CP60: A Microtubule-associated Protein that Is Localized to the Centrosome in a Cell Cycle-specific Manner

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Submitted June 23, 1995; Accepted September 26, 1995
Monitoring Editor: J. Richard McIntosh

DMAP190 is a microtubule-associated protein from *Drosophila* that is localized to the centrosome. In a previous study, we used affinity chromatography to identify proteins that interact with DMAP190, and identified a 60-kDa protein that we named DMAP60 (Kellogg and Alberts, 1992). Like DMAP190, DMAP60 interacts with microtubules and is localized to the centrosome, and the two proteins associate as part of a multiprotein complex. We now report the cloning and sequencing of the cDNA encoding DMAP60. The amino acid sequence of DMAP60 is not homologous to any protein in the database, although it contains six consensus sites for phosphorylation by cyclin-dependent kinases. As judged by in situ hybridization, the gene for DMAP60 maps to chromosomal region 46A. In agreement with others working on *Drosophila* centrosomal proteins, we have changed the names for DMAP190 and DMAP60 to CP190 and CP60, respectively, to give these proteins a consistent nomenclature. Antibodies that recognize CP60 reveal that it is localized to the centrosome in a cell cycle-dependent manner. The amount of CP60 at the centrosome is maximal during anaphase and telophase, and then drops dramatically during late telophase or early interphase. This dramatic disappearance of CP60 may be due to specific proteolysis, because CP60 contains a sequence of amino acids similar to the "destruction box" that targets cyclins for proteolysis at the end of mitosis. Starting with nuclear cycle 12, CP60 and CP190 are both found in the nucleus during interphase. CP60 isolated from *Drosophila* embryos is highly phosphorylated, and dephosphorylated CP60 is a good substrate for cyclin B/p34^{cdc2} kinase complexes. A second kinase activity capable of phosphorylating CP60 is present in the CP60/CP190 multiprotein complex. We find that bacterially expressed CP60 binds to purified microtubules, and this binding is blocked by CP60 phosphorylation.

INTRODUCTION

The centrosome nucleates microtubules and plays a central role in the organization of dynamic microtubule arrays in all animal cells (for reviews see Karsenti and Maro, 1986; Vorobjev and Nadezhdina, 1987; Kalt and Schliwa, 1993; Kellogg *et al.*, 1994). Unfortunately,

we know very little about this important organelle at the molecular level. The proteins that are responsible for microtubule nucleation remain largely unknown, as do the proteins that are responsible for centrosome assembly, duplication, and positioning. An important first step toward understanding the centrosome is to identify and characterize the many different proteins that function as its components.

We recently described the cloning of a partial cDNA encoding a *Drosophila* microtubule-associated protein that interacts with microtubules and is localized to the

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centrosome in a cell cycle-specific manner (Kellogg and Alberts, 1992). We called this protein DMAP190 to indicate that it is a *Drosophila* microtubule-associated protein with an apparent molecular mass of 190 kDa. We later found that DMAP190 is identical to the protein recognized by the Bx63 monoclonal antibody (Whitfield *et al.*, 1988), which was originally isolated in a screen for monoclonal antibodies that recognize nuclear antigens in the early *Drosophila* embryo (Frasch *et al.*, 1986). To give this protein a consistent name in the literature, we have agreed with the other researchers in the field to rename the protein CP190 (centrosomal protein of 190 kDa).

We used a fusion protein produced from our CP190 clone to generate affinity-purified antibodies that recognize the CP190 protein, and these antibodies allowed us to construct an immunoaffinity column. When *Drosophila* embryo extracts were passed over such an anti-CP190 immunoaffinity column, we found that CP190 was specifically retained and could be eluted in highly purified form with 1.5 M MgCl₂ (Kellogg and Alberts, 1992). Additional proteins that bound specifically to the anti-CP190 column could be detected by washing the immunoaffinity column with 1 M KCl before the MgCl₂ elution. This wash released approximately 10 additional proteins, which represent candidates for proteins that interact with CP190 within the cell (Kellogg and Alberts, 1992).

To further study these potential centrosomal proteins, we initiated a characterization of a 60-kDa protein that is retained on the anti-CP190 immunoaffinity column. Antibodies that recognize the 60-kDa protein revealed that it colocalizes with CP190 at the centrosome. Western blotting experiments show that this 60-kDa protein is quantitatively retained on an anti-CP190 immunoaffinity column, indicating a tight association with CP190, and that it is retained on microtubule affinity columns in a manner identical to CP190 (Kellogg and Alberts, 1992). These experiments provide strong evidence that the 60-kDa protein interacts with CP190 as part of a multiprotein complex within the cell. We originally called this protein DMAP60 to indicate that it is a 60-kDa *Drosophila* microtubule-associated protein, but we now refer to it as CP60 in accordance with the new name for DMAP190.

In the present study, we have used mouse polyclonal antibodies against CP60 to isolate a cDNA clone from a *Drosophila* ovarian cDNA library, which has served as a starting point for further characterization of the CP60 protein.

MATERIALS AND METHODS

Buffers

The following buffers were used. Phosphate-buffered saline (PBS): 14 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.2, 138 mM NaCl, 2.7 mM KCl. Lysis buffer: 50 mM N-2-hydroxyethylpiperazine-N'-2-ethane-

sulfonic acid (HEPES)-KOH, pH 7.6, 1.0 M NaCl, 2 mM Na₃EGTA, 2 mM Na₃EDTA, 0.25% Tween-20, 1 mM phenylmethylsulfonyl fluoride. Kinase buffer: 50 mM HEPES-KOH, pH 7.6, 1 mM MgCl₂, 1 mM Na₃EGTA, 0.1% Tween-20. Phosphatase buffer: 50 mM Tris-HCl, pH 7.8, 2.0 mM MnCl₂, 5 mM dithiothreitol (DTT), 100 µg/ml acetylated bovine serum albumin (New England Biolabs, Beverly, MA). Sample buffer: 65 mM Tris-HCl, pH 6.8, 3% SDS, 5% β-mercaptoethanol, 10% glycerol. Cosedimentation buffer: 50 mM HEPES-KOH, pH 7.6, 1 mM MgCl₂, 1 mM Na₃EGTA, 50 mM KCl. BRB80 buffer: 80 mM piperazine-N,N'-bis(ethanesulfonic acid) (Pipes)-KOH, pH 7.6, 1 mM MgCl₂, 1 mM Na₃EGTA.

