Cofactor D Functions as a Centrosomal Protein and Is Required for the Recruitment of the \(\gamma\)-Tubulin Ring Complex at Centrosomes and Organization of the Mitotic Spindle*NS

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Microtubules are highly dynamic structures, composed of \(\alpha/\beta\)-tubulin heterodimers. Biosynthesis of the functional dimer involves the participation of several chaperones, termed cofactors A–E, that act on folding intermediates downstream of the cytosolic chaperonin CCT (1, 2). We show that cofactor D is also a centrosomal protein and that overexpression of either the full-length protein or either of two centrosome localization domains leads to the loss of anchoring of the \(\gamma\)-tubulin ring complex and of nucleation of microtubule growth at centrosomes. In contrast, depletion of cofactor D by short interfering RNA results in mitotic spindle defects. Because none of these changes in cofactor D activity produced a change in the levels of \(\alpha\)- or \(\beta\)-tubulin, we conclude that these newly discovered functions for cofactor D are distinct from its previously described role in tubulin folding. Thus, we describe a new role for cofactor D at centrosomes that is important to its function in polymerization of tubulin and organization of the mitotic spindle.

Centrosomes are the major microtubule-organizing center in animal cells and are composed of two centrioles (barrel-shaped structures composed of \(\alpha/\beta\)-tubulin heterodimers) surrounded by a dense fibrillar network of proteins called the pericentriolar material (PCM). A key step in the initiation of new microtubule growth is the regulated recruitment to the PCM of \(\gamma\)-tubulin ring complexes (\(\gamma\)-TuRCs), consisting of \(\gamma\)-tubulin and \(\gamma\)-complex proteins (GCPs) (3–7), that promote the polymerization of \(\alpha/\beta\)-tubulin heterodimers. In addition to nucleating new microtubule growth, centrosomes also organize cytosolic microtubules during interphase, spindle microtubules during mitosis, and axonemes in ciliogenesis (8, 9).

Formation of \(\alpha/\beta\) tubulin heterodimers is promoted by five tubulin-specific co-chaperones, termed cofactors A–E, that act on \(\alpha\)- and \(\beta\)-tubulin folding intermediates in a stepwise process that generates polymerizable heterodimers (1). Demonstration of the roles for these five cofactors in folding tubulin heterodimers comes from in vitro folding assays, which also allowed purification of the five co-chaperones (1, 10). This function is consistent with genetic studies in *Saccharomyces cerevisiae* (11, 12), *Schizosaccharomyces pombe* (13–15), *Arabidopsis thaliana* (16, 17), and *Caenorhabditis elegans* (18) in which mutations in cofactor D (CoD) yielded defects in microtubule-dependent processes, including maintenance of chromosome number.

That the tubulin-specific cofactors may play additional roles in regulating microtubule stability or dynamics was first proposed by Tian et al. (2), who found that overexpression of bovine CoD in HeLa cells led to the loss of microtubules and decreased levels of \(\alpha\)-tubulin in cells. With additional in vitro data showing that purified bovine CoD disrupts the tubulin heterodimer, the authors proposed a role for CoD in heterodimer destruction, with a secondary loss of microtubules (19, 20). A regulated destructive pathway was suggested by the observations that increased expression of the small GTPase Arl2 could block the effects of CoD overexpression and could be co-immunoprecipitated with CoD (19). Later, the majority of cellular Arl2 was shown to exist in a stable ~300-kDa complex with CoD and the trimeric protein phosphatase, PP2A (21). Neither the tubulin folding activity of CoD nor the GTP binding activity of Arl2 were detectable in the purified complex (21), so we sought other functions for the complex constituents in cell regulation.

**EXPERIMENTAL PROCEDURES**

**Cloning and Plasmids**—The plasmid directing expression in mammalian cells of bovine CoD fused to GFP was obtained from Nicholas Cowan (New York University) (19) and was used as a template to generate our HA-tagged bovine CoD expression constructs. A plasmid containing the entire open reading frame of human CoD was obtained from ATCC (clone MGCC-1583; Manassas, VA) and used to generate both full-length and truncation mutant expression plasmids by PCR amplification using custom oligonucleotide primers. The parent plasmid in each case was pCDNA3.1/Myc-His (Invitrogen), and each open reading frame was cloned in at the NotI and XbaI sites. When N-terminal tags were added, they were included in the primers used in the PCR. Sequences were confirmed by DNA sequencing. Plasmid-based siRNA experiments targeted four 19-nucle-
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otide stretches within the CoD reading frame (GGCTAAATA- TGGACAAATA, CAACTGTTCCTGTATA, TCTATGATCCTACGTATA, and TGACGCTCCTGACTTTGA) that were synthesized and cloned into the pG-SHIN2 vector, a derivative of pSUPER that expresses EGFP from the constitutive SV40 early promoter, using BgIII and HindIII (22).

Antibodies—We generated rabbit polyclonal antibodies to CoD by injection of a 16-mer peptide (60FRVIMD-KYEQPHLLC74) that was covalently coupled to keyhole limpet hemocyanin through the C-terminal cysteine prior to injection in animals (Bioperformance; Affinity Bioreagents). Affinity purification of CoD antibodies was achieved using an Ultralink Iodoacetyl Gel (Pierce) column to which peptide was conjugated following the manufacturer’s instructions (23, 24). Other antibodies used in this study were monoclonal α-, β-, and γ-tubulin antibodies (Sigma), monoclonal and polyclonal His6 antibodies (Cell Signaling Technologies), monoclonal centrin-2 (a generous gift from Jeff Salisbury (Mayo Clinic, Minneapolis, MN)), and polyclonal antibodies against pericentrin and GCP-WD (gifts from Tim Stearns (Stanford University)).

