Suppression of Tubulin Polymerization by the LKB1-Microtubule-associated Protein/Microtubule Affinity-regulating Kinase Signaling*

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LKB1, a tumor suppressor gene mutated in the Peutz-Jeghers syndrome, encodes a serine/threonine protein kinase. Recent biochemical studies have shown that LKB1 activates 14 AMP-activated protein kinase-related kinases including MARKs (microtubule-associated protein/microtubule affinity-regulating kinases) that regulate microtubule dynamics. Here we show in vitro that LKB1 phosphorylates and activates MARK2, which in turn phosphorylates microtubule-associated protein Tau at the KXGS motif and suppresses tubulin polymerization. In cells, forced expression of LKB1 suppresses microtubule regrowth, whereas LKB1 knockdown accelerates it. We further show that the phosphorylation of Tau by the LKB1-MARK signaling triggers proteasome-mediated degradation of Tau. These results indicate that LKB1 is involved in the regulation of microtubule dynamics through the activation of MARKs.

LKB1 is a tumor suppressor gene encoding a serine/threonine protein kinase. Linkage analyses showed that mutations in LKB1 are responsible for the Peutz–Jeghers syndrome, an autosomal dominant disease characterized by gastrointestinal hamartoma, mucocutaneous pigmentation, and an increased risk of cancer (1–3). Somatic LKB1 mutations have been reported also in some sporadic cancers (4–6). Phenotypes in Lkb1 knock-out mice further support that LKB1 is a tumor suppressor gene. Specifically, Lkb1+/− mice develop gastrointestinal hamartomas after >20 weeks of age and hepatocellular carcinomas after >50 weeks (7–10).

Regarding the normal functions of LKB1, several lines of evidence indicate that LKB1 plays an important role in cell polarity. In Caenorhabditis elegans and Drosophila melanogaster, the LKB1 orthologs par-4 and lkb1, respectively, regulate cell polarity (11, 12). Also in mammalian cells, the LKB1 activity can induce complete cell polarization (13), suggesting the evolutionary conservation of its role in cell polarity.

The molecular mechanism(s) by which LKB1 acts as a tumor suppressor and controls cell polarity is poorly understood. However, recent biochemical studies have provided important clues to the mode of action of LKB1. First, full activation of LKB1 requires two accessory subunits, STE20-related adaptor pseudokinase (STRAD) and scaffolding protein MO25 (14, 15). Second, this complex activates two AMP-activated protein kinases (AMPKs; AMPKα1 and AMPKα2) by phosphorylating the LXT motif in the activation loop (16–19). Furthermore, in silico search of human kinome has revealed that other 14 serine/threonine kinases have the LXT motif in the activation loop (5, 18). In fact, LKB1 does activate 12 of them (MARK1, MARK2, MARK3, MARK4, NUA1K1, NUA2K2, SAD-A, SAD-B, SIK, SNRK, QIK, and QSK) in addition to two AMPKs (5, 18).

Interestingly, 6 of 14 kinases possibly downstream of LKB1, namely MARK1/2/3/4 and SAD-A/B, have been reported to regulate microtubule (MT) dynamics through phosphorylation of microtubule-associated proteins (MAPs; Tau, MAP2, and MAP4) at the conserved KXGS motifs that face the microtubule lattice (20–22). It remains to be determined whether the other eight downstream kinases affect MT dynamics. The phosphorylation of KXGS motifs reduces the activity of microtubule-associated proteins (MAPs) that stabilize MTs, presumably by weakening the electrostatic interaction between MAPs and MTs (20, 23). Genetic analyses of D. melanogaster have suggested that Drosophila lkb1 has a functional link to the Drosophila MARK ortholog par-1 that regulates MT dynamics and cell polarity (12, 24, 25). Moreover, the genetic studies in C. elegans have revealed that the MARK ortholog par-1, in addition to the LKB1 ortholog par-4, is essential for cell polarization (11). Despite such circumstantial evidence, roles of LKB1 in MT dynamics have remained elusive. Here we provide both biochemical and cell biological evidence that LKB1 regulates MT dynamics through MARK, especially MARK2 that has been the best characterized in the MT-dependent cellular processes including establishment of cell polarity (26–31).

