Biochemical re-evaluation of GFP-tubulin expressed in mammalian cells

Daisuke Furuta[1], Ryutaro Moriyama[1] and Nobuyuki Fukushima[2]

[1]*Department of Life Science,* [2]*RIST,*
Kinki University, 3-4-1 Kowakae, Higashiosaka, Japan,

(Received December 24, 2008)

Abstract

Green fluorescent protein (GFP)-tagged tubulin has been widely employed to examine tubulin dynamics in living cells. In the present study, we biochemically re-evaluate the property of GFP-tubulin expressed in cells. When expressed in mammalian cells, antibodies against GFP or the amino-terminal region of tubulin detected GFP-tubulin in western blot analyses. By contrast, antibodies against the carboxyl terminal region of tubulin failed to recognize exogenous GFP-tubulin, but not endogenous tubulin. These results indicate that GFP-tubulin is partially truncated at the carboxyl terminal region and suggest that data of experiments using GFP-tubulin should be carefully analyzed.

Keywords: GFP-tubulin, Myc-tubulin, live cell imaging, posttranslational modifications

Introduction

Microtubules are the component of the cytoskeleton and play a role in regulation of cell morphology, dynamics and division (1,2). Microtubules consist of tubulin subunits formed from α- and β-tubulin. The tubulin subunits are assembled to the end of microtubules at the growing of microtubules, while they are released from the end of microtubules at the shrinking. Such tubulin dynamics has been biochemically and immunocytochemically examined in various types of experimental systems. During the last decade, live cell imaging technologies have been advanced and applied to analyze tubulin dynamics including treadmilling or dynamic instability in living cells. This well-established method employs genetically engineered living cells that express fluorescent-tagged tubulin, such as green fluorescent protein (GFP)-tagged tubulin (3-7). These studies have shown that tagged tubulin is polymerized and incorporated into microtubules, suggesting normal behavior of tagged tubulin in living cells.

Carboxyl terminal (C-terminal) region of tubulin contains unique features involved in regulation of tubulin functions through its posttranslational modifications (PTMs) (8-11). For example, α-tubulin is detyrosinated at the last amino acid tyrosine,

leading to generation of detyrosinated tubulin, whose last amino acid becomes glutamate. Detyrosinated tubulin is though to be associated with microtubule stability. Another type of modification is polyglutamylation at the C-terminal region of α-and β-tubulin. In order to investigate the function of these PTMs of tubulin, we have overexpressed GFP-tubulin in mammalian cells and attempted to analyze PTMs of GFP-tubulin. During our analysis, we surprisingly noticed that GFP-tubulin always lacked the immunoreactivity for several tubulin antibodies against the C-terminal region. Such deletion in GFP-tubulin may overestimate the data obtained from live cell imaging analysis.

Materials and Methods

Expression plasmid

GFP-human α1-tubulin was obtained from Clontech. Myc-tubulin was generated by subcloning of tubulin into pcDNAIII-myc plasmid, which we generated as an expression plasmid for an N-terminal myc-tagged protein.

Cell cultures, transfection and cloning

HEK293 and COS7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. For biochemical assay, cells were cultured in a 35-mm dish. Cells were transfected with expression plasmids using lipofectamineLTX (0.1 μg plasmids and 0.3 μl of lipofectamineLTX per cm^2). Cells were washed next day and further cultured for 1 day. To establish clonal cells, HEK293 cells were transfected with GFP-tubulin and single colonies expressing GFP-tubulin were selected in the presence of 600 μg/ml G418. Two clones, WT-1 and WT-2, were used in this study. For fluorescent analysis, cells were cultured on glass-coverslips precoated with poly-L-lysine.