Cell Culture and Immunofluorescence—HeLa cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 1% penicillin/streptomycin (Gibco). Cells were transfected using either Lipofectamine 2000 or Lipofectamine Plus according to the manufacturer’s directions (Invitrogen). The Smart Pool mixture of four synthetic RNAs targeting human CoD was purchased from Dharmacon, and transfections were performed using Dharmafect 1 according to the manufacturer’s directions (Dharmacon).

For immunofluorescence, cells were fixed using either methanol (−20 °C, 5–7 min) for centrosomal staining or PHEMO buffer (25) (room temperature, 10 min) for α-tubulin staining of microtubules and in the nocodazole washout (aster formation) assay (25). Treatment of cells with nocodazole (15 μM, 2 h) was performed as described (25). Quantification of centrosomal staining intensities for CoD, γ-tubulin, centrin-2, pericentrin, and GCP-WD were performed by comparison with the staining observed in control cells and were scored by eye.

Centrosome Purification—Cells were transfected, and 24 h later, centrosomes were isolated from cells using a modification of the method of Hsu and White (26) as described by Zhou et al. (25). Briefly, cells were incubated with cytochalasin D (1 μg/ml) and nocodazole (0.2 μM) at 37 °C for 1 h. Cells were then washed, followed by incubation with shaking at 4 °C for 10 min in lysis buffer. Insoluble material was removed by centrifugation. Lysates were added to a 60% sucrose solution, and tubes were centrifuged at 25,000 × g for 30 min to sediment centrosomes into the sucrose cushion. The bottom layer was collected, loaded onto a discontinuous sucrose gradient, and spun at 120,000 × g for 1 h. Fractions (0.2 ml each) were collected from the top, diluted in 2 ml of 10 mM PIPES, pH 7.2, and sedimented by centrifugation at 25,000 × g for 30 min. Supernatant was removed, and pellet protein was resuspended and denatured in SDS sample buffer.

Data Analysis—All experiments reported have been repeated at least three times with similar results. Fluorescence images were collected with an Olympus BX60 microscope with a ×100 oil immersion objective (Melville, NY), and images were taken using a Q-Imaging RETIGA 1300R camera. Images were processed using Adobe Photoshop. Graphs were generated using GraphPad Prism.

Standard epifluorescence images were obtained in each case, to facilitate viewing of structures or staining in different focal planes. This results in lower resolution in several of the images shown in the figures, as compared with the use of confocal data. In addition, and only in Fig. 7, some of the images shown were merged from epifluorescence images taken at different focal planes to allow depiction of the multiple pole/spindle phenotypes.

RESULTS

CoD Is a Component of Centrosomes—Rabbit polyclonal antibodies were raised against a 15-mer peptide, located near the N terminus of human CoD and conserved in several mammalian CoD orthologs. The CoD antisera specifically recognized a ~130-kDa protein in HeLa cell lysates (Fig. 1A, middle), and this band was clearly increased in immunoblots of cells expressing His6-tagged CoD (Fig. 1A). For this experiment only, we chose conditions of transfection that would allow low expression levels of CoD-His6 in order to visualize both endogenous and overexpressed protein at similar exposure times. Preimmune sera did not react with this protein (Fig. 1A, left), and the signal was effectively competed by preincubation of antiserum with the immunizing peptide (Fig. 1A, right). Affinity-purified antibodies yielded similar results, with a decrease in nonspecific binding and specific antibody titer so that there was substantially reduced CoD signal intensity (data not shown). Fractionation of HeLa cell lysates by centrifugation at 100,000 × g revealed that an estimated 90% or more of the protein fractionates in the S100 (data not shown), suggesting that the vast majority of CoD is cytosolic, as expected from the fact that CoD has twice been purified from cytosol (1, 21, 27). Indirect immunofluorescence of cultured mammalian cells revealed that CoD is also present at centrosomes, as seen by its co-localization with γ-tubulin in both interphase and mitotic HeLa cells (Fig. 1B). The specificity of this localization of endogenous CoD was confirmed, since centrosomal staining was absent when preimmune serum was used or when the immune serum was preincubated with the peptide antigen (data not shown). Because of the presence in immunoblots of a cross-reactive band of ~75 kDa, we performed two additional tests of the presence of CoD at centrosomes. Human CoD carrying a N-terminal His6 tag was transiently expressed in HeLa cells prior to fixation and localization with antibodies directed against the His6 tag. We found that His6-CoD was also at centrosomes at early time points after transfection (Fig. 1C) and was also found to co-sediment with the peak of γ-tubulin in our centrosome purification protocol (Fig. 1D). Together, these data show that CoD is a component of the centrosomes. Endogenous CoD was also observed at centrosomes in U2OS, COS-7, or MCF-7 cells (data not shown). The intensity of staining of CoD at centrosomes increased during the cell cycle, similar to that of γ-tubulin, and appeared to occupy a larger area than that of centrin-2 at the centrioles (28, 29). Thus, CoD is found in the PCM and is probably increasingly recruited during the course of the cell cycle.
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Human and Bovine CoD Display Differences in Effects on Microtubules When Expressed in HeLa Cells—Because CoD is involved in tubulin biosynthesis and localizes to centrosomes, we sought to determine if it also functions to regulate assembly of microtubules at the microtubule organizing center. Expression in HeLa cells of GFP-tagged bovine CoD was shown previously (19) to cause a dramatic reduction in the number of microtubules at the microtubule organizing center. We sought to determine if it also functions to regulate assembly of microtubules when expressed in HeLa cells of GFP-tagged bovine CoD was shown previously (19) to cause a dramatic reduction in the number of microtubules at the microtubule organizing center. We expressed this protein in HeLa cells and observed neither an impact on the microtubule integrity nor any change in the levels of α-tubulin, despite the fact that the protein was expressed to levels comparable with those of wild type bovine CoD. These data reveal that the C terminus of bovine CoD is required for microtubule disruption (Fig. 2, A (middle) and B). In an effort to determine if the bovine residues were sufficient to confer the ability to disrupt microtubules onto human CoD, we also constructed an N-terminal HA-tagged human CoD with these 4 amino acids changed to the bovine sequence (CoD-HsΔα(4)). When expressed transiently in HeLa cells, we again did not see any changes in the density of microtubules (data not shown); nor were changes in the levels of α-tubulin detected by immunoblot analyses (Fig. 2B). These data indicate that these 4 residues are critical for microtubule disruption, we constructed an N-terminal HA-tagged bovine CoD with these 4 amino acids changed to the bovine sequence (BovΔα). We expressed this protein in HeLa cells and observed neither an impact on the microtubule integrity nor any change in the levels of α-tubulin, despite the fact that the protein was expressed to levels comparable with those of wild type bovine CoD. These data reveal that the C terminus of bovine CoD is required for microtubule disruption (Fig. 2, A (middle) and B). In an effort to determine if the bovine residues were sufficient to confer the ability to disrupt microtubules onto human CoD, we also constructed an N-terminal HA-tagged human CoD with these 4 amino acids changed to the bovine sequence (HsΔα(4)). When expressed transiently in HeLa cells, we again did not see any changes in the density of microtubules (data not shown); nor were changes in the levels of α-tubulin detected by immunoblot analyses (Fig. 2B). These data indicate that these 4 residues are necessary for the microtubule disruption activity of bovine CoD but are not sufficient to confer this activity upon human CoD.

