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Tau-isoform dependent enhancement of taxol mobility through microtubules

HyunJoo Park [a,b,*], MahnWon Kim [a,1], Deborah K. Fygenson [b,2]

[a] Physics Department, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea
[b] Physics Department and Biomolecular Science and Engineering Program, University of California, Santa Barbara, CA 93106, USA

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ABSTRACT

Tau, a family of microtubule-associated proteins (MAPs), stabilizes microtubules (MTs) and regulates their dynamics. Tau isoforms regulate MT dynamic instability differently: 3-repeat tau is less effective than 4-repeat tau at suppressing the disassembly of MTs. Here, we report another tau-isoform-dependent phenomenon, revealed by fluorescence recovery after photobleaching measurements on a BODIPY-conjugated taxol bound to MTs. Saturating levels of recombinant full-length 3-repeat and 4-repeat tau both cause taxol mobility to be remarkably sensitive to taxol concentration. However, 3-repeat tau induces 2.5-fold faster recovery (~450 s) at low taxol concentrations (~100 nM) than 4-repeat tau (~1000 s), indicating that 3-repeat tau decreases the probability of taxol rebinding to its site in the MT lumen. Finding no tau-induced change in the MT-binding affinity of taxol, we conclude that 3-repeat tau either competes for the taxol binding site with an affinity of ~1 μM or alters the MT structure so as to facilitate the passage of taxol through pores in the MT wall.

The microtubule (MT)[3] is a hollow, crystalline assembly of the heterodimeric α,β-tubulin protein and the stiffest element of the cellular skeleton [1]. Many important cellular functions of MTs, including mitosis and locomotion, depend on the regulation of dynamic instability by microtubule-associated proteins (MAPs) [2]. Tau is one of several MAPs that stabilize MT dynamics. Tau has received a great deal of attention since it was discovered to be the major constituent of neurofibrillary tangles that are the hallmark of a variety of neurodegenerative diseases [3–5]. The prospect of clarifying the origins of these diseases motivates much research into the mechanism of tau's MT-stabilizing effect.

Tau is a soluble and natively unfolded polypeptide in solution [6,7] that acquires structure as it binds the microtubule [8]. Its MT-binding domain contains either three or four conserved repeat sequences, strongly suggesting that a single molecule of tau bridges multiple dimers in the MT lattice [9]. Exactly where tau binds on the MT exterior remains to be resolved [10–12]. Recent experiments indicate that tau can also bind to the MT interior, at a location on near β-tubulin that overlaps the binding site for taxol [13].

Taxol is a small, hydrophobic molecule that has proven to be an effective therapeutic for certain cancers due to its MT-stabilizing properties [14,15]. MT-mediated interactions between taxol and tau may point to a common mechanism of MT stabilization, which could shed light on the mechanism of MT dynamics as well as improve clinical protocols. To date there are four types of evidence: (i) taxol displaces weakly bound tau from MTs *in vitro* [13]; (ii) taxol mobility becomes sensitive to taxol concentration in tau-saturated MTs *in vitro* [16]; (iii) taxol causes detachment of tau from MTs *in vivo* [17]; and (iv) taxol restores tau-linked functions, like axonal transport, in neurodegenerative thauopathies [18].

Given that different isoforms of tau have different effects on MT dynamics both *in vitro* and *in vivo* [19,20], it is natural to ask whether the interaction between taxol and tau evidenced above is isoform-specific. Evidence of type (i) reveals differences that correlate most strongly with the presence or absence of one of the conserved peptide repeats and its adjoining sequence in the C-terminal half of the protein [13].

Here, we document yet another isoform-specific effect of tau on MTs by studying the mobility of taxol. These experiments use a taxol analog with a fluorescent label and measure fluorescence recovery after photobleaching (FRAP) on tau-decorated MTs. The results reveal a significant quantitative and qualitative difference between isoforms with three (3rTau) or four (4rTau) of the C-terminal repeats. At low taxol concentrations, 3rTau speeds taxol's recovery nearly 2.5-fold, while 4rTau has no discernable effect.

To interpret this result, we model how recovery relates to taxol-binding kinetics using a first-order reaction scheme. The model

* Corresponding author. Address: Physics Department, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea. Fax: +82 42 869 8150.
E-mail addresses: phj78@kaist.ac.kr (H. Park), mwkim@kaist.ac.kr (M. Kim), deborah@physics.ucsb.edu (D.K. Fygenson).
[1] Fax: +82 42 869 8150.
[2] Fax: +1 805 893 3307.
[3] *Abbreviations used:* MT, microtubule; MAPs, microtubule-associated proteins.

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motivates further experiments, which rule out explanations based on a tau-dependent change in the MT-binding affinity of taxol or changes in the large-scale structure of the MTs. We conclude that 3rTau interferes locally with the accessibility of the taxol site for re-binding and discuss possible mechanisms.

Materials and methods

Tau

Recombinant full-length adult human brain 3-repeat tau and 4-repeat tau were the kind gifts of Dr. Stuart Feinstein (University of California, Santa Barbara). Both were purified by the procedures described in [20] from Rosetta (DE3) pLacI cells induced with 1 mM isopropyl-β-D-thiogalactoside. Stock tau concentrations were determined by the Bradford assay in comparison with a tau standard. The latter was established by SDS–PAGE and Coomassie blue staining (Pierce, Rockford, IL) against a primary tau standard, calibrated by mass spectrometry.

Stock tau solutions were stored at −20 °C in BRB-80 buffer (80 mM Pipes, pH 6.8, 1 mM EGTA, 1 mM $MgSO_4$ and 10% β-mercaptoethanol) and then kept for 10 min at room temperature and vortexed before use.

