CP250, a Novel Acidic Coiled Coil Protein of the *Dictyostelium* centrosome, Affects Growth, Chemotaxis, and the Nuclear Envelope

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The *Dictyostelium* centrosome is a nucleus associated body consisting of a box-shaped core surrounded by the corona, an amorphous matrix functionally equivalent to the pericentriolar material of animal centrosomes which is responsible for the nucleation and anchoring of microtubules. Here we describe CP250 a component of the corona, an acidic coiled coil protein that is present at the centrosome throughout interphase while disappearing during prophase and reappearing at the end of late telophase. Amino acids 756–1148 of the 2110 amino acids are sufficient for centrosomal targeting and cell cycle-dependent centrosome association. Mutant cells lacking CP250 are smaller in size, growth on bacteria is delayed, chemotaxis is altered, and development is affected, which, in general, are defects observed in cytoskeletal mutants. Furthermore, loss of CP250 affected the nuclear envelope and led to reduced amounts and altered distribution of Sun-1, a conserved nuclear envelope protein that connects the centrosome to chromatin.

INTRODUCTION

Centrosomes are the main microtubule organizing centers (MTOCs). They nucleate, anchor and organize microtubules. In animal cells they comprise a pair of centrioles and a surrounding pericentriolar matrix (PCM). The PCM is a key structure of the centrosome and is responsible for microtubule nucleation and anchoring. Its principal components are large coiled coil proteins like pericentrin and proteins of the AKAP450 family that provide docking sites for γ -tubulin ring complexes and regulatory molecules. They allow the centrosome to function as MTOC and to carry out its regulatory functions during cell cycle transitions, cellular responses to stress, and organization of signal transduction pathways (Keryer *et al.*, 2003; Doxsey *et al.*, 2005). Accordingly, the protein composition of the centrosomes is complex and highly dynamic.

In protozoa, algae, and fungi, the centrosome morphology varies greatly. In *Dictyostelium discoideum* it is a nucleus associated body consisting of a box-shaped core surrounded by the corona, an amorphous matrix functionally equivalent to the PCM (Ueda *et al.*, 1999). Only a few centrosomal proteins have been characterized in *D. discoideum* so far.

Most of them were identified based on the homology to their mammalian counterparts such as γ -tubulin (Euteneuer *et al.*, 1998); DdCP224, an XMAP215-family protein (Gräf *et al.*, 2000); the centrin-related DdCrp (Daunderer *et al.*, 2001); EB1 (Rehberg and Gräf, 2002); Lis1 (Rehberg *et al.*, 2005); Spc97 and Scp98 (Daunderer and Gräf, 2002); and the kinase DdNek2 (Gräf, 2002). A 350-kDa protein, that has not been further characterized, was isolated using monoclonal antibodies generated against a nucleus-centrosome complex (Kalt and Schliwa, 1996). A further 70 candidates of the *Dictyostelium* centrosome were identified in a proteomic approach (Reinders *et al.*, 2006). These proteins are components of the corona; however, during mitosis they relocate to the centrosomal core structure and stay associated with the centrosome throughout mitosis. Exceptions are DdNek2, a permanent component of the core that is also present in the cytoplasm, and DdCrp, which disappears during prometaphase and reassociates with the centrosome after telophase. DdCrp resembles with its behavior the corona that dissociates from the centrosomal core at the transition from prophase to metaphase and reforms at the end of telophase (Ueda *et al.*, 1999).

Here we describe CP250, a novel component of the *D. discoideum* centrosome, and localize it to the corona. We follow its dynamic association with the centrosome during interphase and mitosis. Loss of the protein leads to defects in growth, cell polarity, and development. The results point toward a role for CP250 in centrosome-microtubule cytoskeleton interactions during interphase. Furthermore, we observed in the mutant alterations in the nuclear envelope (NE) using antibodies specific for the UNC-84 homolog Sun-1 and the KASH-domain protein interaptin pointing out

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the close interaction between centrosome and NE (Rivero *et al.*, 1998; Xiong *et al.*, 2008; Starr, 2009).

MATERIALS AND METHODS

Strains, Cell Culture, and Vector Construction

Strain AX2-214 is the parent of all strains generated in this study. The CAPbsr mutant has been described previously (Noegel *et al.*, 1999; 2004). For expression of green fluorescent protein (GFP)-tagged α -tubulin a plasmid was used described by Koonce *et al.* (1999). Growth and development were done as described (Khurana *et al.*, 2002). For generation of CP250-deficient cells a knockout vector was generated by amplifying gDNA sequences (DDB 0233900) from positions 1648–2229 and 2781–3348 and cloning them into the Cre-loxP vector pLPBLP carrying a blasticidin resistance conferring gene (Faix *et al.*, 2004). For expression of CP250 GFP fusion proteins, sequences encompassing amino acids 756–1148 corresponding to nucleotides 2236–3444 (GFP-CP250 D1) and amino acids 447–1257 corresponding to nucleotides 1338–3770 (GFP-CP250 D2) were cloned into pBsr GFP N2 and pBsr GFP N1, respectively. Expression was under the control of the constitutively active *Dictyostelium* actin15 promoter. The plasmids were introduced into AX2 cells by electroporation following standard procedures (Faix *et al.*, 2004). Selection of the transformants was with blasticidin (3.5 μ g/ml). Mutant cells in which the CP250 gene was successfully targeted were identified by PCR followed by Southern blotting. Mutant analysis was done as described (Khurana *et al.*, 2002; Noegel *et al.*, 1999; 2004). Cell size was determined using cells that had been treated with 20 mM EDTA in Soerensen phosphate buffer (17 mM Na⁺/K⁺-phosphate buffer, pH 6.0) in order to obtain perfectly round cells. For expression as glutathione S-transferase (GST)-fusion proteins, cDNA sequences encoding amino acids 756–1148 (GST-CP250 D1) were cloned into pGEX4T (GE Healthcare, Waukesha, WI).

