

The Cell Cycle–dependent Localization of the CP190 Centrosomal Protein Is Determined by the Coordinate Action of Two Separable Domains

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Abstract. CP190, a protein of 1,096 amino acids from *Drosophila melanogaster*, oscillates in a cell cycle–specific manner between the nucleus during interphase, and the centrosome during mitosis. To characterize the regions of CP190 responsible for its dynamic behavior, we injected rhodamine-labeled fusion proteins spanning most of CP190 into early *Drosophila* embryos, where their localizations were characterized using time-lapse fluorescence confocal microscopy. A single bipartite 19–amino acid nuclear localization signal was detected that causes nuclear localization. Robust centrosomal localization is conferred by a separate region of 124 amino acids; two adjacent, nonoverlapping fusion proteins containing distinct portions of this region show weaker centrosomal localization. Fusion proteins that contain both nuclear and centrosomal localization sequences oscillate between the nucleus and the cen-

trosome in a manner identical to native CP190. Fusion proteins containing only the centrosome localization sequence are found at centrosomes throughout the cell cycle, suggesting that CP190 is actively recruited away from the centrosome by its movement into the nucleus during interphase. Both native and bacterially expressed CP190 cosediment with microtubules in vitro. Tests with fusion proteins show that the domain responsible for microtubule binding overlaps the domain required for centrosomal localization. CP60, a protein identified by its association with CP190, also localizes to centrosomes and to nuclei in a cell cycle–dependent manner. Experiments in which colchicine is used to depolymerize microtubules in the early *Drosophila* embryo demonstrate that both CP190 and CP60 are able to attain and maintain their centrosomal localization in the absence of microtubules.

CENTROSOMES are the major microtubule organizing centers found in animal cells. Properties intrinsic to centrosomes include the capacity to duplicate as well as the ability to nucleate and organize microtubule arrays. These arrays, in turn, are essential for a variety of cellular processes including cell division and chromosome segregation, directed cell movement, and general cytoplasmic organization (for reviews see Mazia, 1987; Vorobjev and Nadezhdina, 1987; Schatten, 1994; Kellogg et al., 1994). Despite their importance and the fact that they have been studied for over a century, centrosomes remain a mystery. We still do not understand how centrosomes nucleate microtubules, how they duplicate and separate, or how the changes in centrosome composition and structure that accompany the transition from interphase to mitosis occur.

Our knowledge of the centrosome is largely phenome-

nological. A molecular characterization of the centrosome has been elusive due to its small size and paucity, which make biochemical purification difficult, and to its involvement in very general organizational processes, which makes genetic approaches problematic. Nevertheless, progress is being made. A number of centrosomal components have already been identified and their cDNAs cloned. Components of the spindle pole body, the centrosome equivalent in yeast and *Aspergillus*, have also been identified and clues to their function have been obtained from the analysis of mutants (for reviews on known centrosomal components see Kalt and Schliwa, 1993; Kimble and Kuriyama, 1992).

We identified a protein originally called DMAP190 (*Drosophila* microtubule associated protein of 190 kD) using a combination of microtubule affinity chromatography and immunocytology in *Drosophila* embryos (Kellogg et al., 1989). DMAP190 is a microtubule-associated protein that is recruited to centrosomes at the onset of mitosis. DMAP190 was found to be identical to the antigen recognized by the Bx63 antibody, which was uncovered in a bank of monoclonal antibodies made to *Drosophila* nuclei (Whitfield et al., 1988; Frasch et al., 1986). In agreement

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with other workers in the field, we now call this protein CP190 (for centrosomal protein of 190 kD).

CP190 has been cloned and sequenced (Whitfield et al., 1995; GenBank/EMBL/DBJ accession number Z50021); the sequence predicts a novel protein of 1,096 amino acids with an isoelectric point of 4.5 and a molecular weight of 120 kD. CP190 shares a low level of amino acid identity (<15%) over a considerable portion of its length with a class of proteins including neurofilaments, myosin heavy chain, and MAP-2. This homology is thought to result from the presence of extensive tracts of α helical structure that CP190 is predicted to contain. Although most of the proteins in this class possess coiled-coil structural motifs, CP190 does not contain the heptad repeats expected in a coiled-coil structure. Sequence comparisons have also identified a cluster of four putative zinc fingers between amino acids 472 and 590, roughly in the middle of the predicted protein (Whitfield et al., 1995). Native CP190 is found in nuclei during interphase. At prophase, upon nuclear envelope breakdown, CP190 rapidly accumulates at centrosomes where it remains throughout mitosis; beginning at telophase, CP190 is again imported into reforming nuclei (Frasch et al., 1986; Whitfield et al., 1988; Oegema, K., B. Alberts, J. W. Sedat, and W. S. Marshall, manuscript in preparation).

Immunoaffinity chromatography using columns constructed from anti-CP190 antibodies identified a group of proteins that interact with CP190 (Kellogg et al., 1992). One of these, CP60 (centrosomal protein of 60 kD) has been cloned and sequenced (Kellogg et al., 1995). CP60 exhibits behavior similar to CP190; CP60 is found in eluates from microtubule affinity columns and localizes to nuclei and to centrosomes in a cell cycle-dependent manner (Kellogg et al., 1992, 1995). However, CP60 shares no significant amino acid homology with CP190 or with any other known proteins (Kellogg et al., 1995).

Our objectives in this work are twofold: to determine how CP190 achieves its dynamic cell cycle-dependent pattern of centrosomal and nuclear localization and to gain insight into the function of CP190 and CP60 in the *Drosophila* embryo. These studies are a first step towards understanding the cell cycle-dependent changes in structure and function that occur at centrosomes. In this work, we focus on the identification of regions of CP190 important for its dynamic localization pattern and on an examination of the mechanism by which CP190 and CP60 localize to centrosomes *in vivo*.

Materials and Methods

Expression and Purification of CP190 Fusion Proteins

Two types of fusion proteins were used in these experiments: 6XHis tagged fusion proteins were made using the QIA express pQE9 vector (Stuber, 1990) from Qiagen (Chatsworth, CA) and fusions with glutathione-S-transferase (GST)¹ were made using the pGEX-2T vector (Smith, 1988) with a modified polylinker. An oligonucleotide was synthesized and ligated between the BamHI and EcoRI sites of pGEX-2T to create the final sequence GGATCCGGTACCAGATCTCGAGTCGACAA-

GCTTGGAAATTC. The new polylinker thus contains the following restriction enzyme cutting sites in order: BamHI, KpnI, BglII, XhoI, SalI, HindIII, and EcoRI. To generate the fragments of the CP190 DNA sequence that were cloned to produce fusion proteins, we performed nested PCR using a cDNA library as the template (Brown and Kafatos, 1988; Sambrook et al., 1989) and Vent DNA polymerase (New England Biolabs, Beverly, MA). The primers for PCR were derived by reference to the CP190 cDNA sequence (Whitfield et al., 1995) and they contained BglII and HindIII sites at their 5' ends. The PCR products were cloned into either the BamHI/HindIII sites in the pQE9 vector or the BglII/HindIII sites in the modified pGEX-2T vector. The 6XHis fusion proteins therefore begin with the sequence MRGSHHHHHHGS. Transformation was into *Escherichia coli* M15(pREP4) for pQE9-CP190 constructs or *E. coli* TG-1 for pGEX-2T-CP190 constructs.

A Ni-NTA resin (Qiagen) was used to purify the 6XHis fusion proteins. Chromatography was carried out according to the manufacturer's specifications with some modifications. The extract buffer was often supplemented with 2 M urea, since it significantly improves fusion protein solubility. Columns were eluted in 1-ml fractions in a buffer containing 250 mM imidazole (Sigma Chemical Co., St. Louis, MO). GST fusion proteins were purified as in (Smith, 1988) with some buffer modifications. Proteins were eluted in 50 mM Na phosphate, pH 8.0, 500 mM NaCl, 10 mM 2-mercaptoethanol, 5 mM reduced glutathione (Sigma Chemical Co.). Both 6XHis and GST fusion proteins were further purified on a Superose 12 gel filtration column equilibrated into FPLC buffer (50 mM Na phosphate, pH 8.0, 250 mM NaCl, 1 mM 2-mercaptoethanol) using a fast protein liquid chromatography (FPLC) system (Pharmacia Fine Chemicals, Piscataway, NJ).

The 6XHis fusion protein concentrations were determined by measuring their OD₂₈₀ in FPLC buffer using extinction coefficients calculated from their primary amino acid sequence (Gill and von Hippel, 1989). The GST fusion protein concentrations were determined relative to bovine serum albumin using the Bradford assay (Bradford, 1976).

