STOP Proteins

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ABSTRACT. Microtubules assembled from pure tubulin *in vitro* are labile, rapidly depolymerized upon exposure to the cold. In contrast, in a number of cell types, cytoplasmic microtubules are stable, resistant to prolonged cold exposure. During the past years, the molecular basis of this microtubule stabilization in cells has been elucidated. Cold stability is due to polymer association with different variants of a calmodulin-regulated protein, STOP protein. The dynamic and hence the physiological consequences of STOP association with microtubules vary in different tissues. In neurons, STOP seems almost permanently associated with microtubules. STOP is apparently a major determinant of microtubule turnover in such cells and is required for normal neuronal differentiation. In cycling cells, only minor amounts of STOP are associated with interphase microtubules and STOP does not measurably affect microtubule dynamics. However, STOP is associated with mitotic microtubules in the spindle. Recent results indicate that such an association could be vital for meiosis and for the long-term fidelity of the mitotic process.

Key words: microtubules/stability/STOP proteins

Introduction

Microtubules assembled from pure tubulin *in vitro* are dynamic structures that exhibit both spontaneous length fluctuations and treadmilling behaviour (19, 20). A similar dynamic behaviour is observed *in vivo* (3, 15). A natural hypothesis is therefore that the intrinsic dynamic properties of microtubules are conserved in the cytoplasmic environment. However, cytoplasmic microtubules are markedly different from pure tubulin microtubules with regard to their apparent stability properties. Pure tubulin microtubules disassemble rapidly upon exposure to the cold or when suspensions are diluted in large volumes of buffer. In contrast, cold and dilution resistant microtubules are a common occurrence in living cells (1, 4, 13, 22, 28). The origin and significance of such microtubule stability have been uncertain. Microtubules interact with a large variety of cell components and microtubule stabilization could be a relatively unspecific event, triggered by the cumulative effect of polymer association with many different

ligands. On the other hand, microtubule dynamics are thought to be central to microtubule organization and function. In this case, only specific and regulated effectors would be expected to affect microtubule stability. The present review concerns the elucidation of the origin of microtubule cold-stability, an extreme state of microtubule stabilization. It is shown that, in a variety of cell types, microtubule cold stability results from polymer association with a family of calmodulin-regulated proteins called STOP proteins. These proteins can block reversibly microtubule dynamics. The significance of such molecular switches that can suppress or release microtubule dynamic activity in cells is only beginning to be unraveled.

Neuronal STOP proteins

Molecular characterization of STOP

Neuronal cells contain abundant subsets of cold-stable microtubules (1, 5, 21, 28). Therefore, microtubule cold stability was initially investigated in brain tissues. The pioneering work of Webb and Wilson (28) showed that microtubule cold stability could be conferred to cold labile microtubules by a factor that could be isolated from cold stable microtubule preparations using

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DEAE ion exchange chromatography. These data were important because they showed that microtubule cold stability did not result from some kind of microtubule denaturation and that the factor(s) responsible for cold stabilization was distinct from tubulin. Further work in the R.L. Margolis laboratory showed that microtubule cold stability could be abolished if the stable polymers were exposed to the action of Ca^{++} -calmodulin (10). Chromatography of microtubule proteins from cold-stable polymers on immobilized calmodulin columns revealed the presence of a calmodulin binding protein, not found in cold-labile microtubule preparations, and for this reason called STOP (Stable Tubulin Only Polypeptide) (11). The purification of STOP using biochemical procedures proved to be difficult, due to both extreme sensitivity of the protein to proteases and low purification yields. Despite such difficulties convincing evidence that STOP was indeed the factor responsible for microtubule cold stability in brain preparations and that STOP was associated with stable microtubules in living cells could be obtained (17, 18). However, the molecular characteristics and significance of STOP remained elusive.

