Cell

A Family of Protein-Deglutamylating Enzymes Associated with Neurodegeneration

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DOI 10.1016/j.cell.2010.10.014

SUMMARY

Polyglutamylation is a posttranslational modification that generates glutamate side chains on tubulins and other proteins. Although this modification has been shown to be reversible, little is known about the enzymes catalyzing deglutamylation. Here we describe the enzymatic mechanism of protein deglutamylation by members of the cytosolic carboxypeptidase (CCP) family. Three enzymes (CCP1, CCP4, and CCP6) catalyze the shortening of polyglutamate chains and a fourth (CCP5) specifically removes the branching point glutamates. In addition, CCP1, CCP4, and CCP6 also remove gene-encoded glutamates from the carboxyl termini of proteins. Accordingly, we show that these enzymes convert detyrosinated tubulin into Δ2-tubulin and also modify other substrates, including myosin light chain kinase 1. We further analyze *Purkinje cell degeneration* (*pcd*) mice that lack functional CCP1 and show that microtubule hyperglutamylation is directly linked to neurodegeneration. Taken together, our results reveal that controlling the length of the polyglutamate side chains on tubulin is critical for neuronal survival.

INTRODUCTION

Polyglutamylation is a posttranslational modification that generates glutamate side chains of variable length on the γ-carboxyl groups of glutamic acid residues within the primary sequence of the target proteins. This modification has been initially discovered on α- and β-tubulin, the building blocks of microtubules (MTs; Eddé et al., 1990; Rüdiger et al., 1992), and later also found on other proteins (Regnard et al., 2000; van Dijk et al., 2008). Polyglutamylation levels are particularly high on stable MT assemblies such as the ones found in neurons (Audebert et al., 1993), axonemes (Bré et al., 1994), centrioles, and basal bodies (Bobinnec et al., 1998) but is also enriched in the highly dynamic mitotic spindle (Regnard et al., 1999). Tubulins are polyglutamylated at their carboxy-terminal tails, which upon assembly of MTs become exposed on the outer surface of the tubules, where they provide binding sites for several MT-associated proteins (MAPs) and molecular motors. Accordingly, MT polyglutamylation has recently been shown to regulate the activity of ciliary dynein (Kubo et al., 2010; Suryavanshi et al., 2010) and of the MT-severing protein spastin (Lacroix et al., 2010).

The enzymes that catalyze polyglutamylation, polyglutamylases, are members of a protein family sharing a homology domain with another tubulin-modifying enzyme, tubulin tyrosine ligase (TTL; Ersfeld et al., 1993), and thus are referred to as TTL-like (TTLL) proteins (Janke et al., 2005; van Dijk et al., 2007). Initial functional studies on polyglutamylases provided first evidence that polyglutamylation plays important roles in vivo. Mice knocked out for TTLL1 polyglutamylase display abnormal beating of airway epithelial cilia leading to respiratory problems (Ikegami et al., 2010). Another polyglutamylase, TTLL6, was also shown to play a role in cilia. Depletion of this enzyme in zebrafish reduced glutamylation in the olfactory placodes resulting in defective assembly of olfactory cilia (Pathak et al., 2007). Surprisingly, also overexpression of TTLL6 enzyme, which caused abnormal elongation of glutamate chains, produced ciliary defects in *Tetrahymena* (Janke et al., 2005; Wloga et al., 2009). Taken together, this suggests that maintaining the correct levels of tubulin polyglutamylation by the coordinated action of polyglutamylases and deglutamylating enzymes is essential. The aim of the present work was to identify deglutamylating enzymes and analyze their functions in vivo.

Recent studies of a family of cytosolic carboxypeptidases (CCPs) have suggested that one of the members of this family, CCP1 (also known as Nna1), is the enzyme that removes the C-terminal tyrosine from α-tubulin (Kalinina et al., 2007; Rodriguez de la Vega et al., 2007); however convincing evidence has not been provided. The possibility that CCP1 is involved in tubulin modifications was exciting because loss of function of

CCP1 had been associated with the phenotypes observed in *Purkinje cell degeneration* (*pcd*) mice (Fernandez-Gonzalez et al., 2002). *pcd* mice display multiple defects including degeneration of several types of neurons. The first neurons to be lost are Purkinje cells, which die during early adulthood leading to ataxia. The loss of Purkinje cells is followed by degeneration of cerebellar granule neurons (CGNs), and later some populations of thalamic neurons also disappear (Mullen et al., 1976). Moreover, mitral cells from the olfactory bulb as well as retinal photoreceptors degenerate progressively over 1 year. Finally, the males are sterile and the females experience difficulties giving birth (reviewed in Wang and Morgan, 2007). Recent studies demonstrated that Purkinje cell loss as well as retinal degeneration observed in the *pcd* mice could be rescued by wild-type (WT) CCP1 but not an inactive CCP1 (Chakrabarti et al., 2008; Wang et al., 2006). This demonstrated that the lack of CCP1 carboxypeptidase activity is responsible for the phenotypes observed in the *pcd* mice; however, the molecular mechanisms underlying the numerous defects remain unclear.