Expression and Purification of CP60 Full-length Fusion Protein

Full-length 6XHis CP60 fusion protein was produced using the pREST vector from Invitrogen (San Diego, CA). Nested PCR was performed as above using primers from the CP60 cDNA sequence (Kellogg et al., 1995). The primers contained BglII and HindIII sites at their 5' ends and the resulting fragment was cloned into pRSETB. Transformation was into *E. coli* BL21(DE3)plysS. Purification was as above except that a Superose 12 gel filtration column was used in place of the Superose 12 column.

Fluorescent Labeling of Fusion Proteins

To label fusion proteins, 0.75 μ l of 12.5 mg/ml tetramethyl-rhodamine-NHS ester (Molecular Probes, Eugene, OR), dissolved in either *N,N*-dimethylformamide or dimethylsulfoxide, was added to 75 μ l of fusion protein (1–5 mg/ml) in FPLC buffer. The mixture was incubated on ice for 5 min, and 7.5 μ l of 2 M potassium glutamate, pH 8.0 and 0.75 μ l of 0.5 M dithiothreitol were added to stop the reaction. To remove free rhodamine, each labeled fusion protein was then transferred into injection buffer using a small spin column of Bio-Gel P-6 resin that excluded the protein (Bio-Rad Laboratories, Hercules, CA). The injection buffers used were 50 mM Hepes, pH 7.6, 250 mM KCl for the 6XHis fusion proteins and 50 mM Hepes, pH 7.6, 100 mM KCl for the GST fusion proteins. The extent of labeling was assayed by spectroscopy; if the protein was over or under labeled the procedure was repeated varying the amount of rhodamine added. (Proteins were considered over labeled if the absorption peak at 522 nm, due to rhodamine dimers, was equivalent to or higher than the absorption at 556 nm). Labeling stoichiometries were determined using a value of 50,000 M⁻¹ cm⁻¹ for tetramethyl rhodamine (Molecular Probes, Eugene, OR) and were generally between 0.15 and 0.5 rhodamine/protein monomer.

Embryo Injection and Confocal Microscopy

Embryos were manually dechorionated and injected at 50% egg length according to standard procedures (Santamaria, 1986). The concentrations of the injected labeled fusion proteins were between 2 and 15 mg/ml, and the volume injected was approximately 1–2% of the embryo's total volume (Foe and Alberts, 1983). When added as a second marker, 40,000-mol wt fluorescein dextran (Molecular Probes, Eugene, OR) was injected at con-

1. Abbreviations used in this paper: FPLC, fast protein liquid chromatography; GST, glutathione-S-transferase; MAP, microtubule-associated protein; NLS, nuclear localization signal.

centrations between 0.15 and 2 mg/ml. Time-lapse confocal microscopy was performed using a Nikon Optiphot fluorescence microscope equipped with the Bio-Rad MRC 600 laser scanning confocal attachment. All images were collected using a Nikon 60× Plan Apo lens with a numerical aperture of 1.4. Embryo injection and screening was performed on a Nikon Diaphot inverted microscope equipped with an epifluorescence attachment.

Fixation and Immunofluorescence

Embryos were fixed in 37% formaldehyde as described (Theurkauf, 1992). Vitelline membranes were removed with methanol. The rabbit anti-CP60 antibody used has been described (Kellogg et al., 1995). The rabbit anti-CP190 antibody used was prepared by immunizing a rabbit with a total of 1.5 mg of a 6XHis fusion with CP190 amino acids 385–508, prepared as described above. Immunizations and bleeds were carried out by the Berkeley Antibody Company (Richmond, CA). The antibodies were affinity purified on a column of immobilized 6XHis CP190 amino acids 385–508, prepared as described (Kellogg and Alberts, 1992) according to standard techniques (Harlow and Lane, 1988). Donkey Cy5 anti-rabbit and fluorescein anti-mouse antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Bead Coseimentation Assays (Native CP190)

Antibodies were coupled to Affi-prep protein A beads (Bio-Rad Laboratories, Hercules, CA) at 0.5 mg/ml using dimethylpimelimidate as described (Harlow and Lane, 1988). Rabbit antibodies to CP190 amino acids 2299–2554 were prepared as described above. Random rabbit IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). A total of 100 μ l of beads were incubated for 1 h at 4°C with 3 ml of *Drosophila* embryo extract prepared as in (Kellogg and Alberts, 1992) except that only two volumes of buffer containing 100 mM KCl were used to re-suspend the embryos. The beads were then extensively washed with 50 mM Hepes, pH 7.6, 1 M KCl, 1 mM Na₃ EGTA, 1 mM MgCl₂, 10% glycerol, 0.05% NP-40, plus 1:200 protease inhibitor stock (Kellogg et al., 1989). Cycled tubulin at 15 mg/ml in BRB80 (80 mM potassium Pipes, pH 6.8, 1 mM MgCl₂, 1 mM Na₃ EGTA) was polymerized by the addition of an equal volume of BRB80, 20% dimethylsulfoxide, 2 mM GTP at 37°C. The microtubules were stabilized following polymerization by the addition of taxol to 100 μ M. Microtubules (5 μ l) were diluted into 125 μ l of equilibration buffer (20 mM potassium Pipes, pH 6.8, 50 mM potassium acetate, 1 mM MgCl₂, 1 mM Na₃ EGTA) plus 0.1% Tween 20 before incubation with 50 μ l of beads for 20 min at room temperature. The beads were centrifuged through 10 ml sucrose step gradients consisting of 5 ml of 70% sucrose and 5 ml of 30% sucrose in equilibration buffer plus 0.1% Tween 20 using a table top centrifuge (International Equipment Company, Needham Heights, MA) at top speed for 10 min. Proteins pelleted with beads were analyzed by SDS–polyacrylamide gel electrophoresis.

Microtubule Coseimentation Assays (Bacterially Expressed Fusion Proteins)

To 30 μ g of each fusion protein in FPLC buffer, we added 13 μ g of the T4 bacteriophage gene 45 protein (Morris et al., 1979) as a carrier. The volume of each sample was brought up to 66 μ l with FPLC buffer, which was then exchanged for equilibration buffer using spin desalting columns. The fusion proteins were centrifuged at 100,000 rpm for 10 min in the TLA 100 rotor (Beckman Instruments, Fullerton, CA). Cycled tubulin at 18.5 mg/ml in BRB80 was used to prepare taxol stabilized microtubules as described above. Fusion protein (10 μ l) was mixed with 80 μ l of equilibration buffer containing 200 μ g/ml gene 45 protein (as carrier) and either 10 μ l of microtubules or 10 μ l of control buffer. The mixtures were layered over 100 μ l cushions of 80 mM potassium Pipes, pH 6.8, 1 mM MgCl₂, 1 mM Na₃ EGTA, 50% glycerol, and were then centrifuged at 100,000 rpm for 10 min in the TLA 100 ultracentrifuge. Supernatants and pellets were then analyzed by electrophoresis on 13.5% polyacrylamide gels.

Microtubule Bundling Assays

Cycled tubulin (8 μ l of 10 mg/ml) and rhodamine-labeled cycled tubulin (2 μ l of 10 mg/ml) were added to 10 μ l of BRB80 plus 2 mM GTP. The tubulin was allowed to polymerize at 37°C for 20 min. Labeled microtubules were stabilized by the addition of 80 μ l of BRB80 plus 20 μ M taxol. Fusion protein (1 μ l of 0.5 mg/ml in 50 mM potassium phosphate, pH 8.0, 300 mM KCl, 1 mM 2-mercaptoethanol) was mixed with 2 μ l of rhodamine-

labeled microtubules. The mixture was incubated at room temperature for 10 min and then diluted 1:25 into BRB80, 60% glycerol, 0.1% glutaraldehyde, plus an oxygen scavenging system (50 μ g/ml catalase, 100 μ g/ml glucose oxidase, 12.5 mM glucose), and mounted for viewing under the fluorescence microscope.

The tubulin used in the experiments described in this paper was purified from bovine brain according to Mitchison et al. (1984), through the phosphocellulose chromatography step. As judged by SDS–polyacrylamide gel electrophoresis, it is free of detectable microtubule associated proteins (MAPs).