Generation of Antibodies

Mouse monoclonal antibodies were generated against CP250 D1 (amino acids 756–1148) as described (Xiong *et al.*, 2008). For immunization of mice the GST-part was removed by thrombin cleavage. The identity of the CP250 polypeptide was confirmed by mass spectrometry. mAbs K68-332-3 and K68-439-8 were used in this study. They recognized the bacterially produced recombinant protein, the GFP-tagged fusion proteins and the endogenous protein in Western blots of whole cell lysates and the GFP-tagged fusion proteins and the endogenous protein in immunofluorescence analysis and immunoelectron microscopy. Rabbit polyclonal antibodies specific for Sun-1 were generated against a GST fusion protein containing the N-terminal 266 amino acids of Sun-1 (N800, Xiong *et al.*, 2008; Pineda, Berlin, Germany) and affinity purified.

Immunofluorescence Analysis

Approximately 1×10^6 cells were transferred onto a coverslip (10-mm diameter) and allowed to adhere for 20 min at room temperature. If not mentioned otherwise, standard immunofluorescence staining was carried out using ice-cold methanol as fixative (45 min, -20°C). Cells were treated twice for 15 min (room temperature) with blocking solution (1 $\times$ PBS containing 0.5% (wt/vol) BSA and 0.1% (vol/vol) fish gelatin). The appropriate antibodies were diluted in the blocking solution and applied for 1 h at room temperature; the excess of antibodies was removed by washing with the blocking solution before the 1-h incubation with the according secondary antibodies.

For cell cycle synchronization, cells were kept for 3 h in a Petri dish in Soerensen phosphate buffer, at room temperature. The buffer was then replaced by growth medium, and incubation was continued for 4 h. Fixation was with cold methanol. For the staging of the mitotic phases we used the disappearance or reappearance of microtubules as well as the size of the centrosomes.

Primary antibodies used in this study were mouse monoclonal anti-GFP antibody K3-184-2 (Noegel *et al.*, 2004), mAb 260-60-10 against interaptin (Rivero *et al.*, 1998), Sun-1 specific mAb K55-432-2 (Western blot analysis) and K55-460-1 (immunofluorescence; Xiong *et al.*, 2008) as well as polyclonal antibodies (this study), rat mAb YL1/2 against α -tubulin (Kilmartin *et al.*, 1982), mouse monoclonal antibodies K29-359-31 and K29-359-10, which are subclones of a single clone, that were generated against a tubulin-fraction from the green alga *Spermatozopsis similis* and that recognize specifically the *D. discoideum* centrosome (gift from Dr. K. Herkner and Dr. M. Melkonian, (University of Cologne; Xiong *et al.*, 2008). Polyclonal antibodies against DdCP224 were kindly provided by Dr. R. Gräf (University of Potsdam; Gräf *et al.*, 1999). The appropriate secondary antibodies were Cy3-conjugated sheep anti-mouse IgG (Sigma, St. Louis, MO) and Alexa 488- or 647-conjugated goat anti-mouse or donkey anti-mouse and Alexa 488 and 647 donkey anti-rabbit IgG or Alexa 568-coupled goat anti-rabbit IgG (Molecular Probes, Eugene, OR). DNA was stained with 4'-6-diamidino-2-phenylindole (DAPI). Analysis was done by confocal laser scanning microscopy (Leica TCS SP5). Actin was recognized by mAb act1 (Simpson *et al.*, 1984), CAP by mAb 230-18-8 (Gottwald *et al.*, 1996).

Electron Microscopy

Nuclei of AX2 cells were isolated using Nucleopore membranes (5 μ m, Corning Glass, Corning NY). The cells were washed twice in cold phosphate buffer and then resuspended in Dicty-PHEM (Cox *et al.*, 1995) containing a protease inhibitor cocktail and PMSF. They were pressed through the filter assembly three to five times. The resulting mixture was layered onto a 30% sucrose cushion and centrifuged at 3500 rpm for 10 min at 4°C . The pellet containing mainly nuclei was resuspended in Dicty-PHEM and centrifuged onto 12-mm coverslips at 3500 rpm for 5 min. The nuclei were fixed with 2% formaldehyde in Dicty-PHEM for 15 min. They were washed and treated with mouse monoclonal antibodies K68-332-3. The antibodies were detected using 6 nm goat anti-mouse antibody (Aurion, Biotrend, Wageningen, the Netherlands). Dehydration and embedment were carried out according to standard procedures. Alternatively, methanol fixed nuclei were embedded in Lowicryl (Euteneuer *et al.*, 1998). mAb K29-359-31 was applied to thin sections followed by a bridge (rabbit anti-mouse) Ab (rabbit anti-mouse) and 10 nm protein A gold. The samples were sectioned using a Reichert Ultracut E and viewed in a JEOL 1200C (Peabody, MA) equipped with a TEM camera Keenview-10/12 and iTEM imaging system (Soft Imaging System, Münster, Germany).

Analysis of Cell Shape and Cell Migration

Aggregation competent AX2 and CP250⁻ cells were plated onto glass coverslips and allowed to settle for 15 min, and chemotaxis experiments performed with micropipettes filled with 10^{-4} M cAMP attached to a micromanipulator system. Images were recorded at intervals of 6 s using a Leica DM-IL inverse microscope (Deerfield, IL; 40 $\times$ objective) and a conventional CCD video camera and analyzed using Dynamic Image Analysis Software (DIAS, Soll Technologies, Iowa City, IA; Wessels *et al.*, 1998).

