The Tumor Suppressor CYLD Regulates Microtubule Dynamics and Plays a Role in Cell Migration*

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The familial cylindromatosis tumor suppressor CYLD is known to contain three cytoskeleton-associated protein glycine-rich (CAP-Gly) domains, which exist in a number of microtubule-binding proteins and are responsible for their association with microtubules. However, it remains elusive whether CYLD interacts with microtubules and, if so, whether the interaction is mediated by the CAP-Gly domains. In this study, we data demonstrate that CYLD associates with microtubules both in cells and in vitro, and the first CAP-Gly domain of CYLD is mainly responsible for the interaction. Knockdown of cellular CYLD expression dramatically delays microtubule regrowth after nocodazole washout, indicating an activity for CYLD in promoting microtubule assembly. Our data further demonstrate that CYLD enhances tubulin polymerization into microtubots by lowering the critical concentration for microtubule assembly. In addition, we have identified by wound healing assay a critical role for CYLD in mediating cell migration and found that its first CAP-Gly domain is required for this activity. Thus CYLD joins a growing list of CAP-Gly domain-containing proteins that regulate microtubule dynamics and function.

The microtubule cytoskeleton plays a critical role in many cell activities, such as cell shaping, intracellular trafficking, cell division, and cell motility. The structure and function of microtubules are regulated exquisitely in cells by a repertoire of microtubule-binding proteins, including proteins that regulate the assembly and organization of microtubules and motor proteins that mediate the transport of organelles and vesicles (1). These proteins bind microtubules either at the microtubule ends or on the outside of the microtubule wall or bind to soluble tubulin. Different structural motifs present in the microtubule-binding proteins are responsible for their interaction with microtubules. For example, the microtubule end-binding protein EB1 interacts with microtubules through the calponin homology domain (2), whereas the cytoplasmic linker protein 170 (CLIP-170) and the dynactin subunit p150glued interact with microtubules through the cytoskeleton-associated protein glycine-rich (CAP-Gly) domains (3, 4).

The CAP-Gly domain is an evolutionarily conserved motif composed of about 70 amino acids with a large number of glycine residues. This domain has been identified in a wide range of microtubule-binding proteins apart from CLIP-170 and p150glued, such as the motor protein kinesin-73, the CLIP-170-like protein BIK1, and the tubulin folding cofactors B and E (5–7). These CAP-Gly proteins either play critical roles in regulating microtubule dynamics or are involved in microtubule-mediated cellular processes (1). A common function for the CAP-Gly domains in the CAP-Gly proteins is to mediate their colocalization and association with microtubules. A growing body of evidence suggests that the CAP-Gly domains themselves are responsible for binding to microtubules (8–10).

The tumor suppressor for human familial cylindromatosis (CYLD) is a recently identified CAP-Gly protein that consists of 956 amino acid residues with three CAP-Gly domains in the amino terminus and a ubiquitin hydrolase domain in the carboxyl terminus (11). CYLD has been shown to inhibit NF-κB pathway activation by removing lysine 63-linked polyubiquitin chains from upstream signaling molecules, including tumor necrosis factor receptor-associated factor 2 (TRAF2), TRAF6, NF-κB essential modulator, and Bcl-3 (12–15). Several other functions for CYLD have been proposed recently, such as the regulation of cellular entry into mitosis (16), although the precise molecular mechanisms remain to be elucidated.

CYLD is the first protein reported to have three CAP-Gly domains, whereas most other CAP-Gly proteins have only one or two. The first and third CAP-Gly domains of CYLD are quite similar to the previously reported CAP-Gly domains in the amino acid sequence, whereas the second CAP-Gly domain shows weaker similarity (11). It was found recently that ectopically expressed CYLD colocalizes with microtubules in cells (16). However, it is unknown whether CYLD indeed interacts with microtubules like other CAP-Gly proteins and, if so, whether the interaction is mediated by the CAP-Gly domains. In addition, it is unclear whether CYLD plays a role in regulating microtubule dynamics or microtubule-mediated cellular events as other CAP-Gly proteins do. In this study, we show that CYLD binds microtubules primarily through its first CAP-
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Gly domain, regulates microtubule dynamics, and is important for cell migration.

