Short Communication
Exposure of lumenal microtubule sites after mild fixation

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High-resolution analysis of tubulin structure and docking the structure of tubulin dimer into a map of microtubules led to a prediction that sites for tubulin acetylation are in the interior of microtubules. This is somehow difficult to reconcile with their susceptibility to proteases and acetylation in assembled microtubules. To assess the availability of acetylated α-tubulin for antibodies, immunofluorescence on detergent-extracted cells, on cells fixed under various conditions and in microinjected cells was performed with monoclonal antibodies of known epitope locations. The presented data indicate that acetylated α:Lys40 is not exposed on unfixed microtubules but that this region of lumenal microtubule surface becomes easily exposed under mild fixation conditions.

Introduction

The structure of the tubulin dimer at 3.7-Å resolution, determined by electron crystallography of zinc-induced two-dimensional tubulin sheets stabilized with taxol, revealed that the tubulin monomer showed a compact molecular structure with three functional domains: the amino-terminal domain containing the nucleotide-binding region, an intermediate domain containing the drug-binding site, and the carboxy-terminal domain containing the region for binding the microtubule-associated/motor proteins (Nogales et al., 1998). The docking of the crystal structure of the tubulin dimer into a 20-Å map of the microtubules led to a prediction that the sites for taxol and tubulin acetylation are in the interior of microtubules (Nogales et al., 1999). The lumenal surface could be thus important for the binding of biologically relevant molecules. On the other hand limited proteolysis of taxol-induced microtubules showed that the α-tubulin region containing acetylatable α:Lys40 was exposed in taxol-induced microtubules (de Pereda and Andreu, 1996) and that polymerized microtubules were a better substrate for α-tubulin acetyltransferase (Maruta et al., 1986). Moreover, different antibodies against acetylated α-tubulin decorated microtubules in fixed cells (Piperno et al., 1987; Woods et al., 1989).

To assess the availability of acetylated α-tubulin for antibodies in cells, we performed immunofluorescence on detergent-extracted cells, on cells fixed under various conditions and on microinjected cells. An antibody recognizing a different epitope on the amino-terminal domain of α-tubulin and another antibody reacting with an epitope on the carboxy-terminal end of α-tubulin were used as controls. The presented data indicate that acetylated Lys is not exposed on unfixed cytoplasmic microtubules but this region of the lumenal microtubule surface is easily exposed under mild fixation conditions.

Materials and methods

Antibodies

The following antibodies directed against α-tubulin were used: mouse monoclonal antibody 6–11B-1 (IgG2b) (Piperno and Fuller, 1985) recognizing an antigenic determinant located in the phylogenetically conserved region α25–50 and reacting with α-tubulin acetylated at Lys 40 (LeDizet and Piperno, 1987) (Sigma, Prague, Czech Republic), mouse monoclonal antibody TU-01 (IgG1) (Viklický et al., 1982) recognizing an antigenic determinant located in the region α65–79 (Grimm et al., 1987) and rat monoclonal antibody YL1/2 (IgG) (Kilmartin et al., 1982) recognizing an antigenic determinant located in the region α444–451 and reacting with α-tubulin tyrosylated at the C-terminus (Wehland et al., 1983). The specificity of antibodies was confirmed by immunoblotting. In Western blots of whole cell lysates of 3T3 cells the antibodies reacted only with a protein with relative mobility corresponding to α-tubulin and no cross-reactivity with other proteins was observed. In double label fluorescence experiments microtubules were detected by affinity-purified rabbit antibody against the αβ-tubulin dimer (Dráber et al., 1991). Monoclonal antibody HTF-

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14 (IgG1) (Bártek et al., 1984) specific for human transferrin served as negative control.