A turning point in the process of STOP characterization was the development of STOP monoclonal antibodies (26). These antibodies were subsequently used to clone the STOP cDNA (6). The analysis of this cDNA showed that STOP was a 100-kDa protein, with no homology to any known protein (Bosc *et al.*; and Fig. 1). STOP contains two domains of repeated motifs. The central region of the protein comprises 5 tandem repeats of 46-amino acids. Four of these repeats share 98% homology with the consensus sequence. Such identity between long repeated sequences seems to be unprecedented. The STOP C-terminus contains 28 imperfect repeats of an 11-amino acid motif. Adjacent to this C-terminal repeat region, STOP contains an arginine and lysine rich region, encompassing about 40 amino acids, that we use to call the KR region of the protein. The domain structure of the protein is paralleled by the exon structure of the STOP gene. The STOP gene in mice contains four exons (7). Exon 1 comprises the nucleotide sequences corresponding to the N-terminal domain of STOP, including the central

repeats. Exon 2 corresponds to the linker domain of the protein lying between the central repeat region and the KR domain. Exon 3 and 4 correspond to the KR domain and to the C-terminal repeat domains, respectively. We used the cloned STOP cDNA to test the ability of STOP to induce microtubule cold stability in living cells. When STOP was expressed in cells that lack cold stable microtubules, STOP associated with microtubules and induced complete polymer stabilization (6). These data showed that STOP had similar action on microtubules *in vivo* as *in vitro*. Recently, several calmodulin binding domains and microtubule binding domains have been identified on STOP (Bosc *et al.* Unpublished observations).

STOP function in neuronal cells

Microtubule stabilization in neuronal cells is expressed in different ways. Microtubules show extensive cold-stability. Microtubules are largely drug-resistant (1, 2). Many microtubules are long-lived, showing a slow subunit turnover (12, 14, 23, 27). As an apparent consequence of this slow polymer turnover, tubulin is extensively modified, in particular with regard to its tyrosination status. Normal tubulin carries a tyrosyl residue at the C-terminal end of its α -subunit. Long-lived polymers are exposed to the continuous action of a carboxypeptidase and this results in the detyrosination of the constitutive tubulin to generate detyrosinated, Glu-tubulin, microtubules (16). In very long-lived polymers, α -tubulin loses a second C-terminal amino acid and this generates $\Delta 2$ -tubulin polymers (24, 25). Neuronal microtubules are extensively detyrosinated and show a highly specific enrichment in $\Delta 2$ -tubulin (25). It has been unclear whether the different manifestations of polymer stabilization in neuronal cells have a common origin or result from the action of many different effectors. Clearly, STOP appeared as a reasonable candidate for an important role in microtubule cold stabilization. However, it remained to be found whether this role was real and unique, and whether STOP activity was also responsible for the observed microtubule drug stability and slow subunit turnover as determined by the extent of polymer detyrosination.

Fig. 1. Schematic representation of STOP and E-STOP showing domain structure of the two proteins. Both proteins contain a central domain composed of 5 (STOP) or 6 (E-STOP) repeats. STOP also contains a C-terminal repeat domain that is lacking in E-STOP. The E-STOP sequence ends at a position corresponding to α 614 in the STOP sequence (Bosc *et al.*, 1996).

We used dorsal root ganglia cells from rat embryos (DRG cells) to investigate the sub-cellular distribution of STOP in differentiating neuronal cells (Guillaud *et al.*, and Fig. 2). An unexpected outcome of this study was the discovery of new isoforms of STOP. DRG cells contained a major STOP variant that was also dominant in rat embryonic brain tissue. This STOP variant, E-STOP, lacked the C-terminal repeat domain found in the adult STOP and corresponding to exon 4 in the mouse gene. Despite this deletion, E-STOP (Fig. 1) has the same microtubule stabilizing activity as the complete adult STOP. Immunoelectron microscopy examination of DRG cell axons showed STOP association with microtubules (Fig. 2). No detectable amount of STOP was present in the soluble cell compartment. At least 60% of neuronal microtubules were associated with significant amounts of STOP. Furthermore, STOP proteins were specifically enriched on cold-stable, drug-resistant, and detyrosinated microtubules. These results were compatible with a general role of STOP in the regulation of microtubule stability and turnover in neuronal cells. However, it might have been the case that STOP accumulated on polymers stabilized by other