Western blot analysis

Western blotting was performed as described previously (12). Cells were washed with cytoskeletal buffer (10 mM Pipes, 150 mM NaCl, 5 mM $MgCl_2$, pH6.8) and incubated in 50 μl cytoskeletal stabilizing buffer (cytoskeletal buffer containing 20% glycerol, 5% DMSO, 0.5% Triton X-100, and protease inhibitor cocktail) for 5 min at room temperature. Cell extracts were harvested and spun at 20,000 x g for 10 min. Resultant soluble (supernatant) and cytoskeletal (precipitate) fractions were mixed with 4 x Laemmli buffer, containing dithiothreitol. Proteins (20~50 μg) were separated on 7 or 10% acrylamide gels, and then transferred onto PVDF membranes, followed by incubation with primary antibodies (Fig. 1). Primary antibody concentrations were the following: anti-GFP-antibody (Santa Cruz) at 100 ng/ml, anti-α-tubulin against its C-terminal region (DM1A, Sigma) at 100 ng/ml, anti-α-tubulin against its N-terminal region at 100 ng/ml, anti-tyrosinated α-tubulin (TUB-1A2, Sigma) at 1:1000, anti-glutamylated tubulin (Chemicon) at 100 ng/ml, and anti-myc antibody (9E10, Santa Cruz) at 100 ng/ml. Then, blots were incubated with the peroxidase-labeled anti-mouse or rabbit IgG antibody (100 ng/ml) and bound antibodies were visualized with the ECL plus detection system.

Immunoprecipitation

Cells were washed with PBS and extracted with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1% SDS, and protease inhibitor cocktail). Cell extracts were harvested and spun at 20,000 x g for 10 min. Resultant supernatants were incubated

with agarose-conjugated anti-Myc antibody (4 μg/ml, MBL) at 4 °C for 1hr. Agarose beads were collected by centrifugation at 3,000 x g for 1 min and further washed with RIPA buffer three times. The immunoprecipitates were mixed with 2 x Laemmli buffer containing dithiothreitol and subjected to western blot analyses, as described above.

Results and Discussion

We generated two stable HEK293 cell lines expressing GFP-tubulin (WT-1 and WT-2, Fig. 2). Western blot analysis of these cells using anti-GFP antibody showed that these clones expressed a single 77 kDa band, as expected (Fig. 3). Antibody against the N-terminal domain, 65-79, of α-tubulin also detected the product. Half amounts of expressed GFP-tubulin were present in the cytoskeletal fraction, similar to the ratio for endogenous tubulin. However, GFP-tubulin was hardly detected by anti-α-tubulin antibody (DM1A), which recognizes the C-terminal region spanning amino acid 413 and 434 (13) (Fig. 3). We further examined PTMs of GFP-tubulin using several antibodies. The employed antibodies included anti-tyrosinated tubulin antibody (TUB-1A2), which recognizes tubulin containing tyrosine at the C-terminal end 451(14), and anti-glutamylated tubulin antibody, which recognizes glutamylated (detyrosinated) tubulin containing glutamic acid at the C-terminal end 450 (14) (Fig. 1). These antibodies recognized endogenous tubulin, but failed to detect GFP-tubulin (Fig. 3). These findings demonstrated that GFP-tubulin lacks immunoreactivities against these antibodies against the C-terminal region, suggesting degradation of GFP-tubulin at the C-terminal region in cells.

The possibility that molecular size of

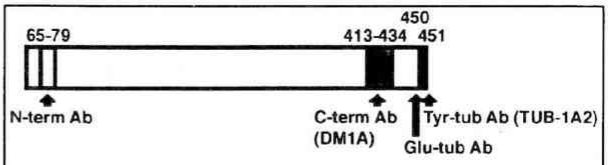

Figure 1 Mapping of anti-α-tubulin antibodies. Epitopes of the antibodies used in this study are shown.

27 kDa GFP affected the stability of GFP-tubulin was also examined. We constructed Myc-tubulin whose tag was an approximately 1 kDa peptide and transiently expressed in COS cells. Because Myc-tubulin overlaps with endogenous tubulin, we immunoprecipitated Myc-tubulin using anti-Myc antibody and then analyzed in western blot analyses. Immunoprecipitated Myc-tubulin was clearly recognized by anti-Myc antibody as well as DM1A (Fig. 4). However, anti-glutamylated antibody failed to detect Myc-tubulin (Fig. 4). Whether TUB-1A2 detects Myc-tubulin remains to be determined. Taken together, these results suggested that GFP-tubulin may be labile due to high molecular mass of GFP, compared with Myc-tubulin.