Immunofluorescence

Immunofluorescence microscopy was performed on either fixed or unfixed cytoskeletons of 3T3 cells as described (Dráber et al., 1989). Shortly, extraction and fixation steps were carried out in microtubule stabilizing buffer (MSB; 0.1 M Mes, pH 6.9, 2 mM EGTA, 2 mM $MgCl_2$, 4% polyethyleneglycol 6000) at 37 °C. To prepare fixed samples, cells grown on coverslips were rinsed with MSB and then extracted for 1 min with 0.2% Triton X-100. The resulting cytoskeletons were routinely fixed for 20 min in a 3% formaldehyde solution. Alternatively 1, 0.5, 0.4, 0.3, 0.2 and 0.1% formaldehyde was used. In some cases cytoskeletons were fixed in 3% formaldehyde for 1, 2 and 5 min. Samples were thereafter washed three times in MSB and incubated at 37 °C with primary antibodies. Unfixed cytoskeletons were prepared under standard conditions. Cells were rinsed in MSB, extracted for 4 min with 0.2% Triton X-100 in MSB containing 10 μM taxol and rinsed twice in MSB. Coverslips with unfixed samples were incubated at 37 °C with primary antibodies diluted in MSB. After washing in MSB at 37 °C, the cytoskeletons with bound antibodies were fixed for 20 min in 3% formaldehyde and washed again. Some unfixed samples were prepared by 0.2% Triton X-100 extraction in the absence of taxol. Such preparations were fixed after incubation with primary antibodies and used for double labelling with polyclonal anti-tubulin antibody. Ascitic fluids were diluted 1:200, anti-mouse antibody conjugated with lissamine rhodamine (Jackson Immunoresearch Laboratories, West Grove, PA) was diluted 1:100. In double-label fluorescence experiments, polyclonal anti-tubulin antibody was diluted 1:5 and anti-rabbit antibody conjugated with fluorescein isothiocyanate (Jackson Immunoresearch Laboratories, West Grove, PA) was diluted 1:100. The preparations were examined with an Olympus A70 Provis microscope. Neither the control antibody nor the conjugates alone gave any detectable staining.

Microinjections

Microinjection of the cells with antibodies was carried out as described (Dráber et al., 1989) using an inverted Zeiss Axiovert microscope and Zeiss micromanipulator. Antibodies in injection buffer (Klymkowsky, 1981) were injected at a concentration of 0.6–2 mg/ml. After injection the cells were incubated at 37 °C for 15 min, 1 h or 6 h before extraction with Triton X-100 and fixation with formaldehyde. Samples were then incubated with the affinity-purified rabbit anti-tubulin antibody and processed for double-label immunofluorescence.

Results

To assess the role of fixation on the exposure of lumenal α:Lys40 in cytoplasmic microtubules, experiments on fixed and unfixed mouse fibroblasts 3T3 were performed with well characterized monoclonal antibodies. Two antibodies (6–11B-1, TU-01) are directed against epitopes on the amino-terminal domain of α-tubulin, and the control antibody YL1/2 recognizes an epitope on the carboxy-terminal end of α-tubulin. Antibody 6–11B-1 reacts with acetylated α:Lys 40 that is located within the H1-S2 loop (H, α helix; S, β strand) and antibody TU-01 reacts with an epitope that is located in the α65–79 region that is included in the S2, H2 and S2-H2 loop. The predominant features of the inside surface of microtubules are long loops H1-S2, H2-S3 and S9-S10 (Nogales et al., 1999). The TU-01 antibody is not directed against posttranslationally modified α-tubulin. The antibody YL1/2 reacts with tyrosylated α:Glu450 that is located on the C-terminus of the subunit. The reactivity of antibodies 6–11B-1, TU-01 and YL1/2 in fixed and unfixed cells is shown in Figure 1. While the

