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Characterisation of polyglutamylases in trypanosomatids

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ABSTRACT

Microtubules are subject to post-translational modifications, which are thought to have crucial roles in the function of complex microtubule-based organelles. Among these, polyglutamylation was relatively recently discovered, and was related to centrosome stability, axonemal maintenance and mobility, and neurite outgrowth. In trypanosomatids, parasitic protozoa where microtubules constitute the essential component of the cytoskeleton, the function of polyglutamylated microtubules is unknown. Here, in order to better understand the role of this conserved but highly divergent post-translational modification, we characterised glutamylation and putative polyglutamylases in these parasites. We showed that microtubules are intensely glutamylated in all stages of the cell cycle, including interphase. Moreover, a cell cycle-dependent gradient of glutamylation was observed along the cell anteroposterior axis, which might be related to active growth of the microtubule 'corset' during the cell cycle. We also identified two putative polyglutamylase proteins (among seven analysed here) which appeared to be clearly and directly involved in microtubule polyglutamylation in in vitro activity assays. Paradoxically, in view of the importance of tubulins and of their extensive glutamylation in these organisms, RNA interference-based knockdown of all these proteins had no effect on cell growth, suggesting either functional redundancy or, more likely, subtle roles such as function modulation or interaction with protein partners.
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1. Introduction

Microtubules (MTs) are highly conserved dynamic structures made of α- and β-tubulin heterodimers that are encoded by a family of polymorphic genes and are involved in a variety of essential cell processes such as intracellular transport, mitosis and mobility. In addition to their length and structure variations, MTs can be subject to a series of reversible post-translational modifications (PTMs) (acetylation, phosphorylation, polyglutamylation, polyglycylation, palmitoylation and detyrosination), some of which are very unusual (reviewed in Wloga and Gaertig (2010) and Janke and Bulinski (2011)). Polyglutamylation is a PTM that generates glutamate side-chains of variable length on the gamma-carboxyl group of glutamate residues within the primary sequence of the target protein (reviewed in Janke et al. (2008)). It occurs in a variety of proteins, essentially α- and β-tubulins (Eddé et al., 1990; Rüdiger et al., 1992), but also the nucleosome assembly proteins NAP1 and NAP2 (Regnard et al., 2000) and other nucleo-cytoplasmic shuttling proteins (van Dijk et al., 2008) that, in higher eukaryotes, are proposed to function in a broad array of physiological activities, through molecular mechanisms as diverse as phosphatase inhibition, chromatin regulation, caspase activation and intracellular transport (Okada et al., 2011; Reilly et al., 2011; Kuryan et al., 2012). The modification is generated by relatively recently discovered polyglutamylating enzymes, members of the Tubulin Tyrosine Ligase-Like (TTLL) family (Janke et al., 2005). TTLLs constitute a large family of proteins that share a homology domain with TTL, another tubulin-modifying enzyme, and whose members can catalyse the ligation of glutamate amino acids to tubulins or other substrates (van Dijk et al., 2008). In mammals, 13 TTLL enzymes exist, among which nine are glutamylases (van Dijk et al., 2007) and three are catalysing a related modification, glycylation (Rogowski et al., 2009; Wloga et al., 2009). Each enzyme displays defined reaction preferences for modifying the α- or β-tubulin and for generating short or long sidechains

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(van Dijk et al., 2007; Rogowski et al., 2009). Both the polyglutamylation and the polyglycylation reactions consist of two biochemically distinct steps: initiation and elongation, which are often mediated by distinct TTLLs. Initiation is the formation of an isopeptide bond with the gamma-carboxyl group of the glutamate acceptor site, whereas side chain elongation consists of the formation of regular peptide bonds (Redeker et al., 1991; Wolff et al., 1994). It has been proposed that the range of signals generated by the variable length of the added sidechain may allow a fine tuning of the function of MTs and of their interactions with protein partners (Lacroix and Janke, 2011) (reviewed in Janke et al. (2008) and Janke and Kneussel (2010)). Thus, tubulin PTMs, and more specifically polymodifications, are thought to have crucial roles in the assembly, maintenance and function of complex MT-based organelles (reviewed in Janke and Bulinski (2011)).

Polyglutamylation is an ancient phenomenon evolutionarily conserved from protists to mammalian cells; it is present in sperm flagella of mammals, in sea urchins as well as in several protists including *Giardia*, *Tetrahymena*, *Crithidia* and *Trypanosoma* (Eddé et al., 1990; Seebeck et al., 1990; Bré et al., 1994; Rüdiger et al., 1995; Moulay et al., 1996; Plessmann and Weber, 1997; Schneider et al., 1997; Weber et al., 1997; Westermann et al., 1999). In mammalian cells, tubulin polyglutamylation is related to centrosome stability, axonemal maintenance and mobility in cilia and flagella, and neurite outgrowth (Gagnon et al., 1996; Bobinnec et al., 1998; Million et al., 1999; Westermann and Weber, 2003; Janke et al., 2005; Ikegami et al., 2006; Pathak et al., 2007; Vogel et al., 2010) (reviewed in Janke et al. (2008)). In protists, knowledge of the biological function of polyglutamylated MTs is limited (Wloga et al., 2008; Wloga and Gaertig, 2010) and, in view of the extreme genetic and biological diversity encountered in these organisms (Berriman et al., 2005), probably much more diverse than in mammals.