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3 The abbreviations used are: STRAD, STE20-related adaptor pseudokinase; Adv, adenovirus; AMPK, AMP-activated protein kinase; KD, kinase-dead type; MARK, microtubule-associated protein/microtubule affinity-regulating kinase; MEF, mouse embryonic fibroblast; MT, microtubule; WT, wild-type; TA, T208A; GST, glutathione S-transferase; MOPS, 4-morpholinepropanesulfonic acid; siRNA, small interfering RNA.
EXPERIMENTAL PROCEDURES

Antibodies—Antibodies against C-TAK1 (clone 183.A10.A3), MARK2 (clone 5C12D2), LKB1 (sheep polyclonal, and mouse monoclonal 5c10; Upstate, Charlottesville, VA), AMPKα1/2, phospho-AMPKα1/2 (Thr-172), phospho-MARK1/2/3 (Cell Signaling Technology, Danvers, MA), Tau (clone Tau-5; Chemicon, Temecula, CA), β-actin (clone AC-74), γ-tubulin (clone GTU-88; Sigma), V5 (Invitrogen), and β-tubulin (clone YL1/2; Chemicon, Temecula, CA) were obtained from commercial sources. Note that the mouse monoclonal antibody C-TAK1 and Tau-5 recognize MARK2/3 (18) and total Tau protein, respectively. The mouse monoclonal antibody 12E8, specific to phospho-Tau (clone Tau-5; Chemicon, Temecula, CA) were obtained from commercial sources. Note that the mouse monoclonal antibody C-TAK1 and Tau-5 recognize MARK2/3 (18) and total Tau protein, respectively.

cDNA—The following cDNAs were isolated by standard PCR-based cloning techniques; human LKB1 (NCBI accession number NM_000455), human STRADα (NCBI accession number NM_001003787), human MARK2 transcript variant 2 (NCBI accession number NM_004954), and human Tau transcript variant 4 (NCBI accession number NM_016841). Hemagglutinin, FLAG, and V5 tags were attached to the amino termini of LKB1, STRADα, and MARK2, respectively, by PCR-based methods. LKB1 (kinase dead (KD), a catalytically inactive version of LKB1 with a D194A mutation (33)), MARK2 (T208A (TA)) (18, 34), MARK2 (KD, a catalytically inactive mutant with T208A/S212A substitutions (this double mutant has no detectable kinase activity, probably because the S212A mutation changes the conformation of the ATP binding site)) (34, 35), and mutant Tau (S2A, a KXS motif-free variant with S262A/S356A substitutions (S2A, i.e. S262A/S356A)) (36) were generated with QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol.

Recombinant Adenoviruses—Recombinant adenoviruses were constructed by Adeno-X Expression System 1 (Clontech Laboratories, Mountain View, CA) according to the manufacturer’s protocol. The titers of the recombinant adenoviruses were determined by the method previously reported by others (37). Every recombinant adenovirus was infected at a multiplicity of infection number NM_000455), human STRADα (NCBI accession number NM_001003787), human MARK2 transcript variant 2 (NCBI accession number NM_004954), and human Tau transcript variant 4 (NCBI accession number NM_016841). Hemagglutinin, FLAG, and V5 tags were attached to the amino termini of LKB1, STRADα, and MARK2, respectively, by PCR-based methods. LKB1 (kinase dead (KD), a catalytically inactive version of LKB1 with a D194A mutation (33)), MARK2 (T208A (TA)) (18, 34), MARK2 (KD, a catalytically inactive mutant with T208A/S212A substitutions (this double mutant has no detectable kinase activity, probably because the S212A mutation changes the conformation of the ATP binding site)) (34, 35), and mutant Tau (S2A, a KXS motif-free variant with S262A/S356A substitutions (S2A, i.e. S262A/S356A)) (36) were generated with QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol.