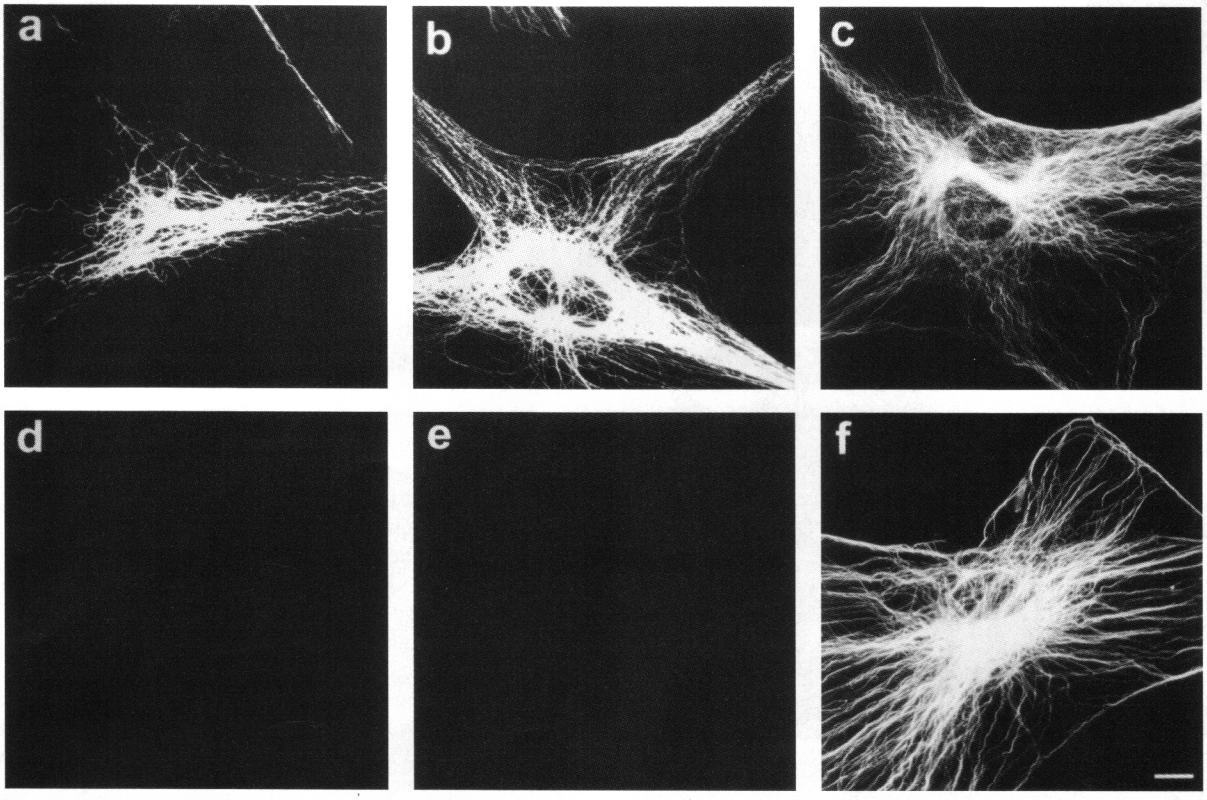

Fig. 1. a–f. Immunofluorescence staining of microtubules with antibodies against α-tubulin on fixed (**a–c**) and unfixed, detergent-extracted (**d–f**) cells. Staining with antibody 6–11B-1 (**a, d**), antibody TU-01 (**b, e**) and antibody YL1/2 (**c, f**). Bar, 10 μm.

antibodies 6–11B-1 and TU-01 reacted only with fixed samples, the antibody YL1/2 decorated microtubules under both conditions. Double-label experiments with monoclonal antibodies and the polyclonal anti-tubulin antibody confirmed undamaged microtubules in fixed and unfixed cells (not shown). As expected, the 6–11B-1 antibody reacted only with some subpopulation of fixed microtubules that represented the more stable microtubules (Schulze et al., 1987). When unfixed cytoskeletons were prepared in the absence of taxol, a smaller number of microtubules were present in such samples, and were often fragmented (Fig. 2a). The antibody YL1/2 decorated remaining unfixed microtubules along their whole length. On the other hand, antibodies 6–11B-1 and TU-01 stained only small fragments in some microtubules (Fig. 2b). Stained fragments could reflect local openings in unstabilized microtubules. Increased diffuse staining was also observed. Staining of microtubules with 6–11B-1 and TU-01 antibodies was observed after as low as 0.2% formaldehyde fixation. Under standard fixation conditions (3% formaldehyde), microtubules were decorated with 6–11B-1 antibody already after 1 min. This indicates that the binding of antibodies to microtubles is rapid and even mild fixation leads to the exposure of the epitope located on the lumenal H1-S2 loop.

As the unfixed cytoskeletons were routinely prepared in the presence of 10 μM taxol which could possibly influence the exposure of corresponding epitopes, antibodies were also injected into living cells and incubated for various time intervals. Of the injected antibodies only YL1/2 decorated the microtubules, whereas 6–11B-1 and TU-01 did not bind to them and gave only diffuse staining in the cytoplasm. Double-label experiments with injected monoclonal antibody 6–11B-1 or YL1/2 and polyclonal anti-tubulin antibody confirmed undamaged microtubules in injected cells. These data document that, similarly as in unfixed cells, the acetylated α:Lys 40 is not accessible on microtubules in vitro and that the inability of 6–11B-1 antibody to decorate microtubules in unfixed cells is not attributable to the stabilizing effect of taxol.