Results

Identification of Protein Domains Responsible for the Centrosomal and Nuclear Localizations of CP190

After PCR was used to amplify six overlapping fragments of the CP190 cDNA, these fragments were cloned into the vector pQE9 to construct a series of fusion proteins (Fig. 1), each with a 12–amino acid tag containing 6-histidine residues at its amino terminus. Each of the fusion proteins is denoted by its CP190 amino acid numbers; (Whitfield et al., 1995). These fusion proteins were soluble and could be purified under native conditions; however, fusion proteins including amino acids 1–166 were insoluble and were

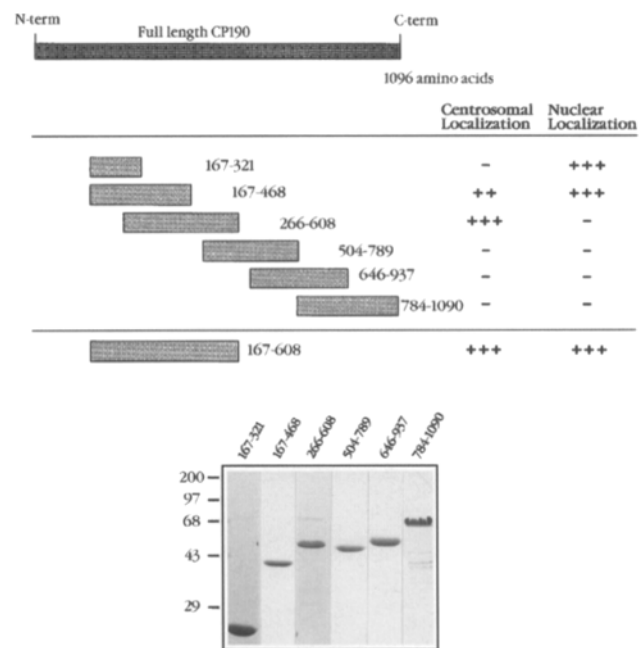


Figure 1. A map of the 6XHis tagged fusion proteins used in injection experiments. Purified fusion proteins containing the indicated sequences from CP190 (amino acid numbers are shown) were rhodamine-labeled and injected into syncytial *Drosophila* embryos. The columns to the right summarize the localization of each fusion protein, as observed by time-lapse confocal microscopy. In the inset, each of the purified fusion proteins has been analyzed by SDS–polyacrylamide gel electrophoresis (13.5% polyacrylamide) and detected by staining with Coomassie blue. The size of markers in kilodaltons is indicated in the left margin. For the injection experiments, the highest concentration tested was often limited by the solubility of the fusion protein. The maximum concentration injected was 4.6, 4.5, 5.6, 7.3, 13.6, 15.0, and 3.0 mg/ml for each fusion protein, as listed from top to bottom in the figure: each protein showing positive localization gave consistent results at concentrations of 1.5–2.0 mg/ml and higher.

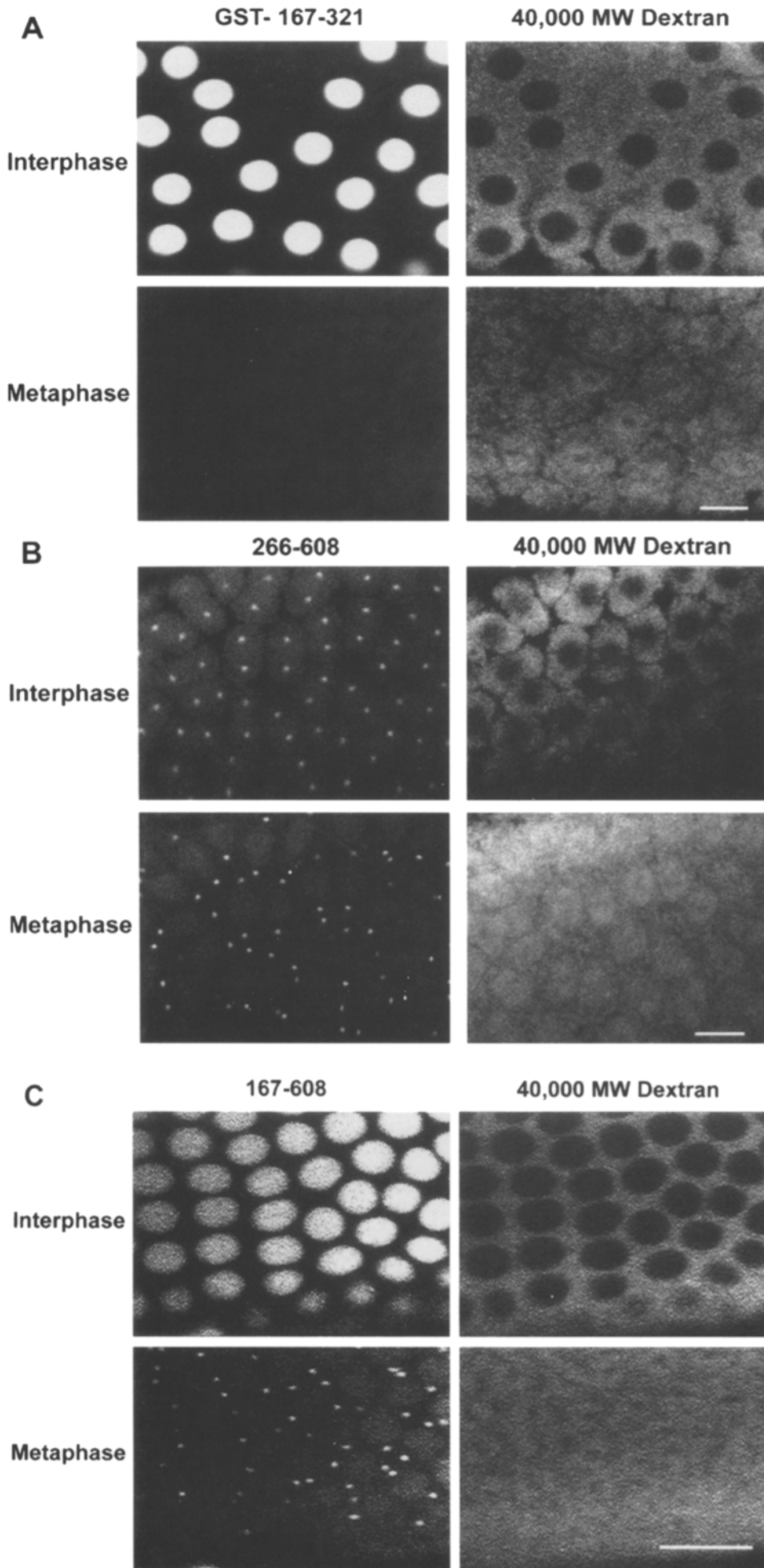


Figure 2. Injection of CP190 fusion proteins reveals three different localization patterns. Confocal micrographs of living embryos co-injected with rhodamine-labeled fusion protein and fluorescein-labeled 40,000-mol wt dextran. Each set of micrographs was selected from a time-lapse series taken of a portion of the embryo's surface during several cell cycles. The localization patterns are classified according to the localization of the fusion proteins during interphase and mitosis (interphase localization/mitotic localization). Shown here for each fusion protein are interphase (*top*) and metaphase (*bottom*) of the same cell cycle. Rhodamine-labeled fusion protein is on the left and fluorescein-labeled dextran is on the right. The 40,000-mol wt dextran was injected at concentrations of 0.15–0.3 mg/ml; the fusion proteins were injected at a concentration of 1.7 mg/ml. Examples of the three localization patterns obtained are shown: (a) Co-injection of rhodamine labeled GST-167-321 and 40,000-mol wt fluorescein dextran. GST-167-321 has a nuclear/cytoplasmic localization pattern; it is imported into nuclei as they reform in telophase and is completely localized in nuclei by interphase. Upon nuclear envelope breakdown, the fusion protein immediately disperses into the cytoplasm. Because the 40,000-mol wt dextran is excluded from nuclei as they reform, the nuclei appear as black holes against a cytoplasmic background of dextran. Upon nuclear envelope breakdown, the dextran immediately diffuses into the nuclei. (b) 266-608 is an example of a centrosomal/centrosomal pattern of localization; it is found at centrosomes at constant levels throughout the cell cycle. (c) 167-608 is an example of a protein that cycles between nuclei and centrosomes giving a nuclear/centrosomal localization pattern. This protein accumulates in nuclei during telophase and remains there throughout interphase; upon nuclear envelope breakdown, it immediately begins to accumulate at centrosomes. The centrosomal localization peaks by metaphase and remains relatively constant until the subsequent telophase. Bars, 10 μ m.

therefore not pursued. We purified the overlapping fusion proteins in Fig. 1 by Ni-NTA agarose affinity chromatography followed by passage over a FPLC Superose 12 sizing column. The sizing column step removed aggregated fusion protein and truncated products and was often essential to get functional proteins. After fluorescent labeling of the purified fusion proteins with *N*-hydroxysuccinimidyl rhodamine at low stoichiometry, we injected them into *Drosophila* embryos during or just prior to the late syncytial nuclear divisions (cycles 10–14); fluorescein-labeled 40,000-mol wt dextran was co-injected to serve as a cell cycle marker (Kalpin et al., 1994).

At early stages of *Drosophila* development, the embryo is a syncytium, so injected protein can diffuse via the common cytoplasm to the hundreds of nuclei present. By cycle 10, the nuclei have migrated to form a uniform monolayer just beneath the cortex (Foe and Alberts, 1983), simplifying their visualization. We followed these nuclei and their associated centrosomes using fluorescent time-lapse confocal microscopy. During interphase, the nuclei appear in the fluorescein channel as black holes that exclude dextran. At prometaphase, when the nuclear envelope breaks down, dextran floods into the nucleus, thus allowing us to accurately assess the cell cycle state of the injected embryos (Fig. 2).

