GCP6 Binds to Intermediate Filaments: A Novel Function of Keratins in the Organization of Microtubules in Epithelial Cells

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In simple epithelial cells, attachment of microtubule-organizing centers (MTOCs) to intermediate filaments (IFs) enables their localization to the apical domain. It is released by cyclin-dependent kinase (Cdk)1 phosphorylation. Here, we identified a component of the γ -tubulin ring complex, γ -tubulin complex protein (GCP)6, as a keratin partner in yeast two-hybrid assays. This was validated by binding in vitro of both purified full-length HIS-tagged GCP6 and a GCP6(1397–1819) fragment to keratins, and pull-down with native IFs. Keratin binding was blocked by Cdk1-mediated phosphorylation of GCP6. GCP6 was apical in normal enterocytes but diffuse in K8-null cells. GCP6 knockdown with short hairpin RNAs (shRNAs) in CACO-2 cells resulted in γ -tubulin signal scattered throughout the cytoplasm, microtubules (MTs) in the perinuclear and basal regions, and microtubule-nucleating activity localized deep in the cytoplasm. Expression of a small fragment GCP6(1397–1513) that competes binding to keratins in vitro displaced γ -tubulin from the cytoskeleton and resulted in depolarization of γ -tubulin and changes in the distribution of microtubules and microtubule nucleation sites. Expression of a full-length S1397D mutant in the Cdk1 phosphorylation site delocalized centrosomes. We conclude that GCP6 participates in the attachment of MTOCs to IFs in epithelial cells and is among the factors that determine the peculiar architecture of microtubules in polarized epithelia.

INTRODUCTION

Microtubule-organizing centers (MTOCs) are complex multiprotein structures necessary to assemble microtubules (MTs) in vivo (Urbani and Stearns, 1999). MTOCs can stabilize and anchor the minus ends of the MTs they organize (Wiese and Zheng, 2000). Therefore, by acting as minus end caps, the distribution of MTOCs may determine the localization of minus ends, and, ultimately, the overall polarity of MTs within a cell. The best-known MTOC, the centrosome, contains an array of γ -tubulin ring complexes (γ -TurC; Urbani and Stearns, 1999) in the amorphous pericentriolar material, each of which can nucleate a single MT (Dietenberg *et al.*, 1998). In simple epithelial cells, MTs are not connected to the centrosome (Meads and Schroer, 1995), but rather they are distributed in a parallel array with the minus ends oriented toward the apical domain (Bacallao *et al.*, 1989; Mogensen *et al.*, 1989). Borisov and coworkers described a pathway for the formation of cytoplasmic MTs by release from the centrosome (Keating *et al.*, 1997). This MT release operated in the “ventral” region (i.e., between the nucleus and the basal domain), and it did not apply for cortical MTs. In the same year, however, two groups independently found a second MT nucleation pathway, independent of centro-

somes, possibly mediated by γ -TurCs located in the cytoplasm (Vorobjev *et al.*, 1997; Yvon and Wadsworth, 1997). Determining which of these mechanisms operates in polarized epithelial cells is important to understand how the architecture of MTs in an apicobasal orientation is developed. More specifically, the localization of the minus ends will depend on different mechanisms if they are released from centrosomes and moved to distant minus end-stabilizing molecules, as proposed by Mogensen *et al.* (2000), or if they are nucleated and stabilized by noncentrosomal γ -TurCs. In the first model, the localization of minus ends may depend entirely on the distribution of the stabilizing molecule. In contrast, in the second model it will largely depend on the distribution of the nucleating activity of noncentrosomal γ -TurCs.

Many reports suggest an important physiological role for MT nucleation based on noncentrosomal γ -tubulin-containing structures, possibly γ -TurCs, in polarized epithelial cells. First, γ -TurCs are known to nucleate and cap isolated MTs in the absence of centrosomes (Moritz *et al.*, 2000; Keating and Borisov, 2000). Second, many epithelial cells in vivo lack centrosomes, but they still have polarized MTs in an apicobasal orientation. For example, in the small intestine, all crypt cells have centrosomes, but only one in five of the more differentiated quiescent villus enterocytes displays centrosomes (Komarova and Vorobjev, 1993). Furthermore, MTs are nucleated in cells lacking canonical centrosomes that still show γ -tubulin aggregates in early vertebrate embryos (Gueth-Hallonet *et al.*, 1993) and in *Drosophila* (Szollosi *et al.*, 1986; Mogensen *et al.*, 1989). Third, vertebrate intestine and kidney epithelia show a layer of noncentrosomal apical γ -tubulin (Ameen *et al.*, 2001; Wald *et al.*, 2003), also observed in *C. elegans* intestinal epithelium (Bobinnec *et al.*, 2000). Finally, under conditions that perturb the integrity of

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Abbreviations used: γ -TurC, γ -tubulin ring complex; Cdk, cyclin-dependent kinase; dox, doxycycline; HA, hemagglutinin; IF, intermediate filament; K, keratin; MT, microtubule; TET, tetracycline; TX-, Triton X-.

those noncentrosomal γ -tubulin layers, such as K8-null mutation (Ameen *et al.*, 2001) or ischemia/reperfusion of the kidney (Wald *et al.*, 2003), substantial perturbations of the MT architecture are observed. Therefore, the possible role of noncentrosomal γ -tubulin in nucleation and capping of MTs in vivo may have been broadly overlooked.

Whether epithelial MTs are nucleated by isolated γ -TurCs or by canonical centrosomes, the apical distribution of γ -tubulin-containing structures in simple epithelial cells in interphase (Buendia *et al.*, 1990; Rizzolo and Joshi, 1993; Apodaca *et al.*, 1994; Meads and Schroer, 1995; Salas, 1999) may contribute to determine cytoskeletal polarity. In previous publications, we showed that detergent-insoluble γ -tubulin-containing structures are attached to intermediate filaments (IFs) (Salas, 1999) and that this attachment is responsible for the apical distribution of MTOCs in simple epithelial cells (Salas, 1999; Ameen *et al.*, 2001). The attachment of MTOCs to IFs has been shown in other cell types as well (Trevor *et al.*, 1995; Pockwinse *et al.*, 1997; Mulari *et al.*, 2003) as well as the recruitment of overexpressed keratins around centrosomes (Blouin *et al.*, 1990). Furthermore, we found that cyclin-dependent kinase (Cdk)1-mediated phosphorylation separates centrosomes and noncentrosomal γ -tubulin from IFs, consistently with the physiological events at the onset of mitosis. Moreover, a 190-kDa protein that coimmunoprecipitates with keratins (Ks) and γ -tubulin was found to be a substrate of Cdk1 (Figueroa *et al.*, 2002). These observations lead us to focus on the structure of the γ -TurC and possible molecular mechanisms that attach γ -tubulin-containing structures to the IF scaffold.

γ -TurCs contain γ -tubulin and a family of γ -tubulin complex proteins (GCPs)2-6, conserved in eukaryotes (Jeng and Stearns, 1999). Recent genetic studies in fission yeast have shown that orthologues of GCP2-3 (Alp4, 6) are essential components of the MTOC and that their mutations are lethal. Deletion of the GCP6 orthologue (Alp16), however, is not lethal, but rather results in a phenotype characterized by longer MTs (Fujita *et al.*, 2002). The GCP6 (Xgrip210) protein in vertebrates is much larger than its yeast counterpart because of the appearance of a long central domain, that comprises multiple repeats (Murphy *et al.*, 2001). This domain is conserved among vertebrates, but missing in single-celled eukaryotes (Fujita *et al.*, 2002). In this work, we identified GCP6 as a partner of keratins and explored the possibility that GCP6 is a linker protein involved in the attachment of insoluble γ -tubulin-containing structures, including noncentrosomal MTOCs, to IFs as well as the implications of the distribution of γ -tubulin in the architecture of MTs in epithelial cells.

MATERIALS AND METHODS

Cells and Vectors

CACO-2 (human colon carcinoma) and COS-1 cells were originally obtained from American Type Culture Collection (Manassas, VA) and have been kept in the laboratory, frozen at low passages. Stable transfectants of U2OS (human osteosarcoma) cells constitutively expressing a 6xHis (HIS)-tagged GCP6 under the cytomegalovirus (CMV) promoter were a kind gift from Dr. Tim Stearns and reported previously (Murphy *et al.*, 2001). Stable CACO-2 cells expressing the tetracycline (TET)-inducible element were obtained in the laboratory and described previously (CACO-2 TET-ON; Wald *et al.*, 2005). Vectors were as follows: pSilencer 3.0-H1 and pSilencer 3.0-U6 (Ambion, Austin, TX), and pEGFP-N1, expressing enhanced green fluorescent protein (EGFP) under the CMV promoter (Clontech, Mountain View, CA). pRevTRE (Clontech) was used to generate retroviral vectors. For protein expression in mammalian cells, a pcDNA3.1 Directional TOPO expression kit (Invitrogen, Carlsbad, CA) was used. Bait and prey vectors for yeast two-hybrid assays pGADT7 and pGBKT7 were obtained from Clontech as part of the Matchmaker Gal4 yeast two-hybrid system. Purified myosin and tropomyosin were a generous gift from Dr. Danuta Szczesna-Cordary (Department of Molecular

and Cellular Pharmacology, Miller School of Medicine, University of Miami, Miami, FL).