Cloning and In Situ Hybridization

Mouse polyclonal antibodies that recognize CP60 (Kellogg and Alberts, 1992) were used to screen an ovarian lambda gt11 cDNA library (Steinhauer *et al.*, 1989), as described by Kellogg and Alberts (1992). We obtained six positive plaques by screening 250,000 plaques. The sequence of the largest DNA insert (1.3 kb) contained a polyadenylation site at one end and a large open reading frame extending to the other end. To test whether this clone encodes CP60, we used the polymerase chain reaction (PCR) to amplify a 0.7-kb fragment, using two PCR primers (primer 1, GCGAATTCCTGTC-CGCCGCCGC; primer 2, CGCGAATTCGCTCCAGCTTGGCGG). The PCR fragment was cloned into the *Eco*RI site of the vector pGEX.1, which directs expression of proteins fused to the enzyme glutathione-S-transferase (GST; Smith and Johnson, 1988). We thereby obtained a 45-kDa fusion protein, which was purified and used to generate affinity-purified antibodies according to the methods described by Kellogg and Alberts (1992). These antibodies recognized CP60 on Western blots and in fixed embryos in a manner identical to the original mouse polyclonal sera, demonstrating the correct identity of the clone.

Northern blot analysis was used to determine the size of the CP60 mRNA in RNA isolated from 0- to 3-h embryos. Based on the size of the mRNA (1.8 kb), and the presence of a polyadenylation site at one end of the partial CP60 cDNA, we concluded that our clone was lacking approximately 0.5 kb from the 5' end of the cDNA. We used a nested PCR approach to clone this missing DNA from a plasmid cDNA library (Brown and Kafatos, 1988; Gibbons *et al.*, 1991). A primer complementary to the cloning vector used for construction of the cDNA library (Brown and Kafatos, 1988), and a pair of nested primers taken from the sequence of the partial CP60 cDNA were used. A single 0.5-kb DNA fragment was obtained in the nested PCR reaction. The fragment was subcloned and sequenced, and the overlap of its sequence with that of the partial cDNA clone demonstrated that it was indeed the 5' end of the CP60 cDNA.

To confirm that the open reading frame of the full-length cDNA encodes a protein of the expected size (60 kDa), PCR was used to amplify the open reading frame and clone it into the vector pTrcHis (Invitrogen, San Diego, CA), which directs expression of proteins fused to six histidines. The fusion protein was expressed in bacteria according to the protocol supplied by Invitrogen.

The location of the CP60 gene on polytene chromosomes was determined using a digoxigenin-dUTP-labeled probe as previously described (de Frutos *et al.*, 1989).

Sequencing

Both strands of the CP60 cDNA were sequenced by using either the Sequenase protocol (United States Biochemical, Cleveland, OH), or the fluorescent dye-labeled terminator chemistry employed by the Biomolecular Resource Center at UCSF.

Immunofluorescence

Embryos were fixed with formaldehyde and stained according to the method of Theurkauf (1992). The affinity-purified rabbit antibodies against CP190 and CP60 were prepared as described by

Kellogg and Alberts (1992), and were used for immunofluorescence experiments at a concentration of 1–2 $\mu\text{g}/\text{ml}$. These antibodies stain only CP190 or CP60 in Western blotting experiments using crude embryo extracts, and the affinity-purified anti-CP60 antibodies used for this study stain the centrosome in a manner identical to the mouse serum used in our previous study (Kellogg and Alberts, 1992). For the triple label experiment shown in Figure 4, we used the same anti-CP60 mouse serum that we used previously (Kellogg and Alberts, 1992). Photomicrographs were taken on a Nikon Microphot FXA using a Nikon 60X plan apo objective and Kodak technical pan film at ASA 400 (see Figure 2A).

Immunoaffinity Chromatography

Immunoaffinity chromatography was carried out according to the methods described in our previous study (Kellogg and Alberts, 1992), with several modifications. High affinity polyclonal antibodies, rather than low affinity antibodies, were used, and the extract and wash buffers contained 70 mM KCl rather than 50 mM KCl (see Figure 5). Gel electrophoresis was carried out as previously described (Anderson *et al.*, 1973).

Immunoprecipitation, Dephosphorylation, and Phosphorylation of CP60

To prepare beads for the immunoprecipitation of CP60, 100 μl of protein A-Sepharose (Bio-Rad, Richmond, CA) were mixed with 100 μg of affinity-purified anti-CP60 antibody in a volume of 1 ml of PBS for 1 h. The bound antibody was then covalently linked to the beads with dimethylpimelimidate according to the method of Harlow and Lane (1988). After the coupling reaction, the beads were washed three times with 0.1 M glycine (pH 3.0) before use to remove any antibody not covalently linked, followed by PBS to remove the glycine buffer.

To immunoprecipitate CP60, a *Drosophila* embryo extract was made by homogenizing 1 g of embryos (age 1 to 2.5 h) in 2 ml of lysis buffer using a motor-driven Dounce homogenizer. The extract was centrifuged at 4°C for 1 h at 100,000 $\times g$, and 1 ml of the supernatant was mixed with 100 μl of anti-CP60 beads. After mixing for 1 h at 4°C, the beads were pelleted and washed three times with 15 vol of lysis buffer, followed by two times with 15 vol of kinase buffer. For dephosphorylation of CP60, 10 μl of beads are washed once with 20 vol of phosphatase buffer, and then brought to a total volume of 25 μl with the same buffer. Lambda phosphatase (200 U; New England Biolabs) was added and the reaction was incubated at 30°C for 30 min. The beads were then washed once with 50 vol of kinase buffer, resuspended in 250 μl of sample buffer, and incubated at 100°C for 3 min to release the CP60 from the antibody. For Western blots, 10 μl of the CP60 released from the beads was loaded onto a 10% polyacrylamide gel, which was blotted onto nitrocellulose and probed with anti-CP60 antibody.

