Motor-dependent microtubule disassembly driven by tubulin tyrosination

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In cells, stable microtubules (MTs) are covalently modified by a carboxypeptidase, which removes the C-terminal Tyr residue of α-tubulin. The significance of this selective detyrosination of MTs is not understood. In this study, we report that tubulin detyrosination in fibroblasts inhibits MT disassembly. This inhibition is relieved by overexpression of the depolymerizing motor mitotic centromere-associated kinesin (MCAK). Conversely, suppression of MCAK expression prevents disassembly of normal tyrosinated MTs in fibroblasts. Detyrosination of MTs suppresses the activity of MCAK in vitro, apparently as the result of a decreased affinity of the adenosine diphosphate (ADP)–inorganic phosphate- and ADP-bound forms of MCAK for the MT lattice. Detyrosination also impairs MT disassembly in neurons and inhibits the activity of the neuronal depolymerizing motor KIF2A in vitro. These results indicate that MT depolymerizing motors are directly inhibited by the detyrosination of tubulin, resulting in the stabilization of cellular MTs. Detyrosination of transiently stabilized MTs may give rise to persistent subpopulations of disassembly-resistant polymers to sustain subcellular cytoskeletal differentiation.

Introduction

Tubulin is subject to posttranslational modifications that principally affect the C terminus of its α subunit. In one of these modifications, the C-terminal Tyr residue of α-tubulin is cyclically removed from the peptide chain by a carboxypeptidase and then subsequently religated to the chain by tubulin Tyr ligase (TTL; Hammond et al., 2008). This cycle generates pools of tyrosinated and detyrosinated microtubules (MTs) in cells. As a rule, dynamic MTs are tyrosinated, whereas stable polymers are detyrosinated (Schulze et al., 1987). Detyrosination does not, per se, stabilize MTs (Khawaja et al., 1988). However, MT stabilization in cells induces MT detyrosination, which is thus considered as a consequence, not a cause, of MT stabilization (Webster et al., 1987).

In recent years, important functions of tubulin tyrosination have been discovered. Thus, TTL loss and the resulting tubulin detyrosination confer selective advantage to cancer cells during tumor growth (Mialhe et al., 2001). TTL suppression in mice, which induces massive tubulin detyrosination, leads to lethal disorganization of neuronal circuits (Erick et al., 2005). Cells derived from TTL-deficient (TTL knockout [KO]) mice display morphogenetic and polarity anomalies (Peris et al., 2006). Tyrosination has turned out to be crucial for tubulin interaction with cytoskeletal-associated protein (CAP)–Gly plus end–tracking proteins (Badin-Larcon et al., 2004; Peris et al., 2006; Bieling et al., 2008; Steinmetz and Akhmanova, 2008), suggesting that the phenotypes observed after TTL suppression may arise from mislocalization of CAP-Gly proteins at detyrosinated MT plus ends. However, a mechanistic explanation for the long-recognized correlation between MT stability and tubulin tyrosination remains elusive. This prompted us to reexamine the relationship of tubulin tyrosination with...
MT dynamics using TTL KO cells in which MTs are exten-
sively detyrosinated. We found that the tyrosination status of
the MT had a profound effect on the MT-depolymerization
activity of kinesin-13 family members.

Results and discussion

We initially observed decreased MT sensitivity to the depoly-
merizing drug nocodazole in TTL KO mouse embryonic fibro-
blasts (MEFs) compared with wild type (WT; Fig. 1 A),
suggesting that MTs are stabilized in TTL KO MEFs. We have
previously shown that the interaction of MTs with stabilizing
factors such as structural microtubule-associated proteins or
plus end–binding proteins is either unaffected or inhibited by
tubulin detyrosination (Saoudi et al., 1995; Peris et al., 2006).
Therefore, we hypothesized that in TTL KO cells, detyrosinated
MTs might be a poor substrate for MT destabilizing factors
such as the kinesin-13 protein mitotic centromere-associated kinesin
(MCAK), which is an important depolymerizing motor in
cycling cells (Newton et al., 2004; Mennella et al., 2005;
Gupta et al., 2006; Manning et al., 2007; Ohi et al., 2007; Hedrick
et al., 2008). Thus, MCAK overexpression should rescue the
loss of dynamic MTs in TTL KO cells. Conversely, suppression
of MCAK expression should prevent disassembly of normal
tyrosinated MTs in fibroblasts. To test these possibilities, individ-
ual MT dynamics were monitored in WT or TTL KO MEFs.
Cells were either untreated or transfected with MCAK cDNA
or with MCAK siRNAs (Fig. S1 D shows MCAK depletion
by siRNAs). MT behavior was scored close to the membrane in
lamellipodial extensions.