Stable Transfectants and Expression of Tagged Fragments of the C-terminal Region of GCP6

GCP6(1397-1819) fragment cloned in pACT vector was obtained from Molecular Interaction Facility (Biotechnology Center, University of Wisconsin, Madison, WI) as a product of a yeast two-hybrid screen. This construct was further divided into two fragments, which were subcloned into pRevTRE vector (Clontech) containing an HA C-terminus tag. The accuracy and orientation of the inserts cloned in each construct were verified by restriction analysis and polymerase chain reaction sequencing. The retrovirus packaging PT67 cells were transfected with the fragment prevTRE constructs and selected in 0.4 mg/ml hygromycin, and then viral supernatants were collected to infect subconfluent CACO-2 TET-ON cells. These cells were then selected in 0.2 mg/ml Geneticin (G-418) (for the TET-inducible element) and 0.4 mg/ml hygromycin (for the GCP6 fragment under TET promoter) for 2 wk (3 passages). All the experiments reported here were performed within the following 10 passages, because it was observed that the expression of the GCP6 fragment faded after several passages, even when the cells were continuously kept in selection media, possibly because of genetic instability of the cells (Tsushimi *et al.*, 2001).

Site-directed mutagenesis of GCP6 S1397 was performed using QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Transient expression of these mutants tagged with V5 and expression of C-terminal fragments of GCP6 in COS-1 and CACO-2 cells was achieved by transfection of these constructs cloned into the pcDNA3.1 vector.

Antibodies used were as follows. The anti-GCP6 polyclonal antibody (Ab) was raised against a synthetic peptide (24: GQRVNRKRKRSLKK) at Synpep (Dublin, CA). This sequence was selected because it is highly specific for GCP6 and conserved in mouse and human. There is, however, a repeat within the GCP6 molecule at aa 700 with 81% similarity. Commercially available antibodies were as follows: anti- α -tubulin and anti- γ -tubulin monoclonal antibodies (mAbs) (Sigma-Aldrich, St. Louis, MO), polyclonal (rabbit) anti- γ -tubulin antibody (Sigma-Aldrich), anti-K8 B22.1 mAb (Biomedica, Foster City, CA) and TROMA I (Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, IA), anti-K18 B23.1 mAb (Biomedica), anti-K19 RCK108 mAb (Accurate Chemical & Scientific (Westbury, NY), anti-pan keratin mAb C11 (Sigma-Aldrich), polyclonal (chicken) anti-green fluorescent protein (GFP) antibody (Chemicon International, Temecula, CA), mAb anti-HIS tag (QIAGEN, Valencia, CA), rat mAb anti-hemagglutinin (HA) tag (Roche Diagnostics), polyclonal antibody anti-pericentrin (Abcam, Cambridge, MA), and mAb anti-V5 tag (Invitrogen). Secondary antibodies were all affinity-purified and purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Yeast Two-Hybrid Assays

Full-length cDNA clones of K8 and K19 were purchased from American Type Culture Collection and ResGen (Carlsbad, CA), respectively. The full open reading frame (ORF) of K19 was subcloned and used as a bait to screen three premade cDNA expression libraries (from lymphocyte, prostate, and breast in pACT and pACT2) at the Molecular Interaction Facility (Biotechnology Center, University of Wisconsin). Ninety-six wells tested positive via selection on adenine dropout and α -galactosidase (gal) assay. All the prey isolates were validated positives and bait specific in the validation assay. For confirmation of possible interactions with K8, the full K8 ORF was cloned into the pGBKT7 vector (Clontech) between the EcoRI and BamHI sites. The in-frame sequence encoding a hybrid fragment of GCP6 was rescued from positive colonies and used as prey. *Saccharomyces cerevisiae* (AH109 strain) were cultured in YPDA medium and transformed by the lithium acetate-mediated method (Ito *et al.*, 1983). Prey and bait were selected in $-Leu$ or $-Trp$ media, respectively, and tested for α -gal activity for self-activation on 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside (X - α -gal) plates. Two-hybrid interactions were selected in $-Trp$, $-Leu$, $-His$, $-adenine$ media and tested on X - α -gal plates as well.

Short Hairpin RNA (shRNA) Expression

Two synthetic oligonucleotides containing GCP6 sequences, 258:aagatgagact-caacagctgc and 3905:aacacccatgtaccatccct, were used to prepare shRNA-expressing constructs in the pSilencer 3.0 vector under either the H1 or the U6 promoters. The four resulting constructs were sequenced to assess integrity and orientation. For transfection, 0.6 μ g of each purified vector was mixed with 0.6 μ g of the pEGFP vector (2:1 M ratio), and 1.8 μ l of FuGENE (Roche Diagnostics) in 1 ml of DMEM per well. CACO-2 cells at ~80–90% confluence (plated 4 d in advance) growing on Transwell filters, were transfected with this mixture for 48 h. Then, the monolayers were washed with regular culture medium, refed one more time 24 h later, and fixed for immunofluorescence 3 d after transfection.

Purification of HIS-tagged GCP6, HIS-V5-tagged GCP6(1397-1819), lacZ, and Keratins

6xHIS-tagged GCP6 was extracted from UO2S cells growing in 850-cm² roller bottles. The cells were washed in cold phosphate-buffered saline (PBS), and extracted in 5 ml of radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 50 mM Tris-HCl, pH 8, 1 mM Na₃VO₄, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a cocktail of antiproteases containing, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.8 μ M aprotinin, 20 μ M leupeptin, 40 μ M bestatin, 15 μ M pepstatin A, 14 μ M E-64, final concentrations; P8340, Sigma-Aldrich]. LacZ-6xHIS (β -galactosidase) and GCP6(1397-1819)-6xHIS-V5 were expressed in COS cells transfected with the expression control plasmid pcDNA3.1 D/V5-His/lacZ (Invitrogen) or pcDNA3.1 directional TOPO vector, respectively, and extracted as described above. The extract was spun at 9000 $\times$ g for 10 min, and the supernatant was loaded on a Ni²⁺-resin column (ProBond purification system; Invitrogen). The column was extensively washed in phosphate buffer and 50 and 200 mM imidazole, and eluted in 350 mM imidazole according to manufacturer's specifications. The eluates were dialyzed against Cdk1 buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM EGTA, and 0.01% Brij) for phosphorylation experiments, and, in all cases, concentrated by ultrafiltration on Centricon 10 concentrators (Amicon, Beverly, MA). Keratins were isolated from CACO-2 cell cultures by Triton X-100 extraction in 1.5 M KCl, and cycles of solubilization of the pellet in 8 M urea and dialysis against PBS as described by Steinert *et al.* (1982), a preparation that yields highly purified K8, K18, and K19, as determined by two-dimensional gel electrophoresis previously (Salas, 1999). Recombinant purified keratins expressed in *Escherichia coli* were obtained from BioWorld (Dublin, OH).

For phosphorylation with Cdk1, 40 U/ml recombinant Cdk1 kinase and human cyclin B expressed in a baculovirus expression system and activated by cyclin-activating kinase phosphorylation (BIOMOL Research Laboratories, Plymouth Meeting, PA) were added to 200 μ l of concentrated GCP6 in Cdk1 buffer. In radiolabeled phosphate, 250 μ Ci/ml (3000 Ci/mmol) [γ -³²P]ATP was added to all the samples in the presence or absence (negative control) of 10 U/ml Cdk1–cyclin B. For cold ATP phosphorylation, the kinase was added to all samples, and ATP omitted in the negative controls.

Extraction of HA- or V5-tagged GCP6 Fragments

CACO-2 TET-ON cells expressing either fragment 1 or 2 of C-terminal region of GCP6 were seeded in six-well plates and treated either with 1 μ g/ml doxycycline (dox) containing media to induce gene expression, or tetracycline-free media as negative controls. Alternatively, V5-His-tagged fragments were transiently expressed in COS-1 cells. For immunoblot analysis, the cells were extracted in extraction buffer (10 mM Tris-Cl, pH 7.8, 1% Triton X-100, 5 mM EGTA, 2 mM MgCl₂ and the cocktail of antiproteases described above), incubated at room temperature for 10 min, and spun down at 14,000 rpm for another 10 min. The supernatants were collected. The pellets were resuspended in Tris-HCl, pH 7.8, and sonicated for 10 s and then spun down at 14,000 rpm. The pellets were then solubilized 1% SDS, Tris-HCl, pH 6.8. Equal amounts of protein from the Triton X-100-soluble (supernatants of the first centrifugation) and insoluble (pellets) fractions were incubated in SDS sample buffer for 2 h and HA-tagged protein content was analyzed by SDS-PAGE. For blot overlays, 6xHis-V5-tagged GCP6 fragments were expressed in COS-1 cells by using the pcDNA 3.1 vector, extracted in RIPA buffer and purified on Ni²⁺ columns under nondenaturing conditions as described above.