Human and bovine CoD are 1192 and 1199 residues in length, respectively, and share 81.4% sequence identity. Recently, Shultz et al. (30) reported that deletion of the last 15 amino acids from bovine CoD inhibited its ability to disrupt microtubule integrity. The only differences between human and bovine CoD in this region are in 4 of the last 5 C-terminal residues (Fig. 2C). To determine if the C-terminal residues are critical for microtubule disruption, we constructed an N-terminal HA-tagged bovine CoD with these 4 amino acids changed to the human sequence (BtΔHs(4)). We expressed this protein in HeLa cells and observed neither an impact on the microtubule integrity nor any change in the levels of α-tubulin, despite the fact that the protein was expressed to levels comparable with those of wild type bovine CoD. These data reveal that the C terminus of bovine CoD is required for microtubule disruption (Fig. 2, A (middle) and B). In an effort to determine if the bovine residues were sufficient to confer the ability to disrupt microtubules onto human CoD, we also constructed an N-terminal HA-tagged human CoD with these 4 amino acids changed to the bovine sequence (HsΔαBt(4)). When expressed transiently in HeLa cells, we again did not see any changes in the density of microtubules (data not shown); nor were changes in the levels of α-tubulin detected by immunoblot analyses (Fig. 2B). These data indicate that these 4 residues are necessary for the microtubule disruption activity of bovine CoD but are not sufficient to confer this activity upon human CoD.

Human CoD is predicted to be expressed in as many as five alternatively spliced variants or isoforms in the Expasy UniProtKB data base (available on the World Wide Web) and as two in Entrez Gene (Gene ID: 6904). Variant 1 is predicted to be 1192 residues in length and is supported by over 500 expressed


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![Image of a panel](image)

**FIGURE 2.** Human and bovine CoD have different effects on microtubule integrity and α-tubulin levels. A, HA-tagged human, bovine, and Bt.Hs(4) CoD were expressed in HeLa cells for 24 h before fixation with PHEMO and staining with antibodies to α-tubulin (left) and HA (middle) to identify transfected cells. DNA was visualized using Hoechst 33342. B, whole cell lysates (25 μg/lane) were prepared from untreated cells or 24 h after transfection with plasmids directing expression of the indicated CoD constructs, resolved in SDS gels, and analyzed by immunoblotting with antibodies to the HA epitope; α-, β-, or γ-tubulin; or actin. Transfection efficiencies were typically at least 60% for all experiments. C, alignment of the last 15 residues of human and bovine CoD. Conserved residues are shown in red. Hs, Homo sapiens; Bt, Bos taurus CoD.

![Image of a panel](image)

**FIGURE 3.** Excess CoD causes loss of centrosomal γ-tubulin. HeLa cells were transfected with CoD-His6, and 18 h later they were fixed with methanol and stained with antibodies to His6 (left panels), γ-tubulin (top row, middle panel), or centrin-2 (bottom row, center panel). DNA was visualized using Hoechst 33342. Top row, the arrowheads point to γ-tubulin staining in untransfected cells. Bottom row, centrosomal centrin-2 (arrows) staining is retained in both control cells and those expressing CoD-His6 (bottom middle panel). Scoring was performed using epifluorescence microscopy with focusing throughout the z plane to ensure loss of γ-tubulin staining at centrosomes. Scale bars, 5 μm.

sequence tag sequences. A longer variant, variant 4, is predicted to include one additional exon, encoding 38 amino acids, but a BLAST search of this sequence yielded no matches in the expressed sequence tag data base. The other three predicted variants are much shorter in length (<700 residues), and tblastn searches of unique DNA sequences from each yielded four expressed sequence tag matches to one and only one to each of the others. Orthologs of CoD were also found in a variety of species, including mouse, rat, yeasts, plants, and worms, and each of these proteins is predicted to be between 1024 and 1232 residues in length. Thus, we conclude that human CoD is primarily, and possibly exclusively, expressed in tissues as the 1192-residue protein. All subsequent studies were performed with this variant 1 of human CoD.

