p180 Is Involved in the Interaction between the Endoplasmic Reticulum and Microtubules through a Novel Microtubule-binding and Bundling Domain[□]

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p180 was originally reported as a ribosome-binding protein on the rough endoplasmic reticulum membrane, although its precise role in animal cells has not yet been elucidated. Here, we characterized a new function of human p180 as a microtubule-binding and -modulating protein. Overexpression of p180 in mammalian cells induced an elongated morphology and enhanced acetylated microtubules. Consistently, electron microscopic analysis clearly revealed microtubule bundles in p180-overexpressing cells. Targeted depletion of endogenous p180 by small interfering RNAs led to aberrant patterns of microtubules and endoplasmic reticulum in mammalian cells, suggesting a specific interaction between p180 and microtubules. In vitro sedimentation assays using recombinant polypeptides revealed that p180 bound to microtubules directly and possessed a novel microtubule-binding domain (designated MTB-1). MTB-1 consists of a predicted coiled-coil region and repeat domain, and strongly promoted bundle formation both in vitro and in vivo when expressed alone. Overexpression of p180 induced acetylated microtubules in cultured cells in an MTB-1-dependent manner. Thus, our data suggest that p180 mediates interactions between the endoplasmic reticulum and microtubules mainly through the novel microtubule-binding and -bundling domain MTB-1.

INTRODUCTION

The microtubule (MT) cytoskeleton plays fundamental roles in a variety of cellular processes, including cell polarity establishment and intracellular organelle organization and maintenance. Indeed, the morphology and intracellular distribution of the endoplasmic reticulum (ER) are dependent on the MT network (Terasaki *et al.*, 1986). The ER is organized into a three-dimensional network of interlinked membranous tubules and cisternae throughout the cell and extends tubular structures toward the cell periphery (Baumann and Walz, 2001; Voeltz *et al.*, 2002). Numerous studies have focused on how motor proteins mediate movement of the ER along MTs and provided evidence for their roles in MT-based ER tubule formation (Allan and Schroer, 1999;

Karcher *et al.*, 2002). A protein complex of a motor protein and a cargo receptor is assumed to be required for the transport of ER membranes along MTs (Sheetz, 1999). Although nonmotor MT-binding proteins were shown to be involved in organelle assembly and organization, the underlying molecular mechanism remains unclear. Because only a fraction of the ER in each cell is highly motile (Lee and Chen, 1988), the remaining ER, particularly that in the perinuclear region, is considered to be predominantly stationary. Further “static” association of organelles with MTs must be required for the structural organization and maintenance of the stationary ER. Indeed, accumulating evidence suggests that there are specific nonmotor MT-binding proteins for different organelles. CLIP170 was first identified as a class of cytoplasmic linker proteins that link MTs to endosomes (Pierre *et al.*, 1992). GMAP-210 is a minus-end MT-binding protein that associates with the Golgi apparatus and plays a role in maintaining its structural integrity (Infante *et al.*, 1999). Furthermore, ch-TOG, the human homologue of XMAP215, a previously described MT-associated protein (MAP) in *Xenopus* eggs, is localized at the ER membrane in interphase cells (Charrasse *et al.*, 1998). In addition to these cytosolic factors, a new class of direct interactions between the ER membrane and MTs, which are mediated by the integral ER membrane protein CLIMP-63, has been demonstrated (Klopfenstein *et al.*, 1998). These interactions are supposed to contribute to the positioning of the ER along MTs.

In the present study, we focused on p180, which is an integral ER membrane protein and a member of the ES/130 family. ES/130 was originally reported as a morphogenesis

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Abbreviations used: Cnx, calnexin; Crt, calreticulin; CLIMP-63, cytoskeleton-linking membrane protein of 63-kDa; CLIP, cytoskeleton-linking protein; DMP, dimethyl pimelimidate; ER, endoplasmic reticulum; HCMV, human cytomegalovirus; HEL, human embryonic lung; MAP, microtubule-associated protein; MT, microtubule; PDI, protein disulfide isomerase; TEM, transmission electron microscopy; WT, wild type.

inducer in the embryonic chicken heart (Rezaee *et al.*, 1993), whereas canine p180 was first identified as a ribosome-binding protein on the rough ER (Savitz and Meyer, 1990). ES130/p180 was reported to be expressed in most human tissues, and especially strongly in secretory tissues (Langley *et al.*, 1998), although its precise role in animal cells has not yet been elucidated. Previously, we demonstrated that human cytomegalovirus (HCMV) specifically binds to human p180 via its N-terminal domain (Ogawa-Goto *et al.*, 2002), suggesting that the interaction between p180 and the virus may facilitate intracellular transport of HCMV capsids. Because an intact MT network is required for efficient transport of HCMV capsids to the nucleus (Ogawa-Goto *et al.*, 2003), we examined whether p180 interacts with the MT network in the host cells. Here, we present data that human p180 has a unique microtubule-binding and -bundling domain that induces acetylated MT bundles and may be involved in maintaining the normal distribution of the ER.

MATERIALS AND METHODS

Plasmids and Expression Vectors

Full-length cDNAs of human p180 (DDBJ accession number: AB287347) containing 54 or 24 decapeptide repeats (Ogawa-Goto *et al.*, 2002) were inserted into the pcDNA4 HisMax vector (Invitrogen, Carlsbad, CA) to generate pcDNAp180-54R or pcDNAp180-24R, respectively. pEGFP-C1 and pEGFP-C2 (BD Bioscience Clontech, Palo Alto, CA) were used to construct expression plasmids for truncated forms of human p180. The entire coding sequence of enhanced green fluorescent protein (EGFP) was inserted into pcDNAp180-54R to generate wild-type (WT) p180 fused with GFP (GFP-WT). The signal sequence of calreticulin (Crt) was PCR-amplified from the pEYFP-ER vector (BD Bioscience Clontech) and introduced at the 5' end of GFP-WT to generate GFP-ER-WT. Various truncation mutants were generated by PCR procedures that introduced premature stop codons or by digestion with restriction enzymes that cut within the p180 coding region. The EGFP coding sequence in all the above-described EGFP-tagged constructs was substituted with EGFP-A208K from GFP-VSVG (kindly provided by Dr. Lippincott-Schwartz, National Institute of Child Health and Human Development) to express monomeric GFP (Zacharias *et al.*, 2002). The pQE31 and pQE32 vectors (Qiagen, Valencia, CA) were used to construct bacterial expression plasmids for (His)₆-tagged truncated forms of human p180. For in vitro cosedimentation assays, full-length p180 carrying 24 repeats (24R-p180), rather than 54 repeats, was used because bacterial expression of the latter was unsuccessful.