Immunoblot, Immunoprecipitation, "Far Western" Blot (Blot Overlay), Dot Blot, and Pull-Down

SDS-PAGE and immunoblot were performed as described previously (Salas, 1999) as well as 2D-electrophoresis and immunoblot (Figueroa *et al.*, 2002). For blot overlay, a mixture of purified CACO-2 keratins (20 μ g/lane; K8, K18, and K19), recombinant bacterially expressed keratins, or purified control proteins were separated in a 10% acrylamide gel, and blotted onto nitrocellulose along with colored molecular weight markers (Pierce Chemical, Rockford, IL). The blots were saturated with 1% globulin-free bovine serum albumin (BSA) (Sigma-Aldrich) in 0.1% Tween 20 in PBS. HIS-tagged GCP6, eluted from the Ni²⁺ column, desalted, and concentrated by ultrafiltration was phosphorylated with 10 U/ml Cdk1–cyclin B (recombinant, active; BIOMOL Research Laboratories) in the presence or absence (negative control) of 1 mM cold ATP. Then, phosphorylated or nonphosphorylated GCP6 were added onto the saturated blots and incubated overnight at room temperature. The overlay was extensively washed, and the membranes were processed as for a regular immunoblot with anti-HIS tag or anti-V5 antibodies. A similar procedure was followed for dot blot analysis, except that a suspension of purified polymerized native CACO-2 intermediate filaments was loaded onto the nitrocellulose sheet using a vacuum-driven dot-blot apparatus (5 μ g/dot). For pull-down experiments, the same type of preparation was used to covalently bind intermediate filaments to CNBr-activated Sepharose beads (Pharmacia, Piscataway, NJ). The beads were blocked with 1% casein, incubated with purified GCP6, and extensively washed. The bound protein was extracted in sample buffer. GCP6 ligand biotinylation was performed with sulfo-N-hydroxysuccinimide-biotin (Pierce Chemical) according to the manufacturer's protocol.

In Vivo MT Nucleation Assay

CACO-2 cells were grown on glass coverslips or Transwell inserts for 12 d. The cells were then incubated with the same medium supplemented with 33 μ M nocodazole in the apical chamber only for 5 h. During that period, the medium was replaced by the same medium at 4°C, and the cells were kept in the refrigerator for 15 min. Then, the cultures were moved back to the incubator and allowed to slowly equilibrate back to 37°C. Some cultures were fixed at the end of the 5-h period, whereas others were rapidly washed three times with prewarmed (37°C) medium without nocodazole and further incubated there for 12 more min before fixation. For MT fluorescence, the cells were fixed in prewarmed glutaraldehyde/formaldehyde as described below.

Transgenic Mice, Immunofluorescence, and Confocal Microscopy

The K8-null transgenic mice in the FVB/n background were a generous gift of Hélène Baribault (Baribault *et al.*, 1994) and the procedures for frozen sectioning, blocking endogenous mouse IgG as well as the intestine phenotype were described previously (Ameen *et al.*, 2001). Procedures for immunofluorescence and confocal microscopy have been described previously (Salas, 1999). For MT morphology, the cells were continuously kept at 37°C and fixed in 0.1% glutaraldehyde/3% formaldehyde (freshly prepared from paraformaldehyde). In this case, GFP was visualized by its intrinsic fluorescence. For HIS-tag, γ -tubulin, and GCP6 fluorescence, the cells were fixed in 100% methanol. In those cases, GFP fluorescence was undetectable, and GFP was colocalized by immunofluorescence by using an anti-GFP antibody. Confocal microscopy was performed in a Zeiss LSM 510 microscope keeping the slit closed to reach 0.7–1 Airy units. Confocal stacks were normally collected at 0.4- μ m intervals. The images were acquired under immersion 63 $\times$ (1.4 numerical aperture) objectives at room temperature. Mounting media was 10% polyvinyl alcohol, 30% glycerol in PBS. In some cases, when the entire cell content was to be shown, projections of the complete confocal stack were obtained using the Zeiss image browser. Routinely, the images were converted to TIFF format, and noise was reduced by averaging (2-pixel radius). For three-dimensional (3D) reconstructions in the XY plane, subsets of the stack making up a depth of $\sim$ 2 μ m around the desired region (apical, transnuclear, or basal) were reconstructed using SlideBook 4.2 software (Intelligent Imaging Innovations, Denver, CO) after near-neighbors deconvolution. For XZ 3D reconstructions, the entire stack was cropped in 7-voxel-thick volumes, reconstructed using the same method, and rotated 90°.

RESULTS

Full-Length GCP6 Binds to Keratin Monomers and Fully Assembled Keratin Intermediate Filaments

To identify molecular partners of keratins potentially involved in the organization of the apical domain, a yeast two-hybrid screen of three different libraries was conducted using a full-length K19 bait. In addition to numerous expected positives, such as type II keratins, one of the prey isolates encoded an in-frame C-terminal fragment (1397-1819) of the γ -tubulin complex component GCP6 (AF272887.1), which, interestingly, is a 190-kDa protein in human. That is the same electrophoretic mobility as the Cdk1 target that we found involved in the attachment of MTOCs to IFs in CACO-2 cells (Figueroa *et al.*, 2002).

To validate the yeast two-hybrid assays, we first isolated full-length 6x HIS-tagged GCP6 from stable transfectants of U2OS (human osteosarcoma) cells constitutively expressing it under the CMV promoter (Murphy *et al.*, 2001). These cells showed colocalization of the HIS-tagged GCP6 with centrosomes (Figure 1A, arrows), indicating that the recombinant protein is appropriately incorporated into MTOCs. Extracts of these cells were purified in a Ni²⁺-resin column. The eluates contained only three bands recognized by an anti-HIS tag antibody in immunoblot (Figure 1B, lane 2; 190, 155, and 45 kDa). The 190-kDa band occurred in the 350 mM imidazole elution but not in the 200 mM imidazole wash (lane 1). The smaller His-tagged peptides were interpreted as degradation products of the 190-kDa band. The Ni²⁺ column eluate was divided in equal aliquots and subjected to phosphorylation with [γ -³²P]ATP in the presence or absence of Cdk1–cyclin B (Figure 1B, 3 and 4, respectively). The 190- and 155-kDa bands were phosphorylated (Figure

Figure 1. GCP6 binds directly to keratins *in vitro*. (A) 6xHIS-tagged GCP6 expressed as a stable transfectant in U2OS (human osteosarcoma) cells was detected by anti-HIS tag antibody in centrosomes identified by γ -tubulin immunofluorescence (arrows). Bar, 10 μ m. (B) The same HIS-tagged GCP6 expressed in U2OS cells was purified by Ni^{2+} column, washed at 200 mM (lane 1), eluted at 350 mM imidazole (lane 2), and analyzed by immunoblot with an anti-HIS tag antibody. The same eluate as in lane 2 was subjected to phosphorylation with 250 $\mu\text{Ci}/\text{ml}$ [$\gamma\text{-}^{32}\text{P}$]ATP in the presence (+, lane 3) or absence (–, lane 4) of 10 U/ml Cdk1/cyclin B and analyzed by SDS-PAGE and PhosphorImager. The HIS-tagged protein eluted from the Ni^{2+} column was also analyzed by immunoblot with anti-GCP6 antibody (lane 5). The same antibody preincubated (+, lane 6) or not (–, lane 7) with the corresponding antigen peptide was used on blots from total extract of CACO-2 cells (50 μg total protein/lane). The arrow points at the apparent molecular weight of 190 kDa in all blots. Standards, 200, 128, 80, and 40 kDa. (C) The same purified full-length HIS-tagged GCP6 was phosphorylated with 10 U/ml Cdk1 but in the presence (+, lane 2) or absence (–, lane 1) of 1 mM cold ATP. These preparations of full-length GCP6 were used to overlay nitrocellulose sheets previously blotted with a mixture of purified CACO-2 keratins (K8, K19, and K18, 20 μg of total protein/lane) and blocked with BSA. After washes, the sheets were developed with an anti-HIS tag Ab (lanes 1 and 2). Then, the

1B, lane 3), not surprisingly, because GCP6 has a single Cdk1 consensus phosphorylation site in S1397 and multiple cyclin binding sites. This site is expected to be included in a His-tagged C-terminal 155-kDa fragment but not in a 45-kDa fragment. Furthermore, in parallel immunoblots, the 190- and 155-kDa bands were recognized by a polyclonal antibody (Figure 1B, lane 5) raised against a synthetic peptide from the N-terminal region of GCP6, which is conserved among mammalian homologues but not shared by other members of the GCP family. Recognition of the second band was unexpected because the 155-kDa fragment that contains the His tag should not include the N-terminal region of the full-length molecule. However, a closer analysis of GCP6 amino acid sequence showed that the peptide used to raise the antibody is repeated with 81% similarity starting at aa 700, which is, therefore, likely to act as a second epitope for the antibody. To further test the specificity of the antibody, it was used in immunoblot of CACO-2 cell total extracts obtained by rapidly boiling the cells in SDS sample buffer to avoid protein degradation. One of the two identical blots (Figure 1B, lane 6) was treated with the antibody supplemented with its synthetic epitope peptide and the other with antibody alone (lane 7). A nonspecific band in the front serves as loading control. In this case, only the 190-kDa band was specifically recognized (Figure 1B, arrow).