Injection of each of the labeled fusion proteins resulted in either no localization or one of three localization patterns that we have designated nuclear/cytoplasmic, nuclear/centrosomal, and centrosomal/centrosomal, according to their

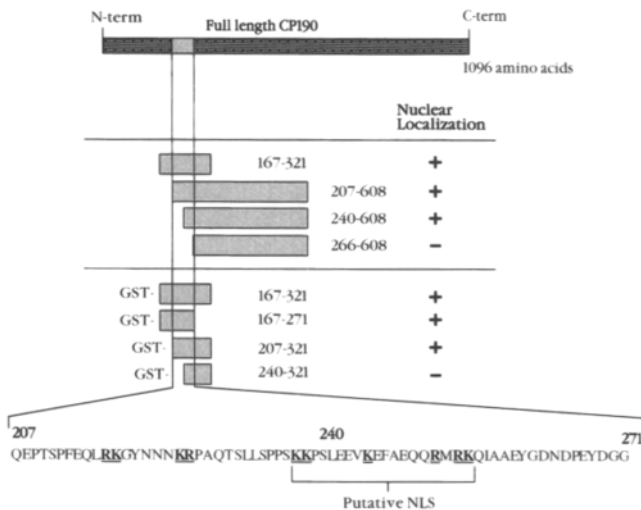


Figure 3. Characterization of the region of CP190 responsible for nuclear localization. Fusion proteins in the region of the nuclear localization domain are diagrammed here. The top 4 fusion proteins were 6XHis tagged and were injected at concentrations of 4.6, 1.3, 3.1 and 2.7 mg/ml (in order from top to bottom); the bottom four were fusions of small pieces of CP190 with glutathione-S-transferase and were injected at concentrations of 3.3, 1.5, 2.3, and 3.8 mg/ml (in order from top to bottom). Purified fusion proteins were rhodamine-labeled and injected into syncytial *Drosophila* embryos. Nuclear localization was scored by subsequent observation on an inverted fluorescence microscope. From this data, the region that we believe to be responsible for nuclear localization is found between amino acids 207 and 271. The basic residues are underlined. A bipartite NLS is found between amino acids 237 and 255.

locations during interphase and mitosis (interphase localization/mitotic localization). The first pattern, nuclear/cytoplasmic, is exemplified by fusion protein 167-321 (Fig. 1). This fusion protein is localized to the cytoplasm during mitosis, and is imported into reforming nuclei in telophase where it remains throughout interphase. Upon nuclear envelope breakdown, fusion protein 167-321 disperses evenly throughout the cytoplasm where it remains throughout mitosis. The localization of this portion of CP190 when injected as a GST fusion protein was identical to that of the smaller 6XHis fusion protein (see Fig. 2 a, GST-167-321).

Injection of fusion protein 167-468 resulted in a nuclear/centrosomal pattern of localization, labeling nuclei during interphase and showing weak centrosomal localization during mitosis. Fusion protein 266-608 gave a centrosomal/centrosomal pattern of localization (Fig. 2 b), strongly localizing to centrosomes with equal intensity during both interphase and mitosis.

We constructed fusion protein 167-608 (Fig. 1) to see if we could enhance the centrosomal localization of fusion protein 167-468 while maintaining its nuclear/centrosomal pattern of localization. This protein localized to nuclei during interphase and gave a robust centrosomal localization during mitosis, mimicking the localization pattern of the native protein (Fig. 2 c).

Characterization of a Region of CP190 Responsible for Nuclear Localization

Based on our injection data, the region responsible for the nuclear localization of our fusion proteins is located between amino acids 167 and 321. Closer examination of the amino acid sequence in this region identified a potential bipartite nuclear localization signal (NLS) between amino acids 237 and 255 (Fig. 3). A bipartite NLS consists of two

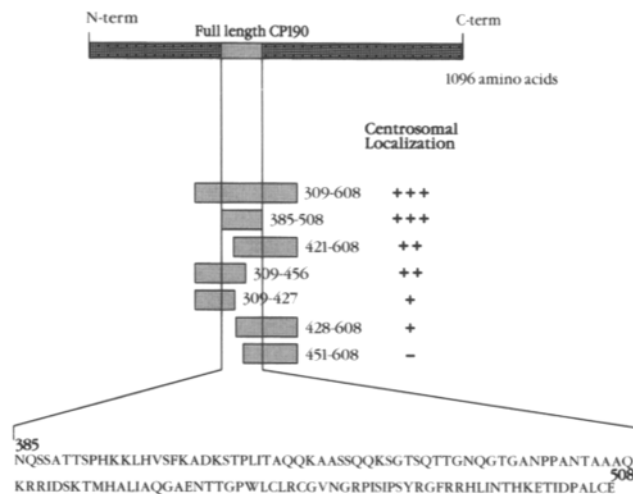


Figure 4. Characterization of the domain(s) of CP190 responsible for its centrosomal localization. The 6XHis tagged fusion proteins diagrammed were rhodamine-labeled and injected into syncytial *Drosophila* embryos. Centrosomal localization was scored by fluorescence confocal microscopy. Two non-overlapping fusion proteins, 309-427 and 428-608, localize to centrosomes, suggesting that the centrosomal localization domain contains multiple independent elements which together cooperate to give robust centrosomal localization.

basic amino acids, followed by a spacer region of approximately 10 amino acids, followed by another region of 5 amino acids, 3 of which are basic; in many cases, the spacer can vary considerably in length (Dingwall and Laskey, 1991). The potential NLS in CP190 has a spacer of 13 amino acids.

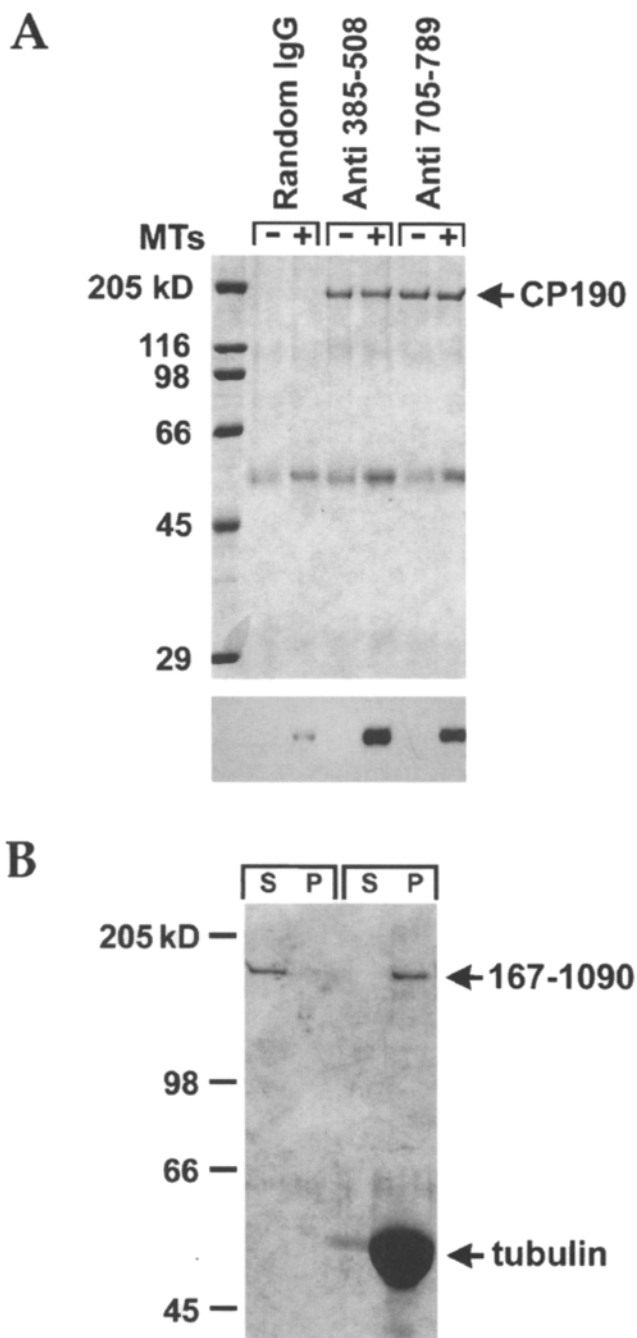
The fusion proteins shown in Fig. 3 were labeled with rhodamine and injected into embryos to test whether this potential NLS is responsible for the nuclear localization of our CP190 fusion proteins. Fusion protein 207-608 gave a nuclear/centrosomal localization pattern, confirming that it contains sufficient sequences to target the fusion protein to nuclei during interphase. Fusion protein 240-608 also gave a nuclear/centrosomal localization pattern, even though this construct deletes the upstream element of the bipartite NLS (Fig. 3). The slightly smaller fusion protein 266-608, representing an additional NH₂-terminal deletion of 26 amino acids, however, fails to concentrate in the nucleus. Thus, sequences essential for the nuclear localization of CP190 appear to lie between amino acids 240 and 266.