**Increased Expression of CoD Causes Loss of Centrosomal γ-Tubulin and Aster Formation**—Although the increased expression of human CoD in HeLa cells did not produce changes in α-tubulin levels or microtubule density, we did observe the loss of γ-tubulin staining at centrosomes (Fig. 3, top row). The effect of His6-CoD expression on γ-tubulin staining was thought to be dose-dependent, because at early times after transfection (e.g. 5 h, when protein expression is still low), γ-tubulin and His6-CoD were still retained on centrosomes (Fig. 1C). We also found that HA-tagged human, HsΔBt(4), and BtΔHs(4) CoDs each localized to centrosomes, although the staining of BtΔHs(4) at centrosomes was noticeably weaker than that of the other constructs. We did not detect any centrosomal staining of bovine HA-CoD, which may indicate that CoD localization to this organelle requires microtubules, since this is the only construct that resulted in their loss in cells.

Cellular CoD-His6 staining was noticeably increased by 8 h post-transfection. By this time, the centrosomal staining of γ-tubulin was largely lost, and it was completely absent in transfected cells after 18 h (Fig. 3, top row). Similarly, expression of any of the HA-tagged bovine, human, HsΔBt(4), or BtΔHs(4) CoD proteins also resulted in the loss or reduction in γ-tubulin staining at centrosomes (data not shown). In contrast, expression of CoD-His6 did not alter centrin-2 staining at centrosomes at any of these times, compared with control cells (Fig. 3, bottom row). Centrin-2 is associated with centrioles and is required for centriolar and centrosomal duplication (28, 31, 32). The retention of centrin-2 at the centrosomes in cells transfected with CoD indicates that core components of centrosomes are not altered under these conditions and that, despite the loss of γ-tubulin, the integrity of the centrosomes is otherwise intact.

We next determined whether cells overexpressing CoD were able to nucleate the growth of new microtubules as efficiently as control cells. Cells were treated with nocodazole to depolymerize microtubules and then washed to remove the drug, and the growth of new microtubules was observed by following the formation of microtubule asters emanating from centrosomes at early times after washout of the drug, by labeling fixed cells with α-tubulin antibodies. Cells overexpressing CoD failed to produce asters under these conditions and thus appear to be compromised in their ability to nucleate the growth of microtubules from centrosomes (Fig. 4A, top) but were able to nucleate cyto-
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CoD Contains Two Centrosomal Targeting Domains—CoD is a large (133-kDa) protein that is predicted to fold almost entirely into a series of α-helices with loops that form HEAT or armadillo repeats (33). Recombinant human CoD is insoluble when expressed in bacteria, and we also failed to obtain soluble protein from insect or yeast (Pichia pastoris) cell expression systems. In efforts to define functional domains within CoD involved in the effects described above, we generated a series of plasmids directing expression of truncation mutants and tested them for effects on centrosomal proteins and functions. The typical levels of expression achieved in HeLa cells of the truncation mutants used in our study and pictorial representations of the mutants are shown in Fig. 4B. We first assayed the mutants for the ability to prevent aster formation in the nocodazole washout experiment, and each half (residues 1–610 and 611–1192) of CoD prevented aster formation as effectively as the full-length protein (Fig. 4C). Further truncations of CoD revealed that there are two different domains, found within residues 310–610 and 803–1054 (shaded bars in Fig. 4B), that are independently capable of preventing aster formation when expressed in HeLa cells. Other regions of the protein had either no effect or exhibited only very weak activity, despite their expression to comparable levels in cells (Fig. 4B). Note that expression of neither CoD (Fig. 2B) nor any of the truncation

solic microtubules (arrows in 4A). Similar results were obtained with CoD overexpression in human osteosarcoma (U2OS), breast adenocarcinoma (MCF-7), and monkey kidney (COS-7) cells (data not shown). Even at 1 h after drug washout, we failed to observe asters in HeLa cells overexpressing CoD, despite the fact that the density of microtubules in these cells continued to increase over time (Fig. 4A, middle and bottom). We interpret these data as evidence that the presence of excess CoD in cells prevents the centrosome from acting as the primary site of microtubule nucleation but does not inhibit the machinery involved in such nucleation. This conclusion is also consistent with the fact that there was no evident difference in microtubule densities in these cultures prior to treatment of cells with nocodazole.

Because such a profound loss of microtubule nucleation at centrosomes might be expected to have a more severe impact on cell survival or mitosis, cells overexpressing CoD were examined for 2 days post-transfection. Mitotic indices were scored as the percentage of transfected cells with condensed DNA. One day after transfection, we noted that the mitotic index had decreased to zero in cells staining positively for CoD expression, compared with mock-transfected cultures (0% versus 4.4% ± 0.3, with at least 100 cells counted in each of three independent experiments). However, by 48 h, mitosis had resumed in transfected cells, and no difference was observed in the mitotic index. Thus, the excess expression of CoD causes the loss of aster formation activity at centrosomes that probably contributes to a block in mitosis, but the transient nature of this arrest, due to the transience of the protein expression, is ample cause for caution in interpretation of these data. The construction of stable cell lines with inducible expression of CoD would probably shed additional light on this issue.

CoD-His6 and 18 h later were treated with nocodazole (15 μm) for 2 h at 37 °C, followed by washout with fresh medium and continued incubation. At the times shown on the left (8 min (top), 30 min (middle), and 60 min (bottom)) the cells were fixed using PHEMO buffer and stained with antibodies to His6 (left) and α-tubulin (middle), as described under “Experimental Procedures.” Note the return of microtubules in cells expressing CoD at later times, despite the lack of aster formation. DNA was visualized using Hoechst 33342. Formations of asters were quantified (n = 90 for each construct) in mock-transfected cells and cells expressing truncation constructs. Error bars, the range of triplicate determinations. Scoring was performed using epifluorescence with focusing throughout the z plane to ensure loss of asters.