Miscellaneous Methods

For the yeast-two-hybrid screen residues 1–700 of the CAP cDNA sequence were cloned into pAS2 (Durfee *et al.*, 1993; Noegel *et al.*, 2004). A *Dictyostelium* cDNA library kindly provided by Dr. A. Kuspa (Baylor College of Medicine) was used for the screen (Knuth *et al.*, 2004). The interactions were further confirmed by testing the recombinant proteins in vitro (Knuth *et al.*, 2004) using GST-CP250 D1 and N-CAP (residues 1–226) expressed as his-tagged fusion protein. The binding assay was done in 10 mM MES, pH 6.0, 138 mM KCl, 2 mM EDTA, 3 mM MgCl₂, 1% Triton X-100, and 5 mM DTT. To enrich for centrosomal proteins, the procedure published by Gräf (2001) was essentially followed. The sucrose density gradient centrifugation step was omitted. Proteins were separated on 8% SDS-polyacrylamide gels or gradient gels (3–15% acrylamide) and transferred to nitrocellulose membrane using wet blotting methods. For detection of CP250 transfer was done for 48 h at 140 mA.

For immunoprecipitation of GFP-CP250 D2* protein cells were lysed in lysis buffer (10 mM Tris/HCl, pH 8.0, 50 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, complete protease inhibitor cocktail [Roche, Indianapolis, IN]), 10 mM benzamide, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin). GFP specific polyclonal rabbit antibodies were coupled to protein A Sepharose and incubated with the lysate. Bound proteins were separated on 3–15% gradient gels and analyzed by Western blotting. Alternatively gels were stained with Coomassie Brilliant Blue R, bands were cut out, and the proteins were identified by LC-MS/MS.

For analysis of the effect of microtubule-specific drugs cells were treated with nocodazole (5 and 10 μ M), vinblastine (1 μ M), taxol (1 μ M), thiabendazole (5 and 10 μ M), benomyl (50 μ M), and colchicine (0.5 μ M) for 2 up to 45 h. The distribution of proteins after drug treatment was assayed in immunofluorescence studies, and the impact on growth was followed by determining the cell number.

RESULTS

Identification of CP250 as an Interactor of CAP

CP250 was identified in a yeast two-hybrid screen as a potential interaction partner of CAP (cyclase-associated protein) using the N-terminal 233 amino acids of CAP (N-CAP; Noegel *et al.*, 1999). Among others we identified four clones ranging from 1504 to 2106 base pairs in length and overlapping in a 1176-base pair stretch. These clones were derived from a single gene, DDB0233900, located on chromosome 6 and their sequences corresponded to the central part of this gene (Figure 1A). Gene DDB0233900 codes for a protein of 2110 aa with a molecular mass of 248,355. The protein was named CP250 based on its location and molecular mass. It has a pI of 4.4 and contains stretches for which a coiled coil structure is proposed by the Smart prediction program (Schultz *et al.*, 1998). Several involucrin repeats and an Nt-DnaJ domain signature at position 965–984 are predicted

Figure 1. CP250 and its interaction with CAP. (A) CP250 domain structure and location of CP250 clones that were used. The amino acid position is indicated. Clones 1–4 were isolated in a yeast two-hybrid screen using the N-terminal domain of CAP (N-CAP) as bait. CP250 D1 and CP250 D2 were expressed as GST and GFP fusion proteins for further studies. The polypeptide (antigen) used for production of monoclonal antibodies is indicated. (B) Summary of the results of a yeast two-hybrid screen of CP-250 clones with several CAP plasmids (Noegel *et al.*, 2004). BD, Gal4 DNA-binding domain fusion; AD, Gal4 activation domain fusion. Presence or absence of interaction is given by “+” or “–.” The strength of the interaction is indicated by the number of “+” signs. (C) The direct interaction of N-CAP polypeptide with GST-CP250 D1 was assayed in in vitro pull-down experiments. A, his-tagged N-CAP; B, GST-tagged CP250 D1; C, control, binding of his-tagged N-CAP to GST-Sepharose beads; D, binding of his-tagged N-CAP (asterisk) to GST-CP 250-D1 bound to GST-Sepharose beads.

(Figure 1A). Blast searches showed weak homologies to myosins, hook-related proteins, the *Schizosaccharomyces pombe* protein SPAC1F3.06c that colocalizes with the Sun-domain protein Sad1, a component of the spindle pole body that is the yeast equivalent of the centrosome, and CENPE, reflecting the coiled coil structure (Earnshaw and Cleveland, 1991; Saito *et al.*, 2005).

Further yeast two-hybrid assays showed that all CP250 clones isolated interacted also with full-length CAP and CAP300 encoding the N-terminal 100 amino acids of CAP but not with the C-terminal domain of CAP proving the specificity of the interaction (Figure 1B). The smallest region in common between the four clones codes for a 46-kDa polypeptide (aa 756–1148; Figure 1A). It was expressed as GST-fusion protein (GST-CP250 D1) and could pull down recombinant N-CAP in in vitro interaction assays supporting the interaction of CP250 with CAP (Figure 1C). GST-CP250 D1 showed an unusual migration behavior in SDS-PAGE. Instead of the expected 76,000 it migrated as a protein of nearly 95,000. Abnormal migration behavior in SDS-PAGE is frequently observed for proteins with an extended structure (Noegel *et al.*, 1989).

CP250 Is a Centrosomal Protein and Localizes to the Corona

To determine the subcellular localization of CP250, we generated monoclonal antibodies against recombinant CP250 D1 (Figure 1A). The antibodies recognized the recombinant CP250 polypeptide, the GFP-tagged CP250 fusion proteins, and the endogenous protein in Western blots of whole cell homogenates. In AX2 lysates we detected CP250 as a large protein migrating at ~280–300 kDa. In cells expressing GFP-CP250 D1 we detected with mAb K68-439-8 a strong signal at ~95 kDa that was also recognized by GFP-specific mAb K3-184-2, whereas in lysates from cells that express GFP-CP250 D2 we observed a signal above 250 kDa both with mAb K3-184-2 and mAb K68-439-8 (Figure 2B and data not shown). We designate this protein GFP-CP250 D2* to indicate that it is the result of a knock in. Endogenous CP250 was absent. Further analysis revealed that in these cells a knockin

event had occurred and that the vector had integrated into the CP250 gene leading to the production of a GFP fusion protein of a calculated molecular weight of ~220 kDa (Figure 2, A and C). The identity of the protein was confirmed in immunoprecipitation experiments with polyclonal GFP antibodies and subsequent identification using LC-MS/MS.