Trypanosoma and *Leishmania* are flagellated parasitic protozoa of the Trypanosomatidae family. They have a simple but precisely ordered cytoskeleton, primarily made of stable MTs (Schneider et al., 1997) and, with MTs constituting the most abundant part of their cytoskeleton, they show a reduced dependence on the acto-myosin network (Kohl and Gull, 1998; Berriman et al., 2005). MTs constitute four sub-structures in trypanosomatids: the mitotic spindle, the flagellar axoneme, the basal body of the flagellum and, most importantly, the subpellicular 'corset'. This corset is made exclusively of a dense network of MTs cross-linked to each other and to the plasma membrane, forming a helical pattern along the long axis of the cell (reviewed in Robinson et al. (1995)). It is responsible for the cell shape and plays a major role in events such as positioning of organelles, mitosis and cytokinesis (Sasse and Gull, 1988).

Similar to other organisms, MTs in these parasites are subject to a series of PTMs (detyrosination, acetylation, polyglutamylation) (Wolff et al., 1992; Gull, 1999). Using mass spectrometry analysis, trypanosomatid tubulin has been shown to be extensively glutamylated and non-glutamylated tubulins were reported to be almost absent (Schneider et al., 1997). In contrast, the closely related modification glycylation was not detected in *Trypanosoma brucei* (Schneider et al., 1997). The discovery that TTLL proteins catalyse tubulin glutamylation (Janke et al., 2005; van Dijk et al., 2007) led us to identify eight TTLL genes in silico in the genome of *T. brucei*. These enzymes belong to five of the diverse subtypes of this protein family (TTLL1, TTLL4, TTLL6, TTLL9 and TTLL12). To understand the role of this evolutionarily conserved but highly divergent PTM (Wloga and Gaertig, 2010) and to better characterise tubulin glutamylation in trypanosomatids, the distribution of polyglutamylated MTs during the cell cycle in *Leishmania major* and *T. brucei* was examined using specific antibodies (Abs). The putative TTLL genes of *T. brucei* and *L. major* were then cloned and the subcellular localisation of the gene products determined using GFP-fused recombinant proteins; their enzymatic activities were analysed in vitro and an insight into their putative biological function obtained using RNA interference (RNAi).

2. Material and methods

2.1. Parasites

Leishmania major 'Friedlin' promastigotes (MHOM/IL/81/Friedlin) were grown as previously described (Dubessay et al., 2004). Procyclic forms of the 29–13 line of *T. brucei* were grown at 27 °C in SDM 79 (Sigma®, MO, USA) supplemented with FCS (10%), hemin (7 μg/ml), hygromycin (30 μg/ml) and geneticin (10 μg/ml) for continuous culture, plus phleomycin (5 μg/ml) for RNAi experiments.

2.2. Bioinformatics

The sequences of genes encoding putative TTLL proteins in *L. major* and *T. brucei* were searched in the MapView database (http://www.genedb.org/genedb/leish/). The corresponding trypanosomatid protein sequences were aligned using Clustal X and corrected manually as described previously (Wloga et al., 2008).

2.3. Construction of L. major cell lines expressing GFP fused proteins

The genes encoding putative TTLLs were PCR-amplified from genomic DNA. The PCR products were cloned into a pGEM-T-Easy vector (Promega®, WI, USA) and then inserted into the expression vectors pTH6cGFPn and pTH6nGFPc (Dubessay et al., 2006) which place the GFP fusion at the N-terminus and C-terminus, respectively. The presence of the reading frame of the recombinant proteins was confirmed by sequencing.

Transfection in *L. major* was performed as described previously (Casanova et al., 2009). Briefly, 5×10^7 cells with 80 μg of plasmid DNA were electroporated using a Bio-Rad GENE Pulser II and a Pulse Controller (Bio-Rad, Hercules, USA) at 25 μF and 1500 V with two pulses of 0.5 ms each and 10 ms between each pulse. Hygromycin B (Sigma®) was added at 30 μg/ml for selective pressure.

2.4. Microscopy and immunofluorescence imaging

For the intracellular localisation of GFP-fused proteins or for immunofluorescence of whole cells, transfected cells grown to mid-log phase were fixed in 4% paraformaldehyde (PFA) and air-dried on microscope fluorescence slides (Dubessay et al., 2006). For immunofluorescence analysis of cytoskeletons, cells were washed in PBS and deposited on 8-well slides. Cytoskeletons were extracted in 0.25% Nonidet P40 (NP40), 100 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), 1 mM $MgCl_2$, pH 6.9, for 5 min at room temperature (Pradel et al., 2006) and then fixed in 4% PFA for 20 min for *L. major* or for 5 min for *T. brucei*.

In both immunofluorescence preparations (whole cell or cytoskeleton), fixed cells were incubated with either the GT335 (1:10,000, Adipogen®, CA, USA), the PolyE (1:10,000 for *L. major* and 1:5,000 for *T. brucei*, made by the authors) or the YL1/2 (1:200, Santa Cruz Biotechnology®, CA, USA) Abs for 45 min, followed by 45 min with anti-mouse Alexa 546 or 488 (1:500, Molecular Probes®, OR, USA), anti-rabbit Alexa 488 (1:500, Molecular Probes®) or anti-rat conjugated with TRITC (1:500; Santa Cruz Biotechnology®) Abs, respectively.

To study the mitochondrion, cultivated cells were primarily incubated for 10 min at 27 °C with Mitotracker Red CMXRos (500 nM, Molecular Probes®) and washed in culture medium without

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