EXPERIMENTAL PROCEDURES

Reagents—Nocodazole, 4',6-diamidino-2-phenylindole, and antibodies against FLAG, α-tubulin, acetylated α-tubulin, and β-actin were purchased from Sigma. The antibody against detyrosinated α-tubulin was from Abcam, and the antibody against CYLD was generated by immunization of mice with a glutathione S-transferase (GST) fusion protein containing amino acids 1–555 of human CYLD. Horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Biosciences, and fluorescein- or rhodamine-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories.

Cell Culture and Transfection—HeLa and CV1 cells in Dulbecco’s modified Eagle’s medium and human umbilical vein endothelial cells (HUVEC) in RPMI 1640 medium were cultured in the indicated media supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37 °C in a humidified atmosphere with 5% CO₂. Plasmid transfection was performed using the polyethylenimine reagent (Sigma). CYLD and enhanced green fluorescent protein (EGFP) small interfering RNAs (siRNAs) were synthesized by Ribobio (Guangzhou, China) and transfected into cells with the Lipofectamine 2000 reagent (Invitrogen).

Plasmids and Proteins—The pEGFP-C1-CYLD and pcDNA3-FLAG-CYLD plasmids were kindly provided by Dr. George Mosialos (A. Fleming Biomedical Sciences Research Center, Vari, Greece). The plasmids pEGFP-C1-CYLDΔG1 and pEGFP-C1-CYLDΔCG1 (deletion of amino acids 115–209) encode silent mutants that are not targeted by CYLD siRNA and were generated by PCR. The bacterial expression plasmids for GST-tagged CYLDsm and pEGFPC1-CYLDsm encode silent CYLD mutants that are not targeted by CYLD siRNA and were generated by PCR. The bacterial expression plasmids for GST-tagged CYLDsm and pEGFPC1-CYLDsmΔCG1 (deletion of amino acids 115–209) encode silent mutants that are not targeted by CYLD siRNA and were generated by PCR. The BL21 (DE3) strain of Escherichia coli (99% pure) was obtained from Cytoskeleton Inc.

Immunofluorescence Microscopy—Cells grown on glass coverslips were fixed with methanol for 5 min at −20 °C or fixed with 4% paraformaldehyde for 30 min at room temperature followed by permeabilization in 0.5% Triton X-100/phosphate-buffered saline (PBS) for 20 min. Cells were then blocked with 2% bovine serum albumin in PBS and incubated in succession with primary and fluorescein- or rhodamine-conjugated secondary antibodies followed by staining with 4',6-diamidino-2-phenylindole for 5 min. Coverslips were mounted with 90% glycerol in PBS and examined with an Olympus fluorescence microscope.

Western Blotting—Proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore) as described previously (17). The membranes were blocked in Tris-buffered saline containing 0.2% Tween 20 and 5% fat-free dry milk and incubated first with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies. Specific proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer’s instructions (Pierce).

Immunoprecipitation and GST Pulldown—For immunoprecipitation, cells were transfected with pcDNA3-FLAG-CYLD or the empty vector. The cell lysate was incubated with anti-FLAG-agarose beads at 4 °C for 2 h. Alternatively, the cell lysate was incubated with anti-α-tubulin antibody at 4 °C for 2 h, and protein A-agarose beads were then added to incubate for another 3 h. The beads were washed extensively and boiled in SDS loading buffer, and the precipitated proteins were detected by SDS-PAGE and Western blotting. For GST pulldown in vitro, GST or GST-CYLD fusion protein immobilized on glutathione-agarose beads was incubated with purified tubulin at 4 °C for 2 h. The beads were washed extensively, and the proteins were analyzed by Western blotting.

Microtubule Cosedimentation Assays—To examine CYLD-microtubule interaction in vivo, cells were homogenized, added to 100 mM PIPES, pH 6.8, and centrifuged at 4 °C for 1 h at 50,000 rpm. The supernatant was added to 20 μM paclitaxel and 1 mM GTP, incubated at 37 °C for 5 min, and then placed on ice for 15 min. The mixture was loaded over the PEMG buffer (100 mM PIPES, 1 mM EGTA, 1 mM MgSO₄, 1 mM GTP, pH 6.8) containing 15% sucrose and 20 μM paclitaxel and centrifuged for 30 min at 30,000 rpm. The pellet (containing microtubules and associated proteins) and supernatant fractions were then collected individually, and the proteins present in each fraction were examined by Western blotting. To examine CYLD-microtubule interaction in vitro, microtubules were assembled from bovine brain tubulin at 30 °C in the presence of 1 mM GTP and 20 μM paclitaxel. The reaction mixture was layered over PEMG buffer containing 50% sucrose and 20 μM paclitaxel and centrifuged for 30 min at 30,000 rpm. The pellet and supernatant fractions were then collected and analyzed by Western blotting.