In immunofluorescence studies of AX2 cells mAbs K68-439-8 and K68-332-3 detected a spot near the nucleus. In coimmunofluorescence analysis with polyclonal antibodies specific for the centrosomal protein DdCP224 the CP250 staining overlapped with the DdCP224 staining, indicating that CP250 localizes to the centrosome (Figure 2D).

To corroborate these results, we carried out immunoelectron microscopy using mAb K68-332-3 (Figure 3A). This approach confirmed the localization of CP250 at the centrosome. K68-332-3 decorated the more open matrix of the corona of the centrosome but did not stain the core. Thus, CP250 is most likely a component of the corona. CP250 was also recently described as a centrosomal component in the course of a proteomic characterization of the *Dictyostelium* centrosome, and our data confirm and extend the proteomic data (Reinders *et al.*, 2006).

Requirements for the Centrosomal Localization

To determine the region of CP250 that mediates the centrosome association of CP250, we examined the localization of GFP-CP250 D1 (residues 756–1148) encompassing one involucri repeat and the DnaJ domain (Figure 1A) in AX2 cells. In immunofluorescence analysis GFP-CP250 D1 behaved like a centrosomal protein and appeared as a single dot near the nucleus. This dot was recognized by mAb K68-439-8 and colocalized with mAb K29-359-31 and pAbs specific for DdCP224 as well as the MTOC when cells were stained for α -tubulin (Figure 3B and data not shown).

mAb K29-359-31 recognizes specifically tubulin at the centrosome (Xiong *et al.*, 2008) and localizes in immunoelectron microscopy to the corona (Figure 3A, bottom panels). Gold particles formed clusters within the corona, but were not present in the electron-dense, layered core structure of the centrosome. The staining was similar to the one of γ -tubulin

Figure 2. Integration of GFP-CP250 D2* encoding sequences into the endogenous gene by homologous recombination. (A) Southern blot analysis demonstrating the homologous recombination event. AX2 (1) and DNA of the knockin strain (2) was digested with *AccI* and hybridized with probe A and B. (B) Western blot analysis showing endogenous CP250 in whole cell lysates of AX2 (a, arrow) and the shortened GFP-tagged CP250 protein generated through homologous recombination (b). The lysates were also used for immunoprecipitation of GFP-CP250 D2* (b', arrow) using polyclonal GFP-specific antibodies. Note the absence of a signal in the immunoprecipitation employing the AX2 lysate (a'). The blot was probed with mAb K68-439-8. (C) The recombination event and the location of probes A and B are shown as well as the fragments generated in the wild type (1) and in the knock in mutant (2) after *AccI* digestion. (D) mAb K68-439-8 detects the centrosome in AX2 cells and colocalizes with the centrosomal marker DdCP224. Bar, 5 μ m.

that is detected in regularly spaced clusters within the corona (Euteneuer *et al.*, 1998).

The data show that amino acids 756-1148 are sufficient for correct localization of CP250. Expression of the GFP-fusion protein did not affect centrosome position, centrosome number, and the nucleus number in the cells (Figure 3B). GFP-CP250 D2* was also seen at the centrosome (Figure 3B).

CP250 Is a Permanent Resident of the Corona

Next we followed the fate of CP250 during the cell cycle. The fate of the corona during mitosis was previously revealed in electron microscopy studies (Ueda *et al.*, 1999). It disappears at the end of prophase and reassembles during late telophase. Most of the known components of the corona do not follow this pattern as they relocate to the core of the centrosome during mitosis and stay associated throughout the cell

cycle. CP250 behaved differently. It was present throughout interphase, still seen in prophase but was no longer detectable during meta- and anaphase and reappeared at the centrosome at the end of late telophase (Figure 4A). The GFP-tagged polypeptide CP250 D1 behaved in the same manner and was not present at the centrosome during metaphase until late telophase (Figure 4B), whereas K29-359-31 staining was always associated with the centrosome (Figure 4C).

CP250-deficient Cells Are Viable, But Are Impaired in Growth and Development and Have an Altered Chemotaxis Behavior

To analyze the role of CP250 in vivo, we generated cells lacking the protein using a replacement vector (Figure 5A). Correctly targeted clones were identified by PCR and con-

Figure 3. Identification of CP250 as centrosomal protein. (A) Localization of CP250 and the K29-359-10 epitope at the centrosome in immunoelectron microscopy studies. The CP250 specific mAb K68-332-3 decorates the corona of the centrosome. mAb K29-359-31 generated against a tubulin fraction from the green alga *S. similis* is also seen in the periphery of the centrosome. Two examples are shown in each case. (B) The GFP-tagged CP250 polypeptides are present at the centrosome indicating that the CP250 D1 (residues 756-1148) encoded sequences contain the information for centrosomal localization. GFP-CP250 D1 is present in a single spot within the cells that is recognized by mAb K68-439-8 and colocalizes with the MTOC in cells stained with α -tubulin-specific mAb YL1/2. GFP-CP250 D2* colocalizes with DdCP224 recognized by specific polyclonal antibodies. DNA is stained with DAPI. Confocal images are shown. Bar, 5 μ m.

firmed by Southern and Western blotting (Figure 5B and data not shown). Furthermore, mutant cells had lost CP250 staining in immunofluorescence analysis (Figure 5C). On *Klebsiella aerogenes* lawns, cell growth was reduced whereas growth in suspension was not affected (Figure 6A and data

not shown). CP250 cells were significantly smaller than the parent strain with the majority of cells having a diameter $\leq 12 \mu$ m, whereas AX2 cells were between 12 and 16 μ m (Figure 6B). The smaller diameter was not due to an increased thickness of the cells for two reasons. First, we determine the diameter using perfectly round cells. This is achieved by EDTA treatment before the measurement. Second, we analyzed the cells using confocal microscopy and did not observe an increase in thickness. The mutant cells were mostly mononucleated.

