nated in the town of Contursi in the Salerno province of southern Italy (*11*); some members emigrated to the United States, Germany, and other countries. The extended family pedigree consists of 592 members with 60 individuals affected by PD (Fig. 1). High molecular weight genomic DNA was isolated from whole-blood lysate as described [G. I. Bell, J. Karam, W. Rutter, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5979 (1981)]. Genotyping was performed as described [J. M. Gyapay *et al.*, *Nature Genet* **7**, 262 (1994)]. Pairwise linkage analysis was performed by means of the MLINK program of the FASTLINK package. [G. M. Lathrop, J. M. Lalouel, C. Julier, J. Ott, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3442 (1984); R. W. Cottingham, R. M. Idury, A. A. Schaffer, *Am. J. Hum. Genet.* **54**, 252 (1984); S. K. Gupta, A. A. Schaffer, A. L. Cox, S. Dwarkadas, W. Zwaenepoel, *Comp. Biomed. Res.* **28**, 116 (1995).] Allele frequencies were used as reported in the Genome Data Base (http://gdbwww.gdb.org) and the Cooperative Human Linkage Consortium (CHLC) database (http://www.chlc.org). Multipoint analysis was performed with the LINKMAP program of the FASTLINK package. For the multipoint analysis allele frequencies were set to 1/*n* where *n* equals the number of alleles observed. In the two-point analysis, lod scores were calculated for both the reported and the 1/*n* allele frequencies with minimal effect on the maximum lod score observed. Simulations of multipoint analysis in a subset of the pedigree with different allele frequencies similarly indicated no significant effect on the scores calculated. Maximum lod scores as shown were observed for the heterozygote and homozygote disease allele penetrance set to 0.99 which is similar to the PD allele penetrance previously reported ranging from 0.88 to 0.94 (*11*). All unaffected individuals used in the study were of age above the mean for onset of illness. Disease allele frequency was set to 0.001. Distances and order of genetic markers were set as reported in the CHLC database. Overlapping three point analysis was performed for markers *D4S2361*, *D4S1647*, *D4S421*, and the PD locus. The 12-allele *D4S2380* locus was not included because of prohibitive run time. Multipoint analysis was performed on an IBM SP2 parallel computer and the SGI Challenge machine. Changes have been made in the pedigree shown to protect patient confidentiality.

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Identification of BIME as a Subunit of the Anaphase-Promoting Complex

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The initiation of anaphase and exit from mitosis require the activation of a proteolytic system that ubiquitinates and degrades cyclin B. The regulated component of this system is a large ubiquitin ligase complex, termed the anaphase-promoting complex (APC) or cyclosome. Purified *Xenopus laevis* APC was found to be composed of eight major subunits, at least four of which became phosphorylated in mitosis. In addition to CDC27, CDC16, and CDC23, APC contained a homolog of *Aspergillus nidulans* BIME, a protein essential for anaphase. Because mutation of *bimE* can bypass the interphase arrest induced by either *nimA* mutation or unreplicated DNA, it appears that ubiquitination catalyzed by APC may also negatively regulate entry into mitosis.

Mitotic cyclins bind to the protein kinase $p34^{cdc2}$ to promote entry into mitosis in all eukaryotic cells (*1*). During anaphase, mitotic cyclins are rapidly degraded by ubiquitin-dependent proteolysis, leading to the inactivation of Cdc2 and allowing exit from mitosis (*2*). This proteolytic system is also thought to initiate anaphase by ubiquitinating a hypothetical inhibitor of chromosome segregation (*3*, *4*). Cyclin B ubiquitination can be reconstituted in reactions containing the ubiquitin activating enzyme E1, either one of the ubiquitin conjugating enzymes UBC4 or UBCx (*5*, *6*), and APC (*5*, *7*). *Xenopus* APC contains homologs of CDC27 and CDC16 (*5*), proteins that are essential for the metaphase-anaphase transition in yeast and mammalian cells (*4*, *8*). However, the precise subunit composition of APC and the mechanisms of its activity and cell-cycle regulation remain unknown.