Fig. 2. Immunoelectron microscopy localization of STOP proteins in DRG cell axons. After ten days of culture, DRG cells were stained using an affinity-purified STOP antibody and a Cy3- (A) or gold-labeled (B) secondary antibody. DRG cell axon: microtubules were specifically decorated with gold particles. Bar, 50 nm.

factors. We used inhibitory STOP antibodies to test whether STOP association with stable polymers was the cause or the consequence of the polymer stabilization (9). These antibodies were directed against peptide sequences localized within the central repeat region of STOP and were able to inhibit STOP binding onto microtubules *in vitro*. The antibodies were injected in differentiated PC12 cells. PC12 cells normally contain cold-stable and nocodazole-resistant microtubules. This microtubule stabilization was completely lost in cells injected with inhibitory STOP antibodies (Fig. 3). These results showed that STOP proteins were truly responsible for the high degree of microtubule stabilization observed in neuronal cells, not simply associated with stable polymers.

An important question concerned the consequences of STOP inhibition on cell behaviour, in neuronal cells. We found no evidence for neurite retraction or for an inhibition of neurite elongation in differentiated PC12 cells injected with inhibitory STOP antibodies. Clearly, microtubule stabilization is not absolutely required for the persistence of neurite extensions, at least during the relatively limited time of observation compatible with antibody microinjection experiments. We tested whether STOP proteins were required for the formation of neurite extensions in non-differentiated PC12 cells treated with NGF. Undifferentiated PC12 cells were either injected with STOP antibodies or exposed to STOP antisense oligonucleotides. In both cases, STOP inhibition blocked neurite outgrowth. The mechanisms involved in this inhibition remain to be determined but, apparently, STOP proteins are important for normal neuronal differentiation in cultured cells (9).

Taken together, our results provided strong evidence that the various manifestations of microtubule stabilization in neuronal cells, including microtubule cold stability, detyrosination and drug resistance, were all caused by well-defined effectors, the STOP proteins. Short-term observations on cells in culture yielded limited information concerning the physiological significance of STOP function in development and/or neuronal function. Mice with STOP knockout genes clearly need to be developed to address this question.

Non neuronal variants of STOP proteins

A number of cycling mammalian cells, such as NIH3T3 cells, contain abundant subsets of cold-stable microtubules (13). The origin and significance of this polymer stability have been intriguing. Given the highly dynamic behaviour of cytoplasmic microtubules in cycling cells, the presence of stable polymers seems paradoxical. At the time of the studies reported below, two main possibilities were considered. One possibility was that microtubule cold stability was an artifact due to

Fig. 3. STOP inhibition suppresses microtubule cold and drug stability in fused PC12 cells. Fused PC12 cells injected with control IgGs (A to F), showing IgG (A to C) and microtubule (D to F) staining. A and D: untreated cells; B and E: cold-treated cells; C and F: nocodazole-treated cells. Fused PC12 cells injected with affinity-purified STOP antibody (G to L) showing STOP antibody (G to I) and microtubule (J to L) staining. G and J: untreated cells; H and K: cold-treated cells; I and L: nocodazole-treated cells. Bar, 50 μ m. Note that following cell lysis, enough microinjected IgGs remains associated with insoluble cell structures to allow clear identification of injected cells. STOP antibody injection suppressed microtubule cold and drug stability.

cell exposure to the cold. Another possibility was that cycling cells indeed contained potent MAPs, able to induce complete microtubule stabilization, and subjected to strong regulation in normal conditions. In this case, an attractive possibility was that such putative MAPs were related to the STOP proteins previously characterized in neuronal cells. To test the presence of STOP analogs in cycling cells, we probed NIH3T3 cell extracts with a battery of STOP antibodies directed against various domains of the protein (8). On immunoblots of NIH3T3 cells extracts, several bands reacting with several independent STOP antibodies, were identified. The major STOP species (F-STOP) had an apparent molecular weight of 42 kDa, considerably lower than that of either N-STOP or E-STOP. To determine the structure of F-STOP the corresponding cDNA was cloned. Results (Denarier *et al.* 1998b, and Fig. 4) showed that the F-STOP message differed from the N-STOP message with regard to both exon composition and transcription start site. In the F-STOP message, the nucleotide sequences corresponding to exon 3 were lacking, obviously due to mRNA splicing. As a result, the sequences corresponding to exon 2 and to exon 4 were adjacent. The F-STOP message contained a stop codon located exactly at the junction between the exon 2 and the exon 4 sequences. Therefore, F-STOP contains the entire protein domain encoded by exon 2 whereas the exon 4 domain is absent. The F-STOP cDNA also contained exon 1 sequences, including the nucleotide sequences coding for the STOP central repeat domain. However, quite surprisingly, the nucleotide sequences corresponding to the 5' region of exon 1 were lacking. In fact, the translation start site used for the synthesis of the F-STOP message, corresponded to an ATG located closely upstream of the sequences coding for the central repeats and different from the ATG used in the case of the neuronal STOP messages (8). We are currently engaged in the characterization of the F-STOP promoter. Curiously, it seems that nucleotide se-