Microtubule Depolymerization/Regrowth—The microtubule regrowth experiment was performed as described previously (18). Briefly, HeLa cells were treated with 5 μM nocodazole to depolymerize microtubules, and the drug was then washed out and microtubules were allowed to regrow. To visualize microtubules with immunofluorescence microscopy, cells were fixed in 4% paraformaldehyde in PBS for 30 min and stained with anti-α-tubulin antibody. To examine tubulin partitioning between the polymer and soluble dimer by Western blotting, cellular soluble proteins were extracted with PEMG buffer containing 0.1% Triton X-100 and 4 μM glycyrrettinic acid. The remaining polymer (cytoskeletal) fraction was dissolved in 0.5% SDS in 25 mM Tris, pH 6.8.

Tubulin Turbidity Assay—Spectrophotometer cuvettes held a solution consisting of PEMG buffer and in vitro translated CYLD. The cuvettes were kept at room temperature before the addition of purified tubulin and shifted to 37 °C in a temperature-controlled spectrophotometer (Amersham Biosciences). The assembly was monitored by measuring the changes in absorbance (350 nm) as described previously (19). To examine the effect of CYLD on microtubule stability, microtubules were
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FIGURE 1. CYLD interacts with microtubules both in cells and in vitro. A, immunofluorescent images of CV1 cells stained with anti-α-tubulin (anti-tub) and anti-CYLD (or IgG control) antibodies and the DNA dye 4′,6-diamidino-2-phenylindole (DAPI) are shown. B, cells were transfected with pcDNA3-FLAG-CYLD, an expression vector encoding FLAG-tagged CYLD, and microtubules and associated proteins were then prepared from the cell lysate, in the absence or presence of paclitaxel and GTP (P/G), and pelleted by centrifugation. Proteins present in the pellet (P) and supernatant (S) fractions were detected by Western blotting. 

 RESULTS

CYLD Interacts with Microtubules Both in Cells and in Vitro—We first assessed the subcellular localization of ectopically expressed CYLD tagged with GFP. In agreement with the previous data (16), we found that the GFP-CYLD fusion protein colocalized with microtubules in both CV1 and HeLa cells (data not shown). In addition, with a CYLD-specific antibody, we found that endogenous CYLD also colocalized with the microtubule cytoskeleton in CV1 cells (Fig. 1A) and HeLa cells (data not shown).

We studied CYLD-microtubule association further by microtubule cosedimentation assays. HeLa cells were transfected with an expression vector encoding FLAG-tagged CYLD, and microtubules and associated proteins were then prepared from the cell lysate, in the absence or presence of paclitaxel and GTP, and pelleted by centrifugation. Proteins present in the pellet (P) and supernatant (S) fractions were detected by Western blotting.

Wound Healing Assay—HUVEC grown in 24-well plates as confluent monolayers were mechanically scratched using a 20-μl pipette tip to create the wound. Cells were washed with PBS to remove the debris, and complete culture media were then added to allow wound healing. Phase-contrast images of the wound were taken at three random locations, first immediately after wounding and then at the same location after 24 h, to examine wound closure by migrating cells. Cells that migrated into the wound area were then counted.

The reactions were diluted 1:1 by addition of PEMG buffer, and the temperature was then shifted to 4 °C. Microtubule disassembly upon dilution and cooling was examined by measuring the absorbance at 350-nm wavelength.