The full-length His-tagged GCP6 ligand characterized above was used in far Western analyses on highly purified preparations of IFs from CACO-2 cells separated by SDS-PAGE and blotted onto nitrocellulose, loading identical amounts of protein in all lanes. A parallel sample blot stained with Ponceau S red for total protein is shown in Figure 1C, lane 9. The blots were overlaid with full-length His-tagged GCP6, purified, and phosphorylated or not by Cdk1–cyclin B as described above (Figure 1C, lanes 2 and 1, respectively). Then, the blots were assayed with anti-HIS-tag Ab and the label was found mostly on K8. This selectivity cannot be explained by differences in the amount of keratins loaded (lane 9). More importantly, Cdk1–cyclin B phosphorylation almost abolished binding of HIS-tag GCP6 to isolated keratins (Figure 1C, lane 2). The specificity of keratins was additionally controlled by stripping off the same membranes and reprobing them sequentially with antibodies against K8, K19, and K18 (Figure 1C, lanes 3–8). The purity of the keratin preparation was also assessed by 2D-electrophoresis, and has been shown in a previous publication (Salas, 1999). Negative controls were performed on blots of various irrelevant proteins, including myosin, tropomyosin, and fumarase as well as overlaying keratin blots with a

same sheets were sequentially stripped off and reprobed with anti-K8 (lanes 3 and 4), K19 (lanes 5 and 6), and K18 (lanes 7 and 8) monoclonal antibodies as load controls. One aliquot of the same preparation (10 μg) was run, blotted on a separate lane (lane 9), and stained with Ponceau S red dye. Standards, 80, 40, and 31 kDa. (D) A suspension of highly purified, native intermediate filaments from CACO-2 cells (top) or BSA as control (bottom) were loaded onto nitrocellulose sheets in a dot blot apparatus (10 $\mu\text{g}/\text{dot}$). After extensive washes and blocking protein binding with BSA, the dots were overlaid with the purified HIS-tagged GCP6, phosphorylated (+) or not (–) with Cdk1 as described above. The membranes were then washed and developed for chemiluminescence with the anti-HIS tag antibody. (E) For pull-down, highly purified native polymerized IFs (K8–K18 and K8–K19) were covalently coupled to CNBr-activated Sepharose and incubated in the presence of the same purified Cdk1-phosphorylated (+) or not (–) GCP6. After washes, the beads were eluted in sample buffer and analyzed by immunoblot with anti-HIS tag antibody.

Figure 2. The C-terminal fragment GCP6(1397-1819) binds directly to keratins and can be competed by smaller fragments. The diagram of GCP6 was modified from Murphy *et al.* (2001), including the conserved GCP domains I–V to show the alignment of the GCP6(1397-1819) peptide. Potential coiled-coil forming domains were predicted using software at the European Molecular Biology network (http://www.ch.embnet.org/software/COILS_form.htm). (A) The V5-6xHis-tagged GCP6(1397-1819) fragment was expressed in COS cells, where it could be detected with anti V5-tag antibody in the total extract (input lane 1) and in the purified eluates of Ni^{2+} columns (input lane 2). Blots of highly purified keratins (lanes 3–5), tropomyosin or myosin (lanes 6 and 7 and 8 and 9 respectively) were overlaid with V5-6xHis tagged lacZ (lane 3) or V5-6xHis GCP6(1397-1819) purified on Ni^{2+} columns (lanes 4, 6, and 8). After washes, the nitrocellulose strips were developed with anti-V5 and reprobed with anti-pan-keratin antibodies (lane 5). Blots with tropomyosin or myosin (arrows) were stained with Ponceau red to show total protein (P, lanes 7 and 9, respectively) before the overlay and immunoblot to show protein load. Standards 210, 86, 40, and 31 kDa. (B) GCP6(1397-1819) is a partner of keratin 8 in yeast two-hybrid assays. *S. cerevisiae* were transformed with pGBKT7-K8 and pACT-GCP6(1397-1819) in high stringency media, or with either vector separately, in the selection media indicated below each panel, containing X- α -gal in all cases. Also, pGBKT7-lamin C and pACT-GCP6(1397-1819) were cotransfected as a control for non-specific interactions of GCP6(1397-1819), in moderately stringent media (–Leu –Trp). (C) V5-tagged GCP6(1397-1513) (f1-V5), GCP6(1510-1819) (f2-V5) or the entire C-terminal domain GCP6(1397-1819) were used as ligands in blot overlays on bacterially expressed purified K8, K18, and K19 loaded in different lanes (2 $\mu\text{g}/\text{lane}$) and shown by Ponceau S red staining as protein load. After the overlays, binding of ligands was shown by V5 reactivity. (D) The same type of experiment described in C was repeated using biotinylated GCP6(1397-1819) as ligand, supplemented (Suppl.) or not (–) with nonbiotinylated f1 or f2. After the overlays, binding of ligands was shown by streptavidin (SAvidin) reactivity.

6xHis-tagged β -galactosidase. All those controls showed negative results indicating that GCP6 binding to keratins is specific (data not shown; similar controls are shown in Figure 2A). Because the keratins blotted onto nitrocellulose for far Western analysis are likely denatured, the question remained of whether the binding of HIS-tag GCP6 to keratins is also observed on native, fully polymerized IFs, where steric hindrances may prevent simple protein–protein interactions. To test this, we repeated the same binding assay but transferring purified native polymerized IFs onto nitrocellulose in a dot blot apparatus. Binding of His-tagged GCP6 to highly purified native polymerized intermediate filaments was substantially above background (parallel membrane blotted with BSA) and also inhibited by Cdk1–cyclin B-

mediated phosphorylation (Figure 1D). To independently confirm this result, pull-down experiments were also conducted. Highly purified native IFs were covalently coupled to Sepharose beads. Only GCP6 not phosphorylated by Cdk1–cyclin B was pulled down by IFs (Figure 1E, –).

The original keratin 19 partner in the yeast two-hybrid screen was the in-frame C-terminal fragment of GCP6(1397-1819). Therefore, we wanted to verify that this fragment bears the same keratin-binding features as the full-length GCP6. To that end, we expressed a 6xHis- and V5-tagged construct of the fragment in COS cells and purified a protein of the expected size in Ni^{2+} columns as a single band (Figure 2A, input, lane 2). This GCP6(1397-1819) fragment was overlaid on highly purified keratin blots and developed, this

time, with an anti-V5 epitope antibody. It was found to bind to K8 and K19, but not to K18 (Figure 2A, lane 4). A 6x-His- and V5-tagged lacZ isolated in the same way, failed to bind to keratins. Likewise, V5-GCP6(1397-1819) failed to bind to irrelevant proteins (tropomyosin and myosin; Figure 2A, lanes 6–9) that contain large coiled-coil domains, thus confirming the specificity of the binding to keratins. Because the Cdk1 consensus phosphorylation site is incomplete, we did not attempt to phosphorylate the GCP6(1397-1819) fragment.

The results in Figures 1C and 2A suggested that GCP6 interacts directly with K8 better than with K19, the original bait used in the yeast two-hybrid screen. To independently corroborate this result, we conducted another yeast two-hybrid assay but used a full-length K8 bait and the fragment GCP6(1397-1819) as prey. Neither K8 nor GCP6(1397-1819) self-activated in low-stringency media, nor did GCP6(1397-1819) interact with a lamin C control (Figure 2B, white colonies), but blue-colored two-hybrid K8+GCP6(1397-1819) colonies grew in high-stringency media showing activation of α -gal (Figure 2B, far left panel). To further verify the specificity of binding for keratins, purified K8, K18, and K19 individually expressed in bacteria were used for the overlays (Figure 2C). In addition, smaller fragments of the C-terminal domain, GCP6(1397-1513) and GCP6(1510-1819) (hereafter referred to as f1 and f2, respectively), tagged with V5 were also used as ligands. This experiment confirmed the selectivity of the GCP6(1397-1819) for K8 and K19, which was also shown by f2. In contrast, f1 bound to K8 and only faintly to K19. However, both f1 and f2 fragments were able to compete the binding of GCP6(1397-1819) to keratin (Figure 2D). Altogether, these results confirmed direct protein–protein interactions between the C-terminal domain of GCP6 and K8 or K19 monomers.