Some protein fragments were also expressed as fusions with GST. The GST fusion with amino acids 240-321 of CP190 does not localize to the nucleus. How can we explain these results? One possible explanation is that the complete bipartite NLS between amino acids 237 and 255 of CP190 is normally required for the nuclear localization of our fusion proteins, but, in the case of fusion protein 240-608 fused to 6XHis, the amino terminus with its positive charge can fill in for the missing upstream pair of basic amino acids.

Characterization of the Domain of CP190 Responsible for Centrosomal Localization

To determine if we could further delineate the domain of CP190 required to obtain centrosomal localization, smaller fusion proteins were constructed (Fig. 4). A 124-amino acid 6XHis fusion protein corresponding to amino acids 385-508 of CP190 was able to localize well to centrosomes when fluorescently labeled and injected into embryos. As seen previously for the larger fusion protein 266-608, this fusion protein localized to centrosomes constitutively, with equal intensity throughout the cell cycle. Fusion proteins 421-608 and 309-456 contain NH₂- and COOH-terminal deletions, respectively, of part of the 124-amino acid region of 385-508 (see Fig. 4). These protein fragments also localize to centrosomes, although the ratio of centrosomal staining to background cytoplasmic staining is decreased (data not shown). Two nonoverlapping fusion proteins, 309-427 and 428-608 were then constructed. Although both of these proteins localize to centrosomes, this localization is comparatively weak (data not shown). There is no obvious amino acid homology between these two independent regions of CP190. These results suggest that the centrosomal localization domain is complex, spanning at least the 124-amino acid region which allows robust centrosomal local-

Figure 5. Native and bacterially expressed CP190 bind microtubules. (a) Protein A beads coupled to random IgG, anti-CP190 amino acids 385-508 or anti-CP190 amino acids 705-789 were mixed with *Drosophila* embryo extract. The beads were washed



extensively with buffer containing 1 M KCl to remove any proteins associated with CP190 and were then mixed with taxol-stabilized microtubules or control buffer before layering on 10 ml sucrose step gradients and pelleting in a table top clinical centrifuge. Pelleted beads were boiled in sample buffer and released proteins were analyzed by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide). (Top) Coomassie-stained gel. (Bottom) Western blot of the central portion of the gel with the anti- α -tubulin mouse monoclonal DM1 α . Western blotting was necessary because IgG heavy chain that leached off the beads runs at the same molecular weight as tubulin. (b) The bacterially expressed 6XHis fusion with CP190 amino acids 167-1090 was tested for its ability to cosediment with taxol stabilized microtubules. The supernatants and pellets from sedimentations done in the absence (left two lanes) or presence (right two lanes) of taxol-stabilized microtubules were analyzed by SDS-polyacrylamide gel electrophoresis.

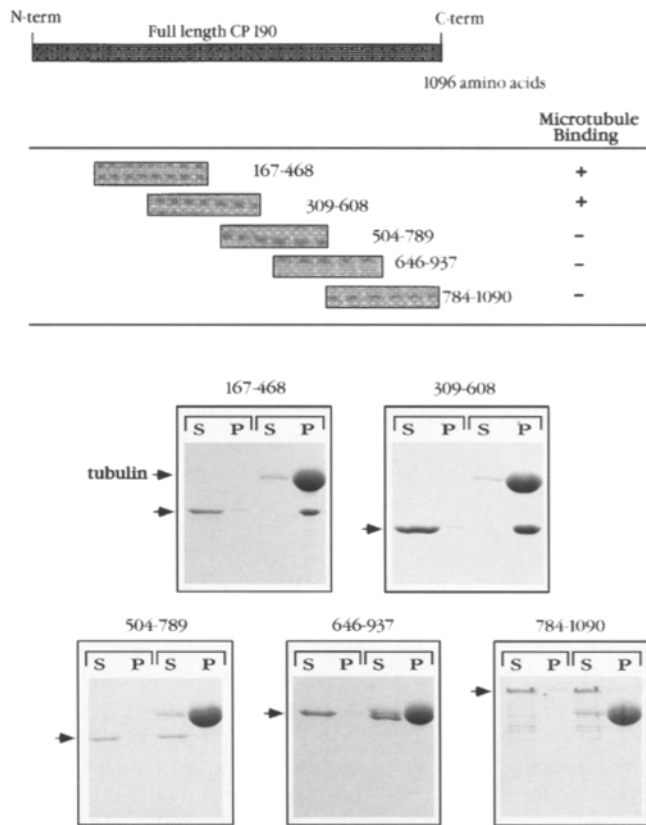


Figure 6. Identification of the region of CP190 important for its microtubule binding. (Top) Map of the fusion proteins spanning CP190 that were tested for their ability to cosediment with microtubules. (Bottom) The supernatants and pellets from sedimentations done in the absence (left two lanes in each box) or presence (right two lanes in each box) of taxol-stabilized microtubules. The samples were separated by electrophoresis on 13.5% polyacrylamide gels and stained with Coomassie blue. The large band in the right-most lane in each box is tubulin. Fusion proteins 167-468 and 309-608 pellet only in the presence of microtubules. Fusion proteins 504-789, 646-937, and 784-1090 remain in the supernatant with or without microtubules.

ization. No significant homology was found when these 124 amino acids (the amino acids between 385 and 508) were compared alone, or as a part of larger fragments, to known protein databases using the BLAST comparison tool (Atschul et al., 1990). The centrosomal localization domain does overlap slightly with the central domain of CP190 predicted to encode four zinc fingers (Whitfield et al., 1995), since the fusion protein with amino acids 385-508 contains one putative zinc finger. However, this zinc finger can not be required for centrosomal localization since the fragment 309-456, which contains no zinc fingers, also gives good localization to centrosomes.

The Domain of CP190 Responsible for Its Centrosomal Localization Cannot Be Separated from a Region That Confers an Ability to Cosediment with Microtubules

Since CP190 was originally identified by microtubule-affinity chromatography (Kellogg, et al., 1989), we wanted to determine if native CP190 could bind directly to microtubules. To do this, we used protein A beads coupled to anti-

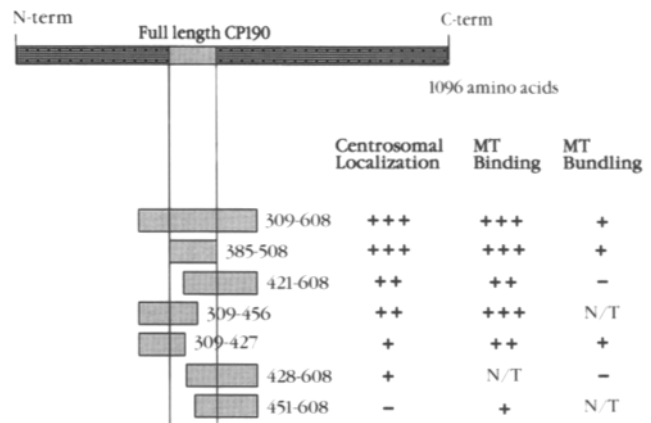
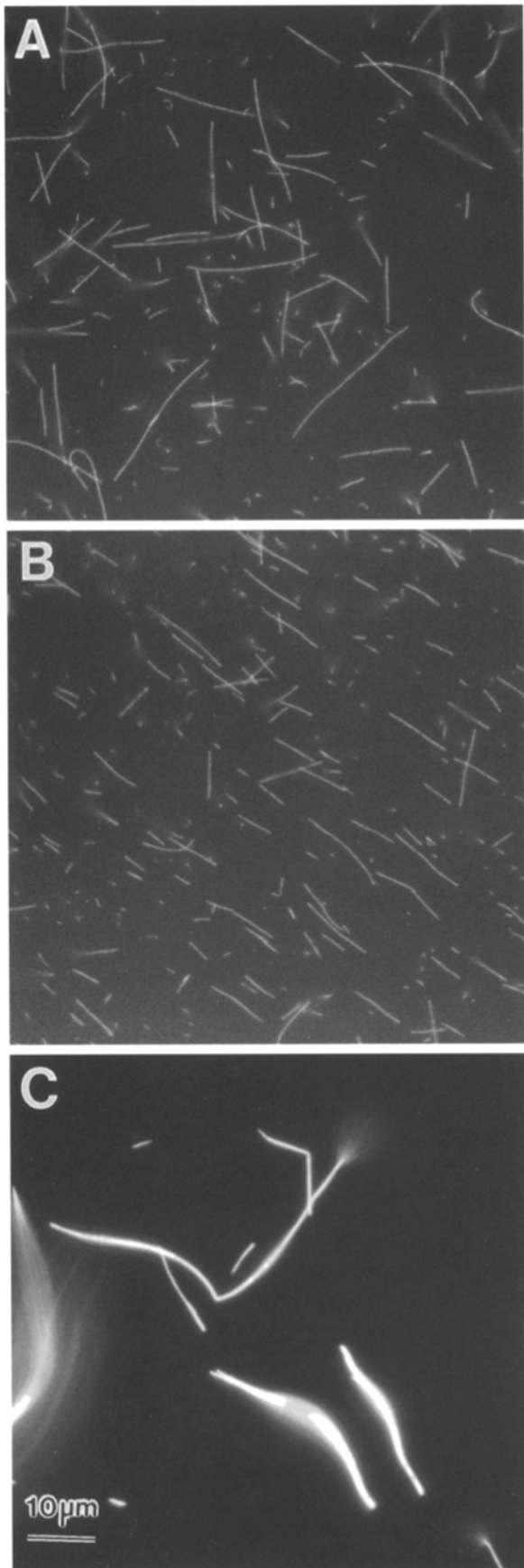