In WT MEFs, most MTs depolymerized upon contact
with the membrane (Fig. 1 B). In contrast, many MTs continued
to grow tangential to the leading edge after touching the mem-
brane in TTL KO MEFs (Fig. 1 B and Videos 1 and 2). The time
spent by MTs close to the membrane (persistence time) was mark-
edly increased in TTL KO MEFs as compared with WT MEFs.
In TTL KO MEFs, MT persistence dropped dramatically after
MCAK overexpression and became similar to that observed in
WT MEFs (Fig. 1 C and Videos 3 and 4). MCAK depletion
dramatically increased MT persistence time, which overran the
observation time in >80% of the cases, in both genotypes
(Videos 5 and 6).

We compared MT dynamic instability in WT and TTL KO
(Fig. 1 D and Fig. S2). MTs in TTL KO MEFs displayed a
higher frequency of rescues (threefold), an increase of the time
spent growing (twofold), and a reduction of the time spent shrinking (twofold). In TTL KO cells, MCAK overexpression
resulted in a decrease in the frequency of rescues (twofold) and a
decrease in the time spent growing with a corresponding in-
crease in the time spent pausing. MCAK depletion by siRNA
increased the rescue frequency in WT cells (threefold) and in-
creased the time spent pausing at the expense of the time spent shrinking in both genotypes. Interestingly, the two genotypes no longer exhibited significant differences in MT dynamics in the
presence of MCAK siRNA.

These results indicate a reduction of MT disassembly and
abnormal MT persistence after membrane contact in TTL KO cells. MCAK overexpression partially rescued MT dynamic param-
eters in TTL KO MEFs, and MCAK suppression erased the inherent differences between the genotypes. These data are
compatible with a simple model in which MCAK activity is im-
paired on detyrosinated MTs. Although no sizeable differences
in MCAK expression were observed in TTL KO cells as com-
pared with WT (Fig. S1, A–C), it is a formal possibility that
MCAK activity could be affected indirectly.

To circumvent this, we assayed recombinant MCAK
activity directly on the exposed MTs of the two MEF genotypes
after cell lysis in a large volume of Triton X-100–based buffer
to remove the cytosol. During lysis and further processing,
MTs were stabilized with 2 μM taxol to prevent spontaneous
depolymerization. During the time course of experiments, in
the absence of added exogenous MCAK, no detectable depoly-
merization of MTs occurred, indicating negligible endogenous
MCAK activity in lysed cells (unpublished data). WT MEFs
contained tyrosinated MTs, and TTL KO MEFs exhibited ex-
tensively detyrosinated MTs and variable amounts of tyro-
sinated tubulin originating from tubulin synthesis (Fig. 2 A; Peris
et al., 2006). Fully detyrosinated MTs could be generated by
treating WT lysed fibroblasts with carboxypeptidase A (CPA;
WT + CPA; Fig. 2 A). WT and WT + CPA lysed MEFs con-
tained similar quantities of MT polymer (Fig. 2, B and C).
Lysed TTL KO fibroblasts also exhibited similar levels of MT
polymer to that of WT fibroblasts (Fig. 2, B and C). After incu-
bation with recombinant MCAK, WT MTs displayed extensive
depolymerization. In contrast, CPA-treated WT MTs or MTs in
TTL KO lysed fibroblasts were minimally affected by the addi-
tion of recombinant MCAK (Fig. 2, B and C). MT depolymer-
ization was greater when TTL KO or WT + CPA MT arrays
were incubated with a neck + motor domain mutant of MCAK
instead of full-length MCAK (Fig. 2 C), but the extent of de-
polymerization was still less than that observed with WT MTs
(Fig. 2 C). MT exposure to the motor domain alone, which is
devoid of sizeable depolymerizing activity (Maney et al., 2001),
did not induce any detectable depolymerization of any MT
array (Fig. 2 C). These results strongly indicate a direct relation-
ship between MT detyrosination and MCAK inhibition, which
is evident even in the presence of the residual amounts of Tyr
ubulin typically seen in TTL KO cells.