Apical Localization of GCP6 In Vivo Depends on the Integrity of IFs

To study endogenous GCP6 in polarized epithelial cells, we used the polyclonal antibody raised against a synthetic peptide in GCP6 that recognizes the 190-kDa protein (Figure 1B, lane 7). In confluent CACO-2 cell monolayers, this antibody showed strong reactivity for centrosomes and also in multiple smaller puncta (Figure 3A). This signal was blocked by free peptide (Figure 3B, corresponding phase contrast, C). Because the antigen peptide is conserved in mouse GCP6, we used the antibody in murine small intestine villus sections obtained from K8-null mice in the FVB/n background (Baribault *et al.*, 1994) where IFs are knocked out (Ameen *et al.*, 2001), and from heterozygous littermates. Although K7, a redundant type II keratin that can replace K8, is not expressed in human (Moll *et al.*, 1982; Cheng and Wang, 2004) or Balb-C mouse small intestine (Ramaekers *et al.*, 1987), we (Ameen *et al.*, 2001) and Baribault *et al.* (1994) found that the FVB/n mice express K7 only in the crypts, where the K8-null epithelium still presents IFs. As the cells move along the villus, they lose their IFs, specially the apical layer (Ameen *et al.*, 2001). To assess whether the distribution of MTOC depends on IFs, we colocalized two different MTOC markers (γ -tubulin and GCP6) with IF proteins in the villus of control heterozygous littermates (Figure 3, D, E, H, and I, green channel), and in K8-null animals, focusing at the transitions between cells that still display apical IFs and cells that lack apical IFs (Figure 3, F, G, J, and K, white arrows). In the crypts, MTOC signal was found in apical centrosomes as well as in a continuous apical layer (data not shown). In the villus, centrosomes could not be identified, in agreement with published data (Komarova and Vorobjev, 1993). Instead, a continuous apical layer of γ -tubulin and GCP6

Figure 3. Apical GCP6 localization depends on IFs. (A) The antibody against GCP6 was tested by immunofluorescence in methanol-fixed CACO-2 cells. (B) The same antibody was mixed with the synthetic peptide used as immunogen before staining the monolayer. C is the corresponding phase contrast of the field shown in B. Note that at least one cell is in mitosis. (D–K) Frozen sections of small intestine villi from K8 null (–/–) mice or heterozygous littermates (+/+), treated for immunofluorescence with anti-pan-keratin antibody (D–G, red channel), anti- γ -tubulin antibody (D–G, green channel), or anti-K19 mAb (H–K, red channel) and anti-GCP6 antibody (H–K, green channel). All sections were counterstained with DAPI (blue). White arrows show the transition of the apical IF layer in K8 null mice. Black arrow on top of D–K shows the orientation of the villus and, therefore, the direction of enterocyte migration. Bars, 10 μ m.

signal was found (Figure 3, E and I). This layer was found to colocalize with the apical layer of IFs (Figure 3, D–G). Likewise, GCP6 was found to colocalize with K19 (Figure 3, H–K). More importantly, in K8 –/– villus enterocytes both MTOC apical signals disappeared exactly in the same transition regions where the apical layer of IFs became discontinuous (Figure 3, G and K, white arrowheads).

Down-Regulation of GCP6 with shRNA Transcribed under the H1 Promoter Results in Scattered γ -Tubulin Distribution in the Cytoplasm, Altered MT Architecture, and Ectopic MT Nucleating Activity

To down-regulate GCP6 in CACO-2 cells, two synthetic oligonucleotides (hereafter referred to as 1 and 2, as de-

Figure 4. Knockdown of GCP6 with shRNA redistributes γ -tubulin. (A) Projections (whole cell volume) of confocal optical stacks of CACO-2 cells cotransfected with H1-1 pSilencer 3.0 (shRNA targeting GCP6) and pEGFP-N1 show immunofluorescence with anti-GCP6 antibody (red) or GFP fluorescence (green). (B) XZ (apical side up) 3D 7-voxel-thick reconstructions of confocal optical stacks of CACO-2 cells grown on Transwell filters, cotransfected with pSilencer-H1-1 (H1-1) or empty pSilencer-H1, and pEGFP. Blue, γ -tubulin; green, GFP; and red, K8 (Troma I). White arrows point at transduced cells. Bars, 10 μ m.

scribed in *Materials and Methods*), directed against sequences in the GCP6 mRNA were cloned in pSilencer to transcribe shRNAs under either H1 or U6 polymerase III promoters. The resulting constructs, termed H1-1, H1-2, U6-1, and U6-2, were cotransfected in subconfluent CACO-2 cells along with a pEGFP vector as a transfection reporter. The percentage of transfected cells ranged from 5 to 10% and was, therefore, not sufficient to conduct biochemical assays. At the immunofluorescence level, however, cells showing EGFP expression also showed reduction of the GCP6 signal for the construct H1-1 (Figure 4A) and as well as with the other constructs (data not shown). It must be noted that because differentiated CACO-2 cells cannot be transfected at all, these experiments had to be performed in cells 6 d after seeding, in a stage where polarization is only incipient (Pinto *et al.*, 1983).

Then, we wanted to analyze how GCP6 knock-down affects the distribution of MTOCs and MTs in CACO-2 cells. As shown previously (Salas, 1999), γ -tubulin signal was cortical and codistributed with IFs (Figure 4B, empty vector,

blue and red channels). Cells transfected with H1-1 shRNA to knockdown GCP6 showed γ -tubulin signal (blue channel) distributed throughout the cytoplasm (Figure 4B, H1-1, arrows). These experiments, and similar results observed for H1-2, and U6-2 shRNAs were blindly scored as percentage of transfected cells in three experiments by using yes/no criteria (Table 1). Statistically significant differences were found between empty vector and cells transfected with both shRNAs 1 and 2 and scored for γ -tubulin signal disperse in the cytoplasm (Table 1) like in the cell shown in Figure 4B, H1-1.

To determine whether GCP6 knockdown affects the steady-state distribution of MTs, we repeated the same experiments but used an antibody that labels microtubules (Figure 5, red channel). The images are shown as XY 3D reconstructions from confocal stacks of the apical-most 2 μ m of the monolayer (Figure 5, A and B, G and H), the basal-most 2 μ m (Figure 5, C and D, I and J) as well as XZ reconstructions of the entire stack (Figure 5, E and F, K and L). In nontransfected cells or in cells transfected with an empty pSilencer H1 vector, MTs were highly concentrated in the apical-most one third of the cytoplasm showing oblique orientation (Figure 5A). Isolated MTs extended in the apico-basal orientation below the apical layer (Figure 5E, arrowheads). At the basal domain MT were rarely observed and did not correlate with transfection (Figure 5D). However, in cells transfected with pSilencer-H1-1, MTs were observed at the transnuclear plane, and, in some cells up to the basal domain (Figure 5, I and K, arrows). Blind scores of transfected cells from three experiments confirmed that similar images were obtained targeting GCP6 with H1-1, H1-2, and U6-1 shRNAs in statistically significant proportions (Table 1).

To verify whether the cytoplasmic (noncortical) γ -tubulin signal (Figure 4B) represents potentially active MTOCs detached from the apical domain, the same experiment was performed preincubating the cells for 5 h in nocodazole. After that treatment, no MTs were observed at the transnuclear confocal planes of any cell (Figure 6, A and C, 5-h nocodazole), and only a few MTs were left in the apical cytoplasm (Figure 6C, more examples in Figure 9). However, washing the drug for 11 min resulted in the appearance of short recently nucleated MTs and several nucleation foci at the nuclear level in cells transfected with pSilencer-H1-1 (Figure 6, E and G, arrows) but not in any other cells. This result supports the notion that targeting GCP6 with specific shRNAs results in an abnormal subapical localization of a fraction of the MT-nucleating activity.

Table 1. Percentage of cells positively cotransfected with GFP and anti-GCP6 shRNAs blindly scored for effects on γ -tubulin and MT distribution

Criteria	Promoter-shRNA						n
	H1	H1-1	H1-2	U6	U6-1	U6-2	
Disperse γ -tubulin	2.2 $\pm$ 3.1	72 $\pm$ 10 ^a	31 $\pm$ 23 ^a	3.3 $\pm$ 3.3	54 $\pm$ 45 ^a	60 $\pm$ 35	160
MTs at transnuclear level	0.0 $\pm$ 0.0	45 $\pm$ 1.3	46 $\pm$ 12	0.0 $\pm$ 0.0	48 $\pm$ 1.6	19 $\pm$ 18	101

H1 and U6 alone are empty vectors; -1 or -2 indicate the anti-GCP6 shRNA sequences. Average percentage of cells scoring positive to the morphological criterion with respect to the total EGFP-transfected cells $\pm$ SD. Data are averages of three independent experiments, counting n total cells.