Figure 7. A comparison of microtubule cosedimentation, centrosomal localization and microtubule bundling is shown for fusion proteins in the region of the centrosomal localization domain. The ability of a fusion protein to cosediment with microtubules was qualitatively assessed based on cosedimentation assays done at two salt concentrations. Experiments were carried out in 20 mM K-Pipes, pH 6.8, 50 mM potassium acetate, 1 mM MgCl₂, 1 mM Na₃EGTA and in the same buffer plus 50 mM NaCl. Bundling assays were performed as described in the legend to Fig. 8. *N/T* indicates that the fusion protein was not tested in the assay.

CP190 antibody to immunoprecipitate the endogenous protein from *Drosophila* embryo extracts. The beads were subsequently washed with buffer containing 1 M potassium chloride to remove proteins which associate with CP190. The beads were then mixed with microtubules and sedimented at low speed through a sucrose step gradient. Fig. 5 *a* shows the results of such an experiment. CP190 is cleanly immunoprecipitated by both anti-CP190 antibodies (Fig. 5 *a*, last four lanes) but not with random rabbit IgG. Microtubules co-pellet with the CP190 bound beads but not with control beads coupled to random IgG.

Additionally, we found that a bacterially expressed 6XHis fusion protein containing amino acids 167-1090 of CP190 would cosediment with microtubules in vitro (Fig. 5 *b*); the stoichiometry of this binding at saturation was one CP190 167-1090 monomer to between four and five tubulin dimers (data not shown), suggesting that CP190 binds along the lengths of microtubules in vitro. We localized the region of CP190 responsible for microtubule binding by subjecting a series of smaller fusion proteins to the cosedimentation test (Fig. 6). Fusion proteins 167-468 and 309-608 cosedimented quantitatively with microtubules under our conditions, whereas the more COOH-terminal fusion proteins, (504-789, 646-937, and 784-1090), remained in the supernatant both in the presence and absence of microtubules (Fig. 6). We next tested the fusion proteins that were used to narrow down the centrosomal localization domain (Fig. 4) in our cosedimentation assay. As shown in Fig. 7, we were unable to separate the region important for centrosomal localization from the microtubule binding region; in fact, it seems that the ability of a fusion protein to cosediment with microtubules closely parallels its ability to localize to centrosomes.

We also tested some of these fusion proteins to see if they could bundle rhodamine-labeled microtubules. Fusion proteins 309-608, 309-427, and 385-508 caused microtubules to



form tight bundles (Fig. 8). This bundling was salt insensitive, as bundles would still form in the presence of 500 mM KCl (data not shown). Fusion proteins 421-608 and 428-608, although able to cosediment with microtubules, were not able to bundle microtubules in our assay. Fig. 7 summarizes the centrosomal localization, microtubule binding and microtubule bundling data for fusion proteins derived from the region of the centrosomal localization domain.

Microtubules Are Not Required for the Accumulation or Maintenance of CP190 or CP60 at Centrosomes

Centrosomal proteins can be divided into two groups: those proteins that require microtubules for their centrosomal localization and those capable of localizing to centrosomes independent of microtubules. The first group is likely to include proteins that function to organize the spindle pole, as well as proteins that accumulate at the centrosome due to its role as a hub for intracellular trafficking. Examples of proteins in the first group include NCD (a minus end-directed microtubule motor protein) and NuMA a protein important for spindle pole integrity (also called centrophilin, SP-H, and SPN), both of which localize to spindle poles in a microtubule-dependent manner (Price and Pettijohn, 1986; Kallajoki et al., 1991; Tousson et al., 1991; Endow et al., 1994). The group of proteins that localize to centrosomes independent of a nucleated microtubule array can be considered components of a "core" centrosome, defined as the structure that remains when microtubules have been depolymerized. Known components of "core" centrosomes include γ -tubulin and pericentrin (Stearns et al., 1991; Zheng et al., 1991; Doxsey et al., 1994).

Since there was a correlation between the centrosomal localization of our fusion proteins and their ability to cosediment with microtubules in vitro, we predicted that the association of CP190 with microtubules might function to localize CP190 to centrosomes in vivo. Moreover, not only does CP60 associate biochemically with CP190 (Kellogg and Alberts, 1992) and likewise localize to nuclei and centrosomes in a cell cycle specific manner, but bacterially expressed CP60, like CP190, binds directly to microtubules in vitro (Kellogg et al., 1995). Therefore, we wanted to determine if either CP60 or CP190 require microtubules to accumulate at centrosomes, rather than being components of "core" centrosomes. Previously, we have shown that there is no apparent difference in the amount of CP190 or CP60 detected by immunofluorescence at centrosomes between control mitotic embryos and colchicine-treated embryos arrested in mitosis (Raff et al., 1993). These experiments demonstrated that CP190 and CP60 remain at centrosomes when microtubules are depolymerized, suggesting that microtubules are not required to maintain their centrosomal

Figure 8. Assaying microtubule bundling. To assay MT bundling, 2 μ l of rhodamine-labeled microtubules was mixed with 1 μ l of purified fusion protein and the mixture was incubated at room temperature for 10 min before dilution into fix (see Materials and Methods). (A) Buffer control microtubules are not bundled. (B) fusion protein 428-608 also cannot bundle microtubules. (C) Addition of fusion protein 385-508 causes microtubules to form tight bundles (the apparent fraying of the ends of the bundles is misleading, being due to the bundles leaving the plane of focus).

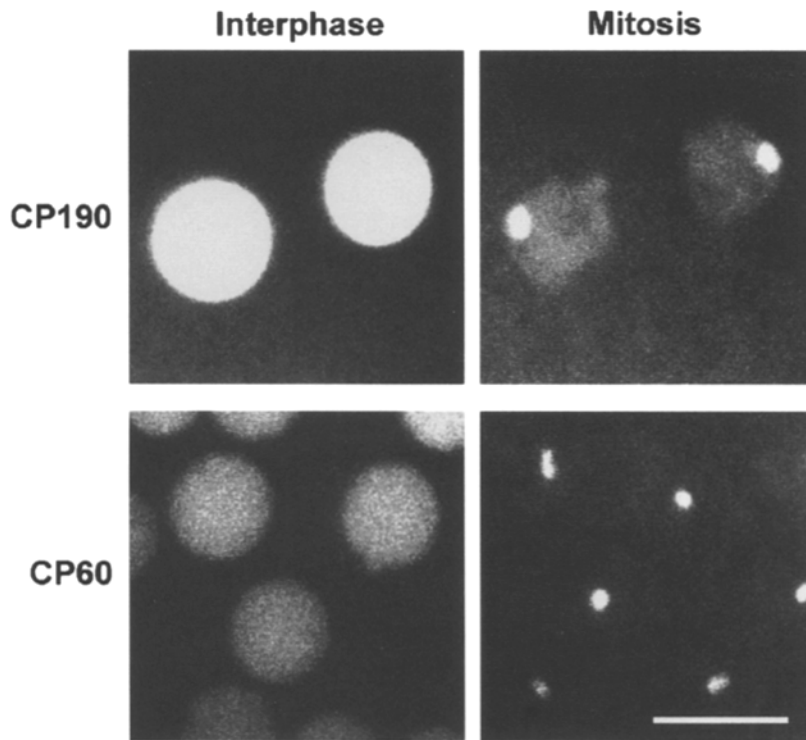


Figure 9. Imaging of CP190 and CP60 in live embryos shows that CP190 and CP60 make the transition from the nucleus to the centrosome even in the absence of microtubules. 6XHis tagged CP60 and 6XHis tagged CP190 (amino acids 167-1090) were purified and labeled with rhodamine. The purified fusion proteins were injected in 50 mM HEPES, 100 mM NaCl, 5 mM colchicine. Immediately following injection, interphase embryos that had imported CP190 or CP60 into their nuclei were selected and followed into mitosis: Z-series were taken on the confocal microscope during interphase and the subsequent colchicine induced arrest. In this way, we were able to watch CP190 and CP60 make the transition from nuclear to centrosomal localization in the absence of microtubules in live embryos. Shown here are projections of these Z-series done using the COMOS software that operates the confocal microscope (BioRad Laboratories). Bar, 10 μ m.

localization. However, microtubules could nevertheless have a role in causing the movement of CP190 or CP60 to centrosomes.