Cell Culture and Transfection

Chinese hamster ovary (CHO), COS-1, K-1034, and human diploid embryonic lung fibroblast (HEL) cells were cultured in DMEM supplemented with 10% fetal bovine serum (Intergen, New York, NY) and maintained in a humid incubator at 37°C in a 5% CO₂ environment. Cells were transiently transfected with 1.5 µg of the p180 constructs or a control plasmid using Fugene-6 (Roche Diagnostics, Mannheim, Germany) or Lipofectamine-plus (Invitrogen), according to the corresponding manufacturer's instructions. Because a well-organized ER structure is most obviously visible after the addition of ascorbic acid (unpublished data), HEL cells were cultured in the presence of 200 µM ascorbic acid for studies of ER morphology. To generate a series of stable transfectants overexpressing human p180, CHO cells were transfected with pcDNAp180-54R and selected with 100 µM zeocin, followed by limited dilution. Concomitantly, control cell lines were selected after transfection with the vehicle plasmid alone. The parental CHO cells contained very little p180, whereas an unknown 160-kDa protein was detected by an anti-p180 C1 antibody (Ogawa-Goto *et al.*, 2002). Among the obtained clones, we used six clones for assays of acetylated tubulin.

Antibodies

Affinity-purified rabbit antibodies against human p180 (C1 and N1) were described previously (Ogawa-Goto *et al.*, 2002). Monoclonal antibodies (mAbs) against the N-terminal domain of human p180 were established by immunization of BALB/c mice with a recombinant (His)₆-tagged polypeptide containing amino acids 27–157 by Nippon Biotest (Tokyo, Japan). The established clones, 4H6 (IgG1, kappa chain) and 1E3 (IgG2b, kappa chain), specifically bound to the N-terminal region of p180 in Western blot and ELISA analyses (data not shown). Rabbit polyclonal antibodies against GFP (Clontech), protein disulfide isomerase (PDI) and calnexin (Cnx; Stressgen, San Diego, CA), mAbs against acetylated tubulin (6-11B-1) and α -tubulin (B-51-2; Sigma-Aldrich, St. Louis, MO), and goat antibodies against Crt and ribo-

phorin II (Santa Cruz Biotechnology, Santa Cruz, CA) were obtained from the respective suppliers.

Immunofluorescence Microscopy

Cultured cells were fixed with paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with antibodies as described previously (Ogawa-Goto *et al.*, 2002). The cells were imaged using an LSM410 confocal microscope system with a 63× 1.4 NA Plan-Apochromatic DIC objective lens (Carl Zeiss, Jena, Germany). In some experiments, an FV100 confocal microscope system with a 40× 1.3 NA lens was used (Olympus, Tokyo, Japan). For measurement of the ER area, fluorescence signals of PDI or Crt staining were captured using the LSM410 system. Signals of control and transfected cells were captured by the same acquisition processes and quantified using the public domain software Image J (<http://rsb.info.nih.gov/ij>). The ER area (in pixels) per cell was profiled and extracted by "particle analysis" with a constant threshold. In a preliminary experiment, the total cell number in the field was simultaneously counted after nuclear staining and indicated that the process could extract appropriate numbers of cells. The average ER area (in pixels) per cell was evaluated in at least 100 cells and compared with that in concomitantly prepared control cells.

Analyses of the Membrane Fraction of p180-transfected Cells

Transiently transfected cells or stable transfectants were washed with phosphate-buffered saline (PBS), scraped and collected by brief centrifugation. The recovered cells were suspended in 50 mM Tris-HCl buffer containing 5 mM MgSO₄, 5 mM EGTA, 1 mM EDTA, and Complete proteinase inhibitor mixture (Roche Diagnostics) and homogenized with a glass homogenizer until there were no intact cells in the suspension, as evaluated by light microscopy. The cell lysate was then centrifuged at 1400 × g for 10 min, and the postnuclear supernatant was ultracentrifuged at 100,000 × g for 1 h to sediment the membrane fraction. Soluble proteins in the resulting supernatant were precipitated by adding trichloroacetic acid solution on ice and collected by centrifugation. The samples were analyzed by SDS-PAGE and Western blotting.

In Vitro MT-Binding Assays with Recombinant Polypeptides

Glutathione S-transferase (GST)-fusion proteins of various regions of p180 were prepared as described previously (Ogawa-Goto *et al.*, 2002). Bacterially expressed (His)₆-tagged polypeptides were purified using Talon resin (BD Bioscience Clontech) according to the manufacturer's instructions. MTs were polymerized by incubating 5 mg/ml bovine brain tubulin (Cytoskeleton, Denver, CO) in 100PEM buffer (100 mM Pipes, pH 6.8, 2 mM MgCl₂, 1 mM EGTA) containing 1 mM GTP and 10% glycerol for 20 min at 35°C. The polymerized MTs were diluted to 0.5 mg/ml, incubated with 20 µM taxol (Sigma-Aldrich) in 100PEM buffer for 10 min, and then mixed with various polypeptides to a final concentration of 2 µM in a total volume of 50–100 µl. The final concentration of NaCl was adjusted to 120 mM unless otherwise described, and the samples were incubated for 30 min. Aliquots of the reaction mixtures were layered onto 100 µl of a cushion buffer (100PEM buffer containing 10 µM taxol and 10% glycerol) and centrifuged in a TL555 or TLA100 rotor (Beckman, Fullerton, CA) at 30,000 × g for 30 min at 25°C. The resulting supernatants and pellets were analyzed by SDS-PAGE. In some experiments, bundle formation was monitored by the OD at 350 nm in a DU530 spectrophotometer (Beckman).