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FIGURE 2. CYLD interacts with tubulin both in cells and in vitro. A, HeLa cells were transfected with pcDNA3-FLAG-CYLD or the empty vector pcDNA3. The interaction of FLAG-CYLD with cellular tubulin was analyzed by immunoprecipitation (IP) of the cell lysate with anti-FLAG antibody and Western blotting of the precipitate with anti-α-tubulin antibody. B, HeLa cells were transfected as in A, and the interaction between FLAG-CYLD and tubulin was examined by immunoprecipitation with anti-α-tubulin antibody (or IgG control) and Western blotting of the precipitate with anti-FLAG antibody. C, experiments were performed as in A, except that CV1 cells were used instead of HeLa cells. D, purified bovine brain tubulin was incubated with in vitro translated CYLD, and the CYLD-tubulin interaction was examined by immunoprecipitation with anti-α-tubulin antibody (or IgG control) and Western blotting of the precipitate with anti-FLAG antibody. E, CYLD, and microtubules and associated proteins were then prepared from the cell lysate, in the absence or presence of paclitaxel and GTP, and pelleted by centrifugation.
CYLD, and the cell lysate was then immunoprecipitated with anti-FLAG antibody. Western blotting of the precipitate with anti-α-tubulin antibody revealed that tubulin was coprecipitated with FLAG-CYLD (Fig. 2A). The interaction between tubulin and FLAG-CYLD in HeLa cells was confirmed by immunoprecipitation of the cell lysate with anti-α-tubulin antibody and Western blotting of the precipitate with anti-FLAG antibody (Fig. 2B). Similarly, we found an interaction between CYLD and tubulin in CV1 cells (Fig. 2C) and 293T cells (data not shown).

We also examined whether CYLD interacts with tubulin in vitro. In vitro translated CYLD was incubated with purified bovine brain tubulin, and the mixture was immunoprecipitated with anti-α-tubulin antibody. SDS-PAGE and autoradiography detected the presence of CYLD in the precipitate (Fig. 2D), indicating an interaction between CYLD and tubulin in vitro. This result was confirmed by incubating bacterially purified GST-CYLD with bovine brain tubulin followed by GST pull-down. As shown in Fig. 2E, Western blotting with anti-α-tubulin antibody showed clearly the presence of tubulin in the pull-down preparation.

**Role of the CAP-Gly Domains in Mediating the Interaction of CYLD with Microtubules and Tubulin**

CAP-Gly domains are known to mediate the association of CAP-Gly proteins with microtubules. Therefore, we asked whether the CAP-Gly domains of CYLD are responsible for its interaction with tubulin.

**FIGURE 3.** Role of the CAP-Gly domains in mediating the interaction of CYLD with microtubules and tubulin. A, a schematic representation of full-length (FL) and truncated forms of CYLD is shown. The three open boxes in the amino terminus represent the three CAP-Gly domains (CG1–3), and the filled box in the carboxyl terminus represents the ubiquitin hydrolase catalytic domain. B, in vitro translated CYLD and its truncated forms were incubated without or with microtubules assembled from 10 μM tubulin. Microtubules were then pelleted by centrifugation, and CYLD proteins in the pellet (P) and supernatant (S) fractions were detected by SDS-PAGE and autoradiography. C, purified tubulin was incubated with bacterially purified GST or various forms of GST-CYLD immobilized on agarose beads. GST pull-down was then performed, and the presence of tubulin in the pulldown preparation was detected by Western blotting using anti-α-tubulin antibody. GST fusion proteins used in the pulldown assay were detected by Coomassie Blue staining.

**FIGURE 4.** Knockdown of cellular CYLD expression retards microtubule regrowth after nocodazole removal. A, Western blot analysis of the expression of CYLD and α-tubulin in HeLa cells transfected with control (enhanced GFP, siControl) or CYLD (siCYLD) siRNAs is shown. The relative level of CYLD was determined by densitometric analysis of the Western blot bands. B, immunofluorescent images of microtubules are shown. HeLa cells transfected with control or CYLD siRNAs were treated with nocodazole to depolymerize microtubules. The drug was then washed out, and microtubules were allowed to regrow for the indicated times. C, HeLa cells were transfected with control or CYLD siRNAs, and microtubule depolymerization-regrowth assay was performed. Tubulin partitioning between the polymer (P) and soluble dimer (S) was then examined after 5 or 15 min of microtubule regrowth. D, experiments were performed as in C, and the ratio of tubulin polymer to soluble dimer (P/S) was measured by densitometric analysis of the Western blot bands. The values shown in this graph represent means ± S.D. of data from three independent experiments. E, immunofluorescence images of microtubules and GFP-tagged proteins are shown. HeLa cells treated by CYLD siRNA were transfected with plasmids expressing GFP-CYLDsm or GFP-CYLDsmΔCG1 (GFP-CYLDsm and GFP-CYLDsmΔCG1 plasmids encode silent mutants that are not targeted by CYLD siRNA). Cells were treated with nocodazole to depolymerize microtubules. The drug was then washed out, and microtubules were allowed to regrow for 5 min. ΔCG1, deletion of the first CAP-Gly domain.
microtubules. By microtubule cosedimentation assay, we found that the amino-terminal region of CYLD, which contains the three CAP-Gly domains, mainly resided in the microtubule pellet. In contrast, the carboxyl-terminal region, which contains the ubiquitin hydrolase domain, was not detectable in the pellet (Fig. 3 A and B). Interestingly, the three CAP-Gly domains of CYLD exhibited distinct microtubule binding profiles; the first CAP-Gly domain showed much stronger binding ability than the other two (Fig. 3B). These results suggest that the first CAP-Gly domain of CYLD is mainly responsible for its interaction with microtubules.

