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Kinesin is involved in protecting nascent microtubules from disassembly after recovery from nocodazole treatment

Jérôme Marceiller[a], Anne Drechou[a], Geneviève Durand[a], Franck Perez[b], Christian Poüs[a,*]

[a]*Laboratoire de Biochimie et Biologie Cellulaire, EAD 1595, Faculté de Pharmacie, 5 rue J.B. Clément, 92296 Châtenay-Malabry cedex, France*
[b]*CNRS UMR 144, Institut Curie Section Recherche, Paris, France*

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Abstract

Upon recovery from nocodazole treatment, microtubules from cultured epithelial cells exhibit unusual properties: they re-grow as fast as any highly dynamic microtubule, but they are also protected against disassembly when challenged with nocodazole like the stable microtubules of steady-state cells. Exploring the mechanism that underlies this protection, we found that it was sensitive to ATP treatment and that it involved conventional kinesin. Kinesin localized at the growing end or along nascent microtubules. Its inhibition using a dominant-negative construct for cargo binding, or by micro-injecting an anti-kinesin heavy chain antibody that impairs motor activity, resulted in the partial or total loss of microtubule protection. Finally, in an ex vivo elongation assay, we found that kinesin also participates in the control of microtubule re-growth. Altogether, our findings suggest that kinesin is involved in an early microtubule protection process that is linked to the control of their dynamics during their early growth phase.

Keywords: Microtubule; Kinesin; Nocodazole; Golgi

Introduction

From a dynamic point of view, the microtubules (MTs) of eukaryotic cells can be roughly categorized into two subpopulations. Dynamic MTs are subject to dynamic instability, switching between growth and depolymerization phases at their plus ends [1]. Due to their frequent transitions between these phases, such MTs are rapidly destabilized by anti-polymerizing drugs such as nocodazole. Stable MTs, which undergo much less frequent transitions, do not vary in length for long periods. They accumulate post-translational modifications on their tubulin subunits and are much more resistant to anti-polymerizing drugs [2,3]. Plus end capping is a major mechanism involved in blocking the plus ends of stable MTs. It prevents them from incorporating or loosing tubulin subunits [4], explaining both their dynamic arrest and their resistance to anti-polymerizing drugs. In contrast, dynamic MTs are expected to exhibit uncapped plus ends, allowing them to grow or shrink.

When cells recover from a prolonged nocodazole treatment that caused a complete MT breakdown, MT sensitivity to anti-polymerizing drugs is not correlated with their dynamics however. In this case, all the nascent MTs form a homogenous population that intriguingly mixes some properties of dynamic and stable MTs, respectively: they grow normally but they also resist challenges with anti-polymerizing drugs or dilution after cell permeabilization [3,5]. This protection against disassembly is likely not to be accounted for by the presence of a plus-end cap similar to that found on stable MTs, as such caps are expected to inhibit MT elongation by blocking MT plus ends [4]. The objective of our study was thus to explore the mechanism that protects nascent MTs in these circumstances. More precisely, we tested the hypothesis that the plus end-directed molecular motor kinesin participates in this protection.

Indeed, we found earlier that MTs that assemble early after nocodazole removal in hepatic cultured cells do not

* Corresponding author. Fax: +33 1 46 83 58 02.
E-mail address: christian.pous@cep.u-psud.fr (C. Poüs).

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only re-grow off centrosomes, but also from scattered Golgi elements [5]. The in vitro assembly of MTs on purified Golgi involved a subset of Golgi-bound γ-tubulin [5], a tubulin that participates in MT nucleation and that is associated with their minus end. This suggested that the growing end of Golgi-based MTs is distal from Golgi elements. Since nocodazole resistance is a plus-end process [6], we reasoned that growing MT protection could involve molecules originating from the Golgi and targeted to MT plus ends. Among these, we considered conventional kinesin as a good candidate as it is concentrated on Golgi membranes at steady state to drive post-Golgi membrane trafficking events [7]. Furthermore, kinesin together with the other kinesin-family proteins KIF3A and KIF3B might function in carrying regulatory proteins towards the growing MT plus-ends such as the anti-oncogene APC [8,9].