Chemical Cross-Linking of (His)₆-tagged Polypeptides

An aliquot (200 µg/ml) of each (His)₆-tagged recombinant polypeptide in 100 mM phosphate buffer (pH 7.4) was cross-linked by adding an equal volume of 20 mM dimethyl pimelimidate (DMP) dihydrochloride (Pierce, Rockford, IL) in 0.2 M triethanolamine (pH 8.2). After a 30-min incubation at room temperature, the reaction was stopped by the addition of 0.5 M Tris-HCl (pH 8.0), and the samples were analyzed by SDS-PAGE.

In Vitro MT-Binding Assays with Full-Length p180

MT-binding assays of immunoprecipitated p180 were carried out as follows. The antibody-antigen complexes were recovered from cell lysates using an anti-C1 or anti-GST antibody and protein A-conjugated magnetic beads (Dynabeads protein A; Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions. After washing, the magnetic beads were blocked with 2% bovine serum albumin in PBS, washed with buffer (50 mM HEPES, pH 7.4, 2 mM EGTA, 100 mM NaCl, 1 mM MgCl₂), and incubated with taxol-stabilized MTs in the same buffer for 1 h at room temperature. After extensive washing, the beads were recovered and subjected to SDS-PAGE and Western blot analyses. The presence of MTs in the bead fraction was detected with an anti- α -tubulin antibody. SDS-PAGE and Western blotting of p180 were carried out as described previously (Ogawa-Goto *et al.*, 2002).

To prepare cell lysates for in vitro cosedimentation assays, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100) containing Complete proteinase inhibitor

mixture for 20 min on ice and then centrifuged at $8000 \times g$ for 30 min. The soluble fraction was further clarified at $25,000 \times g$ for 30 min, and the supernatant was diluted 1:2.5 with MT-binding buffer (80 mM PIPES, pH 6.9, 2 mM EGTA, 100 mM NaCl, 1 mM $MgCl_2$) containing Complete proteinase inhibitor mixture, 2 mM DTT, and 10 μM taxol. For cosedimentation assays, in vitro-polymerized MTs (10 μg /assay, as described above) were added to 100 μl of the diluted lysates and incubated for 30 min at room temperature. The samples were centrifuged at $20,500 \times g$, and the supernatants and pellets were analyzed by SDS-PAGE and Western blotting. In normal assays, the final concentration of NaCl was adjusted to 120 mM unless otherwise described.

Electron Microscopy

For electron microscopic analysis of in vitro-polymerized MTs, samples were prefixed with paraformaldehyde in PBS containing 10 μM taxol, fixed with glutaraldehyde, and negatively stained with 1% uranyl acetate. For transmission electron microscopy (TEM) analysis of cultured cells, cells were treated with medium containing 10 μM taxol for 3 min at $37^\circ C$ and then prefixed with 4% paraformaldehyde in PBS containing 10 mM EGTA and 2 mM $MgCl_2$ for 20 min at $37^\circ C$, followed by a conventional fixation procedure for TEM as described previously (Ogawa-Goto *et al.*, 2002). Ultrathin sections were cut parallel to the substrate. MTs were identified by their characteristic tubular structure with a diameter of 24 nm.

Atomic Force Microscopy

Atomic force microscopic analyses were carried out using an SPM-9500J2 scanning probe microscope (Shimadzu, Kyoto, Japan) equipped with a piezo-electric scanner, of which the maximum widths in the x, y, and z scan range were 125, 125, and 8 μm , respectively. A rectangular cantilever with a tetrahedral silicon tip was used at a force constant of 42 N/m and a resonance frequency of 300 kHz (OMCL-AC160TS; Olympus). Both height and phase images were obtained simultaneously in a dynamic mode in air. The scan speed was generally maintained at 1 Hz. The MT bundles were fixed with 4% paraformaldehyde and deposited on glass coverslips. After extensive washing with PBS and distilled water, the samples were air-dried and processed for atomic force microscopic analyses.

Small Interfering RNA Oligonucleotides and Transfection

RNA duplexes (21 nucleotides) with symmetric 3' (2'-deoxy)thymidine overhangs (two nucleotides) corresponding to nucleotides 153–173 of human p180 were synthesized and annealed (Qiagen). Transfection of the small interfering RNAs (siRNAs) was carried out using Oligofectamine (Invitrogen, Rockville, MD) as previously described (Elbashir *et al.*, 2001), and the cells were incubated for 2–4 d. For control experiments, fluorescein isothiocyanate-labeled nonsilencing oligonucleotides (Qiagen) were used.

RESULTS

Expression of p180 Leads to Increased Amounts of Acetylated MTs in Cultured Cells

To explore the physiological functions of p180 in mammalian cells, we previously cloned a number of stable CHO transfectants overexpressing human p180 (Figure 1A). Most of these clones showed an extended morphology and a more enhanced MT network than control cell lines or the parental cells, although a prominent change in the MT arrays, such as bundle formation, was occasionally observed by light microscopy (data not shown). Distinct effects of p180 were more clearly visible when the cells were stained for acetylated tubulin (Figure 1A), which is a good marker for non-dynamic stable MTs (Bulinski and Gundersen, 1991; MacRae, 1997). Acetylated MTs were mainly detected in the perinuclear region of the control cell lines (Figure 1Aa). In contrast, the p180-overexpressing cell lines exhibited more organized acetylated MTs that extended to the distal region (Figure 1A, b and c). As shown in Figure 1B, Western blotting analysis revealed that acetylated tubulin was mildly increased in the transfectants (lanes 1 and 2) to ~ 1.71 -fold the level in the parental CHO cells (lane 5), whereas acetylated tubulin in the control cell lines (lanes 3 and 4) was 1.14-fold the level in the parental cells. Electron microscopic analyses revealed that laterally coaligned and packed MT bundles were present in a wide area of the cytoplasm in the transfectants (Figure 1C), as has been shown for HEL cells (Ogawa-Goto