We then examined by GST pulldown assay the interaction of various forms of CYLD with tubulin. As shown in Fig. 3C, the amino-terminal region of CYLD could interact with tubulin, whereas the carboxyl-terminal region could not. In addition, we found that only the first two CAP-Gly domains of CYLD were able to interact with tubulin (Fig. 3C).

Knockdown of Cellular CYLD Delays Microtubule Regrowth after Nocodazole Washout—The specific interaction of CYLD with microtubules and tubulin suggested that this protein might play a role in regulating the dynamic property of microtubule assembly. To test this possibility, we transfected HeLa cells with CYLD-specific siRNA and treated cells with nocodazole to depolymerize microtubules. Then we examined microtubule regrowth after nocodazole washout. As shown in Fig. 4A, CYLD siRNA resulted in an apparent decrease of CYLD expression (78.6% decrease). Immunofluorescence staining of cellular microtubules showed that knockdown of CYLD expression dramatically retarded microtubule regrowth after nocodazole removal (Fig. 4B). For example, in cells transfected with control siRNA, microtubule fibers were quite evident after 5 min of regrowth. In contrast, only a few small microtubule asters were detected in CYLD siRNA-transfected cells (Fig. 4B).

To assess quantitatively the effect of CYLD siRNA on microtubule regrowth following nocodazole washout, we prepared cell extracts that contained cytoskeletal (polymeric) and soluble (dimeric) tubulin, respectively, after 5 or 15 min of microtubule regrowth. Consistent with the immunofluorescence staining results, Western blot analysis revealed that CYLD siRNA significantly delayed cellular tubulin polymerization into microtubules (Fig. 4C). Densitometric analysis of the Western blot bands showed a dramatic decrease in the ratio of tubulin polymer to soluble dimer following CYLD siRNA treatment (Fig. 4D).

Furthermore, we found that the inhibitory effect of CYLD siRNA on microtubule regrowth could be significantly reversed by the expression of GFP-tagged CYLD (Fig. 4E). In contrast, expression of GFP-CYLD that does not contain the first CAP-Gly domain, which is mainly responsible for CYLD binding to microtubules, was not quite effective in reversing microtubule regrowth (Fig. 4E). Collectively, these data indicate a role for CYLD in promoting microtubule assembly and demonstrate the functional significance of the first CAP-Gly domain of CYLD in cells.

CYLD Promotes Microtubule Assembly and Stability in Vitro—We then examined the effect of CYLD on the assembly of tubulin subunits into microtubules in vitro by measuring changes in the turbidity produced upon tubulin polymerization at 37 °C. As shown in Fig. 5A, CYLD could increase tubulin turbidity in a concentration-dependent manner, and in the presence of CYLD a larger extent of depolymerization was observed upon cooling the solution to 4 °C. These results indicate that CYLD could promote tubulin polymerization into microtubules in vitro.

We next studied whether CYLD affects microtubule stability. Microtubules were assembled in the presence or absence of CYLD. The assembled microtubules were then rapidly diluted, and microtubule disassembly was followed by measuring the decrease in the absorbance. We found that control microtubules rapidly depolymerized upon dilution, whereas microtubules assembled in the presence of CYLD depolymerized more slowly and retained higher absorbance (Fig. 5B). The remaining absorbance decreased to the base-line level upon cooling the solution to 4 °C in both the presence and absence of CYLD, indicating that the remaining absorbance upon dilution is because of microtubules (Fig. 5B). Thus, CYLD could increase microtubule stability in vitro.