A possibility remained that tubulin detyrosination or CPA
treatment inhibited MCAK by interfering with the interaction
of the MTs with other cellular effector proteins. To control for
this possibility, we assayed MCAK activity on MTs assembled
with purified tyrosinated or detyrosinated tubulin (Fig. 3 A).
MCAK activity on detyrosinated MTs was diminished compared
with tyrosinated MTs, further supporting a direct relationship
between MT tyrosination and MCAK activity (Fig. 3, B and C).
We then used MT-binding assays to identify the point in the
tubulin removal cycle that is affected by tubulin tyrosination.
MCAK’s apparent affinity for MTs is influenced by its nucleo-
tide state (Helenius et al., 2006). Assays were run using the
following nucleotides or nucleotide analogues: the nonhydro-
lyzable nucleotide analogue AMP-PNP (p[NH]ppA, adenosine
5’-[β,γ-imido]triphosphate), which mimics the ATP collision
state; ADP-AIFx (ADP + aluminium + sodium fluoride), which
with the neck + motor domain of MCAK, although differences were smaller (Fig. 4 C). Thus, detyrosination seems to diminish the affinity of the MT lattice for the ADP-Pi– or ADP-bound forms of MCAK.

To test whether the inhibition of depolymerizing motors was unique to TTL KO MEFs, we assayed TTL KO neurons for similar resistance to MT disassembly. When WT or TTL KO neurons were exposed to nocodazole, only residual tubulin staining was detectable in the axons of WT neurons, whereas a persistent MT signal was evident in the axons of TTL-deficient neurons (Fig. 5, A–C). Axonal MT disassembly is largely dependent on the activity of KIF2A (Homma et al., 2003), a neuronal kinesin-13 in the same family as MCAK. KIF2A KO mimics the ATP transition state; ADP-vanadate, which mimics ADP–inorganic phosphate (Pi); and ADP (Fig. 4). By fluorescence microscopy, MCAK proteins yielded a punctuated decorrelation of MTs, with some aggregates in the background (Fig. 4 A), as previously observed (Moore and Wordeman, 2004). In quantitative experiments, the AMP-PNP–bound forms of GFP-MCAK or of the GFP neck + motor domain of MCAK associated to similar extents with tyrosinated or detyrosinated MTs (Fig. 4, B and C). In contrast, the ADP-AlFx–, ADP-vanadate–, and ADP-bound forms of full-length MCAK exhibited a diminished association with detyrosinated MTs compared with tyrosinated polymers (Fig. 4 B). The difference was maximal with the ADP-vanadate (Fig. 4 B). Similar results were observed with the neck + motor domain of MCAK, although differences were smaller (Fig. 4 C). Thus, detyrosination seems to diminish the affinity of the MT lattice for the ADP-Pi– or ADP-bound forms of MCAK.