Figure 1. Increased acetylation of MTs in p180 transfectants. (A) CHO transfectants expressing human p180 (b and c) or control line cells (a) were fixed and stained for acetylated tubulin (a–c) and p180 (a'–c'). Bars, 25 μm . Typical images of two transfectants (clones 5e and 34j) and one control cell line (clone Z3) at 5 h after plating are shown. (B) Western blotting analysis of acetylated tubulin in p180 transfectants (lanes 1 and 2), control line cells (lanes 3 and 4), and parental CHO cells (lane 5). The levels of acetylated tubulin were estimated by densitometric scanning and normalized by the corresponding levels of β -actin. The transfectants contain $\sim 1.71 \pm 0.1$ -fold (mean $\pm$ SD, $n = 6$) higher levels of acetylated tubulin compared with parental CHO cells, whereas the levels in control line cells are not significantly increased (1.14 ± 0.09 -fold, mean $\pm$ SD, $n = 3$). (C) Electron microscopic image of a p180-transfectant (clone 34f) demonstrating lateral alignment of densely packed MTs. Bar, 250 nm. (D) Membrane fractions were prepared from the p180 transfectants and analyzed by Western blotting. p180 cosediments with the membrane fraction, but is not detected in the soluble fraction. Lane 1, total cell lysate; lane 2, membrane fraction; lane 3, soluble fraction. β -actin and ribophorin II were used as cytosolic and membrane protein markers, respectively. (E) CHO cells were transfected with pcDNAp180-54R, fixed at 24 h after transfection and double-stained for p180 (right) and acetylated tubulin (left). Transient p180 overexpression leads to an elongated morphology and heavily enhanced bundles of acetylated MTs. Bars, 20 μm .

et al., 2003). In these transfectants, p180 was found to associate with membranes (Figure 1D, lane 2), but was not detected in the soluble fraction (lane 3). Furthermore, enhanced acetylated MT arrays were observed after transient transfection in CHO cells (Figure 1E, left) and other cell types, including HeLa cells (data not shown). In the transiently p180-overexpressing cells, acetylated MT arrays were more markedly enhanced (Figure 1E, left) than in the stable transfectants (Figure 1A, b and c), probably because a higher level of p180 was expressed in each single cell that had been transiently transfected (Figure 1E, right).

Depletion of Endogenous p180 by siRNAs Leads to Aberrant MTs and ER

To examine the specific role of p180 in establishing acetylated MTs, p180 was depleted by siRNA transfection into HEL cells, which contain abundant endogenous p180 (Ogawa-Goto *et al.*, 2002). At 3 d after transfection, the level of p180 protein was suppressed to $<30\%$ of the level in

Figure 2. Effects of p180 depletion on the ER and MT pattern. (A) HEL cells were transfected with a p180-targeting siRNA and analyzed at 3 d after transfection by Western blotting and densitometry. The level of p180 is reduced to <30% of the level in control cells, whereas the expression levels of other ER markers, Cnx and Crt, are not markedly reduced. The level of acetylated tubulin is reduced to ~55% of the level in control cells. (B) Control (b) or siRNA-transfected (a) HEL cells were fixed at 72 h after transfection and stained for acetylated tubulin (a and b) and p180 (a' and b'). Control cells transfected with a nonsilencing siRNA contain well-developed acetylated MT bundles, whereas p180-depleted cells display immature acetylated MTs. (C) The numbers of cells carrying acetylated MT bundles were counted and expressed as percentages of the total number of p180-depleted or control cells. Cells containing acetylated MT bundles were defined as cells in which the acetylated MT bundles were ≥ 3 -fold longer than the length of the nuclear long axis. Data represent the means $\pm$ SD of three separate experiments. (D) Control (b and d) or siRNA-transfected (a and c) cells were fixed at 72 h after transfection and stained for α -tubulin (green), Crt (red), and p180 (blue). In most of the control HEL (b) and K-1034 (d) cells, MTs are organized in parallel arrays (b2 and d2). In contrast,

control cells (Figure 2A, top). Control cells transfected with a nonsilencing siRNA contained well-developed acetylated MT bundles (Figure 2Bb), whereas p180-depleted cells displayed immature acetylated MTs (Figure 2Ba). The number of cells possessing extended acetylated MTs was markedly reduced to about one-fourth of the number in control cells (Figure 2C). Consistently, decreased amounts of acetylated tubulin were also detected in p180-depleted cells by Western blotting analyses (Figure 2A, bottom). These results suggest a crucial role for p180 in the organization of acetylated MTs.

Given that the ER uses MTs as a framework for extending its reticular network (Terasaki *et al.*, 1986), the next question to be addressed was whether endogenous p180 affected the overall MT organization and ER morphology. The MT network in p180-depleted cells stained for α -tubulin appeared to change to a radial projection or unaligned random pattern (Figure 2Da2), whereas that in control cells showed laterally coaligned arrays (Figure 2Db2). The same results were observed for K-1034 cells (Figure 2D, c2 and d2), which are retinal pigment epithelial cells that also express a high level of p180. For both types of cells, the numbers of cells possessing a radial MT or nonparallel pattern were greatly increased among p180-depleted cells (Figure 2E). Simultaneously, targeted depletion of p180 severely affected the normal ER distribution, which retracted around the nucleus (Figure 2Da3). This ER retraction was also detected using other ER markers, such as PDI and Cnx (not shown), and differing degrees of p180 depletion seemed to be coincidentally associated with the severity of the ER retraction. Semi-quantitative analyses revealed that the transfected cells exhibited less effective ER spreading than control cells (Figure 2F). Thus, depletion of endogenous p180 markedly affects the ER distribution and causes it to retract from the cell periphery, with a concomitant redistribution of MTs to a radial pattern in these cells.

Binding of Full-Length p180 to In Vitro-polymerized MTs

The rearrangement of MTs and the ER in p180-depleted cells suggested a specific interaction between p180 and MTs. To examine whether p180 binds directly to MTs in vitro, bacterially expressed full-length p180 was prepared and subjected to MT-binding assays. For these experiments, we used a splice variant of p180 carrying 24 repeats and the complete N- and C-terminal domains (24R-p180). 24R-p180 was recovered from bacterial lysates by protein A-conjugated magnetic beads using an anti-p180 antibody and incubated with in vitro-polymerized MTs (Figure 3A). The presence of MTs bound to p180 was detected using an anti-tubulin antibody. 24R-p180 exhibited effective interactions with MTs (lane 4), whereas beads with a control antibody did not (lane 6).