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Production of Recombinant MARK2 Proteins in Escherichia coli—To attach GST protein to the amino terminus of MARK2 (wild type (WT)/TA), the Sall/NotI fragment of the full-length cDNA encoding the wild-type or mutant MARK2 (TA) was inserted in the Sall/NotI-digested pGEX-6P-1 vector (GE Healthcare). One liter of LB medium was inoculated with the strain carrying the plasmid pGEX-6P-1-MARK2 WT/TA, and the mixture was used for induction at 20 °C. After induction for 24 hours, the mixture was harvested and the lysates were prepared by sonication. The lysates were then dialyzed against phosphate-buffered saline, 2% (v/v) Triton X-100, 1 mM EDTA, and 1 mM dithiothreitol.

After ultrasonic DNA shearing, the debris was removed from the lysates by centrifugation. The recombinant proteins were purified on Glutathione-Sepharose 4B beads (GE Healthcare) and then eluted from the beads with 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1% (v/v) Triton X-100, 1 mM dithiothreitol at 4 °C for 30 min.

In Vitro Phosphorylation Assay—Phosphorylation reactions were performed at 30 °C in the phosphorylation reaction buffer (8 mM MOPS (pH 7.0), 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 200 μM dithiothreitol, and 100 μM ATP). In Fig. 1A, MARK2 (WT; 800 nM) or MARK2 (TA; 800 nM) was incubated with or without LKB1/STRADα/MO25a (25 nM) (Upstate) for 15 min. The reactions were stopped by the addition of 5× sample buffer (625 mM Tris-HCl (pH 6.8), 10% (v/v) SDS, 0.05% (v/v) bromophenol blue, 5% (v/v) glycerol, and 25% (v/v) 2-mercaptoethanol). In Fig. 1B, after the phosphorylation reaction of MARK2 (WT/TA; 80 nM) by LKB1/STRADα/MO25a (25 nM) for 15 min, the reaction mixtures were incubated with Tau protein (2 μM; Invitrogen) for 3 min. The reactions were then terminated by the addition of 5× sample buffer. Tubulin Polymerization Assay—Tubulin polymerization assay kit (Cytoskeleton, Denver, CO) was used according to the manufacturer’s protocol. Polymerization was performed with 30 μM tubulin and 0.3 μM Tau in the polymerization buffer without glycerol. MARK2 (WT; 800 nM) was incubated with LKB1/STRADα/MO25a (25 nM) in the phosphorylation reaction buffer at 30 °C for 15 min. Tau (3 μM) was added, and the mixtures were incubated for an additional 15 min. The control reaction containing MARK2 protein (800 nM) was incubated without ATP. The samples were boiled for 3 min to inactivate LKB1 and MARK2 and then used for polymerization reaction. Tau is a heat-stable protein and remains active in the enhancement of tubulin polymerization even after the heat treatment. The turbidity was monitored at 340 nm every minute for 60 min at 37 °C with a spectrophotometer Benchmark Plus Microplate Spectrophotometer (Bio-Rad). Each sample was determined in duplicate polymerization assays in three independent experiments.

Reverse Transcription-PCR—Total RNA of MEF3-2 was extracted with ISOGEN solution (Nippon Gene, Tokyo, Japan), and cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). PCR analyses of human Tau and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression were performed with the following primer pairs: Tau sense, 5'-CAAGCTCGCATGGTCAGTAA-3'; Tau antisense, 5'-TCTCAGTGAGCCGGATCTTT-3'; GAPDH sense, 5'- ACCACGTCATGGCCATCAC-3'; GAPDH antisense, 5'-TCCACCACTCTTGGTCTGTA-3'.