We immunopurified APC from interphase and mitotic *Xenopus* egg extracts and analyzed its subunit composition and activity (*9*). Both forms contained eight major polypeptides, that we refer to as APC1 through APC8, in approximately stoichiometric amounts (Fig. 1A and Table 1). The electrophoretic mobilities of APC1, APC3, APC6, and APC8 were reduced in APC purified from mitotic extracts. These mobility differences were apparently caused by phosphorylation because treatment of purified mitotic APC with λ protein phosphatase (λ-PPase) (*10*) produced a subunit pattern resembling the interphase form (Fig. 1D). Mitotic APC supported cyclin B ubiquitination in the presence of either an interphase 100,000*g* supernatant fraction (S100) or a mixture of purified E1 with UBC4 or UBCx (Fig. 1C) (*9*). Interphase APC had approximately 20% of the activity of mitotic APC. Treatment of mitotic APC with λ-PPase (*10*) resulted in a similar reduction of cyclin ubiquitination activity (Fig. 1E), suggesting that phosphorylation of APC subunits is essential for mitotic levels of activity. Similarly, the ubiquitination activity of partially purified cyclosome from clam is inhibited by addition of a phosphatase-containing fraction (*11*).

Protein microsequencing of individual APC subunits revealed that APC1, APC3, APC6, and APC7 are similar to previously described cell-cycle proteins (Table 1) (*12*). Peptide sequences obtained from APC3 and APC6 were 50 to 100% identical to sequences of human CDC27 and CDC16 (*8*), respectively, confirming our previous conclusion (*5*) that these proteins are subunits of APC. A peptide derived from APC7 was identical to a sequence encoded by the human expressed sequence tag H59410, which showed highest similarity to *Saccharomyces cerevisiae* Cdc23p (*13*). This suggests that APC7 is a *Xenopus* homolog of Cdc23p, which has previously only been found in budding yeast, where it interacts with Cdc27p and Cdc16p (*8*) and is required for cyclin degradation (*4*).

None of the peptide sequences revealed any similarity to Cse1p or Cdc26p—proteins required for cyclin degradation in budding yeast (*4*, *14*). We have found that a *Xenopus* homolog of Cse1p does not cofractionate with APC during column chromatography or density gradient centrifugation (*15*). It remains possible that a homolog of Cdc26p, which appears to be a component of yeast APC (*14*), is only loosely associated with the *Xenopus* complex, and is therefore lost during affinity purification. However, in *Xenopus*, neither Cse1p nor Cdc26p is essential for the reconstitution of cyclin B ubiquitination in vitro.

We obtained four peptide sequences from APC1 (*12*), all of which showed 60 to 83% identity to mouse Tsg24, a 216-kD protein that is 30% identical to *Aspergillus nidulans* BIME (*16*). In immunoblot experiments, antibodies to Tsg24 recognized a 210-kD protein in crude *Xenopus* interphase extracts and a

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220-kD protein in mitotic extracts (Fig. 2) and cross-reacted specifically with purified mitotic and interphase APC1 (Fig. 1B). The protein recognized by Tsg24 antibodies precisely cofractionated with CDC27 and CDC16 when either interphase or mitotic S100 fractions were analyzed by anion exchange chromatography and subsequent gel filtration (Fig. 2) (*17*). This suggests that APC1 is a *Xenopus* homolog of BIME and that BIME is associated exclusively with APC in *Xenopus* eggs. A homolog of BIME is also a subunit of APC in *S. cerevisiae*, and yeast BIME mutants are deficient in degradation of the mitotic cyclin Clb2p (*14*), suggesting that BIME is a highly conserved component of APC.

Aspergillus BIME and mouse Tsg24 share three hydrophobic segments that were originally proposed to function as transmembrane sequences (*16*). However, in immunoblot experiments we have been unable to detect BIME, CDC27, or CDC16 in membrane fractions (Fig. 2) (*17*). Therefore, we speculate that the hydrophobic stretches of BIME represent a motif that may be important for interaction with the tetratricopeptide repeats (*8*, *13*) of CDC27, CDC16, and CDC23.