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Figure 1. Impaired MT dynamics in TTL KO MEFs. (A) Analysis of nocodazole effects on WT or TTL KO MEFs. Data are expressed as the ratio of MT signals measured after nocodazole treatment versus control conditions (mean ± SEM). MT signals were estimated for a minimum of 39 MEFs from three independent experiments for each genotype and treatment condition. ***, P < 0.001 with a t test. (B) Video microscopy examination of MTs in WT or TTL KO MEFs expressing m-cherry α-tubulin and GFP-EB3 close to the leading edge of lamellipodial extensions. Colored, closed arrowheads indicate localization of the MT tip at different time points. Open arrowheads of the corresponding color indicate the initial MT tip localization. Most WT MTs underwent extensive depolymerization after membrane contact (white and yellow arrowheads). Some MTs followed the leading edge for a short period of time before depolymerization (pink arrowheads). Most TTL KO MTs continued to grow after membrane touch, running along the plasma membrane or even growing inward (white arrowheads). Occasionally, MTs seemed to push the membrane forward before disassembly, rescue, and regrowth (yellow arrowheads). Similar phenotypes were observed when MT ends were labeled with other TIPs or in experiments run with fluorescent tubulin alone (not depicted). (C) Measurement of the time spent by MTs exploring the area near the leading edge (persistence time). Experiments were run under control conditions or after cell transfection with either MCAK cDNA or MCAK siRNAs. At least 42 MTs were examined in each condition. With MCAK siRNAs, persistence times generally exceeded 100 s and overran the observation time (Videos 5 and 6). Results are shown in other conditions as mean values ± SEM. ***, P < 0.001 with a t test. (D) Analysis of MT dynamic instability. MTs whose growing plus tip was located within 10 µm of the leading edge at time 0 were followed over time after various cell treatments, as indicated. Mean values ± SEM are shown. Statistically significant differences between WT and KO cells are described in Results and discussion; in all cases, p-values were <0.01 with a Mann and Whitney U test. Bars, 10 µm.
The influence of the tubulin tyrosination status on the activity of depolymerizing motors provides an elegant mechanistic explanation for the observed stability of detyrosinated MTs. Tubulin tyrosination affects the activity of the neck + motor mutant of MCAK, which lacks both the N-terminal and C-terminal domains of MCAK. This suggests that neither domain is centrally involved in MCAK interactions with the tubulin C terminus. Both of these domains are implicated in MCAK's ability to track on MT ends, which has been shown to be unaffected by tubulin detyrosination (Moore et al., 2005; Peris et al., 2006). Based on our data, MT detyrosination negatively affects MCAK's tubulin removal activity directly. Thus, tubulin detyrosination, and its negative effect on kinesin-13 family motors, is the most likely explanation for

neurons display morphogenetic anomalies affecting axonal length and branching (Homma et al., 2003). In an analysis of TTL KO neurons morphology, we observed an increase in both the axon length and the total length of axon collaterals. The number of primary collaterals per unit of axonal length was unaffected, whereas the total number of collaterals, including secondary or tertiary branches, was increased (Fig. 5, D–G). These anomalies resemble those observed in KIF2A KO neurons (Homma et al., 2003). Collectively, our data strongly suggest that KIF2A activity is inhibited in TTL KO neurons, and this is supported by experiments in lysed MEFs showing inhibited KIF2A activity on WT CPA or TTL KO MTs compared with WT polymer (Fig. 5 H).

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Impaired MT disassembly has been previously observed in KIF2A-deficient neurons (Homma et al., 2003) or upon MCAK inhibition in cycling cells (Mennella et al., 2005). Obviously, kinesin-13 inhibition provides a highly plausible explanation for the impaired MT disassembly observed in different TTL KO cell types in this study. Additionally, MCAK appears to be important for MT disassembly upon encountering the cell edge, and may be crucial for end-on attachments to specific membrane complexes (Geiger et al., 1984; Morrison, 2007).

Impaired activity of depolymerizing motors may be central to several anomalies observed in TTL KO cells or mice. There is extensive overlap between the phenotypes observed in TTL KO or KIF2A KO neurons or mice (Homma et al., 2003; Erck et al., 2005). Anomalies and adaptations in depolymerizing motor activity may also be important for the facilitating effect of TTL loss on tumor progression (Mialhe et al., 2001). MCAK activity has been observed to be altered in cancer cells (Hedrick et al., 2008; Ishikawa et al., 2008). Interestingly, both CAP-Gly plus end–binding proteins and depolymerizing motors have been identified as factors whose alteration favors genomic instability in aneuploid cells and thus malignant cell invasiveness (Storchova et al., 2006; Pellman, 2007).