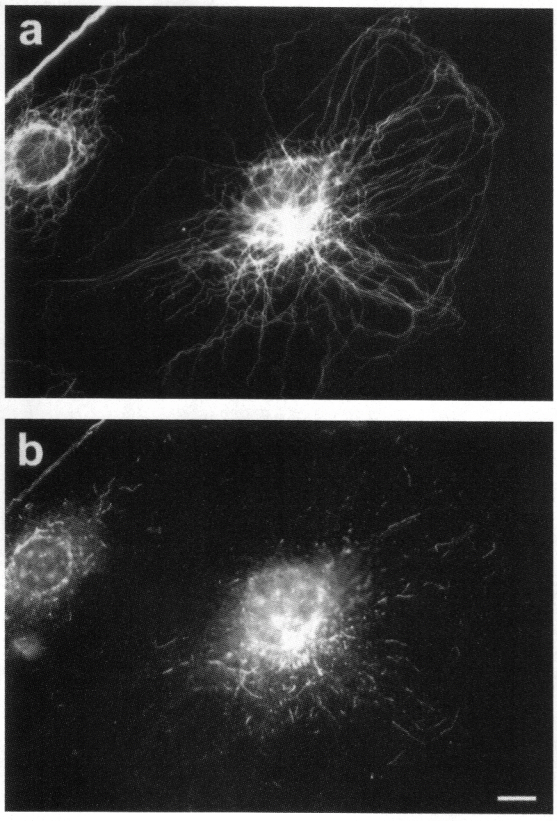

Fig. 2. a–b. Double-label immunofluorescence staining of microtubules on unfixed, detergent-extracted cells prepared in the absence of taxol. Staining with polyclonal anti-tubulin antibody (**a**) and antibody 6–11B-1 (**b**). Bar, 10 μm.

Discussion

The staining of microtubules with antibodies reacting with epitopes located on the lumenal side of microtubules could be due to the transport of the antibodies into the microtubule lumen. According to statistic data it is, however, highly improbable that antibodies can reach these sites by diffusion along the microtubule lumen (Odde, 1998). Because of their size, antibodies also cannot squeeze through the gaps between tubulin subunits in the microtubule lattice. Such mechanism was postulated for the small taxol molecule (Nogales et al., 1999) which rapidly appears on assembled microtubules (Evangelio et al., 1998; Diaz et al., 1998). An alternative explanation of antibody staining is that fixation substantially damages the microtubule lattice and tubulin dimers are lost from the microtubule wall. Although defects in microtubule lattice were described in microtubules assembled in interphase cell extracts (Chrétien et al., 1992), fixation conditions in the presence of MSB are unfavorable for the formation of gaps in the microtubule wall. On the other hand the longitudinal contacts along protofilaments appear to be stronger than the lateral contacts between protofilaments (Mandelkow et al., 1991). But mild fixation could change the conformation of tubulins that influence the interactions between protofilaments. The central element in lateral contacts of protofilaments is the S7-H9 loop (M-loop) that projects out from one side of the protofilament and makes close contacts with H3, the C-terminal part of the H2-S3 loop, and part of H1-S2 loop in the adjacent protofilament (Nogales et al., 1998). A distortion of these contacts could lead to the exposure of α:Lys 40 as well as the epitope for TU-01 antibody. It has been suggested that the microtubule lattice may breathe or open transiently under normal conditions (Diaz et al., 1998; Amos, 2000). It could be that formaldehyde inhibits the reformation of lattice contacts following such openings and enables entering of antibodies. However, in this case all stained, fixed microtubules would have to be open along their whole length at the same time. Therefore, we do not think that this is the case. Experiments on unfixed microtubules, prepared in the absence of taxol, have shown that Triton X-100 is not sufficient to expose corresponding interior sites along the whole length of microtubules. The observed labelling of fragments in some microtubules could be explained by local opening of unstabilized microtubules.

To reconcile various findings with the current model of microtubules it was suggested that acetyltransferase diffuses slowly along the ~150-Å lumen of microtubules (Nogales et al., 1999). Our data indicate that the lumenal site of cytoplasmic microtubules can be exposed for antibody binding after mild fixation conditions. It is possible that conforma-

tional changes of tubulin after binding of other proteins (for example acetyltransferase, proteases), lead to changes in lateral interactions between protofilaments and to an exposure of some regions of the lumenal microtubule surface. Conformational changes of tubulin upon binding of motor proteins were described (Hoenger and Milligan, 1997).

In conclusion, the presented data confirm that α:Lys 40 is not exposed on the surface of microtubules in unfixed cells, but fixation leads to an exposure of this interior microtubule site. The same holds true for TU-01 epitope α65–79. We suggest that lateral interactions between microtubule protofilaments can be changed in cells already by mild fixation. This could explain why antibodies can quickly reach lumenal sites in microtubules. It is possible that by the same way, i.e. by loosening lateral interactions between protofilaments, acetyltransferase can reach α:Lys 40 in cells under physiological conditions.

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