quences that are coding sequences for N-STOP are used as promoter sequences for F-STOP.

Despite extensive deletions compared to the standard N-STOP, we found that F-STOP, as other STOP variants, could induce microtubule cold stability both *in vitro* and *in vivo* (8). However, a major difference between F-STOP and N-STOP concerned their association with microtubules in physiological conditions. Whereas N-STOP appeared to be almost permanently associated with neuronal microtubules, F-STOP was mainly free in the cytoplasm of interphase NIH3T3 cells kept at physiological temperature. During cell exposure to the cold, F-STOP accumulated massively and rapidly on microtubules. F-STOP was associated with the mitotic spindle at both physiological temperature and cold temperature (8). We are currently investigating the molecular basis for the differences observed in the behaviour of F-STOP compared to N-STOP and for the differential association of F-STOP with interphase microtubules compared to mitotic polymers. It seems that these differences are due to a combination of structural factors and of regulatory factors.

To test whether F-STOP was the only factor responsible for microtubule cold stability in NIH3T3 cells, cells were injected with inhibitory STOP antibodies (see above). In microinjected cells, microtubule cold stability was entirely lost, in both mitotic microtubules and interphase microtubules (Denarier *et al.*, 1998b, and Fig. 5). Therefore, in cycling cells as in neuronal cells, microtubule cold stability is caused by microtubule association with a STOP protein species. STOP inhibition had no obvious consequences with regard to cell behaviour, in cultured NIH3T3 cells. Apparently, F-STOP serves a function that is not crucially required for short-term cell survival *in vitro*. Recent experiments in our laboratory indicate that the situation may be quite different in whole animals. We have obtained chimeric mice, using ES cells in which a single STOP allele had been invalidated. Our results indicate that such single

Fig. 4. Structure of the F-STOP cDNA. Schematic representation showing alignment of F-STOP cDNA with mouse brain STOP cDNA. Nucleotides are numbered according to the mouse brain STOP cDNA sequence, a deduced from the mouse STOP gene. The F-STOP cDNA lacks nucleotides 1 to 910 of the brain STOP cDNA, and also lacks the entire sequence of exon 3. A stop codon is introduced exactly at the transition between exon 2 and exon 4 sequences. Therefore, the exon 4 sequences are lacking in F-STOP.

Fig. 5. Interphase and spindle microtubule disassembly in cold-treated cells injected with STOP antibody. NIH3T3 cells were injected with affinity-purified STOP antibody. After a recovery period of two hours, cells were exposed to cold temperature for 30 min. Cells were then fixed and double-stained using Cy3-conjugated anti-rabbit antibody to detect injected 23C antibody (A, C, E) and mAb TUB 2.1/FITC anti-mouse antibody to detect tubulin (B, D, F). (A, B), injected and non-injected interphase cells. (C, D), an injected metaphase cell. (E, F), a non-injected metaphase cell. STOP inhibition abolishes microtubule cold stability. Bars, 10 μ m.

STOP allele knockout is sufficient to induce a meiotic block. The major STOP variant in the testis is F-STOP. Apparently, normal levels of F-STOP are required for the completion of meiosis (Andrieux *et al.* Unpublished observations).