^a Statistically significant differences with the averages for cells transfected with the corresponding empty vector (*t* test, *p* < 0.05).

Figure 5. shRNA knockdown of GCP6 alters MT architecture in CACO-2 cells. CACO-2 cells cotransfected with empty pSilencer-H1 and pEGFP (A–F) or with pSilencer-H1-1 and pEGFP (G–L) 4 d after plating were fixed 3 d later and processed with anti-tubulin (red) antibody, and they show GFP fluorescence in the green channel (B, D, F, H, J, and L). Stacks of confocal images were used to generate XY 2- μ m-thick 3D reconstructions of the apical or basal domains, or XZ reconstructions. Each pair of apical and basal XY 3D reconstructions corresponds to the same field. White arrows point at microtubules in the basal domain. Bars, 10 μ m.

Expression of GCP6(1397-1513) but Not of GCP6 (1510-1819) Affects the Localization of γ -Tubulin, the Steady-State Distribution of MTs, and the Position of MT Nucleation Sites

The GCP6 knockdown results shown above do not rule out the possibility that this protein may have a structural function in the MTOC and that lose fragments of centrosomes or other noncentrosomal MTOCs resulting from the knockdown still have MT-nucleating activity. To test this possibility, we attempted to interfere with the keratin binding function alone, without affecting other functions of GCP6. We reasoned that overexpression of the keratin binding domain alone would likely compete with the endogenous full-size GCP6 for its keratin partners. The results shown in Figure 2 suggest that the keratin binding domain of GCP6 resides within the C-terminal domain. Preliminary experiments expressing the entire GCP6(1397-1819) molecule, however, showed no noticeable effects on the distribution of MTOCs or microtubules. Therefore, we expressed two smaller fragments, f1 and f2, that can compete GCP6 keratin binding in vitro (Figure 2D) under the TET-inducible promoter. For these experiments the fragments were HA tagged.

The expression of each fragment was first assessed by immunoblot by using an anti-HA tag antibody in Triton X (TX)-100-insoluble and -soluble fractions of cells incubated

Figure 6. shRNA knockdown of GCP6 alters the distribution of microtubule-nucleating activity CACO-2 cells. CACO-2 cells were transfected as described in Figure 5. Three days later, and 5 h before fixation, the cells were incubated in 33 μ M nocodazole, and, during the same period, they also were subjected to a 15-min period at 4°C to achieve a more extensive depolymerization of microtubules. At the end of the 5-h treatment in nocodazole, the cells were back at 37°C and were either fixed (A–D) or washed three times in pre-warmed medium and incubated a 37°C for 11 min before fixation (E–H). All the cells were processed as described in Figure 5, and confocal stacks were used to generate XY 3D reconstructions of the transnuclear region of the cell (A, B, E, and F) or XZ reconstructions (C, D, G, and H). White arrows point at newly nucleated microtubules at the transnuclear region of the cytoplasm. Bar, 10 μ m.

with or without dox for 10 d. Peptides of the expected molecular weight were found in cells in dox+ (Figure 7A, arrows). Fragment 1 occurred exclusively in the TX-100-insoluble pellets, whereas f2 occurred in the supernatants and the pellets (Figure 7A). In addition, cells expressing f2 were leaky for the TET regulation. Therefore, these cells were used in the following experiments as constitutive expressors.

To determine the distribution of the GCP6 fragments 1 and 2 within the cells, the same experiment was performed processing filter-grown CACO-2 sublines for immunofluorescence against the HA-tag. In f1-expressing cells, the HA signal (Figure 7B, green) was restricted to the apical domain and the upper part of the lateral domain, overlapping the distribution of IFs as identified by K8 signal (Figure 7B, red). Conversely, in CACO-2 cells expressing f2 (Figure 7B, bottom) the HA signal was widely distributed in the cytoplasm and excluded from the nuclei (Figure 7B, green). Neither fragment showed images compatible with centrosomes. The lack of codistribution with pericentriolar signal was also verified independently (data not shown). Together, the biochemical and morphological data suggest that f1 attaches to the TX-100-insoluble cytoskeleton and codistributes with IFs, whereas f2 is also cytosolic in this CACO-2 clone.

In CACO-2 cells and in tissues in vivo such as kidney tubules, a fraction of γ -tubulin signal is TX-100 insoluble (Figuerola *et al.*, 2002; Wald *et al.*, 2003). To test the possibility

Figure 7. Expression of f1 fragment of GCP6 (diagram on the top right corner) affects γ -tubulin distribution and MT architecture. (A) CACO-2 cells expressing GCP6(1397-1513) (f1) or GCP6(1510-1819) (f2) under a TET-inducible promoter were incubated in DMEM for 2 d and then kept in the same medium in the presence of 1 μ g/ml dox (dox+) or without dox (dox-) for 10 more days. The cells were extracted in TX-100, and equal amounts of total protein from the soluble (supernatants) or insoluble (pellets) fractions were separated in SDS-PAGE, blotted, and identified with an anti-HA or anti-actin (load control) antibodies and chemiluminescence. The apparent mass of the standards is expressed in kilodaltons. (B) The same CACO-2 sublines were cultured on Transwell filters in the presence (dox+) or absence (dox-) of dox as described above. At 12 d after seeding, the cells were fixed and processed with anti-HA tag antibody (green channel) and anti-K8 antibody (red channel) and analyzed by confocal microscopy (XZ sections, apical side up). The images on the right hand side column represent the merge of both channels. Bars, 10 μ m. (C) CACO-2 parental cells (P) and the CACO-2 subline expressing f1 incubated with (+) or without dox (-) as described above were extracted in TX-100 after 12 d of culture. Identical amounts of protein from the TX-100-insoluble pellets or supernatants were separated by SDS-PAGE, blotted, and developed with anti-actin antibody (as load control) and anti- γ -tubulin antibody by chemiluminescence. (D) The CACO-2 cells expressing f1 under the TET-inducible promoter were incubated in the presence (dox+) or absence (dox-) of dox for 12 d, fixed, and processed for immunofluorescence for γ -tubulin (blue) or K8 (red). The images are presented as XZ sections with the apical side up. Bars, 5 μ m. E. CACO-2 cells expressing f1 or f2 under the TET-responsive promoter were cultured on Transwells in the presence (dox+) or absence (dox-) of dox as described above and processed for immunofluorescence for MTs (green) and K8 (red). The XY (top) or XZ (bottom) images show both channels merged. Bar, 10 μ m.

that the expression of GCP6 C-terminal fragments abrogates the attachment of γ -tubulin-containing structures to the cytoskeleton, we analyzed the TX-100-insoluble pellet of parental CACO-2 cells (Figure 7C, P) and f1-expressing CACO-2 cells (Figure 7C, - and +dox) after a brief low-speed centrifugation that would not pellet isolated γ -TurCs. γ -Tubulin was present in the cytoskeletal preparation from parental CACO-2 cells (P) and from f1-CACO-2 cells in the absence of dox (-). However, the expression of f1 resulted in a decrease of TX-100-insoluble γ -tubulin (Figure 7C, pellets dox+). The results were analyzed by densitometry and after normalization by the actin load control, the reduction was found to be in the range of 41–58% in three independent experiments. The analysis of the corresponding supernatants showed a parallel increase of similar magnitude in the Triton X-100 soluble γ -tubulin (Figure 7C, supernatants +), suggesting that γ -tubulin-containing structures had been displaced from their attachments to the cytoskeleton. The fraction of TX-100 insoluble γ -tubulin in f2-expressing cells, in contrast, was identical to that in the parental CACO-2 cells (data not shown). To analyze the effect of the expression of f1 on the distribution of γ -tubulin, f1-expressing CACO-2

cells were incubated in the presence or absence of dox and analyzed by immunofluorescence for γ -tubulin (Figure 7D, blue) and K8 (Figure 7D, red). In the absence of dox, the γ -tubulin distribution (Figure 7D, dox-) was similar to that of the parental CACO-2 cells (Figure 4B), and overlapping the distribution of IFs, that is concentrated in the apical domain, and extending to the upper half of the lateral domain (Figure 7D, red). The centrosomes (Figure 7D, dox-, arrow) were apical with a few exceptions (Salas, 1999). The expression of f1 resulted in three phenotypes deviating from the control pattern: 1) 21% of the centrosomes were separated from the cortical IF apicolateral layer (Figure 7D, dox+, arrow, and Table 2) as opposed to 3.6% (in many cases mitotic cells) in the same f1 CACO-2 subline in the absence of dox (Table 2); 2) 43% of the cells showed decreased or absent γ -tubulin in the apical domain (Figure 7D, arrowheads, and Table 2); and 3) 49% of the cells showed an increase in cytosolic and lateral γ -tubulin signal (Figure 7D, bottom, and Table 2). None of these phenotypes were observed in f2-expressing cells (Table 2).