The MT-binding capacity of p180 was further addressed using HEL cell lysates (Figure 3B). p180 in detergent-ex-

Figure 3. Full-length p180 binds to taxol-stabilized MTs in vitro. (A) Bacterially expressed 24R-p180 (lanes 3–6) or control samples (lanes 1 and 2) were captured by protein A-conjugated magnetic beads using an anti-p180 antibody (lanes 1–4) and incubated in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of MTs. After extensive washing, the samples were analyzed by Western blotting with mAb 4H6 (top panel) or an anti-tubulin antibody (middle). The input tubulin before washing is shown at the bottom. IP, immunoprecipitate. (B) Detergent-extracted HEL cell lysates were incubated with (lanes 1, 2, 5, and 6) or without (lanes 3, 4, 7, and 8) MTs that had been in vitro-polymerized and taxol-stabilized. After a brief centrifugation, the samples were analyzed by Western blotting. In the presence of MTs, endogenous p180 in the HEL cell lysates cosediments with MTs and is detected in the pellet (p) fractions, whereas in the absence of MTs, p180 remains in the supernatant (s) fractions. The final concentration of NaCl was adjusted to 120 mM (lanes 1–4) or 200 mM (lanes 5–8). Cnx was blotted as a nonsedimented control using the same PVDF membrane. (C) Detergent extracts of HEL cells were treated with 2 μ g of an anti-N1 or anti-GST antibody for 30 min, and then taxol-stabilized MTs were added and analyzed as described for B. Addition of the anti-N1 antibody (lane 5), but not the anti-GST antibody (lane 1), disturbs the binding of p180 to MTs.

tracted cell lysates was efficiently cosedimented with MTs (lane 1), whereas it remained in the supernatant in the absence of MTs (lane 4). This binding was barely affected when the NaCl concentration was increased to 200 mM (lanes 5–8).

Binding of p180-derived Polypeptides to MTs and Promotion of Bundle Formation In Vitro

To determine the domain responsible for p180 binding to MTs, a panel of GST-fusion proteins containing different portions of p180 was prepared as shown in Figure 4A. Based on its deduced primary sequence, p180 is predicted to be a membrane protein with a short luminal tail and to consist of a highly basic N-terminal region and an acidic C-terminal region comprising 15 segments of predicted coiled-coil domains (Wanker *et al.*, 1995; Langley *et al.*, 1998). MT cosedi-

Figure 2 (cont). p180-depleted cells (a and c) show aberrant MT organization (a2 and c2). Typical cells displaying radial or nonparallel MTs are shown in panel a, except for a nondepleted cell shown by a small arrow. (E) The numbers of cells displaying radial or nonparallel MTs were counted and expressed as percentages of the total number of p180-depleted or control cells. a, HEL cells; b, K-1034 cells. For this assay, intermediate phenotypes containing a partially parallel pattern were not categorized as radial or nonparallel MTs. Data represent the means $\pm$ SD of three separate experiments. (F) The ER area per cell was measured as described in *Materials and Methods* and expressed as the percentage of the ER area of control cells. a, HEL cells; b, K-1034 cells. Data represent the means $\pm$ SD of three separate experiments.

Figure 4. Binding of truncated p180 polypeptides to MTs in vitro. (A) Schematic representations of full-length and truncated constructs of various regions of p180. Human p180 has a predicted transmembrane domain (TM) close to the N-terminus, a highly basic N-terminal domain consisting of lysine clusters (hatched boxes), a basic tandem repeat domain (solid box), and a C-terminal acidic coiled-coil domain comprised of 15 predicted segments (gray boxes; Langley *et al.*, 1998). The numbers on the left of each truncated mutant denote the amino acid residue numbers of human p180 (DDBJ accession number: AB287347). A summary of the in vitro binding assay data is shown on the right. The epitopes of the antibodies used in this study are shown at the top. (B) The recombinant polypeptides were incubated for 30 min at room temperature in the absence (–) or presence (+) of MTs and then centrifuged on a sucrose cushion. Lanes 1–24, Coomassie brilliant blue staining patterns of GST-tagged proteins (lanes 1–16) and His-tagged proteins (lanes 17–24). N1, N1ΔRp, and 25–157 cosediment with MTs. The large arrows indicate the position of tubulin. Molecular markers are shown on the left. P, pellet; S, supernatant. For the assays for N1ΔRp and N1ΔC, we used (His)₆-tagged polypeptides, because GST-tagged N1ΔRp was extremely unstable at neutral pH under the assay conditions. (C) Negatively stained electron micrographs of taxol-stabilized MTs mixed with N1 (left) and GST (right). Bars, 500 nm. A higher-magnification micrograph of N1 (inset) shows laterally aligned MTs. Bar, 200 nm. (D) Atomic force microscopic images of taxol-stabilized MTs with N1 (left) and GST (right). (E) Taxol-stabilized MTs were incubated with N1 or GST for 30 min. Subsequently, various concentrations of calcium (final concentrations: 0.1–100 mM) were added and briefly mixed at each point shown by the arrows, before the OD at 350 nm was monitored.

mentation assays (Figure 4B) revealed that a bacterially expressed N1 polypeptide effectively bound to taxol-stabilized MTs (lane 1), whereas other more COOH-terminal fragments (residues 853–1340; lane 9) and control GST (lane 5)

did not. An NH₂-terminal fragment, residues 25–157, which resides downstream of the predicted transmembrane domain, was also cosedimented (lane 13), indicating the presence of a second, but less effective, MT-binding domain. To

test the interaction of N1 with MTs in more detail, truncated polypeptides containing only the repeat domain, N1ΔC (residues 623–737), or a flanking coiled-coil region, N1ΔRp (residues 738–944), were prepared. For this assay, we used (His)₆-tagged polypeptides, because GST-tagged N1ΔRp appeared to be extremely unstable under the binding assay conditions. Although N1ΔC failed to bind to MTs (Figure 4B, lane 21), the coiled-coil region N1ΔRp showed MT-binding ability (lane 17).