To phosphorylate CP60, 10 μl of beads carrying dephosphorylated CP60 (prepared as described above) were washed two times with kinase buffer, and then brought to a total volume of 25 μl with kinase buffer containing 300 μM ATP, 0.1 mCi/ml [γ - ^{32}P]ATP (10 mCi/ml, 3000 Ci/mmole), and 1 mM DTT. Purified GST-cyclin B/p34^{cdc2} kinase (5 μl) was then added and the reaction was allowed to proceed for 45 min at room temperature. (The GST-cyclin B/p34^{cdc2} kinase was purified from *Xenopus* embryos as previously described [Kellogg *et al.*, 1995], and was at 0.7 mg/ml in 30 mM Tris-HCl, pH 7.6, 75 mM KCl, 1 mM MgCl_2 , 1 mM EGTA, 5 mM reduced glutathione, 10% glycerol). After the reaction, the beads were washed twice with 50 vol of kinase buffer and resuspended in 40 μl of sample buffer. After incubation at 100°C for 3 min, 15 μl was loaded onto a 10% polyacrylamide gel, which was dried and exposed to film to detect ^{32}P incorporation into CP60. For Western blotting, the CP60 released from the beads was diluted 10-fold and 15 μl was loaded onto a 10% polyacrylamide gel.

Proteins that interact with CP60 and CP190 were prepared for phosphorylation reactions by concentrating the fractions from the immunoaffinity purification experiment shown in Figure 5. To lower the salt concentration in these fractions, 100 μl of the elution from each column was diluted 1 to 5 with 50 mM HEPES, pH 7.6, 1 mM Na_3EGTA , 1 mM MgCl_2 , 0.1% Tween-20, 0.5 mM DTT, and then concentrated fivefold using a Microcon-10 filtration device (Amicon, Beverly, MA). The concentrate was diluted 1 to 5 again with the same buffer, and then concentrated to a final volume of 15 μl . Phosphorylation reactions were carried out as described above, using 7.5 μl of the concentrated elutions for each reaction.

Cosedimentation of CP60 with Microtubules

Purified 6 $\times$ His-tagged CP60 was prepared as described elsewhere (Oegema *et al.*, 1995). The CP60 fusion protein concentration was determined from the absorbance at 280 nm using an extinction coefficient calculated from the primary amino acid sequence (0.41 ml $\text{mg}^{-1} \text{cm}^{-1}$). The purified CP60 fusion protein was prespun at 100,000 rpm for 10 min in a TLA 100 rotor (Beckman, Fullerton, CA). Purified bovine tubulin (Mitchison and Kirschner, 1984) at 10 mg/ml in BRB80 was polymerized by the addition of an equal volume of BRB80 buffer containing 20% dimethyl sulfoxide and 2 mM GTP at 37°C. Microtubules were stabilized following polymerization by the addition of taxol to 100 μM . Fusion protein (7.6 μg) in cosedimentation buffer (10 μl) was diluted to 90 μl with the appropriate amount of either cosedimentation buffer or cosedimentation buffer containing 2 M KCl to give the desired salt concentrations, and either 10 μl of microtubules or 10 μl of control buffer were then added. The mixtures were layered over 100 μl cushions of cosedimentation buffer containing 50% glycerol and spun at 100,000 rpm for 10 min in the TLA 100 rotor. Finally, supernatants and pellets were analyzed by electrophoresis on 11.5% polyacrylamide gels and stained with Coomassie blue.

To determine the stoichiometry of binding at saturation, cosedimentation of CP60 with microtubules was carried out as above at saturating ratios of CP60 to microtubules. Instead of cosedimentation buffer, 80 mM Pipes-KOH, pH 6.8, 1 mM MgCl_2 , 1 mM Na_3EGTA , 50 mM KCl was used. Pellets were electrophoresed on an 11.5% polyacrylamide gel alongside CP60 and tubulin standards and stained with Coomassie blue. Tubulin standards were prepared according to the method of Butner and Kirschner (1991). An image of the gel was transferred into the computer using a CCD camera attached to a Foto/Eclipse imaging system from Fotodyne (Hartland, WI). The resulting bands were quantitated using the histogram function of Adobe Photoshop (Mountain View, CA). Standard curves were generated using the curve fitting functions of Kaleida-Graph (Synergy Software, Reading, PA).

To determine whether phosphorylation affects the ability of CP60 to cosediment with MTs, purified 6 $\times$ His CP60 was phosphorylated with purified *Xenopus* cyclin B/p34^{cdc2} kinase in a reaction containing 20 μl kinase buffer, 2.5 μl 6 $\times$ His CP60, 2.5 μl kinase, and 0.5 mM ATP. The CP60 was at 0.9 mg/ml in 50 mM sodium phosphate (pH 8.0), 250 NaCl, 1 mM β -mercaptoethanol, while the cyclin B/p34^{cdc2} kinase was as described in the previous section. As controls, identical reactions were set up that lacked either the kinase or ATP, and the reactions were incubated at room temperature for 2 h.

For cosedimentation assays, KCl was added to each reaction to 50 mM, as well as 10 μg of carrier protein (the bacteriophage T4 gene 45 protein in cosedimentation buffer containing 0.05% Tween-20 and 1 mM DTT [Morris *et al.*, 1979]). The final volume of each sample was 28 μl . Twelve microliters of each CP60 sample were mixed with 10 μl of microtubules or control buffer; microtubules had been prepared as above but were diluted to 1 mg/ml in cosedimentation buffer containing 0.05% Tween and 1 mM DTT before mixing with the CP60. Samples were layered over 100 μl cushions of cosedimentation buffer containing 0.05% Tween-20, 1 mM DTT, and

50% glycerol, and were spun at 80,000 rpm for 10 min in a TLA 100 rotor (Beckman). Samples of the supernatant and pellet were electrophoresed on an SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with antibodies that recognize CP60.

RESULTS

Cloning and Sequencing of the CP60 cDNA

In a previous study we produced mouse polyclonal antibodies that recognize CP60 by injecting mice with protein purified by chromatography on an anti-CP190 immunoaffinity column (Kellogg and Alberts, 1992). To isolate a cDNA clone for CP60, we used these antibodies to screen a lambda gt11 library constructed with *Drosophila* ovarian cDNAs. We obtained five clones, each of which contained a 1.2-kb insert, and one clone carrying a 1.3-kb insert. Partial sequencing of the 1.3-kb insert and two of the 1.2-kb inserts revealed that they all were derived from a common cDNA. To confirm that these clones encode CP60, we subcloned a fragment of the largest cDNA clone into the vector pGEX.1, which directs expression of proteins fused to the enzyme GST (Smith and Johnson, 1988). Affinity-purified antibodies obtained using the resulting fusion protein recognize a 60-kDa protein that is localized to the centrosome in a manner identical to the protein recognized by the original mouse serum, thus demonstrating the correct identity of the clone.

