdc20 and rabbit CCT. Alternatively, phosphorylation of Cdc20 or additional factors may be required for its efficient release from CCT and subsequent interaction with APC. Because Cdc20 does not appear to be processed properly, we perform most *in vitro* studies with Cdh1.

APC–Substrate Interactions

Similar to the APC–coactivator binding assay described earlier, APC–substrate interactions can be examined using native gels (Passmore *et al.*, 2003). The migration of ^{35}S-labeled APC substrates (Clb2 and a D- and KEN-box-containing domain of Hsl1, $Hsl1p^{667–872}$) changes upon the addition of APC and Cdh1 (Fig. 3B). Both APC and coactivator are required for this band shift. These bands represent APC–substrate complexes, as their migrations are retarded by the addition of APC antibodies (Fig. 3B, lanes 5, 6, 9, and 10). These interactions are specific and likely to be physiologically relevant because they are dependent on the presence of intact D- and/or KEN-boxes (Fig. 3B, lanes 11 and 12) (Passmore *et al.*, 2003).

APC–substrate-binding reactions are performed in 14 μl with the following compositions:

2 μl purified APC (~50 ng) or *CaM elution buffer* (Table I) for negative control
2 μl ^{35}S-labeled substrate (produced in IVT as described earlier)
2 μl unlabeled coactivator (produced in IVT as described earlier) or mock IVT reaction for negative control
0.7 μl 100 m*M* $CaCl_2$

Adjust the volume to 14 μl with binding buffer (10 m*M* Tris-Cl, pH 8.0, 150 m*M* NaCl, 3 m*M* DTT, 1 m*M* Mg-acetate, 2 m*M* EGTA). Incubate samples at room temperature for 15 min, add 1.5 μl native gel sample buffer, and load the entire reaction onto a 5.25% native gel.

We have been unable to detect interactions between APC and Pds1 in the native gel assay, presumably due to a lower affinity of APC^{Cdh1} for Pds1. In addition, the interaction of substrates with APC^{Cdc20} could not be detected, probably due to the poor processing of yeast Cdc20 by rabbit CCT as described previously. The pH of the native gels is high (pH 8.8), which may affect some protein–protein interactions negatively. However, our attempts to run native gels at lower pH have been unsuccessful.

Antibody Shifts

Antibodies to APC subunits induce an antibody shift (Fig. 3B), proving that the complexes visualized on native gels contain APC. For antibody shifts, prepare the binding reactions as described earlier, except make them up to 12 μl instead of 14 μl. After all of the other components have been mixed together, add 2 μl antibody and proceed as described earlier.

Native Gel Composition

We perform native gel analysis using the Mini-PROTEAN 3 system (Bio-Rad) with 1.5-mm spacers and 15-well combs. Prepare native gels using the following gel compositions (Liou and Willison, 1997):

Resolving gel: 0.37 *M* Tris, pH 8.8, 5.25% 37.5:1 acrylamide:bis-acrylamide
Stacking gel: 57 m*M* Tris, pH 8.8, 3.22% 37.5:1 acrylamide:bis-acrylamide
Running buffer: 25 m*M* Tris, 192 m*M* glycine, pH 8.3 (to make a 10× stock solution, dissolve 30 g Tris base and 144.2 g glycine in 1 liter water)

Native gel sample buffer: 125 m*M* Tris, pH 8.8, 84% (v/v) glycerol, bromphenol blue to color

Both the resolving and the stacking gels are made using a 30% acrylamide solution and a 1.5 *M* Tris solution (pH 8.8 at room temperature). Chill native gels and running buffer to 4° before use. Run gels at 110 V and 4° for approximately 2 h, until the dye front reaches the bottom of the gel. Fix, stain, and dry the gels as described previously and expose to BioMax MR–1 film.