Mutation of the *bimE* gene in *Aspergillus* results in arrest of the cell cycle before anaphase (*18*). A similar phenotype is caused by mutation of *Aspergillus bimA* (*19*), a homolog of CDC27 and therefore also a likely subunit of APC. Our findings provide a potential molecular mechanism for the metaphase arrest of these mutants, as they may be unable to ubiquitinate and degrade putative inhibitors of anaphase such as Pds1p and *Cut2*$^+$p (*20*).

In addition to regulating exit from mitosis, BIME may function as a negative regulator of entry into mitosis: *Aspergillus* cells treated with hydroxyurea or carrying a mutation in

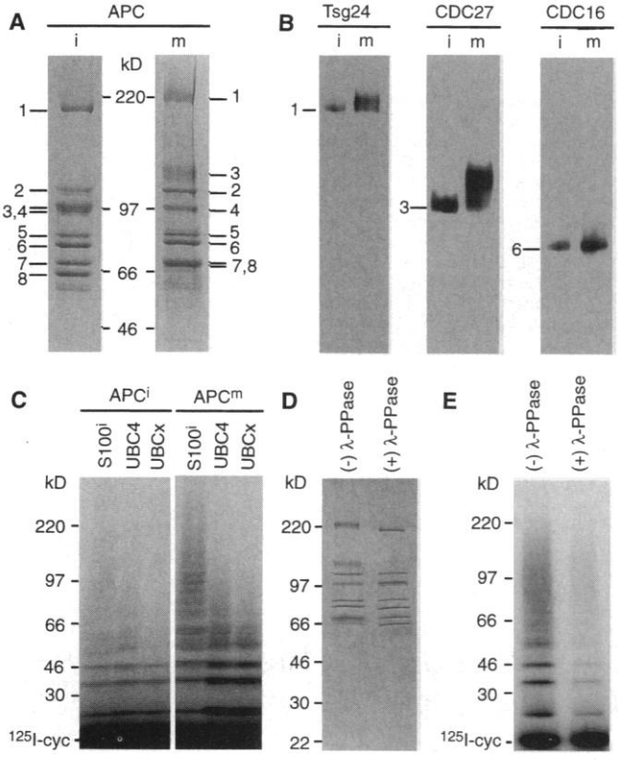

Fig. 1. Subunit composition and cyclin ubiquitination activity of *Xenopus* APC. (**A**) SDS-PAGE analysis of APC purified from interphase (i) and mitotic (m) extracts, stained with Coomassie. (**B**) Immunoblot analysis of interphase and mitotic APC1, APC3, and APC6 with antibodies specific for Tsg24 (mouse homolog of BIME) (*16*), human CDC27, or human CDC16 (*8*). (**C**) SDS-PAGE analysis of ^{125}I-labeled cyclin (^{125}I-cyc)–ubiquitin conjugates generated by interphase or mitotic APC in the presence of either interphase S100 (S100^i) or a mixture of purified recombinant wheat E1 with either *Xenopus* UBC4 or UBCx. (**D**) SDS-PAGE analysis of mitotic APC subunits treated with (+) or without (−) λ-PPase and stained with Coomassie. (**E**) SDS-PAGE analysis of ^{125}I-labeled cyclin-ubiquitin conjugates formed by λ-PPase–treated (+) or untreated (−) mitotic APC in the presence of E1 and UBC4.