Microtubules and taxol with tau

Microtubules (MTs) were prepared by first mixing 5 μL of 5 mg/mL bovine brain tubulin (Cytoskeleton, Denver, CO) with 4 mg/mL 110 kDa dextran (Pharmacia, Uppsala, Sweden) in G-PEM buffer (1 mM GTP, 100 mM Na-Pipes, 1 mM EGTA, 2 mM $MgCl_2$, 5% (w/w) glycerol, pH 6.8) and then incubating for 20 min at 37 °C. These assembly conditions are known to yield well-formed MTs [21], as we verified by TEM (see below).

Red-orange fluorescent BODIPY® 564/570 paclitaxel (Molecular Probes, Eugene, OR), here in referred to as *botax*, was hydrated in 100% dimethylsulfoxide (DMSO) to a stock concentration of 100 μM. To keep DMSO at less than 10% in the final solution, the highest concentration of free taxol, 5 μM, was made using 75% unlabeled taxol (Sigma, St. Louis, MO) and 25% botax. Botax was diluted in G-PEM with 4 mg/mL 110 kDa dextran before being added to the MT samples, which were incubated again at the same temperature for the same amount of time. (Addition of botax was calculated to result in the desired concentration ($100 - 10^4$ nM) free in solution [22] and later verified *in situ* as described below.)

Lastly, 3rTau was mixed with the botax–MT solution to yield a molar ratio of either 1:3 or 1:1 tau-to-tubulin, the resulting solution was again incubated for 20 min at 37 °C. In all cases, the final concentration of tubulin was 2.5 mg/ml (~22.7 μM).

Fluorescence recovery after photobleaching (FRAP)

Briefly, inside a flow cell made of a coverslip, slide and parafilm gasket, melted G75 Sephadex beads (Sigma, St. Louis, MO) stick to the slide and swell to an average size of 40–120 μm when wet. Sample solution is inserted using capillary flow and MTs collect and align around the Sephadex beads. The botax bound to aligned MTs loses its fluorescence (bleaches) when exposed to focused light from a mercury arc lamp (100 W).

During FRAP, epifluorescence images were recorded every few decimal seconds using a shutter (Uniblitz, Rochester, NY) to avoid unnecessary bleaching. Taxol (size ~1 nm) is small enough to diffuse freely through pores in the beads. MTs (diameter ~25 nm) are excluded. The concentration of free botax was determined to within ±26% by measuring the fluorescence intensity *in situ* at the center of a bead, 20 μm above the coverslip, using a cooled CCD camera (Cooke Corp, Romulus, MI) and comparing to standard botax solutions in a similar flow cell without MTs (50–5000 nM).

Images were analyzed using ImageJ (NIH, ver. 1.32j). First, a circular region-of-interest (ROI) was selected around the bleached spot. Then, mean intensity outside the ROI was subtracted from the mean intensity inside the ROI, to correct for any bleaching during data collection, and plotted against time. All plots were well fit by a single exponential

$$I(t) = (I_\infty - I_o) \cdot \exp(-t/\tau_R) \quad (1)$$

where I_∞ represents the amplitude at infinite time, I_o is the amplitude difference between $t = 0$ and infinite time and τ_R is the recovery time (Fig. 1).

In these experiments, the signal is dominated by bound botax. Diffusion of free botax is not directly observed because the fluorescence intensity of botax increases dramatically upon binding to MTs (see Results).

Microtubule bundles

Early experiments, performed without Tau, demonstrated that botax recovery was very sensitive to the density of aligned MTs [22]. The presence of even a small amount of tau (e.g., 1:50 molar ratio with tubulin) separates aligned MTs and results in recovery times that are reproducible throughout a given sample [16].

It has been suggested that the separation between aligned MTs may be due to stiff tau–tau cross-bridges that form between microtubules [23]. We checked, however, that in the absence of dextran, flow-aligned MTs appear equally diffuse with or without full-length 3rTau (Fig. 2). This observation, that tau alone does not compactify MTs, supports the hypothesis that the N-terminal domains mediate a purely repulsive interaction under our conditions and, like bristles in a polymer brush, separate MTs via their time-averaged steric hindrance [24].

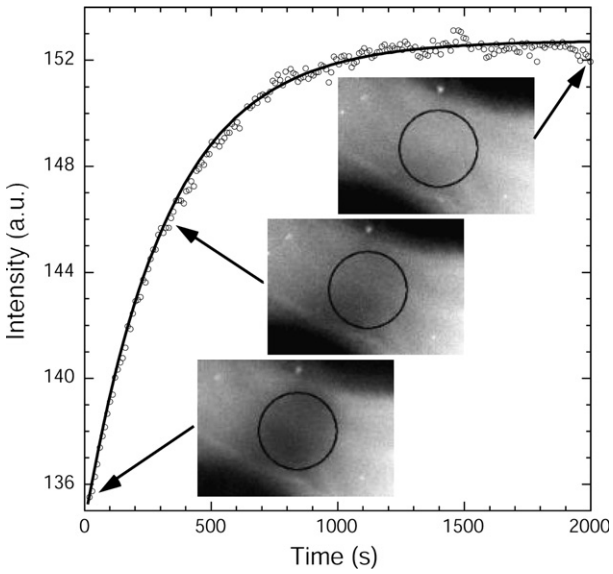

Fig. 1. Mean intensity (open circles) vs. time in a region-of-interest (ROI) that includes the bleached spot on a microtubule bundle around sephadex beads (dark area). The data fit a single exponential (solid line) of the form, $I(t) = I_\infty - I_0 \exp(-t/\tau_R)$. For this sample (300 nM botax, 2.5 mg/ml tubulin and 1:1 3rTau:tubulin), the recovery time τ_R is 331 ± 8 s. Images exemplify the raw data.