When *D. discoideum* cells starve, they enter a developmental cycle that ends with the formation of fruiting bodies. CP250⁻ cells plated on nonnutrient agar aggregated and formed multicellular structures in a timely manner. However, the aggregation streams were more delicate, and the aggregation zones were smaller, resulting in the formation of smaller fruiting bodies (Figure 6, C and D). In contrast, when cells were developed in suspension, development was enhanced shown by the formation of tight aggregates (Figure 6E).

It is generally accepted that the centrosome and microtubules regulate cell polarity and persistence of pseudopods. In mammalian cells, for example, the orientation of the MTOC is a marker for polarization in a wound-healing assay (Lücke *et al.*, 2008). Furthermore, disruption of the microtubule network or mutations in microtubule-associated proteins affect directed cell migration and motility (Williams *et al.*, 2006; Li and Gundersen, 2008; Tang *et al.*, 2008). We therefore analyzed chemotaxis and motility of the mutant cells in a cAMP gradient. We used synchronously developed cells, plated them on a glass surface, and analyzed their chemotactic movement when migrating toward a micropipette filled with 0.1 mM cAMP. Under these conditions wild-type cells elongate, are highly polarized and migrate in a directed manner toward the cAMP source extending and retracting pseudopods in the direction of the cAMP gradient. CP250⁻ cells are also chemotactically active and migrate toward cAMP. The cells are polarized, but they are slightly slower and change their direction more frequently than wild-type cells leading to slightly less directed migration. Notably, the leading pseudopod is less stable, is frequently retracted, and a new pseudopod is formed (Table 1 and Figure 7, A and B; arrow indicates change of direction).

Drug Sensitivity of CP250-deficient Cells

The effect of microtubule inhibitors on the microtubule system in *Dictyostelium* was investigated in detail nearly 30 years ago. In this early work colchicine, vinblastine, nocodazole, and thiabendazole was used to destroy microtubules while leaving the nucleus-associated structures intact (Rubino *et al.*, 1982).

When we tested the effect of several drugs on growth of wild-type and mutant cells, we found that at the concentrations used, nocodazole, vinblastine, and colchicine mildly inhibited growth to a similar extent in wild-type and mutant cells, whereas taxol had no effect on AX2 and a stimulatory effect on the growth of the CP250⁻ strain. Thiabendazole (10 μ M) inhibited growth quite effectively in both strains. When we stained the drug-treated AX2 cells for CP250 the protein was still present at the centrosome, although the microtubule network had been destroyed by the drug treatment (see Figure 10 for nocodazole-treated AX2 cells).

The Centrosome and the Nuclear Envelope in CP250-deficient Cells

Immunofluorescence analysis of the centrosome of the mutant cells by labeling with K29-359-31 showed that each

Figure 4. CP250 as a component of the corona is lost from the centrosome during mitosis and reassociates at the end of late telophase. (A) CP250 staining was followed throughout mitosis. Various stages as indicated are shown. The arrows point at centrosomes positive for CP250, and arrowheads point at centrosomes devoid of CP250. In the top panel, a cell in prophase (asterisk, arrow) and prometaphase (arrowhead) is shown. CP250 is recognized by mAb K68-439-8, α -tubulin by YL1/2. Throughout, arrows point at K68-439-8-positive centrosomes and arrowheads at K68-439-8-negative centrosomes. (B) GFP-CP250 D1 behaves like the endogenous protein. GFP fluorescence was followed for GFP-CP250 D1. Microtubules were visualized using α -tubulin specific mAb YL1/2. Different mitotic stages are shown. The arrowhead points to an MTOC devoid of GFP-CP250 D1, and the arrow indicates reappearance of the protein. (C) The K29-359-10 epitope stays at the centrosome throughout mitosis. Cells expressing GFP-tagged α -tubulin were followed. Arrows point at centrosomes. DNA was stained with DAPI. Bar, 5 μ m.

nucleus was associated with one centrosome. Additional centrosomes were not observed. Also, the distance between the nucleus and the centrosome was not altered, and α -tubulin and K29-359-10 behaved as in wild type during mitosis (Figure 8A). Similarly, the centrosomal marker DdCP224 was present at the centrosome in interphase and in mitotic cells, and we observed that the DdCP224 antibodies labeled the centrosome throughout the entire cell cycle as in AX2. During mitosis, spindle microtubules were also decorated, especially in the midbody region in anaphase and telophase as has been reported for AX2 cells previously (Figure 8B; Gräf *et al.*, 2000).

In general, the centrosome is attached to the nucleus. Proteins that mediate the attachment are KASH-domain proteins present in the outer nuclear membrane (ONM) and SUN-domain proteins present in the inner nuclear membrane (INM). This mechanism of centrosome attachment is highly conserved. In *Caenorhabditis elegans* it is mediated by the KASH-domain protein ZYG-12 and the SUN-domain protein matefin/SUN-1. ZYG-12 can bind to dynein and

ensure centrosome nucleus proximity involving microtubule-dependent translocation of the nucleus toward the centrosome (Malone *et al.*, 2003). In *S. pombe* the corresponding KASH- and SUN-domain-containing proteins are Kms2 and Sad1, and in *Drosophila* it is Klarsicht and Klaroid (Fridkin *et al.*, 2009; Starr, 2009). In *D. discoideum* we have shown that the SUN-domain protein Sun-1 links the centrosome to the NE, whereas a role for the KASH-domain protein interaptin in this process could not be demonstrated. Also, Sun-1 and interaptin do not directly interact (Xiong *et al.*, 2008).