Factors able to promote the differentiation of cellular MTs into distinct stability subclasses have been the subject of sustained interest in cell biology. Decades ago, tubulin tyrosination, which distinguished with striking clarity between stable and dynamic MTs, seemed to be an ideal mechanism responsible for selective MT stabilization (Schulze et al., 1987). This attractive view was discarded when it appeared that tyrosination...
Tubulin tyrosination affects the interaction of MCAK with MTs. (A) Fluorescence images of MCAK binding to MTs. Equal amounts of taxol-stabilized (10 nM) tyrosinated (Tyr) or detyrosinated (Detyr) pure tubulin MTs (red) were incubated with 0.25 μM purified GFP-MCAK (green). The His6-tagged HsKIF2A neck + motor domain (residues 188–537) was prepared as previously described (Erck et al., 2005). HsKIF2A is expressed in the BL21 bacterial strain. His6-tagged full-length MCAK, MCAK182-583, and EGFP-MCAK182-583 were expressed in baculovirus.

Materials and methods

Antibodies
The antibodies used were detyrosinated tubulin (L4), tyrosinated tubulin (clone YLI/2), α-tubulin (clone a3α; Peris et al., 2006), polyclonal anti-GFP (Invitrogen), and polyclonal anti-MCAK (Andrews et al., 2004).

Cell culture and transfection
Hippocampal neurons and MEFs (three different embryos for each genotype) were prepared as previously described (Erick et al., 2005). MEFs were transfected using MEF Nucleofector kits (Amaza Biosystems) and GFP-EB3 (provided by N. Galjart, Erasmus Medical Center, Rotterdam, Netherlands), m-cherry α-tubulin (provided by F. Saudou, Institut Curie, Paris, France), and m-cherry-ΜCAK (Wordeman laboratory). For inhibition of MCAK, Stealth siRNA Negative Control, Stealth Select siRNA 1 MSS232130, siRNA 2 MSS232131, and siRNA 3 MSS232132 (all from Invitrogen) were used. MCAK inhibition was assayed by Western blot analysis, and siRNA 3 was used in MT dynamic analysis.

Recombinant proteins
The His6-tagged HiKIF2A neck + motor domain (residues 188–537) was expressed in the BL21 bacterial strain. His6-tagged full-length MCAK, MCAK182-583, and EGFP-MCAK182-583 were expressed in baculovirus.

Nocodazole susceptibility assay
Cells were treated with carrier alone or with 20 μM nocodazole for 60 min (neurons) or 15 min (MEFs), permeabilized in PHFEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM MgCl2, pH 6.9) with 0.02% saponin and 10 μM taxol, and fixed in PHFEM buffer with 2% paraformaldehyde and 0.05% glutaraldehyde. The MT network was quantified by the α-tubulin fluorescence intensity measured inside the whole cell surface in fibroblasts (determined with F-actin staining) or in three fixed size regions (5 × 5 μm) placed at the proximal, medial, and distal part of each axon.

Immunofluorescence and video microscopy
Fluorescent images of living cells were captured with a charge-coupled device camera (CoolSNAP HQ; Roper Scientific) using a 100× NA 1.3 Plan-Neofluar objective in an inverted motorized microscope (Axiovert 200M; Carl Zeiss, Inc.) controlled by MetaView software (MDS Analytical Technologies). Fixed images were captured with a charge-coupled device camera (CoolSNAP ES; Roper Scientific) in a straight microscope objective (Axioskop 50; Carl Zeiss, Inc.) controlled by MetaView software (MDS Analytical Technologies) using a 40× or 100× NA 1.3 Plan-Neofluar objective.

Analysis of MT behavior
Image capture was every 3 s. MTs displaying a trajectory at a straight angle (70–110°) with the cell membrane were analyzed inside a region of 10 μm from the cell edge. MT persistence time, measured for polymers whose growing end made contact with the membrane, was defined as the time during which at least a segment of MT kept running parallel to the membrane before disassembly. MT dynamic instability parameters were determined and analyzed as previously described (Kline-Smith and Walczak, 2002).