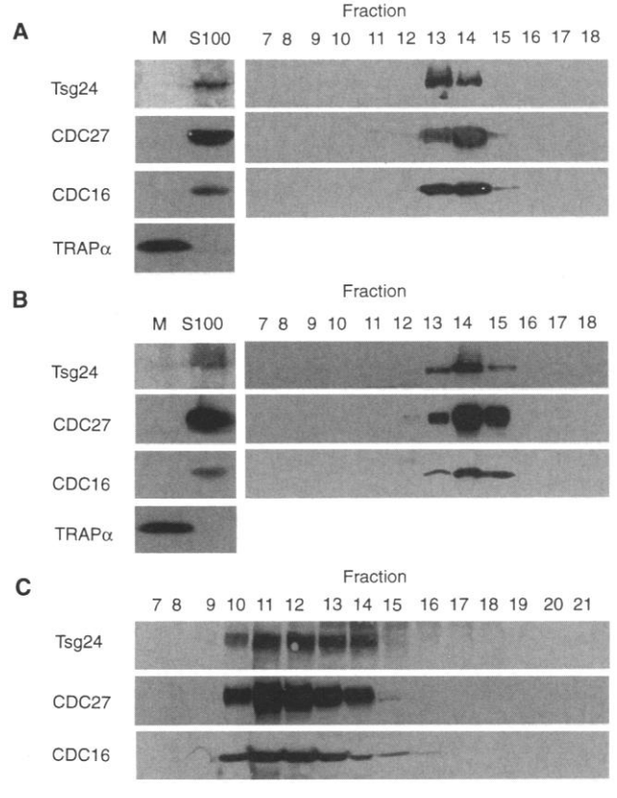

Fig. 2. Cofractionation of a *Xenopus* homolog of BIME with APC. (**A**) Immunoblot analysis of membrane (M), S100, and Resource Q column fractions (7 through 18) obtained from interphase *Xenopus* extracts with antibodies to the proteins indicated. Antibodies to the endoplasmic reticulum transmembrane protein TRAPα (*24*) were used to control for the complete separation of membranous and soluble proteins. (**B**) Immunoblot analysis as in (A) with fractions from mitotic *Xenopus* extracts. (**C**) Immunoblot analysis of Superose 6 fractions obtained by separation of the fractions 13 through 15 in (B).

Table 1. Subunits of *Xenopus* APC purified from interphase (i) and mitotic (m) egg extracts and their homologs in other species. *A.n., Aspergillus nidulans; D.m., Drosophila melanogaster; S.c., Saccharomyces cerevisiae;* and *S.p., Schizosaccharomyces pombe.* The references for each homolog are as follows: mouse Tsg24 and *A.n.* BIME (*16*); human CDC27, *D.m.* CDC27, *S.c.* Cdc27p, human CDC16, and *S.c.* Cdc16p (*9*); *A.n.* BIMA, (*19*); *S.p. Nuc2*$^+$p and *S.p. Cut9*$^+$p, (*23*); and *S.c.* Cdc23p (*13*).

Subunit	Molecular size (kD)		Homologs
	i	m	
APC1	210	220	Mouse Tsg24, *A.n.* BIME
APC2	112	112	–
APC3	100	130	Human CDC27, *D.m.* CDC27, *A.n.* BIMA, *S.c.* Cdc27p, *S.p. Nuc2*$^+$p
APC4	100	100	–
APC5	82	82	–
APC6	75	78	Human CDC16, *S.p. Cut9*$^+$p, *S.c.* Cdc16p,
APC7	69	69	*S.c.* Cdc23p
APC8	66	69	–

the *nimA* gene normally arrest in S and G_2 phase, respectively, but enter mitosis if *bimE* has been mutated (*18*). It is presently unclear why mutation of an APC subunit would cause this phenotype. One possibility is that mitotic cyclins accumulate in abnormal amounts in *bimE* mutants, resulting in premature entrance into mitosis. Alternatively, APC may negatively regulate other activators of mitosis such as NIMA (*21*). Others have proposed that APC may also regulate exit from S phase (*22*). Because APC is composed of eight subunits, it is conceivable that APC ubiquitinates multiple proteins that regulate cell-cycle progression.