Here, we show that CCP1 specifically catalyzes the removal of the penultimate glutamate residue from detyrosinated α-tubulin, thus generating Δ2-tubulin; however, it is not involved in detyrosination itself. Moreover, we demonstrate that the removal of gene-encoded glutamic acids from the C termini of proteins is not specific to tubulin but affects a range of substrates including myosin light chain kinase 1 (MLCK1). Apart from the deglutamylation of protein primary sequence, CCP1 also shortens posttranslationally generated glutamate side chains on tubulin and is therefore a tubulin deglutamylase. We further demonstrate the existence of two functional homologs of CCP1, CCP4, and CCP6, and we provide evidence that another recently described deglutamylating enzyme, CCP5 (Kimura et al., 2010), specifically removes the branching point glutamates generated by polyglutamylation. Finally, we have analyzed the neurodegeneration phenotype in the *pcd* mice in light of the newly identified deglutamylating activity of CCP1. Consistent with the enzymatic activity of CCP1, we show that tubulin polyglutamylation is highly increased specifically in brain areas that degenerate in the *pcd* mice. Moreover, by downregulating polyglutamylation, we were able to partially prevent neurodegeneration, providing direct evidence that abnormally high polyglutamylation levels lead to neuronal degeneration.

RESULTS

CCP1 Generates Δ2-Tubulin and Also Acts as a Tubulin Deglutamylase

Recent studies have suggested that CCP1 is involved in α-tubulin detyrosination (Kalinina et al., 2007). To directly test this idea, we performed immunoblot analysis of protein extracts prepared from HEK293 cells expressing either active or enzymatically inactive (dead, Figure S1 available online) murine CCP1. Although we did not observe any increase in the level of tubulin detyrosination, we detected an ectopic appearance of a related tubulin modification, Δ2-tubulin (Paturle-Lafanechere et al., 1991). Thus, CCP1 seems to catalyze the removal of the penultimate glutamate residue, but not detyrosination itself. To further test whether CCP1 is specifically generating Δ2-tubulin, we expressed this protein in mouse embryonic fibroblasts (MEFs) and stained the cells with antibodies for tyrosinated and Δ2-tubulin. There was no obvious reduction in the level of tyrosinated tubulin, but we observed a strong increase in the labeling for Δ2-tubulin specifically in cells expressing CCP1 (Figure 1B). This increase in the level of Δ2-tubulin was not observed when the enzymatically dead version of CCP1 was expressed (Figure S2A). Taken together these results demonstrate that CCP1 catalyzes the removal of the very C-terminal glutamate residue from detyrosinated α-tubulin, but it is not involved in α-tubulin detyrosination.

Considering that CCP1 was associated with α-tubulin primary sequence deglutamylation, we speculated that it might also catalyze the removal of glutamate side chains, which are added to α- and β-tubulin as a result of posttranslational polyglutamylation (Eddé et al., 1990; Rüdiger et al., 1992). To test this hypothesis, we expressed CCP1 in neurons, which are known to carry a high level of tubulin polyglutamylation (Audebert et al., 1993), and labeled them with polyE antibody that recognizes glutamate chains composed of at least three glutamic acids (Figure S3). Neurons expressing active but not inactive CCP1 showed a strong reduction in the level of polyE signal (Figure 1C and Figure S2B). These results show that CCP1 is indeed involved in the removal of posttranslational polyglutamylation.

To further characterize the deglutamylation activity of CCP1, we set up an in vitro deglutamylation assay in which highly polyglutamylated brain tubulin was incubated with protein extract from HEK293 cells expressing either active or inactive CCP1. The status of polyglutamylation was examined with two polyglutamylation-specific antibodies: polyE that recognizes long glutamate side chains, and GT335 that recognizes all glutamylated forms of tubulin because it is specific to the branching point of the glutamate side chain (Wolff et al., 1992). In combination, these two antibodies allowed us to distinguish between tubulin carrying either short or long glutamate side chains. Brain tubulin treated with active CCP1 was no longer detected by polyE antibodies, while the GT335 signal was not decreased (Figure 1D). This indicated that CCP1 shortens the glutamate side chains of brain tubulin without removing the branching point glutamates. Similar results were obtained with purified 6His-CCP1, confirming that CCP1 directly catalyzes tubulin deglutamylation (Figures S2C and S2D). To further investigate the enzymatic specificity of CCP1 for long side chains, we assayed the enzyme with mono- or polyglutamylated tubulin purified from HeLa cells after overexpression of different TTLL glutamylating enzymes (van Dijk et al., 2007). When monoglutamylated tubulin (modified by TTLL4) was incubated with active CCP1, no changes in glutamylation levels were observed, indicating that CCP1 cannot remove the short side chains generated by TTLL4 (Figure 1D). Surprisingly, when tubulin polyglutamylated with TTLL6 was incubated with CCP1, a strong reduction in both polyE and GT335 signals was observed (Figure 1D). This indicated that besides shortening the polyglutamate side chains, CCP1 also removes the branching point glutamates when the glutamylation is generated by TTLL6 but not by TTLL4 or by TTLL1, which generates most of the glutamylation present on brain MTs (Janke et al., 2005). To validate that the removal of the branching point glutamates by CCP1 depends on the