In the present work, we performed nocodazole washout experiments to synchronize MT re-assembly, and we show that kinesin can be found at both ends or along MTs. We also evidenced that all the nascent MTs, which re-polymerize in these conditions, are protected against disassembly by an ATP-sensitive mechanism that involves conventional kinesin. Our data finally show that kinesin also participates in controlling nascent MT re-growth, suggesting that such growth control and nascent MT protection are intimately linked processes.

Materials and methods

Antibodies, chemicals and plasmids

Anti-α-tubulin (DM1A clone) anti-mouse IgG FITC, and TRITC conjugates, nocodazole, ATP and GTP were purchased from Sigma Chemical Co. (St. Louis, MO). Purified anti-kinesin heavy chain (SUK-4 clone) was purchased from Covance (Princeton, NJ). Phosphocellulose-purified porcine brain tubulin was prepared as described [10]. YFP-tagged tubulin Living-Colors™ vector was purchased from BD Biosciences Clontech (Palo Alto, CA). The DPPIV-GFP construct encodes a fusion protein between GFP and the cytoplasmic and transmembrane domains of dipeptidyl-peptidase IV (DPP-IV). It was a kind gift of Pr. G. Trugnan (INSERM U538, Faculté de Médecine St-Antoine, Paris) and was used as described to label the Golgi [11]. The GFP-tagged TPR repeats from the cargo-binding domain of kinesin light chain 2 was expressed using a construct kindly provided by Dr M. Way (Cell motility laboratory, Cancer Research UK, London) [12]. Cells were transfected using the FuGene 6 reagent (Roche Diagnostics, Switzerland), according to the manufacturer's instructions.

Cell culture

HeLa and MDCK cells were cultured in Dulbecco's minimum essential medium (DMEM) containing an anti-biotic-antimycotic mixture and supplemented with 10% fetal calf serum (Dutscher, Rungis, France). When appropriate, 400 μg/ml geneticin was added into the medium to select stably transfected cells.

Cell treatments

To depolymerize the whole MT network, cells cultured on glass coverslips were treated for 90 min with 10 μM nocodazole at 37°C (starting from a 10-mM stock solution in DMSO), then shifted to ice for 2 h in the presence of nocodazole. Nocodazole washouts consisted first in 3 rinses with ice-cold nocodazole-free culture medium, then in 37°C incubations using pre-warmed nocodazole-free medium (referred to as unconstrained conditions), or pre-warmed culture medium supplemented with 0.5 μM nocodazole (referred to as constrained conditions). Drug-resistance of MTs was tested by performing an additional 10-μM nocodazole treatment (30 min at 37°C) on cells that were first subjected to an unconstrained washout.

To eliminate free tubulin from cells, soluble proteins were extracted by 3 washes with pre-warmed PEM buffer (100 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9) supplemented with 0.1% Triton X-100. After extraction, cells were immediately rinsed with warm Triton-free PEM. When appropriate, cells were then treated with 1 mM ATP or incubated with a 5-μM purified tubulin solution supplemented with 1 mM GTP and 1 mM ATP. After one rinse with warm PEM, cells were fixed prior to immunofluorescence labeling.

Immunofluorescence

Cells were cultured on glass coverslips and after appropriate treatments, they were detergent-extracted, rinsed once in warm PEM and fixed with −20°C methanol for 5 min. When the SUK-4 antibody was used for immunofluorescence labeling, cells were fixed using paraformaldehyde (3.75%, 5 min, room temperature). After one rinse in PBS, cells were subjected either to the single labeling of total α-tubulin or to that of tubulin and kinesin. Incubation with primary antibodies (the anti-tubulin was always used first in the case of double-labeling with two mouse monoclonal antibodies) was performed for 1 h at 37°C. After 3 washes with PBS, cells were incubated with fluorochrome-conjugated secondary antibody (single labeling), or with FITC-labeled F(ab) fragments. After 3 washes with PBS, cells were subjected to a second fixation and then processed for labeling with the second primary antibody (1 h, 37°C). After 3 more washes with PBS, the second primary antibody was detected using Cy5- or TRITC-conjugated anti-mouse IgGs. Samples were mounted in Tris-buffered (80 mM, pH 8.5) 8% Mowiol and 20% glycerol mixture supplemented with di-aza bicyclo-octane (DABCO) as an anti-fading agent. Samples were examined in a Leica DMLB microscope with a 100× objective and a Cohu 4913 CCD camera or on a Zeiss LSM 510 confocal microscope.