We analyzed Sun-1 as well as interaptin and followed their localization during progression of the cell cycle in AX2 and mutant cells. We used GFP- α -tubulin expressing AX2 and CP250⁻ cells and stained for interaptin using mAb 260-60-10 and for Sun-1 using mAb K55-432-2 and polyclonal antibodies. *D. discoideum* undergoes a closed mitosis. Interaptin stays at the NE during mitosis in AX2 cells. During anaphase, interaptin decorates the microtubules linking both asters. In the mutant cells, the interaptin distribution during mitosis followed essentially the pattern seen in wild-

Figure 5. Generation of CP250-deficient cells. (A) A replacement vector was constructed by replacing an internal 552-base pair segment of the CP250 gene with the blasticidin resistance cassette (bsr). The location of relevant restriction enzyme sites and of the probes used for Southern blot analysis is given. (B) Southern blot analysis of XhoI/ScaI digested genomic DNA of AX2 and the CP250⁻ strain. After successful replacement a shift from 5.5 kb for wild type to 2.7 and 3.7 kb occurred as detected by a 5' and a 3' gene-specific probe. (C) In CP250-deficient cells centrosomal staining by K68-439-8 is abolished. The microtubule network was labeled by mAb YL1/2. Bar, 10 μ m.

type cells, but overall interaptin staining at the NE was reduced, whereas the cytoplasmic staining increased indicative of a relocation to endoplasmic reticulum (ER) membranes. Also, staining of the microtubules linking the asters was less obvious (Figure 9A). Sun-1 antibodies strongly label the NE in a continuous manner in interphase in AX2 cells (Figures 9B and 10A; Xiong *et al.*, 2008). During mitosis, Sun-1 stays at the NE, and the intensity of staining increases near the centrosome. In mutant interphase cells, Sun-1 exhibited a strongly reduced NE staining in comparison to AX2, and during mitosis a single spot near the centrosome was detected and nearly no NE stain was observed (Figures 9B and 10A). Western blot analysis revealed a reduction of Sun-1 protein levels in the mutant to ~50% of wild-type levels, confirming the observations of the immunofluorescence analysis (Figure 9C).

On the basis of the data from other organisms where the centrosome NE interaction involves microtubules (Malone *et al.*, 2003), we treated AX2 and CP250⁻ cells with the microtubule-depolymerizing drug nocodazole (10 μ M, 2 h). This led to a redistribution of Sun-1 at the NE and an enrichment of the protein near the centrosome in AX2. In CP250⁻ cells, Sun-1 staining was unaffected by nocodazole treatment, and the protein stayed in its centrosome near position (Figure 10, A and B). The mechanism is not known; however, one proposal is that an intact and dynamic microtubule system maintains the distribution of Sun-1 at the NE and, further, that changes in the microtubule system or its dynamics as they occur during mitosis, drug treatment, or after changes in the corona lead to alterations in Sun-1 distribution.

The Actin Cytoskeleton in the CP250⁻ Strain

Because CP250 was initially identified as an interaction partner of the actin-associated protein CAP, we tested the actin

and CAP distribution in mutant cells. Cortical actin staining was similar in AX2 and mutant cells, whereas cytosolic actin staining appeared increased in the mutant. CAP was unaltered and was observed in a cortical location in both cell lines. In mitotic cells cortical CAP staining was strongly reduced (Figure 11, A and B).

For CAP we have previously characterized a mutant, CAPbsr, which expresses strongly reduced CAP levels (Noegel *et al.*, 1999; 2004). Mutant cells are heterogeneous in cell size and have a cytokinesis defect exhibiting multiple nuclei. Here we stained for the centrosome using mAb K68-439-8. We found that CP250 staining was unaltered; however, CAPbsr showed significant abnormalities in centrosome number. Cells with one nucleus can have two or more centrosomes, and all multinucleated cells had extra centrosomes. In the latter case they were not associated with a nucleus (Figure 11C).

DISCUSSION

CP250 is a novel component of the *Dictyostelium* centrosome identified in a search for partners of CAP. CP250 is an acidic protein with a predicted coiled coil region. Although these are characteristics that it shares with proteins of the PCM of animal centrosomes, CP250 is not directly related to any of these proteins, nor could we identify homologues in other species.

Using immunofluorescence analysis and immunoelectron microscopy, we localized the protein to the corona, which, as the equivalent of the PCM, is responsible for nucleation of microtubules. CP250 not only is a component of the corona, it also behaved like a component of the corona during the cell cycle. It stayed associated with the centrosome during interphase, disappeared at the transition from prophase to

Figure 6. Analysis of CP250-deficient cells. (A) Growth on a lawn of *K. aerogenes*. The diameter of the colonies was measured at the indicated times. (B) Cell size is altered in CP250⁻ cells. Three hundred cells were analyzed in each case. (C) Aggregation on phosphate agar plates. Equal numbers of cells were plated. The pictures were taken at 11 h of development. Bar, 2 mm. (D) Fruiting bodies are smaller in CP250⁻ cells. Cells were allowed to develop on phosphate agar plates. The pictures were taken after 24 h. Bar, 2 mm. (E) Cell adhesion is enhanced in mutant cells. Cells were starved in Soerensen phosphate buffer, and aggregate formation was followed by determining the decrease in OD₆₀₀ at the indicated time points. Absorption at the start of the experiment was set to 100%.

Table 1. Cell motility parameters of AX2 and CP250⁻ cells migrating in a spatial cAMP gradient

	AX2	CP250 ⁻
cAMP gradient		
Speed (μm/min)	9.74 ± 3.18	8.13 ± 3.63
Persistence (μm/min-deg)	3.72 ± 1.47	2.85 ± 1.58
Directionality	0.79 ± 0.18	0.71 ± 0.25
Direction change (deg)	17.52 ± 9.35	27.35 ± 17.74

Images were taken at magnifications of 10× and 40× every 6 s. In all cases cells were analyzed for at least 20 min. The DIAS software was used to trace individual cells along the image series and calculate motility parameters. Persistence is an estimation of movement in the direction of the path. Directionality is calculated as the net path length divided by the total path length and gives a value of 1 for a straight path. Directional change represents the average change of angle between frames in the direction of movement. Values are mean ± SD of 30 cells from at least three independent experiments.

metaphase, a stage where the corona has dissociated from the central core, and reappeared at the end of late telophase (Ueda *et al.*, 1999). In general, the known *Dictyostelium* centrosomal proteins relocate from the corona to the core during mitosis. Only for *Dictyostelium* centrin (DdCnp) a loss from the centrosome was reported at the prophase to metaphase transition, similar to CP250 (Daunderer *et al.*, 2001).