FIGURE 4. Excess CoD or either of the two minimal regions caused the loss of aster formation. A, HeLa cells were transfected with CoD-His6, and 18 h later were treated with nocodazole (15 μm) for 2 h at 37 °C, followed by washout with fresh medium and continued incubation. At the times shown on the left (8 min (top), 30 min (middle), and 60 min (bottom)) the cells were fixed using PHEMO buffer and stained with antibodies to His6 (left) and α-tubulin (middle), as described under “Experimental Procedures.” The numbers indicate the beginning and ending residues; shaded truncations indicate minimal centrosome localization domains (see Fig. 5). C, HeLa cells were transfected with truncation mutants and 18 h later were assayed for aster formation following nocodazole treatment. Formation of asters was quantified (n = 90 for each construct) in mock-transfected cells and cells expressing truncation constructs. Error bars, the range of triplicate determinations. Scoring was performed using epifluorescence with focusing throughout the z plane to ensure loss of asters.
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Increased expression of CoD affects the localization of centrosomal proteins. HeLa cells were transfected with empty vector (control) or with plasmids directing expression of full-length (FL) or truncation mutants of CoD and 18–24 h later were fixed with methanol and stained with the antibodies indicated above the panels. In these experiments, a fragment expressing residues 594–1192 was used rather than the 611–1192 fragment. They yielded the same results in the aster reformation assay (not shown). DNA was visualized using Hoechst 33342. Scale bar, 5 μm. A, top, GCP-WD is lost from centrosomes in cells expressing CoD-His<sub>6</sub>. Bottom, quantification of centrosomal GCP-WD staining intensity in HeLa cells transfected with the indicated plasmid (n ≥ 300 for each condition). Error bars, the range of triplicate determinations. Scoring was performed using epifluorescence with focusing throughout the z plane to ensure loss of the indicated centrosomal signal. B, top, pericentrin staining is lost from centrosomes in cells expressing CoD-His<sub>6</sub>. Bottom, quantification of centrosomal pericentrin staining intensity in HeLa cells transfected with the indicated plasmid (n ≥ 240 for each condition). C, top, endogenous CoD is lost from centrosomes upon expression of CLD1 or CLD2. Bottom, quantification of centrosomal CoD staining intensity in HeLa cells transfected with the indicated plasmid (n ≥ 300 for each condition). D, top, centrosomal centrin-2 staining is unaltered in cells overexpressing CoD or CLD1 but is lost in cells expressing CLD2. Bottom, quantification of centrosomal centrin-2 staining intensity in HeLa cells transfected with the indicated plasmid (n ≥ 250 for each condition). E and F, CoD contains two centrosome localization domains. HeLa cells were transfected with truncation mutant 310–610 (CLD1) (E) or 803–1054 (CLD2) (F), fixed in methanol 5 h after transfection, and stained with antibodies to His<sub>6</sub> and γ-tubulin. Transfection efficiencies were typically at least 60% for all experiments. DNA was visualized using Hoechst 33342. Scale bar, 5 μm.

mutants (Fig. 4B) resulted in a discernible change in the levels of α-, β-, or γ-tubulin in cells.

Increased expression of CoD also resulted in the loss from centrosomes of another component of the γ-TuRC, GCP-WD (6, 7). The same truncation mutants that caused the loss of centrosomal staining of γ-tubulin also decreased that of GCP-WD, which probably indicates that recruitment of the entire ring complex to centrosomes is lost in these cells (Fig. 5A). We next asked if an excess of CoD caused the loss of additional proteins known to interact with the γ-TuRC.

Pericentrin was shown previously to bind directly to components of the γ-TuRC and to be required for its anchoring to centrosomes during mitosis (34). In addition to displacing γ-TuRCs from the centrosome, overexpression of CoD also caused the loss or reduction of pericentrin staining at centrosomes (Fig. 5 B). We also observed a strong correlation between the effects of expression of CoD truncation mutants on aster formation and on the loss of GCP-WD and pericentrin staining at centrosomes (compare Figs. 4C and 5, A and B). In most cases there was a complete loss of centrosomal staining. We also scored separately those cells in which a clear reduction in intensity of staining was observed (see supplemental Fig. 1 for examples of staining intensities).

Because our CoD antibodies were raised against an N-terminal peptide, we were able to assess the retention of endogenous CoD at centrosomes in cells expressing truncation mutants that lacked this epitope. The same two minimal domains, residues 310–610 and 803–1054, that each prevented γ-TuRC recruitment and aster formation also prevented endogenous CoD from binding to centrosomes (Fig. 5C).