To gain more insight into the activity of CYLD in regulating tubulin polymerization, we measured by tubulin turbidity assay

FIGURE 5. CYLD promotes microtubule assembly and increases microtubule stability in vitro. A, effects of 0, 5, 10, or 20 μM in vitro translated CYLD on tubulin polymerization measured by light scattering reflected as the absorbance at a 350-nm wavelength at 37 °C. The open arrow refers to the time point when the temperature was shifted to 4 °C. B, microtubules assembled from tubulin at 37 °C in the presence or absence of in vitro translated CYLD. The reactions were diluted 1:1 by addition of PEMG buffer, and the temperature was then shifted to 4 °C. Microtubule disassembly upon dilution and cooling was examined by measuring the absorbance at a 350-nm wavelength. The solid arrow refers to the time point when the reactions were diluted, and the open arrow refers to the time point when the temperature was shifted to 4 °C. C, determination of the critical concentration for microtubule assembly by tubulin turbidity assay. Tubulin at various concentrations was polymerized at 37 °C in the presence or absence of in vitro translated CYLD or the first CAP-Gly domain, and the absorbance at a 350-nm wavelength was then measured. The base lines at 4 °C were subtracted from the plateau absorbance to obtain the change in absorbance, which was plotted versus the concentration of tubulin. The data were fit by linear regression, and the critical concentration for microtubule assembly was determined from the x intercept. D, determination of the critical concentration for microtubule assembly by microtubule sedimentation assay. Tubulin at various concentrations was polymerized at 37 °C in the presence or absence of in vitro translated CYLD or the first CAP-Gly domain. The reactions were sedimented, and the amount of tubulin assembled in microtubules was derived from quantitative analysis of the SDS-PAGE patterns of tubulin in the supernatants. The concentration of polymerized tubulin was then plotted versus the concentration of total tubulin. The data were fit by linear regression, and the critical concentration for microtubule assembly was determined from the x intercept.

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the effect of CYLD on the critical concentration for microtubule assembly. We found that the critical concentration decreased from 3.01 to 1.54 μM in the presence of 10 μM CYLD (Fig. 5C). The first CAP-Gly domain of CYLD also decreased the critical concentration for microtubule assembly (to 2.15 μM), although its effect was modest compared with that of full-length CYLD. Similarly, microtubule sedimentation assay also revealed a decrease in the critical concentration for microtubule assembly by CYLD (from 2.65 to 1.51 μM) and by the first CAP-Gly domain (to 1.98 μM) (Fig. 5C). Taken together, these data suggest that CYLD promotes tubulin polymerization into microtubules by lowering the critical concentration for microtubule assembly.

**CYLD Plays a Role in Cell Migration**—A number of microtubule-binding proteins that regulate microtubule assembly have been found to be important for the migration of cells (1). To test whether CYLD plays a role in cell migration, we first examined its subcellular localization in HUVEC, which are known to undergo efficient migration upon appropriate stimulation. We found that endogenous CYLD colocalized with a subset of microtubules in HUVEC, and the colocalization pattern was more evident with acetylated microtubules (Fig. 6A). CYLD-specific siRNA was able to knock down CYLD expression effectively in HUVEC (83.2% decrease) (Fig. 6B). The effect of CYLD knockdown on cell migration was then examined using the standard wound healing assay. As shown in Fig. 6C, a complete wound closure was observed 24 h after wounding in HUVEC transfected with control siRNA. In contrast, there was a significant impairment in wound closure in cells transfected with CYLD siRNA. Importantly, the inhibitory effect of CYLD siRNA on wound healing was remarkably reversed by the expression of GFP-CYLDM, but not by the expression of GFP-CYLDM ΔCG1 (Fig. 6D). These results indicate a critical role for CYLD in mediating cell migration and further demonstrate the functional significance of the first CAP-Gly domain of CYLD in cells.