Northern blot analysis revealed that the CP60 protein is encoded by an approximately 1.8-kb mRNA, indicating that our largest cDNA clone was lacking approximately 0.5 kb. We used nested PCR to amplify a clone containing the remainder of the coding sequences for CP60 from a plasmid cDNA library (see MATERIALS AND METHODS). The full length sequence of the CP60 cDNA is shown in Figure 1. The cDNA contains a 1.2-kb open reading frame that encodes a protein with a predicted molecular mass of 48 kDa. Because this is somewhat smaller than the expected size of 60 kDa, we generated a PCR fragment that carried the putative open reading frame and cloned it into a vector that directs the expression of proteins fused to six histidines. This CP60 fusion protein migrates slightly more slowly on polyacrylamide gels than CP60 from *Drosophila* embryos, consistent with the presence of the polyhistidine tag. This confirms the correct identity of the open reading frame.

CP60 is not homologous to any known proteins, although it has six potential sites for phosphorylation by cyclin-dependent kinases or MAP kinases (Figure 1) (Nigg, 1993). In situ hybridization of a CP60 DNA probe to polytene chromosomes reveals that the CP60 gene maps to chromosomal location 46A. We know of no mutations that map to this site, although we have not checked more recently obtained collections of P-element-induced lethal mutations.

CP60 Localizes to the Centrosome in a Cell Cycle-specific Manner

We have used affinity-purified rabbit polyclonal antibodies raised against a CP60-GST fusion protein to follow the localization of CP60 during nuclear cycle 10 in fixed *Drosophila* embryos. As shown in Figure 2, the amount of CP60 that can be detected at the centrosome peaks during late anaphase and telophase, and there is an abrupt loss of most of this centrosome staining in late telophase or early interphase. Figure 2A shows CP60 staining during interphase, when it is barely detectable at the centrosome, while Figure 2I shows CP60 staining during telophase, just before the loss of CP60 from the centrosome. As judged by the observation of many embryos, this loss appears to coincide with the time when the daughter centrosomes complete their migration to opposite sides of the nucleus (the early nuclear divisions in the *Drosophila* embryo take place very rapidly, and the centrosome divides and migrates immediately after chromosome segregation, during late telophase and early interphase (Kellogg *et al.*, 1988). The amount of CP60 at the centrosome remains low through the remainder of interphase and prophase, and then increases somewhat during metaphase before dramatically rising during anaphase. Thus, CP60 is localized to the centrosome in a cell cycle-specific manner. The CP60 antibody also stains the spindle region weakly during metaphase and anaphase.

There are two subtle differences in the centrosomal localizations observed for CP60 and CP190 in the early embryo. First, whereas the amount of CP60 at the centrosome appears relatively low during metaphase, and then rises dramatically during anaphase and telophase, the amount of CP190 at the centrosome is relatively high during metaphase and rises less dramatically during anaphase. The other difference appears only when embryos are fixed according to a different protocol. The embryos shown in Figure 2 were fixed with formaldehyde. If the embryos are fixed using methanol (Kellogg *et al.*, 1988), the weak staining of the spindle region by anti-CP60 during anaphase and telophase is somewhat more pronounced, whereas the anti-CP190 staining is unchanged. The formaldehyde fixation is known to give superior preservation of microtubules (Theurkauf, 1992), but the methanol fixation may be more likely to minimize artifacts due to epitope masking. Because of limitations inherent in immunofluorescence techniques, no strong conclusions can be derived from these subtle differences in the distributions of CP60 and CP190. Techniques utilizing fluorescently labeled proteins are currently being developed to learn more about the relative

TATATAAAACAAGCGGA

TCCTAGTTACATATACCAATTTCGTGTGTCGGTGTATCAAAACAGCCTCCAAAATTATCAGGAACGCGCAACCAGCGGGCCGAAGTCGCTGACTAAG

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1  M A I Q L D K I L Q D I A K E K V L H P N N G G A
1  ATG GCA ATC CAA CTG GAC AAG ATT CTG CAG GAC ATC GCC AAA GAA AAG GTG CTG CAT CCA AAC AAT GGC GGC GCC

26  S K Q E Q E E A W A R I G R L N K L S V H E S R S
76  AGC AAG CAG GAG CAG GAG GAG GCG TGG GCC AGG ATC GGG CGT CTC AAC AAG CTC TCT GTG CAC GAA TCG CGC TCC

51  I F I V L Q K K Y E Q E K L K G N S A W K L F S L
151 ATC TTC ATA GTG CTC CAA AAG AAG TAC GAG CAG GAA AAG CTA AAG GGC AAT TCG GCG TGG AAG CTC TTC AGT CTG

76  M H Q I H Q D P K I S I K E E N D Q V P M E E V E
226 ATG CAT CAG ATC CAT CAG GAC CCT AAA ATC TCA ATC AAG GAG GAG AAT GAT CAA GTA CCC ATG GAG GAG GTG GAG

101 E Y E M L E V Q E P E D E D D A H Q Y G Q H T N S
301 GAG TAC GAA ATG CTG GAG GTG CAG GAG CCT GAG GAC GAG GAT GAC GCC CAT CAG TAC GGA CAG CAC ACC AAC TCT

126 T G S A S S D G E S P T K S H S T G S P E K D E R
376 ACC GGC TCG GCG TCC TCC GAC GGC GAA AGC CCC ACA AAG TCA CAC AGC ACC GGT TCG CCA GAA AAG GAC GAG CGG

151 L A Y A K P N V D T P R P A I E E K K L L N T L A
451 TTG GCG TAC GCC AAA CCC AAC GTC GAC ACT CCT CGC CCA GCC ATC GAG GAG AAA AAG CTG CTC AAC ACA CTG GCT

176 N N T P R S S S L S A A A A V L Q K K G I T V K K
526 AAC AAC ACA CCG CGA AGT AGC TCG CTG TCC GCC GCC GCA GCA GTA CTG CAA AAG AAG GGA ATT ACT GTG AAG AAG