During maintenance of GFP-tubulin expressing HEK293 cells, we also noticed GFP-tubulin distribution within cells varied between cells; some showed tubule-like distribution, other showed scattered localization (data not shown). Moreover, cell populations that lost GFP fluorescence appeared with time. Such fluctuations of GFP-tubulin expression/localization may be attributed to

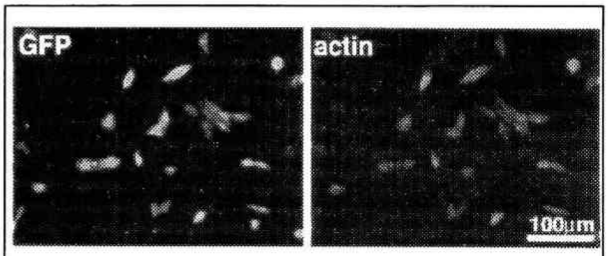

Figure 2 Fluorescence of GFP-tubulin-expressing clonal HEK293 cells (WT-1). Cells were stained for f-actin with rhodamine-phalloidin to visualize all cells in the field.

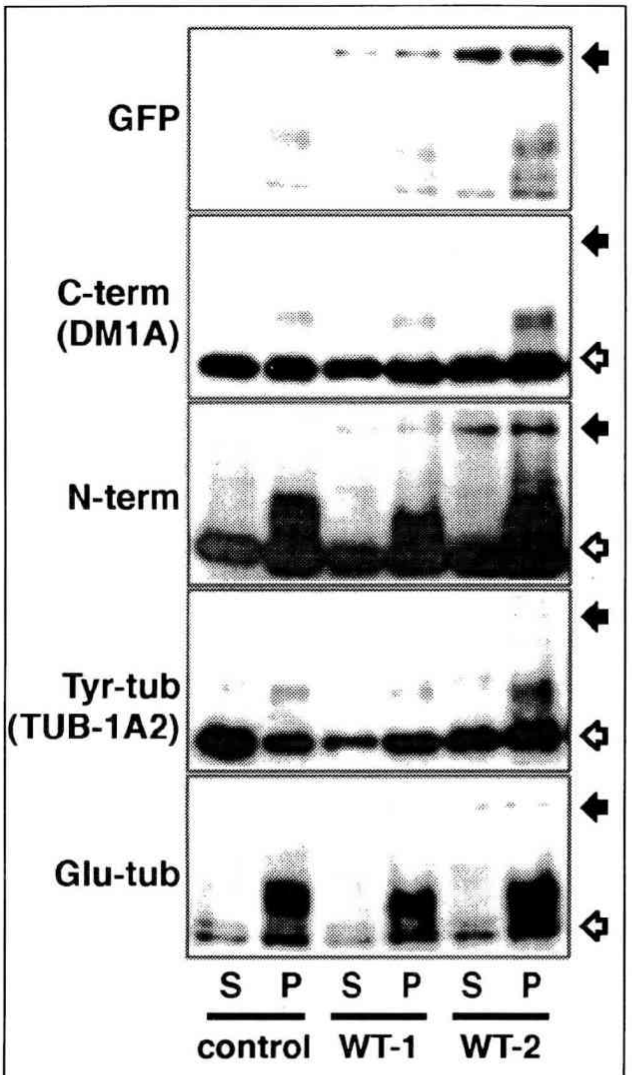

Figure 3 Western blot analysis of GFP-tubulin expressed in clonal HEK293 cells. Non-transfected (control) or two clonal HEK293 cell lines (WT-1 and WT-2) were extracted, and supernatant (S) and precipitated (P) fractions were subjected to western blot analysis. The faint bands observed in the lowest panel (glutamylated tubulin/Glu-tub) were non-specific, which was confirmed by another set of the experiment. Black arrows; GFP-tubulin, White arrows; endogenous tubulin.

protein instability in living cells.

In the present study, we have biochemically re-evaluated the property of GFP-tubulin stably expressed in mammalian cells, and found that GFP-tubulin is partially