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9. S100 prepared from interphase or mitotic *Xenopus* egg extracts was fractionated by Resource Q chromatography as described (*5*), except that the column volume was 50 ml, the volume of the salt gradient was 300 ml, and the fraction size was 15 ml. APC eluted between 345 and 450 mM KCl (corresponding to fractions 13 through 15 in Fig. 2, A and B) and was bound to beads covalently coupled with antibodies to CDC27 (*5*). The beads were washed with 40 volumes of buffer containing 15 mM tris-HCl (pH 7.7), 1 mM $MgCl_2$, 475 mM KCl, and 0.5% NP40. Ubiquitination assays with APC bound to antibody beads and elution of APC from the beads for SDS–polyacrylamide gel electrophoresis (PAGE) analysis were done as described (*5*). APC preparations from two different mitotic extracts (Δ90 extracts and extracts from metaphase II–arrested eggs) (*5*) had identical subunit compositions and ubiquitination activities.
10. Mitotic APC (~16 μg) bound to antibody beads was incubated for 30 min at 30°C in 100 μl of buffer [50 mM tris-HCl (pH 7.8), 1 mM dithiothreitol, 2 mM $MnCl_2$, and 100 μg/ml bovine serum albumin (BSA)] containing either 33 μg λ-PPase (300 units per microgram; Biolabs) or no λ-PPase. BSA and phosphatase were removed by washing the beads as described (*9*).
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12. APC (~250 μg) was purified from 500 ml of interphase *Xenopus* extract (15 g of protein) (*9*). Individual polypeptides were separated by SDS-PAGE, transferred to polyvinyldifluoride membrane (Bio-Rad) and processed for microsequencing at the Harvard Microchemistry Facility as described (*6*). APC1 and APC7 were digested with Lys-C, and APC3 and APC6 were digested with trypsin. The following sequences were obtained: APC1, DYIAPLPFQVANVXP, AEEQNAVLNLDQLGTPQHGMTTSSLTANLR, LSWTRNCDFEGSL, and PMTSIG; APC3, LLHLPAALGPLNPQFGIL, ILFANEK, and ALQELEELK; APC6, EFDFER, CYDFDVHTMK, and GLXLTAQY; APC7, EFFLAHIYTELQLIEEAL. Only high confidence sequences are listed, except for the third APC1 sequence which is derived from an equimolar mixture of two peptides. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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17. Membranes were isolated as described (*5*) with one additional washing step, and S100 and Resource Q column fractions were prepared as in (*9*). Fractions 13 to 15 (Fig. 2B) were pooled and further separated on a Superose 6 HR 10/30 column (Pharmacia) equilibrated in buffer Q-A (*5*). APC eluted with a molecular mass of 1.6 million daltons.
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Identification of Subunits of the Anaphase-Promoting Complex of *Saccharomyces cerevisiae*

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Entry into anaphase and proteolysis of B-type cyclins depend on a complex containing the tetratricopeptide repeat proteins Cdc16p, Cdc23p, and Cdc27p. This particle, called the anaphase-promoting complex (APC) or cyclosome, functions as a cell cycle–regulated ubiquitin-protein ligase. Two additional subunits of the budding yeast APC were identified: The largest subunit, encoded by the *APC1* gene, is conserved between fungi and vertebrates and shows similarity to BIMEp from *Aspergillus nidulans*. A small heat-inducible subunit is encoded by the *CDC26* gene. The yeast APC is a 36*S* particle that contains at least seven different proteins.

Mitotic cyclin degradation is required for the final exit from mitosis (*1*, *2*) and is a prerequisite for S phase in the subsequent cell cycle (*3*). In extracts from *Xenopus* eggs, degradation of cyclin B depends on a particle called the APC, which contains at least eight different proteins. The APC and the cyclosome, a particle found in clam oocytes, function as cell cycle–regulated ubiquitin-protein ligases that mediate destruction box–dependent ubiquitination (*4*, *5*) and thereby target cyclins for proteolysis by the proteasome (*6*). The isolation of mutants defective in cyclin degradation led to the identification of the tetratricopeptide repeat proteins Cdc16p, Cdc23p, and Cdc27p as subunits of the APC (*4*, *7*, *8*). These proteins are required for the onset of anaphase in various organisms (*9*, *10*). Because cyclin proteolysis per se is not required for anaphase (*2*, *11*), it has been suggested that the APC also targets for destruction proteins whose degradation is necessary for sister chromatid separation (*7*, *12*).

Mitotic cyclins such as Clb2p are rapidly degraded in G_1-arrested yeast cells (*7*, *13*). To isolate mutants defective in cyclin proteolysis, we screened mutagenized colonies for β-galactosidase activity resulting from the accumulation of a Clb2-lacZ protein in G_1 at 37°C as described (*7*). We identified 18 mutants that arrested as large, budded cells with a 2C DNA content after cycling cultures were shifted from 25° to 37°C (*14*). Complementation

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