201 T P S S A A A P K P A I G Q Q A P A S G Q R S S S
601 ACG CCC AGC TCC GCA GCA GCA CCG AAA CCG GCT ATT GGG CAA CAG GCG CCA GGC AGT GGG CAA TCA GAG TCC

226 S R T T I Q L P D S L K R K L S E E Y T S S S A Q
676 TCG CGC ACC ACC ATT CAG CTG CCA GAT TCT TTG AAG CGC AAA CTT TCG GAG GAG TAC ACC TCG TCA TCG GCG CAA

251 K K Q T K T T S P K Q A A S S A G A T S V H T S V
751 AAA AAG CAG ACC AAG ACC ACG AGC CCG AAA CAA GCC GCT TCA TCG GCA GGC GCA ACT TCA GTG CAC ACC AGC GTT

276 P D Q L P A G N N V V H T T T A Q L M Q V K K E R
826 CCA GAT CAA CTG CCG GCG GGA AAC AAT GTG GTG CAC ACA ACC ACT GCC CAG CTG ATG CAG GTT AAG AAG GAG CGT

301 E D N Q T P P P G I N I I T I P S E Q Q I N G G G
901 GAG GAC AAC CAG ACA CCA CCG CCG GGT ATC AAC ATC ATC ACC ATT CCA TCG GAA CAA CAG ATA AAC GGT GGT GGT

326 G G T N S S T A A T I A S T S V A S T N G F S P H
976 GGG GGA ACG AAC TCA TCG ACA GCC GCC ACC ATT GCT TCC ACC TCG GTG GCT TCC ACA AAC GGC TTT TCG CCA CAC

351 I E E L D F K N D I I F G P K I N A A S S N T P E
1026 ATC GAG GAG CTG GAC TTT AAG AAT GAC ATT ATC TTC GGG CCG AAG ATC AAC GCC GCC TCC TCC AAT ACA CCC GAG

376 I I N L G N F D N S V P P T F F K N L C N S S R H
1101 ATT ATC AAC CTG GGC AAC TTT GAC AAC TCG GTG CCG CCA ACC TTT TTC AAG AAT CTA TGC AAC TCG TCG CGA CAC

401 E S L G L Y V A N V M N R L S S R A S A K L E L S
1176 GAG TCT CTC GGG CTG TAC GTG GCC AAT GTG ATG AAC AGA CTC TCT TCG CGT GCC TCC GCC AAG CTG GAG CTG TCC
      * * * * *
426 I L R A I L D V Q S D E L E Q *
1251 ATC TTG CGC GCC ATC CTC GAT GTG CAG AGC GAC GAA CTG GAG CAG TAG ACCGACTGATACTCGCAATTTAAATGATTACTCAG
      CTTACTTATTAGACAAACTAAAAAGGTAGTTATGTAGTAGGCGCTGTGAACCATTTCTCGTATTATCGTATTATTTTTCCGTGTTCACAGCTCTGC
      GTTCTGTGCGCCCACTTGTAATATGTTTACTCAGATTCTGGTATGAGTGTTCATAGCATTTACGCATGAATTTTAAAGCTAAATTTAAATGATAT
      TTCTAATATTGAGATTTCGAATTTGTCCTATTATTAAGGTTGAATATGCTACTTGATTTTAAATACGTTTATATGCAATAAATCTACATAACTTAA
      AAAAAA

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Figure 1. The sequence of the CP60 cDNA. Potential sites for phosphorylation by the cyclin-dependent protein kinase p34^{CDC2} are underlined. The potential destruction box near the C-terminus is marked by an asterisk above each of the underlined amino acids. Sequence available through GenBank accession number U38248.

localizations of CP60 and CP190 in living embryos (Oegema *et al.*, 1995).

CP60 Has a Potential Destruction Box

The mechanism by which CP60 is localized to the centrosome in a cell cycle-specific manner is unknown. The fact that CP60 has a number of potential phosphorylation sites for cyclin-dependent kinases or MAP kinases suggests that it is regulated by post-translational modification. Another possibility, however, is that the loss of CP60 at the centrosome at the end of mitosis is due to specific proteolysis. A number of proteins are known to be proteolytically

destroyed at the end of mitosis, including the B-type cyclins, CENP-E, and CENP-F (Evans *et al.*, 1983; Brown *et al.*, 1994; Liao *et al.*, 1995). The existence of additional proteins that are destroyed at the end of mitosis has been inferred from studies using inhibitors of protein degradation (Holloway *et al.*, 1993). In the case of the cyclins, it has been shown that specific proteolysis is mediated by a sequence of amino acids called a "destruction box," which targets the protein for proteolysis by a ubiquitin-dependent pathway (Glotzer *et al.*, 1991). We found that CP60 has a good match to the destruction box sequence near its C-terminus; this sequence is

Figure 2. CP60 is localized to the centrosome in a cell cycle-dependent manner. A double-label immunofluorescence experiment showing the distribution of CP60 (A, C, E, G, and I) and DNA (B, D, F, H, and J). The distribution of the DNA was revealed by staining with Hoechst 33258. The photomicrographs show a small section of the embryo surface during nuclear cycle 10 (see Kellogg *et al.*, 1988, for the distribution of microtubules at this stage). The antibody staining shown in each panel was photographed and printed using the same exposures, with the exception of panel A, which was printed somewhat lighter to help bring out the weak staining of the centrosome. Bar, 10 μ m.

marked in Figure 1, and is compared with destruction boxes from other proteins in Figure 3. The existence of a potential destruction box on CP60 is consistent with the possibility that it is proteolytically destroyed at the end of mitosis.

CP60 Localizes to the Nucleus during Interphase of Later Cell Cycles

During the early nuclear cycles that occur in the *Drosophila* embryo, CP60 is mainly detected at the centrosome, with faint staining over the nuclear region during interphase. Starting with nuclear cycle 12, staining of the nucleus during interphase becomes much more prominent. The intensity of this nuclear staining increases with each nuclear cycle, reaching a maximum during nuclear cycle 13 or 14. From this time of development onward, CP60 is found at the centrosome during mitosis, and within the nucleus during interphase. An example of this distribution is shown in Figure 4. Perhaps the relatively low level of nuclear staining in early embryos reflects the extremely short duration of interphase during these nuclear cycles: the time available may be insufficient for transport of CP60 into the nucleus (Foe and Alberts, 1983).