Western Blotting—Cells were lysed in the lysis buffer (10 mM HEPES (pH 7.4), 50 mM NaCl, 50 mM Na₂PO₄, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 100 mM NaVO₄, and 0.1% (v/v) Triton X-100) containing Complete Mini protease inhibitor mixture (Roche Applied Science) and Phosphatase Inhibitor Mixture 1 (Sigma). Lysates were separated by SDS/PAGE and transferred to Hybond-ECL membrane (GE Healthcare). After blocking with Blocking One or Blocking One-P...
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(Nacalai Tesque, Kyoto, Japan), the membranes were probed with anti-phospho-MARK1/2, anti-MARK2/3, anti-phospho-Tau (Ser-262), anti-LKB1, anti-phospho-AMPKα1/2 (Thr-172), anti-AMPKα1/2, anti-β-actin, anti-V5, Tau-5, or 12E8 antibody. The signals were visualized by Immobilon western detection system (Millipore, Billerica, MA) or ECL Western blotting detection reagents (GE Healthcare).

Establishment of an Lkb1-null Mouse Embryonic Fibroblast (MEF) Cell Line—Lkb1+/− mice (C57BL/6N background) were crossed with outbred ICR mice, and offspring were intercrossed. Lkb1-null embryos at 9.5 days post-coitum were miniced, trypsinized briefly, and placed on 24-well plates. These cells were cultured in RPMI1640 (Sigma) with 10% (v/v) fetal bovine serum (BioWest, Nuaillé, France) and 50% (v/v) conditioned medium from the MEFs that were derived from wild-type embryos at 12.5 days post-coitum. We obtained a spontaneously immortalized cell line (MEF3) by continuous passages (>3 months) of the Lkb1-null MEFs and further established the subclone MEF3-2 cell line. Finally, we cultured MEF3-2 in Dulbecco’s modified Eagle’s medium (Sigma) with 10% (v/v) fetal bovine serum.

Small Interfering RNA (siRNA) Vector and Stable LKB1 Knockdown in HepG2 Cell Lines—The human hepatoblastoma cell line HepG2 was obtained from Cell Resource Center for Biomedical Research, Tohoku University, and cultured in Dulbecco’s modified Eagle’s medium with high glucose (Sigma) supplemented with 10% (v/v) fetal bovine serum. As previously described (13), LKB1-pTER was constructed by inserting the siRNA oligonucleotides against LKB1 (5′-CGAAGAGAGACCGAGAAAATG-3′) into pTER vector (38). LKB1-pTER was converted into LKB1-pTERlox containing the hygromycin B resistance gene and loxP sites. HepG2 cells were transfected with the LKB1-pTERlox by using Effectene Transfection reagent (Qiagen, Hilden, Germany). Stable LKB1 knockdown cell lines were established by the hygromycin B (Invitrogen) selection (250 μg/ml). These clones were infected with recombinant adenovirus containing Cre recombinase or LacZ gene. Clones #15H and #31H (“H” for high level of LKB1 expression) were cultured without hygromycin B, but clones #15L and #31L (“L” for low level of LKB1) were maintained in the presence of hygromycin B (250 μg/ml). To minimize the effect of hygromycin B, #15L and #31L were incubated without hygromycin B for at least 24 h before the microtubule regrowth assays.

Proteasome Inhibitors and Lithium Treatment—MG132 (10 μM; Sigma) or clasto-lactacystin β-lactone (5 μM; Calbiochem) was added to the MEF3-2 cells 4 h before the harvest. For lithium treatment cells were incubated with 20 mM LiCl or NaCl simultaneously with the recombinant adenovirus infection.

Microtubule Regrowth Assay—Serum-starved MEF3-2 cells on the coverslips were treated with 33 μM nocodazole (Sigma) for 2 h at 37 °C. After 3 washes with Dulbecco’s modified Eagle’s medium pre-warmed at 30 °C, the cells were incubated in fresh Dulbecco’s modified Eagle’s medium at 30 °C. In experiments using HepG2, fetal bovine serum (10%; v/v) was added to the medium throughout the entire process. The cells were fixed in −20 °C methanol for 3 min and processed for immunofluorescence staining.