Next, we analyzed the consequences of f1 and f2 expression on the steady-state architecture of MTs by immunoflu-

Table 2. Phenotype of CACO-2 stable transfectants expressing C-terminal fragments of GCP6

Criteria	f2 + dox cells	f1 - dox cells	f1 + dox cells
Disperse γ -tubulin (% of cells)	3.4 $\pm$ 3.4 (91)	3.0 $\pm$ 2.5 (183)	49.5 $\pm$ 22 (208) ^{a,b}
Decreased apical γ -tubulin (% of cells)	3.6 $\pm$ 3.7 (91)	4.5 $\pm$ 2.3 (183)	43.9 $\pm$ 30 (208) ^{a,b}
MTs at transnuclear level (% of cells)	0.4 $\pm$ 1.1 (256)	0.0 $\pm$ 0.0 (160)	29.2 $\pm$ 23 (242) ^{a,b}
Decreased apical MTs (% of cells)	5.3 $\pm$ 6.6 (256)	4.9 $\pm$ 10 (160)	19.0 $\pm$ 17 (242)
Centrosomes detached from cortical IFs (as % of total centrosomes)	3.6 $\pm$ 3.7 (47)	3.6 $\pm$ 3.3 (122)	21.8 $\pm$ 14.7 (157) ^{a,b}

Effects are blindly scored for γ -tubulin and MT distribution and centrosome localization. The data are presented as average $\pm$ SD from four independent experiments (total number of cells counted). The criteria were as follows: disperse γ -tubulin were cells with γ -tubulin signal away from the apicolateral cortical distribution as in Figure 4B, bottom, arrow. Decreased apical γ -tubulin was considered when the average pixel values were <50% of the values in neighboring positive cells (e.g., Figure 7D, arrowheads). MTs at transnuclear level were evaluated in XY confocal images at the lowest extent of the lateral K8 signal, as in Figure 7E, arrows. Decreased apical MTs were evaluated in apical XY confocal images as cells with average pixel values for α -tubulin <50% of the average of neighboring positive cells. Detached centrosomes were verified both in XZ and XY planes to verify that no contact with K8 signal could be found in any plane and expressed as average percent of detached centrosomes $\pm$ SD (total number of centrosomes counted).

^a Statistically significant differences with the averages in the first column.

^b Statistically significant differences with the averages in the second column (*t* test, *p* < 0.05, *n* = 6).

orescence for tubulin (Figure 7E, green) and K8 (Figure 7E, red). In XY sections of f1 cells in the absence of dox, MTs occurred only at the apical level (Figure 7E, green, top). This pattern was identical to the parental CACO-2 cell line (Figure 5, A–E) and f2-expressing cells (Figure 7E, XZ, f2 dox+). When the cells were expressing f1, however, 29% of the cells showed MTs at the transnuclear level in addition to the apical distribution (Figure 7E, arrows, and Table 2). Cells expressing f2 did not show any noticeable change in the architecture of MTs (Figure 7E, XZ). In summary, although both f1 and f2 can compete GCP6 binding to keratins *in vitro*, only f1 displayed dominant-negative qualities respect to MTOC attachment to the cytoskeleton, positioning, and MT architecture *in vivo*.

Because the steady-state architecture of MTs depends upon other molecular mechanisms aside of nucleation, we wanted to assess whether the subcellular distribution of MT nucleation sites was specifically affected by the expression of f1. Although we had already used the nucleation technique in single transiently transfected cells (Figure 6), the question remained to what extent CACO-2 cells display noncentrosomal MT nucleation (Vorobjev *et al.*, 1997; Yvon and Wadsworth, 1997) or centrosomal nucleation followed by MT release and relocation (Keating *et al.*, 1997). Answering this question at this time seemed important because the fraction of centrosomes delocalized by the expression of f1 was relatively modest (21%; Table 2), whereas a substantial fraction of the cells expressing f1 did show scattered noncentrosomal γ -tubulin (Figure 7D). CACO-2 cells were grown on glass coverslips to have flatter cells where all the nucleation events can be detected within a few confocal optical sections. In preliminary experiments, we noted that an additional advantage of using these cultures over the fully polarized filter cultures was that the cells grown on glass lack nocodazole-resistant MTs. The 12-d old cultures were incubated in nocodazole for 5 h, which included a 15-min period at 4°C. Some cells were fixed while still in nocodazole (Figure 8, A, D, and G, 5-h nocodazole), whereas others were rapidly washed in prewarmed medium and incubated for 12 min (Figure 8, B, C, E, F, H, and I, 12-min washout). In preliminary experiments, we had determined that the first MTs occur at 8 min after the washout, but at 12 min at least 60% of the cells showed numerous MT nucleation foci, with very short MTs (Figure 8, red). We reasoned that, if the centro-

somes were the primary source of MT nucleation, even if there is translocation of nascent MTs, all centrosomes must show nucleating activity and a gradient of newly synthesized MTs must be visible around the centrosomes. In contrast with that prediction, 100% of the cells in interphase showing MT nucleation displayed a completely homogeneous distribution of nucleation foci throughout the cytoplasm. The position of the centrosomes was monitored in the same cells by pericentrin immunofluorescence (Figure 8, green). Only 31% of the cells in interphase that displayed MT nucleation after 12-min nocodazole washout showed MT nucleation around one or two of the centrosomes. However, even in those cells, the centrosomes were only one or two out of tens of similar nucleation foci. The most common pattern (69% of the cells showing nucleation), however, was that the centrosome was not surrounded by nascent MTs in interphasic cells (Figure 8, B, E, and H, arrows). The images were completely different in mitotic cells, as identified by chromosome condensation with 4,6-diamidino-2-phenylindole (DAPI) staining (Figure 8I). In those cases, representing ~0.2% of the cells, 100% of the images showed MT signal around centrosomes (Figure 8, C, F, and I, arrows), and, contrarily to the interphasic cells, noncentrosomal foci of MT nucleation were observed only in 8% of the mitotic cells (data not shown). This result indicated that in CACO-2 cells in interphase the bulk of MT nucleation occurs in noncentrosomal MTOCs.

Bearing in mind that MT nucleation is mostly noncentrosomal in these cells, we used the same assay in parental CACO-2 cells and f1-expressing CACO-2 cells (both in the presence or absence of dox), which were grown for 12 d on Transwell filters to achieve full polarization. The cells were incubated for 5 h in nocodazole, including a 15-min period in the cold, and some cells were subjected to a 12-min washout of the drug. Figure 9, A–L, are XY confocal images merging the red channel (K8) and the green channel (MTs) that show either the apical plane or a transnuclear plane right above the basal-most extent of the IFs (approximately at the middle of the lateral domain). As mentioned, monolayers grown on filters did show some cells displaying apical MTs resistant to nocodazole (Figure 9, A, E, and I). Overall, no differences were observed among parental CACO-2 cells or the f1-expressing subline with or without dox after 5 h in nocodazole. More importantly, none of these cells displayed

Figure 8. Microtubule nucleation is noncentrosomal in interphasic CACO-2 cells. The cells were cultured on glass coverslips for 12 d. All the cultures were then subjected to a treatment with 33 μ M nocodazole for 5 h, including 15 min at 4°C (and the rest of the time at 37°C). Some cells were fixed at the end of the treatment (5-h nocodazole; A, D, and G), whereas other monolayers were rapidly washed with standard culture medium prewarmed at 37°C and incubated in the same for 12 additional minutes (12-min washout; B, C, E, F, H, and I) and then fixed. All the monolayers were processed for immunofluorescence with an anti- α -tubulin mAb that stains MT (red, A–C), anti-pericentrin antibody (green, D–F) and DAPI (blue, G–I). The red and green channels are shown separately in the first and second rows, respectively, and all three channels are shown merged in the bottom row of images (G–I). Arrows, centrosomes. Bars, 5 μ m.

MTs at the lateral level (Figure 9, B, F, and J). After 12-min nocodazole washout, extensive MT repolymerization was observed in the apical domain (Figure 9, C, G, and K). In addition, only in f1-expressing cells in the presence of dox-extensive MT nucleation was observed at the transnuclear level (Figure 9L, arrows). This result indicates that at least a fraction of the γ -tubulin delocalized by the expression of GCP6 f1 is indeed part of MT-nucleating structures.