The nuclear staining observed with the CP60 antibody in early embryos appears uniform throughout the nucleus; however, in embryos that are undergoing gastrulation, one often sees a number of bright dots staining within the nucleus (arrowhead, Figure 4A). These dots do not correspond to the centrosome, because one often sees four to six of them scattered

within the nucleus. They also do not correspond to heterochromatic or highly condensed regions of the chromatin (Figure 4, compare A and B with C). Not all nuclei have these spots, and more work needs to be done to determine whether they appear at a specific time during the cell cycle.

It appears that CP60 and CP190 colocalize in the nucleus at all stages, being found in the same intranuclear dots (Figure 4, compare A and B); however, the nuclear localization of CP190 can often be detected in the syncytial blastoderm embryo earlier than can that of CP60.

Immunoaffinity Chromatography with Anti-CP60 Antibodies

CP60 was originally identified in an immunoaffinity chromatography experiment designed to identify proteins that interact with CP190 (Kellogg and Alberts, 1992). In that experiment, we constructed an immunoaffinity column using antibodies that recognize CP190. The column was loaded with a *Drosophila* embryo extract, washed extensively with buffer, and then eluted with 1.0 M KCl to release proteins that associate with CP190, while leaving CP190 itself bound to the antibody on the column. We found that the 1.0 M KCl elution released a number of proteins, including CP60. It was of interest, therefore, to perform the reciprocal immunoaffinity chromatography experiment using a column that contains antibodies that recognize CP60. This would provide further information on the multi-protein complex containing CP190 and CP60, and might also lead to the identification of new proteins. For example, proteins that interact only with the fraction of CP60 that are not complexed with CP190 would not be detected on an anti-CP190 immunoaffinity column.

We constructed immunoaffinity columns with antibodies that recognize CP60 or CP190. As a control, we used a column containing nonimmune rabbit IgG. A *Drosophila* embryo extract was loaded onto each of the three columns, and the columns were then washed extensively with buffer and eluted with 1.5 M KCl to release proteins that interact with CP190 or CP60. Figure 5 shows a polyacrylamide gel analysis of the proteins that were eluted from each of these columns. The group of proteins eluted from the anti-CP190 column is very similar to the group of proteins observed in the eluates from our original experiments (Kellogg and Alberts, 1992). However, we washed the columns in the present experiment under more stringent conditions to select only for more tightly bound proteins, and the pattern of proteins obtained is therefore slightly different. The most notable difference is that CP60 is more prominent. In experiments using even more stringent wash conditions (a greater volume of wash at a higher salt concentration), we find

<u>CP60</u>	R A I L D V Q S D
<u>B -Type Cyclins</u>	
CLB2	R L A L N N V T N
Human B1	R T A L G D I D P
Xenopus B1	R T A L G D I G N
Human B2	R T V L E E I G N
S. purp.	R A A L G N I S N
Arbacia	R A A L G N I S M
Starfish	R G A L E N I S N
Clam	R N T L G D I G N
Drosophila	R A A L G D L Q N
Cdc13	R H A L D D V S N
<u>A Cyclins</u>	
Human	R A A L A V L K S
Xenopus	R T V L G V I G D
Clam	R A A L G V I T N
Drosophila	R S I L G V I Q S

Figure 3. CP60 has a potential destruction box. A comparison of the potential destruction box in CP60 with destruction boxes found in A and B type cyclins from a variety of organisms.

that CP60 is the only protein remaining bound to CP190.

The 1.5 M KCl elution from the anti-CP60 column shows a prominent 190-kDa protein band, and Western blotting confirms that this is CP190, as expected. The fact that CP190 is the major protein that is retained on the anti-CP60 column suggests that these two proteins interact directly. A number of additional proteins are also retained on the anti-CP60 column, which appear to be identical to proteins retained on the anti-CP190 column. We see no major proteins interacting uniquely with the anti-CP60 column. In contrast, a number of proteins are retained on the anti-CP190 column that are not retained on the anti-CP60 column. These could be proteins that interact only with CP190 that is not bound to CP60, or the antibodies against CP60 might block the interaction of some proteins with the CP60/CP190 complex. Alternatively, the additional proteins seen binding to the anti-CP190 column could interact directly with CP190, in which case, the binding of these proteins to the anti-CP60 column would be due to tertiary interactions that might be preferentially lost during the extensive wash used in these experiments (60 column volumes). Further experiments will need to be done to determine whether CP60 and CP190 exist in a number of different complexes. Because none of the proteins are retained on the control column, it is likely that most of the interactions that we observe are biologically significant.

Phosphorylation of CP60 by $p34^{cdc2}$

Antibodies against CP60 recognize a number of closely spaced bands on Western blots, suggesting that CP60 is post-translationally modified within the cell. Because CP60 has six consensus sites for phosphorylation by cyclin-dependent kinases or MAP kinases, we suspected that these different forms were due to phosphorylation. To test this possibility, we immunoprecipitated CP60 from embryo extracts and then treated it with lambda phosphatase. This treatment caused a dramatic shift in the electrophoretic mobility of CP60 (Figure 6, compare lanes 1 and 2), confirming that CP60 is phosphorylated. To test whether CP60 is indeed a substrate for cyclin-dependent kinases, we incubated the phosphatase-treated CP60 with purified cyclin B/ $p34^{cdc2}$ kinase complexes. This caused CP60 to shift up to five different bands (Figure 6, compare lanes 3 and 4), which agrees well with the number of predicted phosphorylation sites for cyclin-dependent kinases on CP60.

We next wanted to determine whether we could identify any additional kinase activities that can phosphorylate CP60. Such kinases might be present in the complex of proteins that interact with CP60 and CP190. We therefore tested the CP60 and CP190-binding proteins isolated by immunoaffinity chromatography for the presence of kinase activity using dephosphorylated CP60 as a substrate. We found that the proteins that interact with CP190 contain a kinase activity that can phosphorylate CP60, whereas the proteins that interact with CP60 and control IgG do

Figure 4. In embryos older than cycle 12, CP60 and CP190 localize to the centrosome during mitosis, and to the nucleus during interphase. A triple-label immunofluorescence experiment showing the distribution of CP60 (A), CP190 (B), and DNA (C) in an embryo undergoing gastrulation. The photomicrographs show a small section of the embryo surface. Staining of the centrosomes in a cell undergoing anaphase is marked by arrows. An example of the staining of dots within the nucleus during interphase is marked by an arrowhead in the upper left corner; these are not centrosomes (see text). A more diffuse staining of the nucleus that is typical of interphase can also be seen in this cell.