We speculated that these truncation mutants may each bind a subset of CoD binding partners and, in so doing, interfere with the function of the endogenous protein. One prediction from this model may be that each of these domains themselves binds to centrosomes. To test this, we expressed each of these two His<sub>6</sub>-tagged mutants, fixed cells at early times after transfection (e.g. 5–7 h), and determined the location of the proteins with the antibody to the His<sub>6</sub> tag. We discovered that each of these two domains contained sequences

FIGURE 5. Increased expression of CoD affects the localization of centrosomal proteins. HeLa cells were transfected with empty vector (control) or with plasmids directing expression of full-length (FL) or truncation mutants of CoD and 18–24 h later were fixed with methanol and stained with the antibodies indicated above the panels. In these experiments, a fragment expressing residues 594–1192 was used rather than the 611–1192 fragment. They yielded the same results in the aster reformation assay (not shown). DNA was visualized using Hoechst 33342. Scale bar, 5 μm. A, top, GCP-WD is lost from centrosomes in cells expressing CoD-His<sub>6</sub>. Bottom, quantification of centrosomal GCP-WD staining intensity in HeLa cells transfected with the indicated plasmid (n ≥ 300 for each condition). Error bars, the range of triplicate determinations. Scoring was performed using epifluorescence with focusing throughout the z plane to ensure loss of the indicated centrosomal signal. B, top, pericentrin staining is lost from centrosomes in cells expressing CoD-His<sub>6</sub>. Bottom, quantification of centrosomal pericentrin staining intensity in HeLa cells transfected with the indicated plasmid (n ≥ 240 for each condition). C, top, endogenous CoD is lost from centrosomes upon expression of CLD1 or CLD2. Bottom, quantification of centrosomal CoD staining intensity in HeLa cells transfected with the indicated plasmid (n ≥ 300 for each condition). D, top, centrosomal centrin-2 staining is unaltered in cells overexpressing CoD or CLD1 but is lost in cells expressing CLD2. Bottom, quantification of centrosomal centrin-2 staining intensity in HeLa cells transfected with the indicated plasmid (n ≥ 250 for each condition). E and F, CoD contains two centrosome localization domains. HeLa cells were transfected with truncation mutant 310–610 (CLD1) (E) or 803–1054 (CLD2) (F), fixed in methanol 5 h after transfection, and stained with antibodies to His<sub>6</sub> and γ-tubulin. Transfection efficiencies were typically at least 60% for all experiments. DNA was visualized using Hoechst 33342. Scale bar, 5 μm.
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sufficient for binding to centrosomes (Fig. 5E and F) and, thus, are termed centrosome localization domains 1 (CLD1, residues 311–610) and 2 (CLD2, residues 803–1054). Phenotypes associated with the expression of CLD1 or CLD2 were very similar, since expression of either domain led to the loss of centrosomal γ-tubulin, GCP-WD, CoD, and pericentrin as well as loss of aster formation after nocodazole washout. One difference between the two domains was that only expression of CLD2 caused the additional loss of centrin-2 staining at centrosomes. Neither full-length CoD nor CLD1 altered centrin-2 staining (Fig. 5D). These data are consistent with a model in which CoD binds to (currently unknown) sites on centrosomes using two different domains, where its presence is required for γ-TuRC recruitment. In addition, the observation that CLD2 expression causes the loss of centriolar centrin-2 staining supports a more central role for CoD in centrosome/centriole integrity. Given that centrin-2 staining was retained upon overexpression of CoD or CLD1, it seems unlikely that the loss of centrosome staining seen with other centrosomal proteins is due to competition with the antibody for binding sites. In efforts designed to test the requirement for CoD in centrosomal integrity, we next examined the consequences of its depletion in cells.

Loss of CoD Causes Mitotic Spindle Defects—Four sequence-independent plasmids that targeted different regions of the open reading frame of human CoD were generated in the pG-SHIN2 vector (22) to drive expression of short hairpin RNAs in mammalian cells. Transient transfection of each of these plasmids into HeLa cells led to similar reductions in the level of CoD, as determined by immunoblot analysis (Fig. 6A) (data not shown). Two plasmids, S1 and S4, were selected for further study in order to protect against off-target effects, as previously described (35). The concentration of plasmids, scheduling of transfections and analyses, and transfection media were each optimized to yield maximal depletion of CoD. Maximal loss of CoD was achieved with two consecutive transfections with pG-SHIN2-based plasmids on days 0 and 2, followed by analysis on day 5. Despite the clear diminution in total cellular CoD levels (Fig. 6A), no changes were evident in the densities of interphase microtubules (Fig. 6B) or in the aster formation assay (data not shown), compared with control cells. Staining of centrosomal CoD was also retained, and this signal was still effectively competed by prior incubation with the immunizing peptide and was absent with the preimmune serum (data not shown). While incomplete, the depletion of CoD resulted in the formation of multipolar spindles (Fig. 6C). The levels of α-, β-, or γ-tubulin were not changed in these cells, as determined by immunoblotting (Fig. 6A), consistent with the conclusion that none of the changes in spindle integrity result from defects in the biosynthesis of the tubulin heterodimer. It is possible that the larger, soluble pool of CoD is more sensitive to depletion than is centrosomal CoD.
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and that spindle integrity fails when the levels of CoD fall below a certain level.

Among efforts to more completely silence expression of CoD, we purchased a pool of four synthetic RNAs designed for knockdown of human CoD and optimized conditions for maximal depletion of the protein in HeLa cells. Immunoblotting data revealed that we achieved a more complete reduction in levels of CoD than with the plasmid-based siRNAs, again with no changes in the levels of tubulins (Fig. 6A). CoD was still retained at centrosomes (data not shown), as has been reported for siRNA of some other centrosomal proteins (e.g. see Ref. 36). These data may indicate that CoD is selectively retained at centrosomes despite its overall diminution in cells, or it may be that the remaining signal is due to the cross-reactive band observed by immunoblot in Fig. 1.

We saw a time-dependent increase in the mitotic index, with a more than doubling in the number of mitotic cells 3 days after transfection of the pooled RNAs (Fig. 6D). Even more dramatic, however, was the increase in the percentage of mitotic cells with spindle defects, since this increased to nearly 60% (Fig. 6, C and E). At the same time, there was evidence of failures in cytokinesis, since the percentage of cells with multiple nuclei was also increased by ~8-fold 3 days after transfection of the pooled siRNAs (Fig. 6F). Note that the plasmid-based siRNAs also produced spindle defects, although in a lower percentage of cells. Based on these observations, we conclude that CoD is required to generate a properly functioning mitotic spindle and that there is a dose-dependent effect in which the lower the CoD levels, the higher the percentage of mitotic cells with spindle defects.