Immunofluorescence Staining—Cells fixed in −20 °C methanol for 3 min were re-hydrated in phosphate-buffered saline for 15 min. After blocking with 10% (v/v) goat serum and 3% (w/v) bovine serum albumin in phosphate-buffered saline for 1 h at room temperature, the coverslips were incubated with anti-β-tubulin and anti-γ-tubulin antibodies overnight at 4 °C. After rinses with phosphate-buffered saline, the coverslips were incubated with Alexa Fluor 488 goat anti-rat and Alexa Fluor 594 goat anti-mouse secondary antibodies (Molecular Probes, Eugene, OR). The coverslips were then examined with Leica DM6000B fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 40× objective. Images were captured with ORCA-ER cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). High magnification images were collected on Leica TCS SL laser scanning confocal microscope (Leica Microsystems) equipped with a 63× oil-immersion objective.

Statistical Analysis—Statistical analyses were performed by using GraphPad PRISM version 4 (GraphPad Software, San Diego, CA). Values of p < 0.05 were considered significant.

RESULTS

Suppression of Tubulin Polymerization by LKB1 in Vitro—To examine the potential role of LKB1 in MT dynamics, we first tested whether LKB1 would affect Tau phosphorylation in vitro. LKB1, together with STRADα and MO25α subunits, phosphorylated the activation loop of protein kinase MARK2 at threonine 208 (Fig. 1A, WT) but not the T208A mutant (Fig. 1A, TA), consistent with a report by others (18). We then examined whether LKB1 activity could modulate the activity of MARK2 in phosphorylating microtubule-associated protein Tau at serine 262 of the KXGS motif (39). In MT dynamics, Ser-262 of Tau is reported to be the most important residue that is phosphorylated by MARK2. More specifically, Ser-262 phosphorylation reduces its affinity to MTs by ~75%, thereby suppressing its effects on tubulin polymerization (39). As expected, LKB1 increased Tau phosphorylation at Ser-262 through MARK2 (Fig. 1B). In contrast, in the absence of LKB1, both wild-type MARK2 (WT) and mutant MARK2 (TA) showed only low and basal-level kinase activity on Tau. In addition, LKB1 failed to activate the mutant MARK2 (TA), and LKB1 by itself did not directly phosphorylate Tau at Ser-262. These results show that LKB1 stimulates Tau phosphorylation through MARK2 in vitro. We, therefore, examined the roles of LKB1 on the in vitro tubulin polymerization reaction in the presence of 0.3 μM Tau protein (Fig. 1C). Under this assay condition, in the absence of Tau, no tubulin polymerization was observed, but the addition of Tau (0.3 μM) into the reaction mixture immediately induced tubulin polymerization (data not shown). As a control, MARK2 in the absence of ATP showed a tubulin polymerization curve with a short nucleation phase followed by a fast growth phase and, finally, a steady phase with the maximum polymer mass (Fig. 1C, open circles). MARK2, in the presence of ATP, significantly inhibited tubulin polymerization due to its basal activity (Fig. 1, C–E, open squares; see below), and further addition of LKB1 markedly enhanced the inhibitory effects of MARK2 in a dose-dependent man-
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Recombinant-adenoviral Expression of LKB1 and MARK2 in Lkb1-null MEFs—To investigate the role of LKB1 on MT dynamics in living cells, we expressed LKB1 and MARK2, respectively, in the Lkb1-deficient MEF3-2 using recombinant adenoviruses (Fig. 2A). We designed them so that a transcription-termination cassette (STOP sequence) (40) should suppress expression of LKB1 or MARK2 during adenovirus production, because LKB1 and MARK2 showed cytotoxic activities in HEK293 host cells. Co-infection of MEF3-2 with Adv-Cre should remove the STOP sequence from Adv-LKB1 and Adv-MARK2, thereby expressing LKB1 and MARK2, respectively (Fig. 2B). As expected, Adv-LKB1 (WT), Adv-LKB1 (KD), Adv-MARK2 (WT), and Adv-MARK2 (KD) all expressed the encoded genes in MEF3-2 (Fig. 2, C and D). The adenovirally expressed LKB1 was functional, because LKB1 (WT), but not LKB1 (KD), phosphorylated the activation loop of the endogenous MARK1/2/3 and AMPKa1/2 in MEF3-2 (Fig. 2C) (16–19).