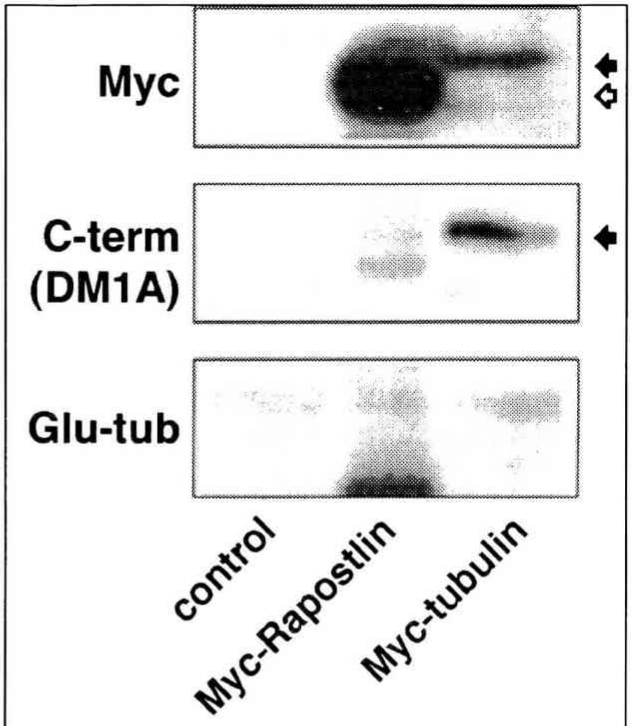

Figure 4 Western blot analysis of Myc-tubulin expressed in COS cells. COS cells were transfected without (control), or with a Myc-tubulin or a Myc-rapostlin plasmid (as a control for immunoprecipitation) and further cultured for 2 day. Cell extracts were subjected to immunoprecipitation combined with western blot analysis. Black arrows; Myc-tubulin, White arrows; Myc-rapostlin.

truncated for the C-terminal regions. Because the binding site for guanine nucleotide is far from in distance their C-terminal region, which is exposed to the outside of microtubules (1), the partially truncated tubulin might still retain normal binding affinity for guanine nucleotide or guanine nucleotide phosphatase activity. Rather, the C-terminal region plays an important role in binding to microtubule-associated proteins or motor proteins, which regulate microtubule stability and dynamics (9). For example, tyrosinated tubulin binds to CAP-Gly proteins, which are members of plus end tracking proteins, whereas glutamylated tubulin preferentially interacts with kinesin. Therefore, GFP-tubulin lacks any interactions

with these regulatory proteins, resulting in instability observed in living cells. Some of the past studies showed excellent live cell imaging of GFP-tubulin (3,4,6,7). However, as far as we know, no studies demonstrated that GFP-tubulin was present as an intact tyrosinated or glutamylated form in living cells. Further evaluation and analyses would be needed to understand usefulness and limitation of tagged tubulin.

Acknowledgment
We thank Mr, Sadahiko Haga and Tohki Tamagawa for generating plasmids.

References

1. Bray, D. (2001) *Cell Movements: From Molecules to Motility*, 2nd Ed., Garland Pub.,, New York
2. Desai, A., and Mitchison, T. J. (1997) *Annu Rev Cell Dev Biol* **13**, 83-117
3. Rusan, N. M., Fagerstrom, C. J., Yvon, A. M., and Wadsworth, P. (2001) *Mol Biol Cell* **12**, 971-980
4. Straight, A. F., Marshall, W. F., Sedat, J. W., and Murray, A. W. (1997) *Science* **277**, 574-578
5. Ludin, B., and Matus, A. (1998) *Trends Cell Biol* **8**, 72-77
6. He, Y., Francis, F., Myers, K. A., Yu, W., Black, M. M., and Baas, P. W. (2005) *J Cell Biol* **168**, 697-703
7. Murray, M. J., and Saint, R. (2007) *Development* **134**, 3975-3983
8. Luduena, R. F. (1998) *Int Rev Cytol* **178**, 207-275
9. Hammond, J. W., Cai, D., and Verhey, K. J. (2008) *Curr Opin Cell Biol* **20**, 71-76
10. Dent, E. W., and Gertler, F. B. (2003) *Neuron* **40**, 209-227
11. Fukushima, N., Furuta, D., Hidaka, Y., Moriyama, R., and Tsujiuchi, T. (in press) *J Neurochem* DOI: 10.1111/j.1471-4159.2009.06013.x
12. Fukushima, N., and Morita, Y. (2006) *Brain Res* **1094**, 65-75
13. Larcher, J. C., Boucher, D., Lazereg, S., Gros, F., and Denoulet, P. (1996) *J Biol Chem* **271**, 22117-22124
14. Kreis, T. E. (1987) *Embo J* **6**, 2597-2606