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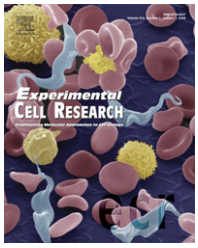

Research Article

Op18/Stathmin counteracts the activity of overexpressed tubulin-disrupting proteins in a human leukemia cell line

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ABSTRACT

Op18/stathmin (Op18) is a phosphorylation-regulated and differentially expressed microtubule-destabilizing protein in animal cells. Op18 regulates tubulin monomer–polymer partitioning of the interphase microtubule system and forms complexes with tubulin heterodimers. Recent reports have shown that specific tubulin-folding cofactors and related proteins may disrupt tubulin heterodimers. We therefore investigated whether Op18 protects unpolymerized tubulin from such disruptive activities. Our approach was based on inducible overexpression of two tubulin-disrupting proteins, namely TBCE, which is required for tubulin biogenesis, and E-like, which has been proposed to regulate tubulin turnover and microtubule stability. Expression of either of these proteins was found to cause a rapid degradation of both α-tubulin and β-tubulin subunits of unpolymerized, but not polymeric, tubulin heterodimers. We found that depletion of Op18 by means of RNA interference increased the susceptibility of tubulin to TBCE or E-like mediated disruption, while overexpressed Op18 exerted a tubulin-protective effect. Tubulin protection was shown to depend on Op18 levels, binding affinity, and the partitioning between tubulin monomers and polymers. Hence, the present study reveals that Op18 at physiologically relevant levels functions to preserve the integrity of tubulin heterodimers, which may serve to regulate tubulin turnover rates.

Introduction

Tubulin α–β heterodimers form polar microtubule polymers that are essential for intracellular transport and cell division in all eukaryotes (reviewed in [1]). Each heterodimer subunit binds a guanidine nucleotide, which is nonexchangeable in α-tubulin (termed the N-site) and exchangeable in β-tubulin (termed the E-site). GTP at the E-site is required for microtubule assembly, and it is hydrolyzed and becomes nonexchangeable upon addition of a subsequent heterodimer to the end of the microtubule. Hydrolysis at the E-site is a consequence of longitudinal heterodimer interactions and involves a catalytic loop located on α-tubulin [2]. The stability of microtubules is dependent on a cap of tubulin-GTP at the ends (termed the GTP-cap) and loss of this cap causes depolymerization. This provides the basis of the dynamic behavior of microtubules, which implies stochastic switches between assembly and disassembly—a phenomenon known as dynamic instability (reviewed in [1]).

Studies in mammals [3–7] and fission yeast [8,9] have shown that biosynthesis of native tubulin involves a complex folding pathway that—besides prefoldin and cytosolic chaperonin—

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Abbreviations: MT, microtubule; Op18, oncoprotein 18/stathmin; shRNA, short hairpin RNA

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requires five tubulin-specific chaperone protein cofactors, denoted TBCA, -B, -C, -D, and -E. The final step in the assembly involves formation of a large complex of α-tubulin/TBCE and β-tubulin/TBCD that (together with TBCC) causes GTP hydrolysis at the E-site and subsequent release of native heterodimers [10]. Besides having a role in tubulin biosynthesis, tubulin-specific cofactors have also been shown to disrupt native heterodimers [7,10–12]. Moreover, based on decay of native tubulin in cells overexpressing TBCD or TBCE, which in the case of TBCD has been shown to be negatively regulated by the small GTP binding protein Arl2 [7], these cofactors have been proposed to be of significance for regulation of tubulin turnover (reviewed in [13]) . In addition, a protein abundant in testis, termed E-like, was recently identified due to its sequence similarity with TBCE [14]. E-like does not assist in assembly of native tubulin but it is still capable of disrupting heterodimers in vitro. Interestingly, it was shown that suppression of E-like expression by RNA interference increased the number of stable microtubules and that overexpressed E-like commits tubulin to proteosomal degradation. It was proposed that E-like represents a cellular activity that regulates tubulin turnover and facilitates cytoskeletal remodeling.