Conclusion

The presence of abundant subsets of cold-stable microtubules in the cytoplasm of different cell types has been an intriguing property of these polymers, in sharp

contrast with the labile nature of pure tubulin microtubules and, in many cases, with the dynamic behaviour of microtubule arrays in cells. The molecular origin of cytoplasmic microtubule cold stability is now elucidated. In neuronal cells as in cycling cells, microtubule cold stability is induced by polymer association with STOP proteins. There are several STOP variants, generated from a single gene through alternative mRNA splicing and through the use of alternative start sites for transcription. These variants are differentially expressed in different tissues and at different stages of de-

velopment. All known STOP variants share ability to interact with calmodulin and microtubules. STOP proteins have a different effect on microtubule turnover in neuronal cells compared to cycling cells. In neuronal cells, STOP proteins are almost permanently associated with microtubules and are major factors responsible for the slow turnover of neuronal microtubules. STOP function is apparently required for neuronal differentiation. In cycling cells at physiological temperature, most STOP molecules are free in the cytoplasm during interphase and do not have measurable influence on microtubule dynamics. In the same cells, STOP proteins are associated with the mitotic spindle and may play a role in the long-term fidelity of mitosis. Many questions concerning the logic and the significance of the STOP system remain open. Crucial information should be yielded by the examination of genetically modified mice with general or targeted disruption of the STOP gene.