Lysed cell experiments
Fibroblasts were lysed in PEM buffer (80 mM Pipes, 1 mM EGTA, and 1 mM MgCl2, pH 6.7) with 0.5% Triton X-100 and 10% glycerol and incubated in PEM with 2 μM of taxol buffer in the presence or absence of 2 μg/ml CPA (Sigma-Aldrich). Lysed cells were then washed in CPA-inactivating buffer (20 mM DDT in PEM-taxol buffer) and extensively washed in PEM-taxol buffer. Lysed cells were incubated with buffer alone (PEM buffer, 2 μM taxol, 1 mM DTT, 75 mM KCl, and 0.25 mM MgATP) or with the same buffer containing purified motor protein (200 nM for MCAK proteins and 800 nM for KIF2A) for 30 min and then fixed in cold methanol. The MT network was quantified by the α-tubulin fluorescence intensity measured inside the whole cell surface of lysed cells.

Motor binding to MTs
Motor binding to MTs was assayed in different nucleotide conditions: AMP-PNP, ADP–aluminum fluoride, ADP-vanadate, and ADP. 1 μM of purified motors was incubated with 2 mM of nucleotides and then with 0.01 μM of tyrosinated or detyrosinated taxol-stabilized MTs (Paturel et al., 1989). Reactions were stopped with PEM, 50% glycerol, and 1% glutaraldehyde and centrifuged on coverslips. MT–motor complexes were fixed in cold methanol and double stained with α-tubulin and GFP antibodies. For quantification,
Figure 5. Impaired MT disassembly and abnormal cell morphology in TTL KO–cultured neurons. (A and B) WT or TTL KO hippocampal neurons 2 d after plating. Neurons were incubated in the absence (control; A) or presence of 20 µM nocodazole (B), permeabilized in saponin-based buffer to extract free tubulin molecules, and double labeled for F-actin (red) and tubulin (green). (C) Quantitative analysis of nocodazole effects. The axonal MT signal was expressed as the ratio of MT signals measured after nocodazole treatment versus control conditions (mean values ± SEM). MT signals were estimated for a minimum of 50 axons from three independent experiments. (D–G) Cell morphology in WT or TTL KO neurons showing length, number of primary or of higher order collaterals, and total length of collaterals. Mean values ± SEM for 93 WT neurons and 123 TTL KO neurons from three independent experiments are shown. (H) Depolymerizing activity of the neck + motor construct of KIF2A on tyrosinated or detyrosinated MTs. The assay was performed as in Fig. 2 C. Mean values ± SEM for 75 cells are shown. ***, P < 0.001 with a t test. Bars, 20 µm.
a line corresponding to each MT in the α-tubulin image was transferred to the GFP images. The amount of motor bound per unit of MT length was calculated as the percentage of positive GFP pixels versus the total number of pixels in the MT line.

In vitro depolymerization experiments
10 nM of taxol-stabilized tyrosinated or detyrosinated MTs were or were not incubated with 10 nM MCAK-ATP. Reactions were stopped with PEM, 50% glycerol, and 1% glutaraldehyde after 10 or 20 min and centrifuged on coverslips. Reactions were fixed in cold methanol and stained with anti-tubulin antibody. Quantification of the total amount of MTs was measured using Image software (National Institutes of Health).

Data processing and analysis
Data were analyzed blind to the genotype or the experimental conditions. For statistical analysis, t tests with unequal variances for samples comprising >30 measures or a parametric Mann and Whitney U test for smaller samples was used.

Online supplemental material
Fig. S1 illustrates endogenous MCAK expression in WT or TTL KO MEFs and its specific suppression with commercial Stealth siRNAs. Fig. S2 represents life history plots of individual MTs from WT or TTL MEFs with endogenous levels or overexpression or suppression of MCAK. Videos 1 and 2 show time-lapse video microscopy of a lamellipodial extension of a WT and TTL KO fibroblast, respectively, expressing m-cherry α-tubulin and GFP-FEB3. Videos 3 and 4 show time-lapse video microscopy of a lamellipodial extension of a WT and TTL KO fibroblast, respectively, expressing m-cherry MCAK and GFP-FEB3. Videos 5 and 6 show time-lapse video microscopy of a lamellipodial extension of a WT and TTL KO fibroblast, respectively, treated with MCAK siRNA for 36 h and expressing m-cherry α-tubulin and GFP-FEB3. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200902142/DC1.

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