To test the effect of microtubule depolymerization on the ability of CP190 and CP60 to accumulate at centrosomes, we co-injected 5 mM colchicine and either purified rhodamine-labeled 6XHis CP60 (full length) or 6XHis CP190 (amino acids 167-1090). The injected embryos were immediately screened to find interphase embryos that had imported the fluorescent CP190 or CP60 into their nuclei. The embryos were then followed using time-lapse confocal microscopy until they entered a colchicine-induced mitotic arrest (Fig. 9). Control embryos were similarly injected and then fixed and stained for microtubules to insure that our treatment was depolymerizing microtubules completely (data not shown). We found that CP190 and CP60 accumulated at centrosomes with normal kinetics in the absence of microtubules, suggesting that microtubule binding plays no role in localizing either CP190 or CP60 to centrosomes.

Neither CP190 nor CP60 Bind along the Length of Microtubules In Vivo

Since neither CP60 nor CP190 require microtubules for transport to centrosomes, we performed double-label immunofluorescence to detect α -tubulin and either CP190 or CP60 in the same embryo. The aim was to determine whether these proteins localize along the lengths of microtubules in vivo, as previously demonstrated for several MAPs (Kreis and Vale, 1993). Neither CP190 nor CP60 could be detected along the lengths of microtubules at any point during the cell cycle (Fig. 10). Although present in the region of the spindle during mitosis, CP190 and CP60 exhibit a granular staining similar in character to their nuclear staining during interphase. If there is an interaction

between CP190 or CP60 and microtubules in the spindle, this interaction is not similar to that of conventional MAPs.

Discussion

This paper presents the beginnings of a molecular characterization of CP190. We have identified the regions of CP190 important for its dynamic pattern of nuclear and centrosomal localization inside the cell, as well as for its ability to cosediment with microtubules in vitro. These data, combined with the results of in vivo experiments that test for a role of microtubules in the centrosomal localization of CP190 and CP60, shed light on the mechanism by which these two proteins localize to centrosomes in vivo.

When a set of 6XHis fusion proteins spanning CP190 were bacterially expressed, purified, rhodamine-labeled, and injected into *Drosophila* embryos, the injected proteins either did not localize or exhibited one of three localization patterns which we have designated nuclear/cytoplasmic, nuclear/centrosomal and centrosomal/centrosomal (to represent localizations during interphase and mitosis, respectively). A fusion protein containing amino acids 167-608, ~40% of full-length CP190, localizes to centrosomes during mitosis and to nuclei during interphase, in a manner that closely mimics the localization pattern of the native protein. The region of CP190 between amino acids 167 and 608 was further divided to identify independent domains responsible for centrosomal or nuclear localization. These results are summarized in Fig. 11.

The region responsible for the nuclear localization of our fusion proteins contains a bipartite NLS (Dingwall and Laskey, 1991) between amino acids 237 and 255, suggesting that the nuclear import of CP190 is signal dependent. We also note that CP190 contains a second potential bipartite NLS between amino acids 125 and 144, in a re-

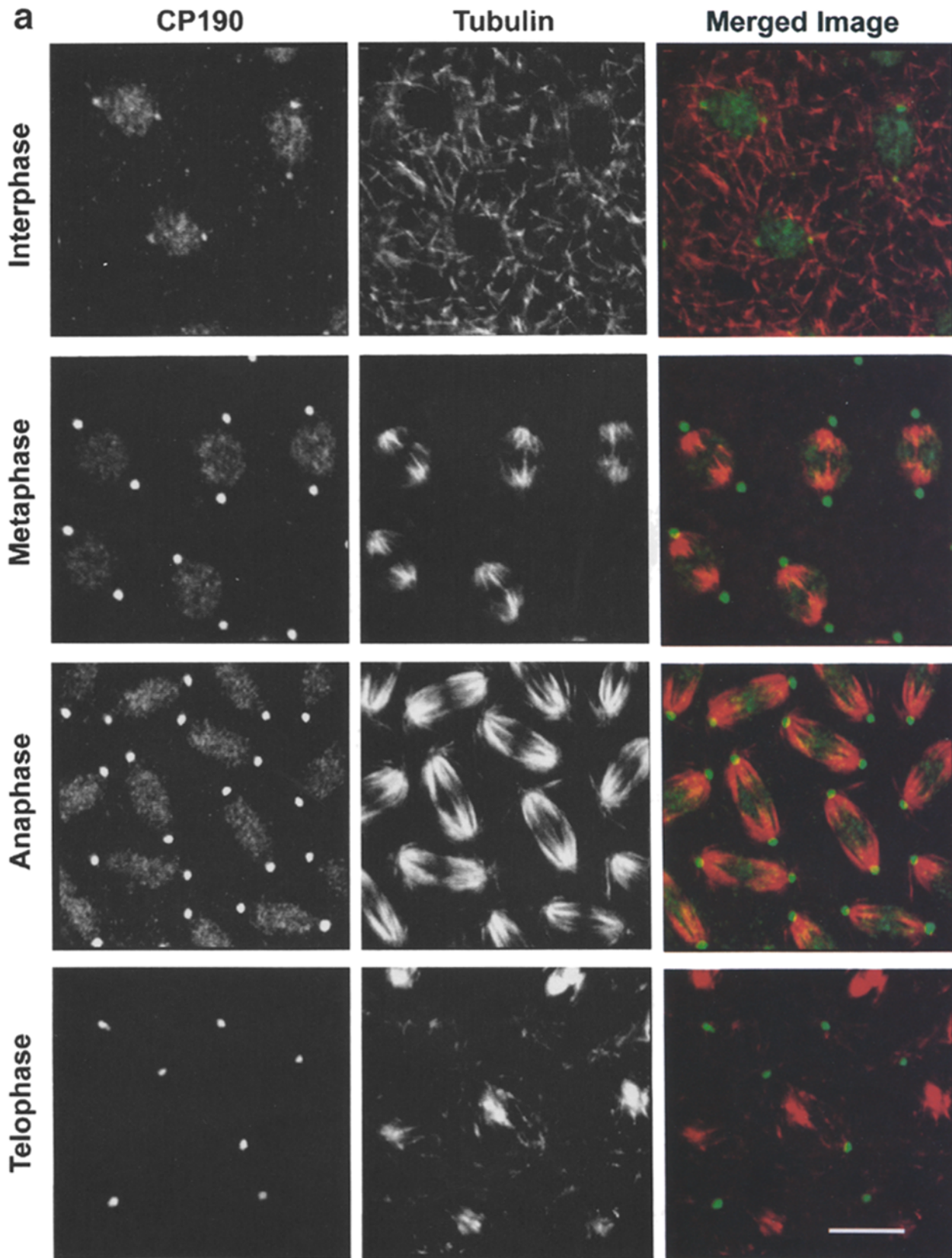
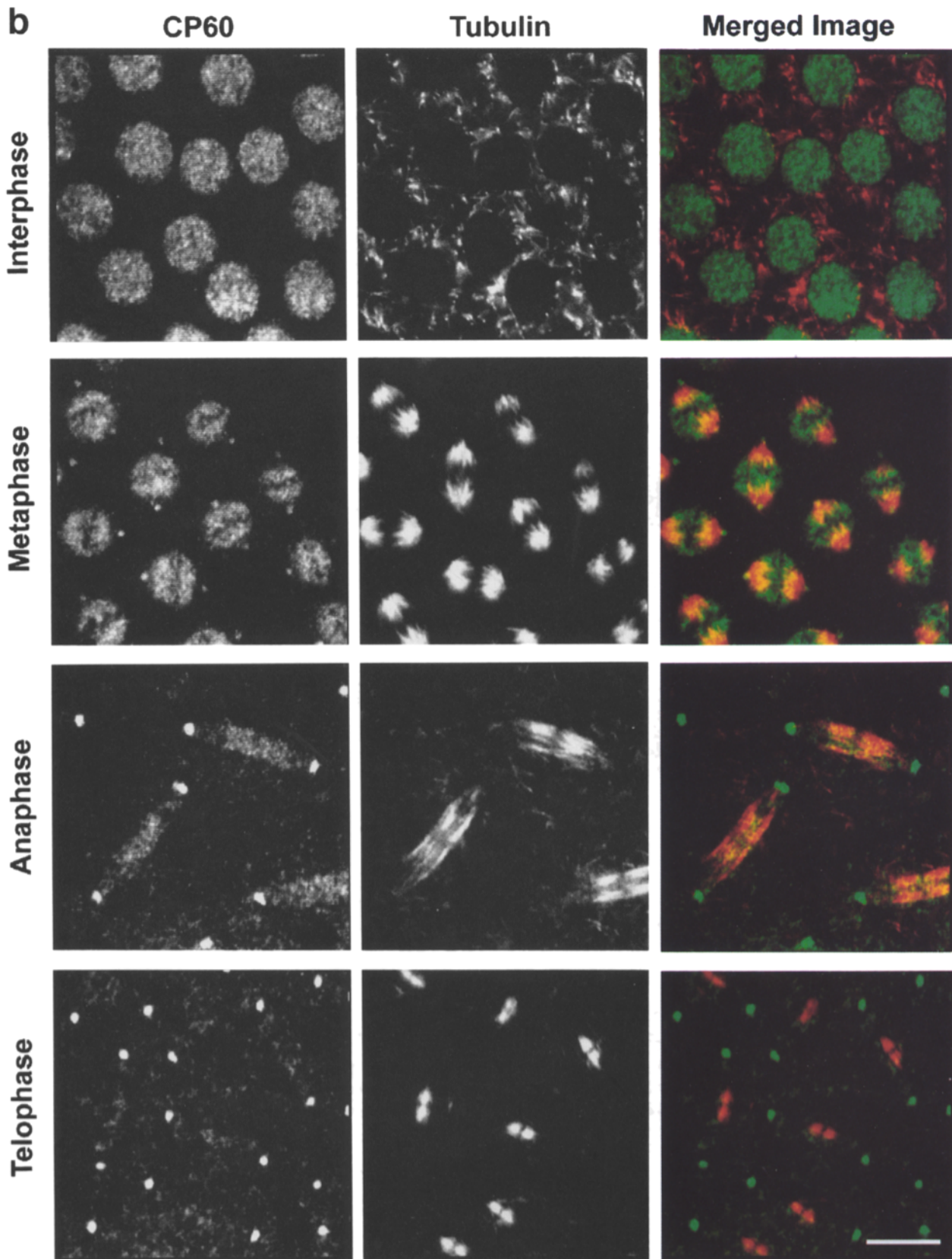