Based on these findings, a role for CP250 in interphase is more likely as compared with a role in centrosome duplication and in mitosis. In human cells and in *C. elegans* an interference with specific PCM proteins affected the PCM and interfered with centriole formation (Keryer *et al.*, 2003; Dammermann *et al.*, 2004). In CP250-deficient cells we did not note defects in centrosome number and positioning when CP250 was absent. Also, the nuclear number is unaltered, indicating that the protein is most likely not involved in centrosome duplication.

We therefore focused on growth, chemotaxis, cell motility, and development that rely on an intact centrosome and microtubule system. CP250-deficient cells were smaller in size and had a disadvantage when grown on a lawn of bacteria. The cells developed and formed mature fruiting bodies that were however smaller in size than wild-type fruiting bodies. The reduced size of the fruiting bodies is probably the result of the smaller aggregation zones formed by the mutant cells. The basis of the growth and developmental defect may result from an altered chemotactic behavior. When we analyzed chemotactic motility toward cAMP, we found that the cells were slightly slower than wild type and migrated in a less directed manner toward the cAMP source. Also, the leading pseudopod seemed less stable and was retracted more frequently than in wild-type cells.

Recently a *Dictyostelium* mutant was described that lacks the *Dictyostelium* homolog of the Fused kinase. The mutant has defects in chemotaxis and development. The cells do not polarize perfectly and are impaired in chemotaxis. Furthermore, development is delayed and fruiting bodies are smaller than in wild type. In this work the kinase was located to microtubules and release of the kinase to the cytosol during microtubule depolymerization caused a loss in cell polarity underlining the importance of the microtubule system for cell polarity and chemotaxis (Tang *et al.*, 2008).

Alterations were also noted in CP250⁻ cells when we stained for the NE components Sun-1 and interaptin. Early

Figure 7. Analysis of cell polarization. (A) Polarization and directed migration of a representative AX2 wild-type cell after 6 h of starvation in a submerged culture were recorded at high magnification over 20 min at a rate of 6 s per frame. The outlines were traced manually, and the changes of direction (arrows) were calculated using the DIAS image analysis software. The panel shows selected images that reflect the behavior of AX2. (B) Shape changes of a CP250-deficient cell. The conditions were as above. CP250⁻ cells are smaller and less polarized. They change their direction more frequently than AX2. At the right, stack images of two cells each are shown.

on a physical association between centrosome and the NE has been suggested from electron microscopic studies (Bornens, 1977; Nadezhdina *et al.*, 1979). However only recently have proteins been described that might mediate this physical association. In *S. pombe* the Sun domain-containing protein Sad-1 together with Kms1 (Miki *et al.*, 2004) and in *S. cerevisiae* the Sun-domain protein Mps3 together with Mps2 provide a physical link to the spindle pole body (Jaspersen *et al.*, 2006). In *C. elegans* Sun-1/matefin functions together with Zyg-12 to attach the centrosome to the NE (Malone *et al.*, 2003). Zyg-12 and Kms1 are KASH-domain proteins and can interact with the SUN-domain proteins. In mammalian cells, SUN2 was detected at the centrosome and at the NE (Wang *et al.*, 2006). We showed that Sun-1 mediates centrosome-nucleus connection in *D. discoideum* and that deletion of the Sun-1 N-terminus (GFP-ΔN-Sun-1) abrogated this close connection. Also, in cells expressing GFP-ΔN-Sun-1 the

centrosomes were often connected to the nucleus by a membrane bridge that was positive for the GFP fusion protein (Xiong *et al.*, 2008).

CP250 might also be involved in the cross-talk between NE and centrosome as its loss led to strongly reduced amounts of Sun-1 and interaptin at the NE, although a direct interaction between these proteins is not likely. The residual amounts of Sun-1 concentrated near the centrosome where the protein might cluster and collect the telomeres as has been described in fission yeast and in mammals. It has been suggested that the prime function of Sun-1 during mitosis and meiosis is near the centrosome where it anchors telomeres, allowing correct distribution of the chromosomes (Schmitt *et al.*, 2007; Ding *et al.*, 2007). In accordance with this, we frequently observed aneuploidy in cells expressing GFP-ΔN-Sun-1 (Xiong *et al.*, 2008). A possible microtubule involvement in this process is indicated by the behavior of

Figure 8. Progression through mitosis is normal in CP250⁻ cells. CP250⁻ cells expressing GFP-α-tubulin were immunolabeled with centrosome-specific mAb K29-359-10 (A) and DdCP224 specific polyclonal antibodies (B). Early anaphase and telophase stages are shown. The arrow indicates the localization of K29-359-10 at the centrosome and the midbody staining by DdCP224 antibodies. Bar, 5 μm.

Figure 9. Distribution of NE proteins in CP250^{-/-} cells. (A) AX2 and CP250^{-/-} cells expressing GFP- α -tubulin were stained with mAb 260-60-10 for interaptin. (B) Sun-1 was detected with mAb K55-460-1. Images of interphase cells and cells at the end of mitosis are shown. Bar, (A and B) 5 μ m. (C) Reduction of Sun-1 in CP250^{-/-} cells as analyzed by Western blot analysis. Equal amounts of proteins were loaded. The blot was probed with mAb K55-460-1.