In order to determine if formation of multipolar spindles was due to abnormal centrosome duplication, we counted the number of centrosomes in interphase cells, using γ-tubulin staining to mark centrosomes and found no increase in centrosome number (not shown). In addition, we found that not all spindle poles in mitotic cells contain centrosomes (Fig. 7, A–C), indicating that excess centriole duplication had not occurred. The intensity of GCP-WD and CoD staining at centrosomes without centrioles appeared to be reduced in comparison with centrosomes containing centrioles (Fig. 7, A (bottom row) and C (middle row)), which may be an indication of centrosomal fragmentation (37–39). However, given that mitotic cells with an abnormal number of centrioles were observed (e.g. see Fig. 7B, bottom row), we cannot completely rule out the possibility that abnormal duplication contributes to the formation of multipolar spindles (see “Discussion”).

DISCUSSION

The data presented here show that CoD is a centrosomal protein with functions critical to the recruitment of the γ-TuRC, initiation of microtubule growth, and organization of the mitotic spindle. We propose that 1) CoD is present in both cytosolic and centrosomal pools, which are only slowly exchangeable; 2) CoD is required for the recruitment from cytosol of a subset of centrosomal proteins including pericentrin and the γ-TuRC; 3) pericentrin, CoD, and γ-TuRC recruitment to centrosomes and initiation of microtubule growth at that site are lost when excess CoD or either of the CLDs is present; 4) centrin-2 is lost from centrosomes in cells expressing CLD2, potentially as a result of a loss of integrity of the centrosome itself; and 5) CoD is required for organization of a functional mitotic spindle as spindle integrity is clearly compromised in cells depleted for CoD. None of the manipulations employed here, including overexpression, knockdown, or expression of dominant acting mutants of CoD, led to any promised in cells depleted for CoD. None of the manipulations employed here, including overexpression, knockdown, or expression of dominant acting mutants of CoD, led to any detectable changes in the levels of α-, β-, or γ-tubulins, indicating the phenotypes observed are not due to changes in tubulin
protein levels. These activities each appear to be distinct from the previously described role for CoD in tubulin heterodimer assembly. Given that there was still CoD staining at centrosomes even after our most rigorous attempts to deplete it, it is plausible that these low levels of CoD are sufficient for maintaining the pool of tubulin dimers. Thus, our data do not address the previously described function of CoD in tubulin folding but instead provide evidence supporting roles at centrosomes in promoting microtubule growth and spindle integrity.

**CoD Is Required for Centrosomal Integrity**—A role for CoD in recruitment of γ-TuRCs and pericentrin to centrosomes is proposed based upon the observations that CoD is a centrosomal protein in all four cell lines examined, and both pericentrin and the γ-TuRC (i.e. γ-tubulin and GCP-WD staining) are lost from centrosomes in cells overexpressing CoD. These losses are accompanied by the inability to initiate microtubule growth (asters) at centrosomes after washout of nocodazole, although the integrity of the centrosome (e.g. centrin-2 staining) appears unaltered. Overexpression of bovine or BtΔHs(4) CoD also caused a loss or reduction of γ-tubulin from centrosomes, indicating that bovine CoD also possesses this activity. However, in the case of bovine CoD, the loss of microtubules may contribute to the centrosomal loss of γ-tubulin, thus complicating interpretation of this observation.

Excess CoD in cells does not block microtubule polymerization as the microtubule network appears normal in cells overexpressing CoD, and microtubules reform over time after washout of nocodazole, despite the lack of aster formation. Our interpretation of these data is that excess CoD prevents the γ-TuRC and pericentrin from binding centrosomes but allows them to remain active in promoting the growth of microtubules from the cytosol or other sites (40). One possible explanation is that the excess CoD binds to partner(s) in the cytosol that normally only bind it at centrosomes, without inhibiting the microtubule nucleation activity of the protein(s). This interpretation is consistent with a growing body of evidence indicating that there are centrosome-independent mechanisms of microtubule nucleation, including those active in cells that have been depleted of γ-tubulin or induced to lose γ-tubulin from centrosomes, or after laser ablation of centrosomes (6, 40–44).

**CoD Contains Two Centrosome Localization Domains**—We defined two domains within CoD that are each sufficient to bind centrosomes and have the same effects as the full-length protein to disrupt the localization of both the γ-TuRC and pericentrin and inhibit aster formation. Expression of either of these CLDs can displace endogenous centrosomal CoD, but one important difference between the two domains is that only expression of CLD2 caused the loss of centrin-2 staining at centrosomes. Neither CLD1 nor full-length CoD had this activity. The simplest model that may explain these data is that CoD acts as an adaptor between the centrioles and components in the PCM, such as pericentrin and the γ-TuRC, to potentially stabilize each complex. These truncation mutants are predicted to compete with the same binding site(s) on centrosomes and act to prevent endogenous CoD from docking productively at centrosomes. High resolution imaging of cells expressing CLD2 may reveal insights into the consequences of this mutant on centrosome morphology but may prove to be difficult to demonstrate if the centrioles are lost.