An S→D Mutation in the Only Cognate GCP6 CDK1 Phosphorylation Site Affects Apical Distribution of MTOCs

Because CDK1-mediated phosphorylation was shown to disrupt the colocalization of MTOCs with IFs (Figuerola *et al.*, 2002) and binding in vitro of full-length GCP6 to keratins was also inhibited by CDK1-mediated phosphorylation (Figure 1C), we asked whether a S→D mutation that mimics phosphorylation in the only consensus CDK1 phosphorylation site of GCP6 (S1397) could affect the distribution of MTOCs. The construct carrying the V5-tagged full-length ORF mutant was transiently transfected in CACO-2 cells (Figure 9M) and COS-1 cells (data not shown). The efficiency of transfection was exceedingly low (<1% cells), possibly due to the large size of the vector (~12,000 base pairs). Even in transfected cells, V5 signal was scarce limited to MTOCs (Figure 9M, arrow) and small cytoplasmic clumps. In cells with two centrosomes, it was not unusual that only one of them was labeled. However, 86% of the V5-labeled centrosomes were found separated from the IFs (Figure 9M, arrow), as opposed to nonlabeled centrosomes (Figure 9M, arrowhead), which were found separated from IFs only in ~2% of the interphasic cells. As a control, a S1397A GCP6 mutant was also transiently expressed in CACO-2 cells with-

out any noticeable change in the position of V5-positive MTOCs (data not shown). This result further indicates that GCP6 plays a role in the positioning of MTOCs in simple epithelia.

DISCUSSION

The results in this work support two major conclusions, that GCP6 participates in the apical localization of MTOCs in CACO-2 cells and that such a localization is important for the architecture of MTs. Overall, the data supports a model in which noncentrosomal MTOCs, perhaps isolated γ -TurCs, are responsible for the nucleation of MTs in simple epithelial cells, consistent with the model originally proposed by Karsenti and coworkers (Bré *et al.*, 1990).

The role of GCP6 in the positioning of MTOCs was shown by three independent approaches, namely, shRNA-mediated knockdown, overexpression of a keratin-binding domain that competitively abrogates GCP6 binding to keratins, and expression of a S1397D GCP6 mutant. In all three cases, γ -tubulin signal, a bona fide reporter of MTOCs, became separated from the apicolateral cortical region where IFs are localized. The expression of f1 in a CACO-2 clone enabled us to quantify the extent of such a release. In agreement with all the morphological observations from the other methods, f1 released only slightly >50% of the TX-100-insoluble γ -tubulin from the pellets (Figure 7C), suggesting the possibility that additional, perhaps redundant, mechanisms may exist to anchor MTOCs.

In K8-null mice, it was shown that IFs are necessary for the apical localization of GCP6 and γ -tubulin (Figure 3), in agreement with previous publications (Salas, 1999; Ameen *et al.*, 2001). Binding of GCP6 to pure keratins in vitro (Figures

Figure 9. (A–L) Overexpression of f1-GCP6 changes the distribution of MT nucleation. Parental CACO-2 cells (A–D) or the subline expressing f1 under the TET-responsive promoter were cultured on Transwell filters in the absence (E–H) or presence of dox (I–L) and treated with nocodazole and cold (5-h nocodazole; A, B, E, F, I, and J) or treated and then washed for 12 min (12-min washout; C, D, G, H, K, and L) as described in Figure 8. Immunofluorescence and red/green channels were also as described in Figure 7E. The images are XY confocal sections at the apical or transnuclear levels and show the red and green channels merged in all panels. (M) Expression of S1397D-GCP6 alters the distribution of MTOCs. CACO-2 cells were transiently transfected with V5-tagged S1387D GCP6 mutant. MTOCs identified by γ -tubulin (blue) that incorporated the mutant reported by V5 (red; arrow) were observed separated from the IFs (TROMA 1; green). Conversely, MTOCs that did not contain V5 signal colocalized with IFs (arrowhead). Both panels are XZ 3D reconstructions with the apical side up. Bars, 5 μ m (A–L) and 10 μ m (M).

1 and 2) and yeast-two hybrid assays (Figure 2) strongly suggest a direct interaction between GCP6 and keratins. A general concern about the specificity of these interactions may be raised because keratins contain extensive coiled-coil domains, and those regions have some potential to sustain nonspecific protein binding. Although full-length GCP6 contains a region with potential for coiled-coil interactions (between aa 600 and 800; Figure 2, diagram), the results in Figure 2 dispel the possibility of such nonspecific coiled-coil binding for two reasons: 1) The C-terminal GCP6(1397–1819) or its fragments f1 and f2 do not display predicted coiled-coil structure; and 2) it is difficult to conceive how nonspecific coiled-coil interactions could discriminate myosin, tropomyosin, and K18 from K8 and K19 in blot overlays (Figure 2A) or K8 from lamin C in yeast two-hybrid assays (Figure 2B), bearing in mind that all control proteins display extensive coiled-coil domains.

The specificity of GCP6–keratin interaction was, however, intriguing. In all blot overlay assays, GCP6 bound better to K8 than to K19 and poorly (Figure 1C) or did not bind at all to K18 (Figure 2, A and C). Although this result supports the notion that this binding is specific, it is difficult to understand because K18 and K19 are much closely related to each other than K8. However, there are small domains for which the similarity between K8 and 19 is larger than that between K8 and 18 (e.g., K8: 300–331, K19: 289–320 compared with their counterpart K18: 355–386). A detailed analysis of the GCP6-binding domain in keratins will be necessary to solve this question. Likewise, although we have generally identified the presence of a keratin-binding domain in the C-terminal region of GCP6, we have not narrowed down the search to a specific domain, and a detailed deletional anal-

ysis will be necessary. Moreover, the difference in selectivity between f1 and f2, with f1 being more specific to K8, highlights the possibility that more than one keratin binding domain may exist in the C-terminal region of GCP6.

Because in simple epithelial cells IFs often do not depolymerize during mitosis (Lane *et al.*, 1982), this attachment poses a problem at the onset of the M phase when centrosomes must migrate to lateral poles to establish a mitotic spindle (Buendia *et al.*, 1990; Figueroa *et al.*, 2002). There is only one Cdk1 consensus phosphorylation site (Holmes and Solomon, 1996) in GCP6 located in S1397, in the central region which is present only in higher eukaryotes but absent in yeast (Fujita *et al.*, 2002). The Cdk1 phosphorylation site is immediately adjacent to f1, which is itself a keratin binding region (Figure 2C), although perhaps only part of a larger keratin-binding domain. Also, there are 10 cyclin ligand recognition sites ([RK]nLn[FYLIVMP]) further enhancing the notion that GCP6 is a bona fide physiological target of Cdk1. That Cdk1-mediated phosphorylation abrogates binding of full-length GCP6 to keratins in vitro (Figure 1) and that an S1397D mutant dislodges centrosomes from the IFs in vivo (Figure 9M) strongly support the notion that Cdk1 phosphorylation site is regulatory for the keratin-binding function. Altogether, this is consistent with a function of GCP6 during the mitotic phase releasing centrosomes from IFs. Alternatively, a large, highly conserved molecule such as GCP6 is expected to have other functions. Zhang *et al.* (2000) showed that the *Xenopus* orthologue of GCP6 (Xgrip210) plays a role in the assembly of γ -TurCs, and the phenotype of Alp16-null (GCP6 orthologue) yeast has shown MTs much longer than normal (Fujita *et al.*, 2002), suggesting a function in the control of nucleating or capping activities.

The MT nucleation experiments highlighted the fact that noncentrosomal nucleation is predominant in interphasic CACO-2 cells, as opposed to mitotic cells, where noncentrosomal nucleation was minimal (Figure 8). Although we cannot distinguish whether noncentrosomal MTOCs actually cap and stabilize the MT minus ends, the results indicate that the position of MTOCs in the apicobasal axis is important for the final polarized localization of the minus ends. The structure of noncentrosomal nucleation sites has not been formally analyzed. We assume that they may represent either single or small clusters of γ -TurCs. However, the different behavior in mitosis/interphase suggests differences at the molecular level that remain to be studied. Recently, Reilein and coworkers showed that a γ -tubulin cap remains attached to the minus ends of the acentrosomal MT network in the basal domain of Madin Darby canine kidney cells (Reilein *et al.*, 2005). It is likely that the same may apply to the MTs in the much denser apical network. That manipulations changing the distribution of γ -tubulin result in changes in the steady-state architecture of MTs strongly suggests that the location of the machinery that nucleates MTs in interphase is important for the final distribution of MTs. In addition, mechanisms that capture and stabilize the plus ends or lateral scaffolding of MTs to other structures of the cytoskeleton are expected to be also essential determinants of MT architecture in epithelial cells. Our data support that notion because, although we induced ectopic MT nucleation with various manipulations of GCP6, we could never substantially decrease the high density of MTs under the apical region. The data presented here, however, show that there is a functional advantage for the apical distribution of noncentrosomal MTOCs in simple epithelia and that GCP6, a component of the γ -TurC, may have acquired Cdk1-regulated keratin binding function recently in evolution.

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