During the cosedimentation assays, we noticed that the MT solutions became extensively turbid in the presence of N1, and to a lesser extent in the presence of N1ΔRp, suggesting bundle formation of the MTs. Electron microscopic analysis revealed that N1 induced dense and laterally aligned MT bundles within 1 min after mixing (Figure 4C, left). By atomic force microscopy, the diameter of the largest N1-induced bundle in Figure 4D (left) was estimated to be $\sim 109.5 \pm 8.7$ nm in height after fixation and dehydration, whereas that of a single filament after incubation with GST (Figure 4D, right) was 10.7 ± 0.49 nm, similar to a previously reported value for air-dried MTs (Vater *et al.*, 1995). These results indicate that at least 10 MTs were assembled in the N1-induced bundle. (His)₆-tagged N1 induced similar MT bundles to GST-N1 (Supplementary Figure S1A, c and d) and its bundling activity appeared to be much higher than that of N1ΔRp (Supplementary Figure S1B). Moreover, monitoring of the turbidity of the N1-induced bundles after calcium addition revealed a remarkable resistance toward calcium-dependent depolymerization (Figure 4E). On the basis of these results, we designated the domain at amino acids 623–944 as MTB-1 and the second potential MTB in the N-terminal region (amino acids 25–157) as MTB-2.

Furthermore, *in vitro* sedimentation assays with full-length p180 (Figure 3C) revealed that pretreatment with an anti-N1 antibody (lane 5), but not with a control anti-GST antibody (lane 1), resulted in reduced p180 binding to MTs, suggesting the importance of this domain. Thus, p180 contains a unique MTB domain consisting of a predicted coiled-coil region and repeat domain that exhibits a direct binding capacity for MTs as well as a strong bundling activity *in vitro*.

Dimer Formation by the MTB-1 Domain

The MT-bundling capacity of the MTB-1 domain implied that it could form a dimer. To address this possibility, we assessed the dimer-forming abilities of (His)₆-tagged N1 and other polypeptides *in vitro* by chemical cross-linking (Figure 5). After treatment with the cross-linker DMP, both His-N1 (Figure 5, lane 2) and His-N1ΔRp (lane 4) moved more slowly upon SDS-PAGE analysis and were detected at their expected dimer positions, whereas His-N1ΔC remained unchanged (lane 6). These data suggest that MTB-1 forms a dimer naturally and that the responsible domain for dimer formation is the predicted coiled-coil region (N1ΔRp).

Overexpression of the MTB-1 Domain Enhances MT Bundling *In Vivo*

To test whether the MT-bundling activities of MTB-1 are exerted *in vivo*, we generated a series of GFP-tagged proteins containing different regions (Figure 6A) and transfected them in COS-1 cells. The GFP sequence of these constructs was mutated to avoid self-dimerization of GFP (Zacharias *et al.*, 2002). After transfection, Western blot analyses of the chimeras revealed that truncated proteins of the correct sizes were expressed (Figure 6B). For assessment of bundling activities, we examined the effect of each construct on MT organization by α -tubulin staining. Overexpression

Figure 5. Dimer formation by the MTB-1 domain. (A) The dimerization capacities of purified His-N1 (lanes 1 and 2), His-N1ΔRp (lanes 3 and 4), and His-N1ΔC (lanes 5 and 6) were examined by chemical cross-linking, because the N1 domain does not contain any cysteine residues. Lanes 1, 3, and 5, in the absence of DMP; lanes 2, 4 and 6, in the presence of DMP. Each polypeptide was separated by SDS-PAGE and stained with Coomassie brilliant blue. Molecular markers are shown on the left.

of GFP-N1 produced a perinuclear fibrillar pattern that was coaligned with MTs (Figure 6Ca). Even at very low expression levels, GFP-N1 was colocalized with MTs (Supplementary Figure S2a). At very high expression levels, GFP-N1 induced extended and wavy MT bundles (Supplementary Figure S2b), which were also positive for acetylated tubulin (Supplementary Figure S3). A truncated construct, GFP-N1ΔRp, exhibited a similar, but less effective phenotype to GFP-N1 (Figure 6Cb), whereas the repeat domain alone, GFP-N1ΔC, did not (Figure 6Cc). None of the other GFP-tagged proteins exerted MT-bundling activities, including GFP-1-157 and GFP-27-157 containing the second potential MTB. The phenotype of GFP-WT seemed to be somewhat similar to that of GFP-N1, although the MTs appeared to be crowded and to cross randomly (Figure 6Ce). These results indicate that the MTB-1 domain consisting of the repeat domain and coiled-coil region is capable of binding to MTs and promoting bundle formation *in vivo* when expressed alone. Again, a specific region of human p180 corresponding to amino acids 738–944 emerged as the minimum domain required for MT binding, although its bundling activity was less than that of MTB-1.

Overexpression of p180 Leads to Enhanced Acetylated MTs in an MTB-1-dependent Manner

We assessed the role of the MTB-1 domain *in vivo* using WT and mutant proteins either possessing or lacking the domain (Figure 7A). GFP-fused chimeras were expressed after transfection and analyzed by Western blotting with mAb 4H6, which confirmed that the chimeric proteins migrated at their expected relative molecular masses (Figure 7B). Again, the chimeras were found to be associated with membranes (Supplementary Figure S4). Confocal laser microscopy revealed that GFP-WT was localized in a perinuclear reticular pattern (Figure 7Ca1). Overexpression of GFP-WT led to rearrangement of the acetylated MTs into broad perinuclear bundles in $\sim 12\%$ of the cells expressing high levels of GFP-WT (Figure 7D, top). In the peripheral region of these cells, the GFP-WT signals showed a fibrillar pattern that was coaligned with an extended meshwork of acetylated MTs (Figure 7Ca). Deletion of the COOH-terminal region led to a striking phenotype. Specifically, the cells expressing high levels of GFP-Δ956 contained long and wavy bundles of acetylated MTs, which mostly overlapped with the GFP-Δ956 signals (Figure 7Cb). When stained for α -tubulin, less clear, but distinct, bundling was observed in cells expressing GFP-Δ956 (Supplementary Figure S5b). In contrast,