Deletion Strains

The functions of individual APC subunits and their interactions within the complex are largely unknown. Because APC subunits are difficult to overexpress and purify, most of them cannot be studied in isolation. Five of the APC subunits are not essential for viability (*DOC1*, *APC9*, *CDC26*, *SWM1*, and *MND2*) and therefore can be deleted in the *CDC16-TAP* yeast strain. Using these deletion strains, the TAP purification approach, the *in vitro* ubiquitination assay, and the APC-binding assay provide excellent tools to delineate the roles of individual APC subunits.

To construct deletion strains, we use PCR-based gene targeting (as described earlier) using the pAG32c vector (Goldstein and McCusker, 1999) instead of pFA6a-TAP-kanMX6. The pAG32c vector contains a hygromycin resistance marker and can be used to transform the *CDC16-TAP* strain, which already has G418 resistance (using 200 μg/ml hygromycin B and 300 μg/ml G418 for selection). Strains with deleted APC subunits should be grown at 25°. Deletion of *DOC1, CDC26*, or *SWM1* results in temperature sensitivity, and the *DOC1* deletion strain grows very slowly even at 25°. APC can be purified from the deletion strains to examine its stability, activity, and ability to interact with coactivators or substrates.

Conclusion

This chapter describes a TAP purification approach to purify endogenous *S. cerevisiae* APC to near homogeneity. In addition, we described an *in vitro* ubiquitination assay and an APC-binding assay. In the ubiquitination assay, the APC forms polyubiquitin chains on yeast substrates (Pds1, Clb2, and Hsl1), but only in the presence of one of the coactivators, Cdc20 or Cdh1. These coactivators confer substrate specificity upon the APC in a D-box and KEN-box-dependent manner, reflecting *in vivo* observations. The native gel APC-binding assay can be used to explore the properties of coactivator and substrate interactions with the APC. In this assay, the

association of yeast APC substrates (Clb2 and Hsl1) with the APC is dependent on coactivator and intact substrate D- and KEN-boxes. These properties reflect the *in vivo* requirements of APC-mediated ubiquitination reactions. Finally, we described how these techniques can be used to study the roles of individual APC subunits.

Acknowledgments

We thank E. A. McCormack and K. R. Willison for assistance in developing the native gel assays. This work is supported by NIH Grant AG11085 to J. W. H. and by Cancer Research UK to D. B.

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[18] Enzymology of the Anaphase-Promoting Complex

By CHRISTOPHER W. CARROLL and DAVID O. MORGAN

Abstract

The anaphase-promoting complex (APC) is an ubiquitin-protein ligase that promotes mitotic progression by catalyzing the ubiquitination of numerous proteins, including securin and cyclin. Its complex subunit composition and extensive regulation make the APC an active subject of investigation for both cell biologists and enzymologists. This chapter describes a system for the reconstitution and quantitative analysis of APC activity from budding yeast *in vitro*. We focus in particular on the measurement of processive ubiquitination, which complements traditional analysis of the reaction rate as a means to elucidate the molecular details of substrate recognition and ubiquitination by the APC.

Introduction

The covalent modification of proteins with ubiquitin is a widespread regulatory mechanism in cell biology. In mitosis, ubiquitin-mediated proteolysis of securin (Pds1 in budding yeast) and mitotic cyclins is required for sister chromatid separation and mitotic exit. The ubiquitination of these proteins is catalyzed by an ubiquitin-protein ligase (or E3 enzyme) called the anaphase-promoting complex.

The APC is a structurally complex E3 containing at least 11–13 subunits, most of which are conserved from yeast to humans (Harper *et al.*, 2002; Peters, 2002). Two subunits, Apc11 and Apc2, contain a RING-H2 domain and a cullin-homology domain, respectively, and are thought to comprise the catalytic core of the enzyme: they form a stable subcomplex, can bind an E2-ubiqutin conjugate, and are sufficient to catalyze some aspects of the ubiquitination reaction (Gmachl *et al.*, 2000; Leverson *et al.*, 2000; Tang *et al.*, 2001). However, the Apc2/11 heterodimer lacks

METHODS IN ENZYMOLOGY, VOL. 398

0076-6879/05 $35.00
DOI: 10.1016/S0076-6879(05)98018-X