Tubulin turnover in cultured cells is reportedly very slow [15,16], which contrasts with the rapid decay of purified tubulin preparations stored under non-polymerizing conditions [17], and references therein). This raises the question of whether an abundant tubulin-binding protein such as Op18/stathmin (Op18) may protect against the heterodimer disrupting activities outlined above and to have the general function of preserving unpolymerized tubulin. Op18 forms ternary complexes with two head-to-tail aligned tubulin heterodimers [18,19] and has been described as a differentially expressed and conserved regulator of microtubule stability in diverse types of animal cells (reviewed in [20,21]). By analysis of three human cell types of hematopoetic origin, we have found that depletion of Op18 by RNA interference results in extensive over-polymerization of tubulin during interphase, but not mitosis, which demonstrates that Op18 is required for normal tubulin monomer-polymer partitioning in the interphase microtubule system [22]. This finding, together with reports of tubulin-disruptive activities in cells, prompted us to investigate whether Op18—through direct interaction with tubulin—might serve a function in both maintaining the pool of unpolymerized tubulin and in preserving the integrity of tubulin heterodimers within this pool. Our approach was based on an inducible expression system to study the immediate consequences of TBCE and E-like overexpression with respect to tubulin disruption, monomer-polymer partitioning, and tubulin protective activities of Op18. The results indicate that at physiologically relevant levels, Op18 protects tubulin heterodimers by directly interacting with them.

Materials and methods

DNA constructs

The pMEP4 shuttle vector, directing inducible expression of FLAG epitope-tagged Op18 (Op18-F), MCAK, and the Op18-like region of SCG10 (residues 35–179, termed sSCG10 since in contrast to native SCG10 the recombinant protein is soluble in detergent-free buffers), has been described previously [23–25]. The pMEP-TBCE-F and pMEP-E-like-F derivatives, both of which encode a C-terminal FLAG epitope tag, were generated by PCR (primer sequences will be provided on request) using TBCE (accession number NM_003193) and E-like (accession number NM_152715) cDNAs, which were kindly provided by Dr Nicholas J. Cowan [14,26]. The EBV-based shuttle vector for constitutive expression of short hairpin RNA (shRNA) targeting Op18 has been described previously [27] and a BLAST search of the NCBI database ensured specific targeting of Op18 mRNA.

Transfections and cell culture

Single transfections and co-transfections of K562 using EBV-based replicating shuttle vectors and subsequent selection of hygromycin-resistant cell lines were performed in a medium specifically designed to support cell growth under conditions that minimize expression from the hMTIIa promoter, as described in detail elsewhere [27,28]. Conditional expression and co-expression in K562 cells was induced from the hMTIIa promoter by addition of 0.5 μM Cd^{2+}. For expression of TBCE-F or E-like-F, cells were transfected with 16 μg of the cognate pMEP derivative or as indicated in the figure legends. For experiments involving co-expression of tubulin-disruptive proteins with Op18-F, 5 μg of pMEP-TBCE-F or pMEP-E-like-F DNA was mixed with 11 μg of pMEP-Op18-F or pMEP-MCAK. To keep DNA concentrations constant in control cells expressing either of the proteins alone, Vector-Co was added up to a total quantity of 16 μg DNA. Transfection with replicating shuttle vectors that direct constitutive synthesis of specific interfering shRNA was performed according to the same basic protocol as described for pMEP vectors, with 2 μg of shRNA-Op18 producing constructs mixed with Vector-Co up to a total quantity of 16 μg DNA. For inducible expression in shRNA synthesizing cells, 2 μg of shRNA-Op18 was mixed with pMEP-TBCE-F or pMEP-E-like-F up to a total quantity of 16 μg DNA. To keep DNA concentrations constant, Vector-Co DNA was used to replace shRNA-Op18 DNA in control cell populations that were not depleted of Op18. Due to the stringent replication control of the EBV-based shuttle vectors, the ratio of transfected DNAs is stable during the 5- to 7-day time course of the present experiments [29].

Immunoblotting and quantification of immunofluorescence by flow cytometry

For immunoblotting, cells were lysed in a Triton X-100 containing buffer and soluble proteins were separated by 12% SDS-PAGE as described [23]. Immunoblotting and subsequent detection using the ECL detection system (Amersham Pharmacia Biotech) were performed using anti-PCNA (PC10, Dakopatts), anti-α-tubulin (B-5-1-2, Sigma), anti-β-tubulin (N357, GE Healthcare), anti-FLAG (M2, Sigma), anti-MCAK [30], and anti-SLEEIQ [31]. Anti-SLEEIQ is a preparation of affinity-purified rabbit antibodies raised against a peptide corresponding to a completely conserved region that allows simultaneous and equivalent detection of all members of the Op18/stathmin family. Quantification of total tubulin content and Op18-like proteins by flow cytometry was performed on cells chilled on