Figure 10. CP60 and CP190 do not co-localize with microtubules in vivo. (a) Double label immunofluorescence of CP190 and α -tubulin in syncytial *Drosophila* embryos. (b) Double label immunofluorescence of CP60 and α -tubulin in syncytial *Drosophila* embryos. Neither CP190 nor CP60 are found along the lengths of microtubules at any stage of the cell cycle. During mitosis CP190 and CP60 appear to stain residual nuclear structures in the region of the spindle. Bars, 10 μ m.



gion of CP190 that we were not able to study due to its insolubility when expressed in bacteria. This additional NLS could also contribute to the nuclear localization of the native protein.

A fusion protein containing the 124 amino acids 385-508 is sufficient for robust localization to centrosomes. Fragments that localize to centrosomes that lack a NLS, such as 266-608 (Fig. 3), remain at centrosomes at constant levels

Map of CP190 domains

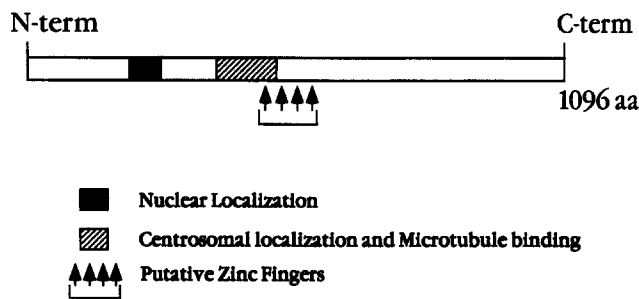


Figure 11. A Map summarizing the locations of the identified domains of CP190. The nuclear localization domain is between amino acids 207 and 271. The region of CP190 between amino acids 385 and 508 is sufficient for both good centrosomal localization and cosedimentation with microtubules *in vitro*. The region of CP190 between amino acids 472 and 590 contains four putative zinc fingers- the arrows point to the center of each putative zinc finger.

throughout the cell cycle. In contrast, fusion proteins that are only slightly longer, and which contain a NLS, such as 240-608, mimic the localization pattern of the native protein. Thus addition of the 26 amino acids containing the NLS confers the ability to be released from centrosomes during interphase as well as the ability to localize to nuclei. This suggests that CP190 is effectively pulled away from centrosomes during interphase by its sequestration into intact nuclei.

Weak centrosomal localization can be achieved by two adjacent, independent fragments of CP190, each containing a distinct portion of the 124-amino acid domain that produces strong centrosomal localization. There is no significant amino acid homology between the two independent fragments, suggesting the absence of a repeated motif for centrosomal binding. It nevertheless appears that separate parts of the localization domain can confer some centrosomal binding and, when put together, they act cooperatively to give the strong centrosomal localization found in fragments containing the entire domain. (A caveat is that we cannot distinguish between weak localization due to poor folding of our fusion proteins and weak localization due to lack of essential sequences.)

When a *Drosophila* embryo extract is passed over a microtubule affinity column, CP190 interacts with microtubules (Kellogg et al., 1989). This result, however, could reflect binding of CP190 to other proteins that bind directly to microtubules. It is therefore interesting that both native CP190 and a bacterially expressed fusion protein corresponding to the COOH-terminal 85% of CP190 can bind directly to microtubules *in vitro*. We have narrowed down the microtubule binding domain of CP190 and find that it is inseparable from the region required for centrosomal localization (see Fig. 7). The microtubule binding domain has no detectable homology to other known microtubule binding domains (Lewis et al., 1988; Himmler et al., 1989; Noble et al., 1989; Yang et al., 1989; Irminger-Finger et al., 1990; Aizawa et al., 1991).

Depolymerization of microtubules *in vivo* using colchicine does not noticeably affect the rate or extent of accu-

mulation of CP190 or CP60 at centrosomes, suggesting that they are members of a very small set of characterized proteins that do not require microtubules to attain or maintain their centrosomal localizations. Many other characterized centrosomal components such as NuMA and NCD require microtubules for their localization to spindle poles or microtubule asters. CP190 and CP60, on the other hand, seem to be cell cycle-dependent components of a "core" centrosome, independent of the nucleated microtubule array. Other known components of core centrosomes include γ -tubulin and pericentrin (Stearns et al., 1991; Zheng et al., 1991; Doxsey et al., 1994).

This raises the important question of the *in vivo* relevance of the *in vitro* binding of both CP190 and CP60 to microtubules. By immunofluorescence, there is no obvious colocalization of CP190 or CP60 along the lengths of microtubules in the spindle or during interphase (Fig. 10). We can think of three possibilities to explain our data: (a) CP190 and CP60 localize to the centrosome by mechanisms independent of MT binding, but function as microtubule binding proteins at the centrosome. (b) The binding of CP190 and CP60 to microtubules observed *in vitro* reflects a related but different association that is important for the binding of these proteins to centrosomes. For example, CP190 and CP60 could bind to γ -tubulin at the centrosome (Raff et al., 1993). (c) The binding of CP190 or CP60 to microtubules could be an *in vitro* artifact, mediated by positively charged regions on the surface of these proteins.

The identification of the nuclear and centrosomal localization domains of CP190 is a first step towards probing its function; these domains can now be mutated and further analyzed. In addition, we can attempt to block the centrosomal localization of native CP190 in *Drosophila* embryos by the injection of antibodies to the centrosomal localization domain, or by the injection of short CP190 fusion proteins. (Some of the fusion proteins that bind centrosomes could have a dominant negative effect.) Finally, by transforming *Drosophila* with a full-length CP190 carrying point mutations designed to disrupt its nuclear localization sequences, we should be able to retain this protein at the centrosome throughout the cell cycle. If CP190 is sequestered in nuclei during interphase to keep it from interfering with centrosome function, such a mutation should produce a clear phenotype.

K. Oegema thanks Doug Kellogg and Chris Field for technical assistance, for many hours of stimulating discussions, and for encouragement and moral support. We also thank Jack Barry for protein purification assistance and advice. In addition, Michelle Moritz, Raffi Aroian, Tim Mitchison, and Arshad Desai made helpful comments and critically read the manuscript.

This work was supported by National Institutes of Health (National Institutes of Health) grant GM 23928 to B. Alberts. K. Oegema was additionally supported by fellowships from the National Science Foundation and the University of California, San Francisco.

Received for publication 23 June 1995 and in revised form 25 August 1995.

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