Expression of LKB1Suppresses MT Regrowth in Lkb1-null MEFs—Adenovirus-induced expression of LKB1 or MARK2 in MEF3-2 did not lead to marked alterations in the steady state MT cytoskeleton morphology, as monitored by the anti-β-tubulin immunofluorescence (data not shown). We then investigated the effects of these kinases on MT regrowth. When incubated for 2 h with 33 μm nocodazole, a MT-depolymerizing reagent, all cells showed depolymerization of MTs (Figs. 3A and 3B). As expected, Adv-LKB1 (WT), Adv-LKB1 (KD), Adv-MARK2 (WT), and Adv-MARK2 (KD) all expressed the encoded genes in MEF3-2 (Fig. 2, C and D). The adenovirally expressed LKB1 was functional, because LKB1 (WT), but not LKB1 (KD), phosphorylated the activation loop of the endogenous MARK1/2/3 and AMPKa1/2 in MEF3-2 (Fig. 2C) (16–19).

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A

Adv-LKB1 (Adv-lox-STOP-lox-STRAD-IRES-LKB1)

ITR CMV lox STRAD-IRES-LK1bpα ITR

Adv-MARK2 (Adv-lox-STOP-lox-MARK2)

ITR CMV lox MARK2α pA ITR

FIGURE 2. Recombinant adenovirus-mediated expression of LKB1 and MARK2 in Lkb1-deficient cells. A, schematic structures of the adenoviral constructs. ITR, inverted terminal repeat; CMV, human cytomegalovirus immediate early promoter; lox, lox site sequence; STOP, transcription termination cassette; IRES, internal ribosome entry site; pA, polyadenylation signal. B, removal of the STOP cassette by Cre recombinase. Co-infection of Adv-Cre should induce Cre-mediated excision of the STOP sequence in the cells. C, Cre-mediated expression of LKB1 (WT/KD) in MEF3-2. The cell lysates were prepared 24 h post-infection and analyzed by Western blotting for the exogenously introduced MARK2 with V5-tag and loading control β-actin. The anti-phospho-MARK1/2 (pMARK1/2), anti-phospho-AMPKα1/2 (pAMPKα1/2) antibodies recognized the phosphorylation sites of LKB1. D, Cre-mediated expression of MARK2 (WT/KD) in MEF3-2 cells. At 24 h post-infection, the cell lysates were prepared and analyzed by Western blotting for the exogenously introduced MARK2 with V5-tag and loading control β-actin.

Degradation of Tau Triggered by LKB1—To further investigate the mechanism by which the LKB1-MARK signaling regulates MT dynamics in vivo as well.

FIGURE 2. Recombinant adenovirus-mediated expression of LKB1 and MARK2 in Lkb1-deficient cells.

 Degradation of Tau Triggered by LKB1—To further investigate the mechanism by which the LKB1-MARK signaling regulates MT dynamics, we constructed a recombinant adenoviral construct containing the cDNA for Tau. As expected, LKB1-MARK2-infected with Adv-Tau expressed Tau protein (Fig. 5A, left panel). However, co-expression of LKB1 (WT) and Tau led to a marked decrease in the Tau protein level (Fig. 5A, left panel). Co-expression of MARK2 (WT) also reduced the Tau protein level (Fig. 5A, middle panel). We then found that expression of MARK2 (KD) restored the Tau protein level in the LKB1-expressing cells in a dose-dependent manner (Fig. 5A, right panel). These results strongly suggested that LKB1 acted as an upstream kinase of MARK2 and decreased the Tau protein level in MEF3-2. We then examined whether the LKB1-MARK2 signaling reduced the mRNA level of Tau and found that neither LKB1 nor MARK2 kinase activity affected it (Fig. 5B). Thus, we speculated that the LKB1-MARK2 signaling might promote degradation of Tau rather than its synthesis. As anticipated, the addition of the proteasome inhibitor MG132 or lactacystin significantly increased the Tau protein level in MEF3-2 cells infected with Adv-Tau alone and in the cells co-infected with Adv-Tau and Adv-LKB1 (WT) (Fig. 5C). On the other hand, the inhibitors hardly increased the Tau level in Adv-LKB1 (KD)-infected cells. These results suggested that the LKB1-MARK-mediated phosphorylation of Tau signaled for its degradation by the proteasome. Consistent with this interpretation, the proteasome inhibitors increased the level of phospho-Tau (Ser(P)-262/356) in the cells co-infected with Adv-Tau and Adv-LKB1 (WT) (Fig. 5C). In addition, the Tau mutant (S2A, namely, S26A/S356A) that lacked the KXGS motifs became refractory to degradation evoked by LKB1 and MARK2 (Fig. 5D). These results collectively indicate that phosphorylation of the KXGS motifs by the LKB1-MARK2 signaling is the prerequisite to Tau degradation in MEF3-2.