**DISCUSSION**

CYLD was initially identified as a tumor suppressor for familial cylindromatosis, an autosomal dominant predisposition to multiple benign tumors of skin appendages called cylindromas.
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(11). It is now known that CYLD is a deubiquitinating enzyme that removes lysine 63-linked polyubiquitin chains from NF-κB signaling components, leading to the inhibition of NF-κB activation, which is thought to underlie the tumor-suppressing function of CYLD (12–15). CYLD harbors three CAP-Gly domains in addition to its ubiquitin hydrolase domain. Whereas it was unclear whether CYLD associates with microtubules like many other CAP-Gly proteins, we have demonstrated in this study, using assays such as colocalization, cosedimentation, immunoprecipitation, and GST pulldown, that CYLD is able to interact with microtubules and tubulin both in cells and in vitro. This finding adds CYLD to the growing list of CAP-Gly domain-containing proteins that associate with microtubules/tubulin. It is worth noting that in cells undergoing apoptosis that have only a few distorted microtubules, CYLD colocalizes with microtubules more evidently (data not shown), suggesting that the interaction of CYLD with microtubules may undergo subtle regulation in response to apoptotic stimuli.

CAP-Gly domains are known to be responsible for the association of CAP-Gly proteins with microtubules. In this study, we present evidence that the interaction of CYLD with microtubules is mediated mainly by its first CAP-Gly domain. Crystallographic studies suggest that the microtubule binding ability of the CAP-Gly domain may depend on a hydrophobic core and some changeable loops, which provide a comfortable conformation for microtubule binding (20). In addition, a conserved sequence located at the loop, GKNLG, is speculated to be responsible for the interaction (20). The corresponding amino acid sequences of the three CAP-Gly domains in CYLD are GFTDG, GNWDG, and GCTDG, respectively (21). It will be interesting to investigate and compare in the future the respective roles of these amino acid sequences in mediating CYLD interaction with microtubules.

Microtubules are intrinsically dynamic polymers, and a number of CAP-Gly proteins, such as CLIP-170 and p150GLUED, are able to modulate the dynamic property of microtubules (1). We have demonstrated that knockdown of cellular CYLD expression dramatically delays microtubule regrowth after nocodazole removal. In addition, by tubulin turbidity assay, we have found that CYLD promotes tubulin polymerization into microtubules and stabilizes microtubules against dilution-induced depolymerization. Furthermore, tubulin turbidity and microtubule sedimentation assays both reveal that CYLD enhances tubulin polymerization into microtubules by lowering the critical concentration for microtubule assembly. These data thus establish CYLD as a novel regulator of microtubule assembly dynamics. It is unknown at present how CYLD exerts its effect on microtubule assembly and stability. Recent studies suggest that CLIP-170 may promote microtubule polymerization and/or nucleation by neutralizing the negative charges of tubulin with the highly positive charges of the CLIP-170 CAP-Gly domains (22). The similarity between CYLD and CLIP-170 in the CAP-Gly domains suggests that CYLD may adopt a similar strategy to promote tubulin polymerization into microtubules.

The dynamic property of microtubules is critical for many of their cellular functions, such as the reorientation of the microtubule network when cells undergo morphological changes or migration and the dramatic rearrangement of microtubules at the onset of mitosis (23–25). Alteration of microtubule assembly and/or stability by abnormal expression of microtubule-binding proteins or by drug treatment can result in serious phenotypes such as defects in cell motility, cell cycle arrest, or even cell death (26–28). Thus, it is not surprising that the alteration of microtubule dynamics by knockdown of CYLD expression results in cell migration defects. In addition, it is stimulating to speculate that the previously observed mitotic entry defects following CYLD siRNA treatment may result at least partially from the alteration of microtubule dynamics (16).