Sun-1 in nocodazole-treated wild-type cells where Sun-1 collects near the centrosome and does not show an overall staining of the NE as in control cells. We previously reported reduced amounts of Sun-1 using siRNAi that resulted in ~40% reduction of Sun-1 (Xiong *et al.*, 2008). In these cells we observed nuclear deformations, but the centrosomes were not affected as in cells that expressed a Sun-1 protein lacking the N-terminus. Normally the N-terminus retains Sun-1 in the inner nuclear membrane. When this location is abandoned, the cells show nuclear deformation, hyperamplification of centrosomes, and an altered centrosome-nucleus distance. These results suggested to us that the residual amounts of Sun-1 are sufficient to tether the chromatin to the centrosome and to maintain the centrosome nucleus connection and mitotic spindle stability, which is in agreement with the results presented here. The absence of nuclear abnormalities in CP250-deficient cells might be explained by the additional loss of one more protein, namely CP250.

In nocodazole-treated wild-type cells Sun-1 also accumulates in NE regions near the centrosome. Nocodazole treatment depolymerizes interphase microtubules very efficiently in *Dictyostelium*. One explanation for the observed changes could be that upon loss of the forces that originate in the cytoskeleton and act on the nucleus, the mechanical strength might be lost and the organization of the NE affected (Starr, 2007, 2009).

Interaptin staining of the NE was also reduced in CP250-deficient cells. In contrast to mammalian cells for which we reported a direct interaction between the C-terminus of SUN-1 with the KASH-domain of Nesprin-1/Enaptin and Nesprin-2/NUANCE (Padmakumar *et al.*, 2005) such a direct interaction does not exist for *D. discoideum* interaptin and Sun-1. Instead, both proteins competed with each other for NE localization (Xiong *et al.*, 2008). The findings here are consistent with the absence of an interaptin Sun-1 interaction.

Figure 10. Sun-1 distribution in nocodazole-treated AX2 and CP250-deficient cells. Cells without (A) and with nocodazole treatment (B) were stained for microtubules (mAb YL1/2), Sun-1 (pAb), CP250 (mAb K68-439-8), and DAPI. Nocodazole treatment (10 μ M) was for 2 h. Confocal images are shown. Bar, 5 μ M.

Identification of CP250 as partner of CAP was rather unexpected as CAP has been described primarily as a cytoplasmic protein present throughout the cell and in regions of high actin dynamics. A clear location at the centrosome could not be demonstrated by immunofluorescence analysis of fixed cells so far. This does not, however, rule out a transient association. In fact, centrosomes can bind many regulatory components that involve the centrosome in a multitude of cellular functions (Doxsey *et al.*, 2005). Among these were proteins that are involved in the regulation of cytoskeleton-related functions like beta-catenin (Bahmanyar *et al.*, 2008) or integrin-linked kinase (Fielding *et al.*, 2008). For the *Dictyostelium* Lis1 homologue an interaction with Rac1A *in vitro* was described through which its observed effects on actin dynamics could be explained (Rehberg *et al.*, 2005). Another link between centrosome and the actin cytoskeleton is provided by the mammalian CENPJ, a protein located at the centrosome throughout mitosis, which associates with a splice variant of protein 4.1 (Hung *et al.*, 2000). Such a connection might explain the phenotypes we observed in CP250-deficient cells that are similar to those frequently observed in cytoskeletal mutants and is supported by the altered actin distribution with increased cytoplasmic levels.

Interestingly, mammalian CAP2 was identified as a protein present in the cytoplasm as well as in the nucleus (Peché *et al.*, 2007). Furthermore, we have described a cytokinesis defect in *D. discoideum* cells deficient for CAP (CAPbsr). Forty-four percent of mutant cells in comparison to 17% of AX2 cells have multiple nuclei (Noegel *et al.*, 1999). We now explored this phenotype and investigated the centrosome by expressing GFP- α -tubulin and staining for CP250. In normal-sized mononucleated CAPbsr cells CP250 distribution was unaltered, and the centrosomes were closely associated with the nuclei in mono- and binucleated cells. In contrast, multinucleated cells had additional centrosomes that were freely present in the cytoplasm. Frequently the cells displayed abnormal nuclei that were larger than normal and had different shapes. The association between CAP and CP250 could also be responsible for the defects in cell polarity and development that are observed in both cell lines (Noegel *et al.*, 1999; 2004; this work). Both the actin and the microtubule cytoskeleton have been implicated in the generation of cell polarity, and it might well be that both systems converge at this point (Azimzadeh and Bornens, 2007). More recently, Srv2, the CAP homologue in *S. cerevisiae*, was identified in complex with Nur1 (Ydf089w), a component of the inner nuclear membrane (Mekhail *et al.*, 2008).

Figure 11. Actin and CAP in CP250⁻ cells and the centrosome in a CAP mutant. (A) AX2 and CP250⁻ cells were stained for tubulin with mAb YL1/2 and for actin with mAb act-1. (B) Staining for CAP was with mAb 230-18-8. Nuclei were stained with DAPI. Bar, 5 μ m. (C) CAPbsr cells exhibit a cytokinesis defect and have extranumerary centrosomes. Microtubuli were stained with mAb YL1/2, centrosomes with mAb K68-439-8. Nuclei were stained with DAPI. Bar, 10 μ m.

In summary, we have identified a component of the corona that affects cell growth and chemotactic motility and is involved in the interaction with the NE. CP250 is present at the centrosome during interphase; however, it disappears during mitosis. We narrowed down the region required for centrosomal association and correct behavior during mitosis to a 46-kDa polypeptide located in the first half of the protein. Loss of CP250 does not interfere with the role of the centrosome as MTOC and for formation of an intact microtubule network nor does it affect centrosome duplication. In this respect it resembles several pericentriolar proteins in higher eukaryotes such as PCM-1 and pericentrin, which appear to be dispensable for centrosome duplication (Balczon *et al.*, 1994; Kleylein-Sohn *et al.*, 2007). By contrast, loss of CP250 leads to disturbance of the NE composition and affects two major proteins of the NE, Sun-1 and interaptin, and causes a reduction of their amounts at the NE. How this is achieved is presently unclear. It might well be that the MTOC as microtubule organizer is not functioning correctly in the mutant. Furthermore, we know that Sun-1 provides a link between centrosome and nucleus. This interaction appears to be altered in the mutant leading to an altered distribution of Sun-1.

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