Figure 6. Overexpression of the MTB-1 domain induces MT bundles in vivo. (A) Schema representing wild-type p180 and various GFP-tagged mutants. A summary of the results from transient transfection experiments in COS-1 cells is shown on the right. The regions of MTB-1 and MTB-2 are shown at the top. In these constructs, the GFP sequence was mutated to avoid self-dimerization. (B) At 16–18 h after transfection, the different GFP-tagged mutants expressed in COS-1 cells were blotted with an anti-GFP antibody. (C) COS-1 cells were transfected with the GFP-tagged mutants or WT p180. (a) GFP-N1; (b) GFP-N1ΔRp; (c) GFP-N1ΔC; (d) nontransfected cells; (e) GFPPer-WT. After 18–20 h, the cells were fixed and stained for α -tubulin (a1–e1). An enlarged image of the boxed region in panel a is shown in a'. Cells marked with asterisks are nontransfected cells. Bars, 10 μ m. (D) Fluorescence images were captured, and cells displaying circular MTs (■) or bundled MTs (□) were counted. A typical example of cells displaying circular MTs is shown in Figure S2c, whereas a typical example of bundled MTs is shown in Figure S2b. Data are expressed as percentages of the total number of GFP-expressing cells and represent the means of two separate experiments. (E) COS-1 cells were transfected with GFP-N1, fixed at 16 h after transfection, and subjected to TEM analysis. In the perinuclear region of these cells, laterally coaligned MTs (arrows in top panel) are observed, which overlap with undefined high-density structures. Loose and twisted MT bundles can also be seen around the structures (bottom). Bars, 250 nm.

the MTB-1 deletion mutant (GFPPer- Δ 623–956) failed to induce perinuclear acetylated MT bundles, even at very high levels of expression (Figure 7C). Because the MTs in some GFPPer-WT-expressing cells were partially resistant to the MT depolymerization reagent nocodazole (data not

shown), p180 overexpression seemed to moderately stabilize MTs, but less efficiently than has been reported for MAP2 or tau-transfected cells (Kanai *et al.*, 1989; Lee and Rook, 1992). Thus, p180 led to enhanced acetylated MTs in an MTB-1-dependent manner.

Figure 7. Acetylated MT patterns after overexpression of WT and mutant p180s. (A) Schema representing WT p180 and expression constructs for GFP-tagged mutants of p180. The regions of MTB-1 and -2 are shown by hatched boxes. S, signal sequence. The epitopes of the antibodies used in this study are shown at the top. (B) COS-1 cells were mock-transfected (lane 1) or transfected with GFPPer-WT (lane 2), GFPPer-Δ956 (lane 3), or GFPPer-Δ623-956 (lane 4). At 48 h after transfection, a Western blotting analysis was performed using mAb 4H6. Cnx was used as a loading control. (C) Immunofluorescence images of cells transfected with (a) GFPPer-WT, (b) GFPPer-Δ956, or (c) GFPPer-Δ623-956, as well as nontransfected cells (d). a1–d1, GFP signals; a2–d2, acetylated tubulin staining. Merged signals are shown on the left. Bars, 10 μ m. (D) Cells displaying bundles of acetylated MTs (top) and circular acetylated MTs (bottom) were counted as described for Figure 6D. Data are expressed as percentages of the cells expressing high levels of GFP and represent the means of two separate experiments.

DISCUSSION

p180 is an integral ER membrane protein that was first identified as a ribosome-binding receptor on the rough ER (Savitz and Meyer, 1990). Although previous studies using heterologous overexpression of canine p180 in yeast indicated its involvement in ER membrane proliferation and secretory pathways (Wanker *et al.*, 1995; Becker *et al.*, 1999), very little is known about its roles in mammalian cells. Here, we have demonstrated a new functional role for p180 in interactions between the ER and the MT cytoskeleton, which are mainly mediated by a newly identified domain designated MTB-1.

MT-Binding Properties of p180

We have presented data that MTB-1 promotes MT bundling both *in vivo* and *in vitro* when expressed alone, similar to other MAPs that are considered to induce MT assembly and stabilization *in vivo* (Mandelkow and Mandelkow, 1995). The bundling activity of MTB-1 was as strong as those of structural MAPs, although the primary sequence of the domain does not contain the typical repeat sequence found in MAPs (Lewis *et al.*, 1988). Motif analyses previously predicted that the carboxyl half of p180 would form a rod-like coiled-coil structure (Leung *et al.*, 1996; Langley *et al.*, 1998). MTB-1 consists of this coiled-coil region and the repeat

domain. Furthermore, the coiled-coil domain appeared to be the domain responsible for the dimeric conformation of p180, which is assumed to be a prerequisite for MT bundling, whereas the repeat region has been reported to act as the ribosome-binding domain (Wanker *et al.*, 1995). MTB-1-induced MT bundles exhibited remarkable resistance toward calcium-induced depolymerization *in vitro*, although the underlying mechanism of this high resistance toward calcium remains to be clarified. Furthermore, full-length p180 was shown to bind directly to MTs *in vitro* using recombinant proteins. Because an anti-MTB-1 antibody abolished the MT-binding activity of p180, the binding is assumed to be domain-dependent, at least partially. Thus, full-length p180 seems likely to exhibit its MT-modulating activity mainly via MTB-1 and to take a dimeric or oligomeric conformation, although we cannot rule out the possibility that other regions could play roles in MT modulation *in vivo*. Indeed, we also identified the MTB-2 domain, which shows a lesser MT-bundling activity.