Tau protein can be phosphorylated not only by MARKs but also by other kinases including cyclin-dependent kinase 5 and glycogen synthase kinase-3β (GSK-3β) (41), and GSK-3β plays a critical role in Tau degradation (42). In addition, several reports suggest cross-talks between the LKB1-MARK pathway and the Wnt pathway in both directions (43–47). We, therefore, evaluated the possible involvement of GSK-3β in the LKB1-mediated degradation of Tau. In the presence of a GSK-3β inhibitor, LiCl, the slowly migrating bands of Tau disappeared, probably due to inhibition of phosphorylation by GSK-3β (Fig. 5E), whereas LiCl treatment did not abolish Tau degradation induced by LKB1. These results suggest that the degradation of Tau promoted by LKB1 is independent of the GSK-3β activity in MEF3-2.

Accelerated Tubulin Polymerization in LKB1 Knockdown Cells—In the gain-of-function experiments using the recombinant adenoviruses, we demonstrated that the LKB1-MARK signaling regulates MT dynamics and Tau degradation in MEF3-2. However, loss-of-function experiments are equally important in elucidating the biological relevance of the LKB1-MARK signaling, because reduction in the LKB1 gene dosage shows serious effects. Specifically, Lkb1 haploinsufficiency in the heterozygous knock-out mice causes gastrointestinal hamartomas, whereas loss of heterozygosity results in
hepatocellular carcinomas (7–10). Thus, we set up experiments using a siRNA against LKB1 in HepG2, a human hepatoblastoma cell line. To minimize the clonal variation, we designed an siRNA construct (LKB1-pTERlox; Fig. 6A) based on the strategy shown in Fig. 6B. Briefly, we first established two stable LKB1-knockdown HepG2 clones (#15 and #31). Transient expression of Cre recombinase by Adv-Cre removed the cassette expressing the siRNA from LKB1-pTERlox in these cells, generating two rescued clones (#15H and #31H). LKB1 expression of #15H and #31H clones were recovered to nearly equal levels to those in the parental HepG2 cells. We also obtained control cell clones with Adv-LacZ that maintained low LKB1 level (#15L and #31L; ~5% of those in #15H and #31H, respectively; Fig. 6C). Using these clonal cell lines, we then performed MT regrowth experiments. The MT network almost disappeared by a 2-h treatment with 33 μM nocodazole in both #31H and #31L clones (Fig. 6D, 0 min). After washing out nocodazole, the LKB1 knockdown clone #31L showed faster tubulin polymerization from the centrosomes than the control clone #31H (Fig. 6D, 4, 8, and 16 min). We obtained similar results using clones #15H and #15L as well (data not shown). These results from the loss-of-function experiments indicate that the endogenous LKB1 activity suppresses MT regrowth in the HepG2 cells, which is consistent with the results of the in vitro tubulin polymerization assay and of the gain-of-function experiments with forced expression of LKB1 in MEF3-2 cells.