References

1. BAAS, P.W. and HIEDEMANN, S.R. 1986. Microtubule reassembly from nucleating fragments during the regrowth of amputated neurites. *J. Cell Biol.*, **103**: 917–927.
2. BAAS, P.W., PIENKOWSKI, T.P., CIMBALNIK, K.A., TOYAMA, K., BAKALIS, S., AHMAD, F.J., and KOSIK, K.S. 1994. Tau confers drug stability but not cold stability to microtubules in living cells. *J. Cell Sci.*, **107**: 135–143.
3. BELMONT, L.D., HYMAN, A.A., SAWIN, K.E., and MITCHISON, T.J. 1990. Real time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. *Cell*, **62**: 579–589.
4. BERSHADSKY, A.D., GELFAND, V.I., SVITKINA, T.P., and TINT, I.S. 1979. Cold-stable microtubules in the cytoplasm of mouse embryo fibroblasts. *Cell Biol. Intern. Rep.*, **3**: 45–50.
5. BLACK, M.M., COCHRAN, J.M., and KURDYLA, J.T. 1984. Solubility properties of neuronal tubulin: evidence for labile and stable microtubules. *Brain Res.*, **295**: 255–263.
6. BOSC, C., CRONK, J.D., PIROLLET, F., WATTERSON, M.D., HAJECH, J., JOB, D., and MARGOLIS, R.L. 1996. Cloning, expression and properties of the microtubule stabilizing protein STOP. *Proc. Natl. Acad. Sci. USA*, **93**: 2125–2130.
7. DENARIER, E., AGUEZZOUL, M., JOLLY, C., VOURC'H, C., ROURE, A., ANDRIEUX, A., BOSC, C., and JOB, D. 1998a. Genomic structure and chromosomal mapping of the mouse STOP gene (*Mtap6*). *Biochem. Biophys. Res. Commun.*, **243**: 791–796.
8. DENARIER, E., FOUREST-LIEUVIN, A., BOSC, C., PIROLLET, F., CHAPEL, A., MARGOLIS, R.L., and JOB, D. 1998b. Non-neuronal isoforms of STOP protein are responsible for microtubule cold stability in mammalian fibroblasts. *Proc. Natl. Acad. Sci.*, **95**: 6055–6060.
9. GUILLAUD, L., BOSC, C., FOUREST-LIEUVIN, A., DENARIER, E., PIROLLET, F., LAFANECHÈRE, L., and JOB, D. 1998. STOP proteins are responsible for the high degree of microtubule stabilization observed in neuronal cells. *J. Cell Biol.*, **142**: 167–179.
10. JOB, D., FISCHER, E.H., and MARGOLIS, R.L. 1981. Rapid disassembly of cold-stable microtubules by calmodulin. *Proc. Natl. Acad. Sci.*, **78**: 4679–4682.
11. JOB, D., RAUCH, C.T., FISCHER, E.H., and MARGOLIS, R.L. 1982. Recycling of cold-stable microtubules: evidence that cold stability is due to substoichiometric polymer blocks. *Biochemistry*, **21**: 509–515.
12. LI, Y. and BLACK, M.M. 1996. Microtubule assembly and turnover in growing axons. *J. Neurosci.*, **16**: 531–544.
13. LIEUVIN, A., LABBÉ, J.C., DORÉE, M., and JOB, D. 1994. Intrinsic microtubule stability in interphase cells. *J. Cell Biol.*, **124**: 985–996.
14. LIM, S.S., SAMMAK, P.J., and BORISY, G.G. 1989. Progressive and spatially differentiated stability of microtubules in developing neuronal cells. *J. Cell Biol.*, **109**: 253–263.
15. MAC NALLY, F.J. 1996. Modulation of microtubule dynamics during cell cycle. *Curr. Opin. Cell Biol.*, **8**: 23–29.
16. MACRAE, T.H. 1997. Tubulin post-translational modifications—enzymes and their mechanisms of action. *Eur. J. Biochem.*, **244**: 265–278.
17. MARGOLIS, R.L., RAUCH, C.T., and JOB, D. 1986. Purification and assay of a 145-kDa protein (STOP₁₄₅) with microtubule-stabilizing and motility behavior. *Proc. Natl. Acad. Sci. USA*, **83**: 639–643.
18. MARGOLIS, R.L., RAUCH, C.T., PIROLLET, F., and JOB, D. 1990. Specific association of STOP protein with microtubules *in vitro* and with stable microtubules in mitotic spindles of cultured cells. *EMBO J.*, **9**: 4095–4102.
19. MARGOLIS, R.L. and WILSON, L. 1978. Opposite end assembly and disassembly of microtubules at steady state *in vitro*. *Cell*, **13**: 1–8.
20. MITCHISON, T.J. and KIRSCHNER, M. 1984. Microtubule assembly nucleated by isolated centrosomes. *Nature*, **312**: 237–242.
21. MORRIS, J.R. and LASEK, R.J. 1982. Stable polymers of the axonal cytoskeleton: the axoplasmic ghost. *J. Cell Biol.*, **92**: 192–198.
22. MOSKALEWSKI, S., THYBERG, J., and FRIBERG, V. 1980. Cold and metabolic inhibitor effects on cytoplasmic microtubules and the Golgi complex in cultured rat epiphyseal chondrocytes. *Cell Tissue Res.*, **210**: 403–415.
23. OKABE, S. and HIROKAWA, N. 1990. Turnover of fluorescently labelled tubulin and actin in the axon. *Nature*, **343**: 479–482.
24. PATURLE-LAFANECHÈRE, L., EDDÉ, B., DENOULET, P., VAN DORSSELAER, A., MAZARGUIL, H., LE CAER, J.P., WEHLAND, J., and JOB, D. 1991. Characterization of a major brain tubulin variant which cannot be tyrosinated. *Biochemistry*, **30**: 10523–10528.
25. PATURLE-LAFANECHÈRE, L., MANIER, M., TRIGAULT, N., PIROLLET, F., MAZARGUIL, H., and JOB, D. 1994. Accumulation of delta 2-tubulin, a major tubulin variant that cannot be tyrosinated, in neuronal tissues and in stable microtubule assemblies. *J. Cell Sci.*, **107**: 1529–1543.
26. PIROLLET, F., RAUCH, C.T., JOB, D., and MARGOLIS, R.L. 1989. Monoclonal antibody to microtubule-associated STOP protein: affinity purification of neuronal STOP activity and comparison of antigen with activity in neuronal and nonneuronal cell extracts. *Biochemistry*, **28**: 835–842.
27. TAKEDA, S., FUNAKOSHI, T., and HIROKAWA, N. 1995. Tubulin dynamics in neuronal axons of living zebrafish embryos. *Neuron*, **14**: 1257–1264.
28. WEBB, B.C. and WILSON, L. 1980. Cold-stable microtubules from brain. *Biochemistry*, **19**: 1993–2001.