Figure 5. Immunoaffinity chromatography with anti-CP60 and anti-CP190 antibodies. Immunoaffinity chromatography was carried out as described in MATERIALS AND METHODS, and protein samples were analyzed on a 10% polyacrylamide gel. The lane marked "supernatant" is 15 μ g of the high-speed supernatant that was loaded onto the columns, while the next three lanes show the proteins that elute from an anti-CP60 column (5 μ g), an anti-CP190 column (5 μ g), and an IgG control column with 1.5 M KCl. The gel is stained with Coomassie blue. For this experiment, three 1-ml columns were prepared, each containing 3 mg of antibody. The columns were loaded at 10 ml/h with extract made from 34 g of 0- to 4-h embryos. After loading, the columns were washed overnight with 60 column volumes of buffer containing 70 mM KCl, and then eluted with buffer containing 1.5 M KCl. The elutions from the anti-CP60 and anti-CP190 columns each contained 100 μ g of protein, while the elution from the IgG control column contained no protein that could be detected by a Bradford assay (Bradford, 1976).

not (Figure 6, lanes 7–9). These results suggest that the CP190/CP60 complex contains a kinase that phosphorylates CP60. The absence of this kinase activity in the proteins that bind to CP60 could be due to any of the reasons discussed in the previous section to account for the differences in the proteins binding to the anti-CP60 and CP190 immunoaffinity columns.

Figure 6. Phosphorylation of CP60. CP60 was immunoprecipitated as described in MATERIALS AND METHODS, treated with phosphatases or kinases, and analyzed by polyacrylamide gel electrophoresis. Lanes 1–4 are Western blots using anti-CP60 antibody to show shifts in electrophoretic mobility, while lanes 5–9 are autoradiographs showing incorporation of 32 P into CP60. (Lane 1) Immuno-precipitated CP60 that has been subjected to no treatment; (lane 2) immuno-precipitated CP60 treated with lambda phosphatase; (lane 3) immunoprecipitated CP60 treated with lambda phosphatase and then incubated in kinase reaction buffer; (lane 4) the same as lane 3 but incubated with purified cyclin B/p34cdc2 kinase; and (lanes 5 and 6) the same as lanes 3 and 4, but the gel has been exposed to film to show that CP60 has incorporated 32 P from ATP- γ - 32 P. In lanes 7–9, dephosphorylated CP60 was incubated in the presence of [γ - 32 P]ATP and the CP60- and CP190-binding proteins isolated by immunoaffinity chromatography in Figure 5. (Lane 7) incubation with the elution from the IgG control column; (lane 8) incubation with CP190-binding proteins; and (lane 9) incubation with CP60-binding proteins. Western blotting reveals that there is a corresponding shift in the electrophoretic mobility of the CP60 in lane 8, confirming that it is CP60 that is incorporating 32 P in this experiment.

CP60 Binds to Microtubules in a Phosphorylation-dependent Manner

In previous studies, we demonstrated that CP60 and CP190 bind to microtubules when a *Drosophila* embryo extract is passed over a microtubule affinity column. This experiment does not, however, tell us whether these proteins bind directly to microtubules, because they could bind by virtue of secondary interactions with other proteins that bind directly. To test whether CP60 is able to interact with microtubules directly, we expressed CP60 as a 6 $\times$ His fusion protein in bacteria, and purified it by metal affinity and gel filtration chromatography. We then determined whether purified CP60 would interact with purified tubulin, using a cosedimentation assay. We found that CP60 binds quantitatively to microtubules at physiological ionic strength, and that significant binding occurs at salt concentrations as high as 250 mM (Figure 7A). We carried out similar cosedimentation experiments to quantitate the stoichiometry of binding at saturating conditions, and found that approximately 1.4 molecules of CP60 are bound per tubulin dimer (not shown).

We next determined whether phosphorylation of CP60 affects its ability to bind to microtubules. We used purified cyclin B/p34^{cdc2} complexes to phosphorylate the purified 6 $\times$ His CP60 fusion protein, and we obtained quantitative phosphorylation of CP60, as seen by a quantitative shift of the CP60 protein to a slower-migrating form on polyacrylamide gels (Figure 7B, compare lanes 1 and 2). This phosphorylation of CP60 completely blocked its ability to

Figure 7. Binding of CP60 to microtubules. (A) Purified 6 $\times$ His CP60 was tested for binding to microtubules using a cosedimentation assay as described in MATERIALS AND METHODS, and samples of the supernatant and pellet were analyzed by polyacrylamide gel electrophoresis. For each pair of lanes, the lane labeled "S" is the supernatant, and the lane labeled "P" is the pellet. The first panel labeled "control" shows the behavior of CP60 alone, demonstrating that it does not pellet in the absence of microtubules. The next panel shows a series of microtubule-binding tests carried out in successively higher concentrations of salt, demonstrating that CP60 binds quantitatively to microtubules at physiological salt concentrations. The indicated salt concentrations are the sum of the buffer (50 mM HEPES-KOH, pH 7.6) and added KCl. The gel is stained with Coomassie blue. (B) Purified 6 $\times$ His CP60 was phosphorylated with cyclin B/p34^{cdc2} and tested for binding to microtubules as described in MATERIALS AND METHODS. The supernatants and pellets were analyzed on an SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with an antibody against CP60. In the first panel, the first pair of lanes shows the behavior of phosphorylated CP60, demonstrating that it fails to cosediment with microtubules. Lanes 2 and 3 show the behavior of CP60 from control phosphorylation reactions that lacked either ATP or kinase, respectively. The second panel shows the results of control binding experiments that lack microtubules.

bind to microtubules in the cosedimentation assay (Figure 7B).