**DISCUSSION**

In this report we have provided evidence that LKB1 suppresses tubulin polymerization through activation of MARK in vitro and in cultured cells. First, we showed in a cell-free system that LKB1 enhanced Tau phosphorylation through MARK2 (Fig. 1B). Second, we demonstrated that activation of the LKB1-
MARK2 pathway suppressed tubulin polymerization through Tau phosphorylation (Fig. 1C). Third, expression of LKB1 or MARK2 suppressed MT regrowth in cultured cells (Figs. 3 and 4), whereas LKB1 knockdown accelerated it (Fig. 6). To our knowledge, this is the first experimental demonstration that LKB1 regulates MT dynamics in vitro and in vivo.

Biological roles of MARK2 have been well documented especially in the MT-dependent cellular processes including intracellular trafficking and cell polarity (26–31). Studies using Madin-Darby canine kidney (MDCK) cells have shown that MARK2 localizes to the lateral domains where it reorganizes MTs that serve as tracks for the intracellular delivery (26, 30). Inhibition of MARK2 activity impairs formation of the apical domain in MDCK cells, probably because of disordered apical delivery (29, 30). LKB1 and its orthologs are considered as key regulators of cell polarity (11–13). Our findings that LKB1 regulates MT dynamics through MARK2 are consistent with the possible role of MARKs in LKB1-induced cell polarization.

Our results suggest that phosphorylation of the KXGS motifs (Ser-262/Ser-356) of Tau by the LKB1-MARK signaling causes proteasome-mediated degradation of Tau (Fig. 5). Recent studies indicate that Tau, only when phosphorylated (42), is recognized and ubiquitinated by the E3 ligase CHIP (carboxyl terminus of the Hsc70-interacting protein) in its degradation process (42, 48). In addition, various lines of evidence suggest that phosphorylation of the KXGS motifs may be an early step toward both the polarization of neurons in neural development and the formation of hyperphosphorylated Tau aggregates in Alzheimer disease (21, 32, 36, 49, 50). It remains to be investigated whether Tau degradation induced by LKB1 plays essential roles in neuronal development and Alzheimer disease.

Regarding the LKB1-MARK signaling in living cells, four issues are worth noting. First, adenovirus-mediated expression of LKB1 (WT/KD) or MARK2 (WT/KD) had no detectable effects on MT regrowth in MDCK epithelial cells and IEC-6 (rat intestinal epithelial cells) endogenously expressing LKB1 (data not shown). The unre sponsiveness of these cell lines to the introduced genes requires further studies. Second, in Lkb1-null MEF3-2, Adv-LKB1 (WT) suppressed MT regrowth substantially as did Adv-MARK2 (WT), although to a lesser extent. The weaker response of MEF3-2 to the exogenous MARK2 than to LKB1 also remains to be investigated further. Third, in Lkb1-null MEF3-2, Adv-LKB1 (KD) accelerated MT regrowth substantially as compared with the Adv-Cre control (Fig. 3). We also found that LKB1 (KD) induced the accumulation of Tau to a level much higher than that induced by the Adv-Cre control (Fig. 5). One possible interpretation is that LKB1 (KD) may have a domi-
nant-negative effect on the basal activity of MARK2. Our reverse transcription-PCR analysis showed that another kinase upstream of MARK, MARK kinase (MARKK), was expressed in MEF3-2.4 MARKK, a member of the Ste20 kinase family, was isolated from pig brain during a search for the kinases upstream of MARK2 (34). MARKK and LKB1 phosphorylate MARK2 at the same residue (Thr-208) (18, 34), whereas it remains to be determined whether MARKK can phosphorylate the other 13 AMPK-related kinases in addition to MARK2 (20). LKB1 (KD) determined whether MARKK can phosphorylate the other 13 upstream of MARK, MARK kinase (MARKK), was expressed in MEF3-2 cells. Further investigation is needed to explain the mechanism behind this phenomenon.

In conclusion, we have shown that LKB1 suppresses tubulin polymerization through the activation of MARK both in vitro and in vivo and that LKB1-MARK signaling is involved in Tau degradation. These results may help elucidate the roles of LKB1 in the MT-dependent cellular processes such as intracellular trafficking and cell polarity.

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