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REFERENCES

1. Amos, L. A., and Schlieper, D. (2005) Adv. Protein Chem. 71, 257–298
2. Hayashi, I., and Ikura, M. (2003) J. Biol. Chem. 278, 36430–36434
3. Pierre, P., Scheel, J., Rickard, J. E., and Kreis, T. E. (1992) Cell 70, 887–900
4. Vaughan, P. S., Miura, P., Henderson, M., Byrne, B., and Vaughan, K. T. (2002) J. Cell Biol. 158, 305–319
5. Guasch, A., Aloria, K., Perez, R., Avila, J., Zabala, J. C., and Coll, M. (2002) J. Mol. Biol. 318, 1139–1149
6. Li, H. P., Liu, Z. M., and Nirenberg, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1086–1091
7. Biernat, J., Mandelkow, E. M., Schroter, C., Lichtenberg-Kraag, B., Steiner, B., Berling, B., Meyer, H., Mercken, M., Vandermeeren, A., Goedert, M., and Mandelkow, E. (1992) EMBO J. 11, 1593–1597
8. Pierre, P., Pepperkok, R., and Kreis, T. E. (1994) J. Cell Sci. 107, 1909–1920
9. Feierbach, B., Nogales, E., Downing, K. H., and Stearns, T. (1999) J. Cell Biol. 144, 113–124
10. Hayashi, I., Wilde, A., Mal, T. K., and Ikura, M. (2005) Mol. Cell 19, 449–460
11. Bignell, G. R., Warren, W., Seal, S., Takahashi, M., Rapley, E., Barfoot, R., Green, H., Brown, C., Biggs, P. J., Lakhan, S. R., Jones, C., Hansen, J., Blair, E., Hofmann, B., Siebert, R., Turner, G., Evans, D. G., Schrander-Stumpel, C., Beemer, F. A., van den Ouweland, A., Halley, D., Delpech, B., Cleveland, M. G., Leigh, I., Leist, J., Rasmussen, S., Wallace, M. R., Fenske, C., Banerjee, P., Osio, N., Chaggar, R., Merrett, S., Leonard, N., Huber, M., Hohl, D., Chapman, P., Burne, J., Swift, S., Smith, M., Ashworth, A., and Stratton, M. R. (2000) Nat. Genet. 25, 160–165
12. Kovalenko, A., Chable-Bessia, C., Cantarella, G., Israel, A., Wallach, D., and Courtois, G. (2003) Nature 424, 801–805
13. Regamey, A., Hohl, D., Liu, J. W., Roger, T., Kogerman, P., Toftgard, R., and Huber, M. (2003) J. Exp. Med. 198, 1595–1964
14. Trompouki, E., Hatzivassiliou, E., Tischritizis, T., Farmer, H., Ashworth, A., and Mosialos, G. (2003) Nature 424, 793–796
15. Massoumi, R., Chmielarska, K., Hennecke, K., Pfeifer, A., and Fassler, R. (2006) Cell 125, 665–677
16. Stegmeier, F., Sowa, M. E., Nalepa, G., Gygi, S. P., Harper, J. W., and Elledge, S. J. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 8869–8874
17. Liu, M., Aneja, R., Liu, C., Sun, L., Gao, J., Wang, H., Dong, J. T., Sarli, V., Giannis, A., Joshi, H. C., and Zhou, J. (2006) J. Biol. Chem. 281, 18090–18097
18. Ezraty, E. J., Partridge, M. A., and Gundersen, G. G. (2005) Nat. Cell Biol. 7, 581–590
19. Xuan, C., Qiao, W., Gao, J., Liu, M., Zhang, X., Cao, Y., Chen, Q., Geng, Y., and Zhou, J. (2007) J. Biol. Chem. 282, 28800–28806
20. Li, S., Finley, J., Liu, Z. J., Qiu, S. H., Chen, H., Luan, C. H., Carson, M., Tsao, J., Johnson, D., Lin, G., Zhao, J., Thomas, W., Nagy, L. A., Shalit, A., and Lu, M. (2002) J. Biol. Chem. 277, 48596–48601
21. Saito, K., Kigawa, T., Koshiba, S., Sato, K., Matsuo, Y., Sakamoto, A.,
Takagi, T., Shirouzu, M., Yabuki, T., Nunokawa, E., Seki, E., Matsuda, T., Aoki, M., Miyata, Y., Hirakawa, N., Inoue, M., Terada, T., Nagase, T., Kikuno, R., Nakayama, M., Ohara, O., Tanaka, A., and Yokoyama, S. (2004) *Structure (Camb.)* 12, 1719–1728

22. Mishima, M., Maesaki, R., Kasa, M., Watanabe, T., Fukata, M., Kaibuchi, K., and Hakoshima, T. (2007) *Proc. Natl. Acad. Sci. U. S. A.* 104, 10346–10351

23. Watanabe, T., Noritake, J., and Kaibuchi, K. (2005) *Trends Cell Biol.* 15, 76–83

24. Desai, A., and Mitchison, T. J. (1997) *Annu. Rev. Cell Dev. Biol.* 13, 83–117

25. Joshi, H. C. (1998) *Curr. Opin. Cell Biol.* 10, 35–44

26. Downing, K. H. (2000) *Annu. Rev. Cell Dev. Biol.* 16, 89–111

27. Jordan, M. A., and Wilson, L. (2004) *Nat. Rev. Cancer* 4, 253–265

28. Mollinedo, F., and Gajate, C. (2003) *Apoptosis* 8, 413–450