DISCUSSION

We have cloned and sequenced a cDNA encoding CP60, a protein that interacts with microtubules and is localized to the centrosome. We originally identified CP60 by virtue of its interaction with CP190, another *Drosophila* microtubule-associated protein that is localized to the centrosome, and these two proteins are components of a multiprotein complex (Kellogg and Alberts, 1992). The fact that CP60 shows no homology to any known proteins is perhaps not surprising, because so few centrosomal proteins have been cloned and sequenced. The centrosome is undoubtedly a complex organelle composed of many different proteins, and the identification and cloning of genes encoding new centrosomal proteins are important steps toward understanding its function and behavior.

Antibodies that recognize CP60 demonstrate that it localizes to the centrosome in a cell cycle-specific

manner. The amount of CP60 at the centrosome is maximal during anaphase and telophase, and then drops dramatically sometime during late telophase or early interphase (see Figure 2). Our results suggest two possible mechanisms for regulating the association of CP60 with the centrosome. One possibility is that the disappearance of centrosomal CP60 at the end of mitosis is due to proteolytic destruction of CP60. A number of other proteins are known to be degraded at the end of mitosis, including the B-type cyclins, CENP-E, and CENP-F (Evans *et al.*, 1983; Brown *et al.*, 1994; Liao *et al.*, 1995). The idea that CP60 is degraded at the end of mitosis is supported by our finding that it has a sequence of amino acids similar to the destruction box that is known to be required for specific degradation of the B-type cyclins at the end of mitosis (Glotzer *et al.*, 1991). The dynamic changes in localization of CP60 might also be due to changes in its phosphorylation state, because our results demonstrate that CP60 has multiple phosphorylation sites for cyclin-dependent kinases or MAP kinases.

Although we observe phosphorylation of CP60 by cyclin-dependent kinases *in vitro*, it remains unclear which kinase or kinases actually phosphorylate CP60 *in vivo*. The substrate specificity of cyclin-dependent kinases and MAP kinases are found to overlap when studied *in vitro* (Nigg, 1993), and members of both of these large kinase families are known to be activated during mitosis (Gotoh *et al.*, 1990; Heider *et al.*, 1994; Minshull *et al.*, 1994). One criteria that can be used to suggest *in vivo* specificity is the finding of a kinase and its substrate together in a complex. For example, in the budding yeast mating pheromone signaling pathway, a number of kinases and their substrates have been identified by genetic and biochemical criteria, and these are found together in a large multiprotein complex (Marsh *et al.*, 1991; Reed, 1991; Choi *et al.*, 1994; Marcus *et al.*, 1994). It is therefore of interest that we found a CP60 kinase activity present in the group of proteins that form a complex with CP190. This kinase activity would seem to represent a good candidate for a protein that phosphorylates CP60 *in vivo*, and identification of this kinase should be possible by further purification followed by peptide sequencing.

The centrosome in animal cells undergoes a number of characteristic changes during mitosis, including a dramatic increase in the amount of pericentriolar material and in the number of microtubules nucleated by the centrosome (Kuriyama and Borisy, 1981; Voronova *et al.*, 1984; Vorobjev and Nadezhdina, 1987). There is also a dramatic increase in the presence of phosphorylated epitopes at the centrosome, as revealed by an antibody that recognizes a mitosis-specific phosphorylated epitope (Vandre *et al.*, 1984). It is interesting to note that the increased abundance of CP60 and CP190 at the centrosome is correlated with these events, suggesting that they may play a role.

Because CP60 is a highly phosphorylated protein, it may contribute substantially to the increase in phosphorylated epitopes seen at the centrosome during mitosis.

The CP60/CP190 complex binds to microtubules when a *Drosophila* embryo extract is passed over a microtubule affinity column. To determine whether CP60 alone is capable of interacting with microtubules, we obtained purified CP60 from a bacterial expression system, and tested whether or not it is capable of binding to purified microtubules in a co-sedimentation assay. We found that CP60 binds quantitatively to microtubules under physiological salt conditions, and that binding is completely blocked by phosphorylation of CP60 with the cyclin-dependent kinase p34^{cdc2}. The fact that the binding of CP60 to microtubules is regulated by phosphorylation argues that it is a genuine *in vivo* activity, rather than the result of an *in vitro* artifact. However, because we do not know when the phosphorylations that inactivate microtubule binding occur during the cell cycle, the function of the microtubule-binding activity of CP60 is unclear.

CP60 and CP190 can be added to a growing list of proteins that localize to both the nucleus and to microtubule structures (Barnes *et al.*, 1992; Kuriyama and Nislow, 1992; Nislow *et al.*, 1992; Yang *et al.*, 1992). The significance of the nuclear localization of these proteins remains unclear, although one can imagine a number of possibilities. Proteins like CP190 and CP60 could be bifunctional, carrying out one function involving microtubules, and an independent function in the nucleus. Alternatively, certain proteins that function in the cytoplasm could be moved into the nucleus to sequester their activity away from cytoplasmic proteins. In our case, when the nuclear envelope breaks down at the beginning of mitosis, CP60 and CP190 would be released into the cytoplasm to play a specific role in the microtubule-based activities of mitosis.

When we used immunoaffinity chromatography to identify proteins that interact with CP60, we found that CP190 is the major protein that interacts with CP60 under the conditions used for our experiment. A number of additional proteins that interact with the anti-CP60 immunoaffinity column also interact with an anti-CP190 immunoaffinity column. These results provide further support for the idea that CP60 and CP190 are components of a multiprotein complex.

The cloning and sequencing of the CP60 cDNA is an important step toward understanding the functions of CP60 and the centrosome. Most recently, we have used the CP60 cDNA sequence in a bacterial expression system to obtain large amounts of purified CP60 protein. The purified CP60 can be fluorescently labeled and injected into embryos to follow its behavior in living cells (Oegema and Alberts, unpublished data). When combined with site-directed mutagenesis,

this approach should allow us to determine how the putative destruction box and specific phosphorylations play a role in the cell cycle-dependent behavior of CP60. This approach may also allow us to create dominant mutations that can be used to disrupt the functions carried out by CP60 within the cell. Finally, the sequence of CP60 provides a first step toward identification of its homologues in other organisms that offer specific technical advantages for centrosome studies.

ACKNOWLEDGMENTS

We thank Christine Field, Nora Kury, Mercedes Rojas, Bill Sullivan, and Bill Theurkauf for providing helpful advice and support. We also thank Christine Field for providing adult supervision. This work was supported by National Institutes of Health grant GM-23928.

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