The dissection of CoD into different functional domains should prove helpful in future studies addressing the question of whether different CoD activities result from the same or different molecular interactions. For example, we believe that the role of CoD in recruitment of γ-TuRC is different from its role in tubulin heterodimer assembly. In different species, however, not all of these activities are inherent in CoD orthologs. In budding yeast (*S. cerevisiae*), CoD (Cin1p) is not essential, but its deletion causes increased sensitivity to microtubule poisons and cold temperatures as well as chromosome instability (11, 12). These results indicate that CoD/Cin1p is not required for heterodimer assembly or initiation of microtubule growth but still influences mitotic segregation in that organism. In contrast, in fission yeast (*S. pombe*), CoD (Alp1) is an essential protein important for the formation of interphase microtubules, required for chromosome segregation, and binds microtubules (14, 15, 45). Defects in microtubules were found to accompany overexpression of Alp1+ (45) or bovine CoD (19) with cell rescue achieved by increased expression of β-tubulin and Atd2, respectively. These effects observed in *S. pombe* were not evident in the human ortholog.

Species differences in the requirement for cofactor A have also been reported. *S. pombe* cofactor A is dispensable for cell viability (13) and is also not required for tubulin folding using an *in vitro* tubulin folding assay (1). In contrast, Nolasco *et al.* (46) found that loss of cofactor A in mammalian cells led to a decrease in soluble tubulin, microtubule alterations, and a loss of cell viability.

**CoD Is Required for Spindle Integrity and Mitotic Progression**—CoD staining was still retained at centrosomes in depleted cells; therefore, the absolute requirement for CoD for the localization of PCM components and initiation of microtubule growth could not be established in our studies. Expression of either CLD removed CoD from centrosomes, and in those conditions pericentrin and γ-TuRC components were absent from centrosomes and centrosomal microtubule initiation was lost. Although we cannot exclude the possibility that the signal retained at centrosomes after CoD depletion results from our CoD antibody cross-reacting with another protein, we believe this to be unlikely, because the signal is completely lost upon expression of either CLD1 or CLD2. If the staining of CoD at centrosomes after siRNA depletion is due to endogenous CoD, we must conclude that centrosomal CoD has a longer half-life in cells than the soluble protein. This could be true if stable binding to centrosomes results from bipartite binding, involving at least the two CLDs. Weaker binding and dissociation of CoD from centrosomes would result from expression of the isolated CLDs competing with the endogenous protein for centrosomal docking site(s). We propose that the expression of either CLD can displace endogenous centrosomal CoD, and consequently any other proteins whose retention at centrosomes requires CoD, by simple competition, yielding a dominant negative effect that more efficiently removes CoD activity from centrosomes than cellular depletion by siRNAs. These data may suggest that different cellular roles for CoD require different minimal or threshold amounts of the protein (e.g. for-
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mation of a mitotic spindle is more sensitive to decreases in CoD levels than is the recruitment of centrosomal proteins. However, we cannot exclude the possibility that the CLDs identified in this study may have activities in addition to competition of CoD binding at centrosomes that could contribute to the phenotypes described.

Formation of multipolar spindles may result from defects in any of several highly regulated processes, including the centrosome duplication checkpoint, cytokinesis, centrosome fragmentation, or some combination of these (8, 47–50). That the two different siRNA strategies each yielded multipolar spindles during mitosis indicates that CoD is required for spindle integrity and that the spindle defects are not due to off-target effects of siRNA. We did not observe an increase in centrosome number during interphase, as would be expected if there was excessive centrosome duplication (51, 52). In contrast, we did observe mitotic cells with more than two poles containing centrioles (as in Fig. 7C, bottom row). More commonly, however, cells with multiple spindles had only two poles that stained positively for centrin-2, a marker of centrioles. Thus, although some aberrant centriole duplication seems to occur, abnormal centrosome duplication is unlikely to be the only mechanism responsible for the multipolar spindles observed in cells depleted for CoD.

In contrast to centrosome amplification, fragmentation generates more than two pericentriolar matrices that may be capable of supporting microtubule growth and organization. The multipolar spindles arising from depletion of CoD were most commonly found to have only two centrin-2 positive poles (Fig. 7, A and C) and are thus thought to have arisen through fragmentation of the centrosomes. Because we did not observe centrosomal fragmentation in interphase cells, it appears likely that centrosomal fragmentation occurs during mitosis to contribute to the formation of multipolar spindles (38). The amount of either CoD or GCP-WD staining at these acentriolar (centrin-2-negative) poles appears reduced in comparison with the amount at poles containing centrioles (centrin-2-positive), consistent with fragmentation.

We also observed an increase in the numbers of cells containing condensed DNA and a loosely organized bipolar spindle in which the DNA had not congressed at the metaphase plate. This was particularly evident in cells depleted of CoD using the plasmid siRNA constructs and may be an indication of abnormal timing of mitotic entry or exit. Additional tests will be required to more fully discriminate between these possibilities.

Finally, we found that the loss of CoD was accompanied by an increase in the number of multinucleated cells (Fig. 6F). We interpret this as evidence of failures in cytokinesis that occurred in cells after aberrant spindles had been generated. Thus, we believe that depletion of CoD results in a number of defects in mitosis and cytokinesis that will require additional studies to identify the underlying mechanisms.

In summary, our data reveal that CoD is a novel component of centrosomes that is involved in the recruitment of other proteins or complexes from a soluble pool to centrosomes. Critical unknowns at this point include the centrosomal binding partners of CoD and the regulatory mechanisms governing the recruitment of these proteins during interphase and mitosis.

GCP-WD has recently been shown to bind centrosomes independently of γ-TuRC and to be phosphorylated in a cell cycle-dependent fashion (6). The fact that CoD was purified from cytosol in a complex with the protein phosphatase PP2A suggests the possibility that CoD activities may also be regulated by phosphorylation, although to date, tests of this possibility have been negative.3 Future studies aimed at discovering the regulatory mechanisms involved in protein recruitment to the centrosome and the specific interactions formed by the different domains of CoD are predicted to provide additional insights into centrosome functions in interphase and mitotic cells.

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