p180 Is an MT-Binding Protein on the ER

Overexpression of p180 led to increased acetylation of α -tubulin, as has been reported for MAPs (Kanai *et al.*, 1989). Consistently, p180 depletion resulted in reduced amounts of acetylated MTs. Because acetylated tubulin is a marker of nondynamic stable MTs, but absent from more dynamic MT structures (Bulinski and Gundersen, 1991; MacRae, 1997), the expression levels of p180 seem to affect the dynamic instability of the MT cytoskeleton. Moreover, our EM study clearly revealed MT bundles in p180-overexpressing transfectants, suggesting that MT bundling and stabilization may be coupled, as reported previously (Chapin *et al.*, 1991). Overexpression of p180 led to the induction of acetylated MTs in an MTB-1-dependent manner. The MT-modulating activity of p180 was further verified by siRNA experiments showing that p180 is required for maintaining the overall parallel MT patterns and ER distribution. It is noteworthy that p180 at its normal expression level was sufficient to maintain the MTs and ER in both HEL (fibroblastic) and K-1034 (epithelial) cells. Moreover, our finding of ER retraction after p180 depletion appears to be reminiscent of the ER phenotype after kinesin suppression (Feiguin *et al.*, 1994). These results imply roles for p180 in linking the ER and MTs and maintenance of the ER, as has been reported for CLIMP-63 (Klopfenstein *et al.*, 1998), although the definitive role of p180 in ER morphology remains to be clarified in further studies. It is also unclear whether the elongated cell morphology is a direct consequence of p180 overexpression. Nevertheless, it is likely that high levels of p180 expression would be involved in maintaining the MT bundles and consequently affect the cell shape via an altered cytoskeleton network. This idea is consistent with our frequent observations that long cellular extensions formed after p180 overexpression were filled with acetylated MT bundles and p180. In cells expressing high levels of p180, effective mass transport of the ER may occur toward peripheral sites where the surface membrane is actively expanding.

Although the ER tubular network can be formed *in vitro* without an MT cytoskeleton (Dreier and Rapoport, 2000), it is clear that the ER is intimately associated with MTs as a framework (Baumann and Walz, 2001; Voeltz *et al.*, 2002), and that a kinesin motor-dependent process is crucial for ER membrane motility in some mammalian cells (Cole and Lippincott-Schwartz, 1995). Recently, several nonmotor MT-binding proteins have been reported (Vedrenne and Hauri, 2006), and these are either integrated into the ER membrane (Klopfenstein *et al.*, 1998) or associated with the membrane

surface like p22 (Andrade *et al.*, 2004). Among these, CLIMP-63 was first identified as a class of direct linkers between the ER and MTs that contribute to the positioning of the ER (Klopfenstein *et al.*, 1998). Based on our data, p180 would be included in this class of protein. The ER phenotype after overexpression of p180 appears to be similar to that after overexpression of CLIMP-63, although their primary sequences show no obvious homology. In particular, the sizes of their cytoplasmic domains differ greatly (106 amino acids for CLIMP-63 vs. more than 1500 amino acids for p180), implying that the two proteins have distinct modes of binding.

The Cytoplasmic Region of p180 Acts as a Multifunctional Domain

Several factors have been reported to bind to the huge cytoplasmic domain of p180, which is comprised of tandem repeats and a coiled-coil rod (Kim *et al.*, 2002; Ogawa-Goto *et al.*, 2002; Diefenbach *et al.*, 2004). Importantly, a potential kinesin-binding domain has been reported downstream of MTB-1 (Diefenbach *et al.*, 2004), although an *in vivo* interaction with kinesin has not been reported. Indeed, we did not detect kinesin in our samples immunoprecipitated with p180 in HEL cells (data not shown). It remains to be clarified whether p180 interacts efficiently with kinesin *in vivo* using other samples containing abundant kinesin.

Interestingly, p180 shares highly homologous regions with kinectin (Leung *et al.*, 1996; Langley *et al.*, 1998), an ER membrane protein that binds to kinesin motors (Toyoshima *et al.*, 1992). We examined whether the conserved domain binds to MTs *in vivo* and *in vitro*, but failed to detect any interactions. Because the primary sequence of p180 predicts many, as-yet unidentified, functional domains, further studies on p180 may provide new insights into our understanding of the association between the ER and the MT network.

Weak Bundling Activities of WT p180 in Cells

The MT-bundling activity of the C-terminal truncation mutant GfPer- Δ 956 was extremely strong and was similar to that of MTB-1. Compared with the mutant, the effects of WT p180 overexpression were unexpectedly low, suggesting the presence of an inhibitory effect of its own C-terminal region. Similarly, the MT-stabilizing effect of GfPer- Δ 956 was apparently higher than that of GfPer-WT (data not shown), although both are less efficient than the reported effects of neuronal MAPs, such as MAP2 and tau (Kanai *et al.*, 1989; Lee and Rook, 1992). The highly stable and rigid MT bundles induced by the neuronal MAPs are assumed to play roles in neuronal functions. However, in the case of fibroblastic or epithelial cells, the formation of highly stable MT bundles may rather impede their normal cellular processes, and the MAP-like activity of p180 may be negatively regulated in order to avoid the formation of MT bundles that are too rigid. Although the underlying mechanism remains to be elucidated, one possibility is that an inhibitory factor that binds to the C-terminal coiled-coil region may negatively control the MT-bundling activity of MTB-1. Alternatively, some modifications, such as phosphorylation, may be involved in the exhibition of its full activity *in vivo*, as has been demonstrated for several structural MAPs (Mandelkow and Mandelkow, 1995).

p180 and ER Morphology

Remarkable proliferation of rough ER membranes has been reported in yeast cells overexpressing canine p180 in a repeat domain-dependent manner (Becker *et al.*, 1999). However, our electron microscopic studies revealed that the ER

phenotypes of p180 transfectants varied among cell lines of different origins (unpublished data). The major findings included proliferation of anastomosing smooth ER along the MT bundles. Occasionally, a mild increase and redistribution of the rough ER were observed. The reason for the variety of phenotypes is unknown at present, but one reason may be possible in-frame splice variants that possess different lengths of repeats (Wanker *et al.*, 1995; Langley *et al.*, 1998). Further studies are currently being undertaken to elucidate the definitive effects of p180 on ER morphology. Interestingly, an early EM study on chondroblasts strongly suggested a close relationship between the rough ER and MTs (Tokunaka *et al.*, 1983). They have clearly demonstrated that ribosomes have been selectively displaced by MTs on the rough ER membrane in chondroblasts after taxol treatment. Our identification of the MT-binding features of p180, which also has a ribosome-binding domain (Wanker *et al.*, 1995), may provide further insights into the intimate interactions between the rough ER and MTs.

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