Cell

Evolutionary Divergence of Enzymatic Mechanisms for Posttranslational Polyglycylation

Krzysztof Rogowski,[1] François Juge,[2] Juliette van Dijk,[1] Dorota Wloga,[3] Jean-Marc Strub,[4] Nicolette Levilliers,[5] Daniel Thomas,[6] Marie-Hélène Bré,[5] Alain Van Dorsselaer,[4] Jacek Gaertig,[3] and Carsten Janke[1,*]
[1]CRBM, CNRS, Université Montpellier 2 and 1
[2]IGMM, CNRS, Université Montpellier 2
Montpellier 34293, France
[3]Department of Cellular Biology, University of Georgia, Athens, GA 30602-2607, USA
[4]LSMBO, Université Louis Pasteur, CNRS, Strasbourg 67087, France
[5]Laboratoire de Biologie Cellulaire 4, CNRS, Université Paris-Sud 11, Orsay 91405, France
[6]Interactions Cellulaires et Moléculaires, CNRS, Université de Rennes 1, Rennes 35042, France
*Correspondence: carsten.janke@crbm.cnrs.fr
DOI 10.1016/j.cell.2009.05.020

SUMMARY

Polyglycylation is a posttranslational modification that generates glycine side chains on proteins. Here we identify a family of evolutionarily conserved glycine ligases that modify tubulin using different enzymatic mechanisms. In mammals, two distinct enzyme types catalyze the initiation and elongation steps of polyglycylation, whereas *Drosophila* glycylases are bifunctional. We further show that the human elongating glycylase has lost enzymatic activity due to two amino acid changes, suggesting that the functions of protein glycylation could be sufficiently fulfilled by monoglycylation. Depletion of a glycylase in *Drosophila* using RNA interference results in adult flies with strongly decreased total glycylation levels and male sterility associated with defects in sperm individualization and axonemal maintenance. A more severe RNAi depletion is lethal at early developmental stages, indicating that protein glycylation is essential. Together with the observation that multiple proteins are glycylated, our functional data point towards a general role of glycylation in protein functions.

INTRODUCTION

Polyglycylation is a posttranslational modification that generates side chains of glycine on the γ-carboxyl groups of specific glutamate residues of target proteins (Bré et al., 1998; Vinh et al., 1999). Similarly to polyglutamylation (Eddé et al., 1990), this posttranslational modification was discovered on tubulin, where it occurs within the carboxy-terminal tail domains (Redeker et al., 1994). While polyglutamylation was observed on many different types of microtubules (MTs), polyglycylation is particularly prominent in cilia and flagella and is mostly found in cells that possess these organelles (Bré et al., 1996; Levilliers et al., 1995).

Functional experiments on tubulin glycylation were difficult to perform since the modifying enzymes were unknown. Initial insights into the potential function of polyglycylation were obtained using modification-specific antibodies. For example, anti-glycylation antibodies affected axoneme beating in vitro, suggesting that tubulin polyglycylation could regulate ciliary dynein (Bré et al., 1996). In *Tetrahymena*, the elimination of the major glycylation sites on β-tubulin was lethal or led to severe axonemal defects, including short axoneme size, absence of the central pair MTs, and incomplete outer doublet MTs (Redeker et al., 2005; Thazhath et al., 2002; Xia et al., 2000). Thus, tubulin glycylation could be an important regulator of both the assembly and functions of axonemal MTs. The recent observations that non-tubulin proteins are also substrates of polyglycylation (Ikegami et al., 2008; Lalle et al., 2006; Xie et al., 2007) indicate that glycylation is a posttranslational modification of general importance.

Here we describe the discovery of a group of enzymes that catalyze tubulin glycylation and show that some of these enzymes have non-tubulin substrates. We demonstrate that glycylases are members of the tubulin tyrosine ligase-like (TTLL) family, which already contains three types of known amino acid ligases, the tubulin tyrosine ligase (TTL; Ersfeld et al., 1993), polyglutamylases (Janke et al., 2005; van Dijk et al., 2007; Wloga et al., 2008), and an enzyme that ligates glycine to nucleosome assembly protein 1 (NAP1; Ikegami et al., 2008). We show that in mouse, TTLL3 and TTLL8 proteins are initiating glycylases with different substrate specificities, whereas TTLL10 is the elongating polyglycylase. In mammals, cooperation of these two types of enzymes generates polyglycine side chains in mammals, whereas in *Drosophila*, bifunctional enzymes catalyze both reactions. We also demonstrate that the previously reported absence of long glycine side chains on human sperm tubulin (Bré et al., 1996) is caused by two inactivating amino acid substitutions in human TTLL10. Finally, we show by RNA

interference (RNAi) that depletion of one of the two glycylases in *Drosophila* leads to strong reduction in polyglycylation, which causes defects in sperm maturation and results in male sterility. Moreover, expression of interfering RNA under a stronger promoter is lethal in early developmental stages. This suggests that polyglycylation is important for whole organism development in *Drosophila*. The identification of additional substrates of glycylation indicates that this modification could have a broad range of functions.

RESULTS

Identification of Murine Glycylases

Our study was initiated with the hypothesis that tubulin glycylases, like two other types of tubulin amino acid ligases, TTL (Ersfeld et al., 1993) and polyglutamylases (Janke et al., 2005; van Dijk et al., 2007), are members of the TTLL protein family. Within the mammalian TTLL family, TTLL3, 8, 10, and 12 remained uncharacterized. According to phylogenetic studies, the *TTLL12* gene is present in species that appear to lack glycylation (Janke et al., 2005; Schneider et al., 1997), and murine TTLL12 has no glycylation activity in vivo and in vitro (K.R., unpublished data). In contrast, the presence of orthologs of *TTLL3*, *8*, and *10* in genomes of diverse species (Janke et al., 2005) correlates with the known presence of tubulin glycylation, and a recent study showed that one of these three genes, *TTLL10*, encodes a glycylase for NAPs (Ikegami et al., 2008).

To test whether mouse TTLL3, 8, and 10 have a tubulin glycylase activity, we expressed the respective cDNAs (van Dijk et al., 2007) in HEK293 cells and tested the cell extracts in an in vitro MT glycylation assay. To distinguish between two possible types of activities, initiation (addition of the first glycine to a γ-carboxyl group of the target glutamate residue) and elongation of glycine side chains, we used substrates differing in the levels of tubulin glycylation: MTs polymerized from brain tubulin (that lack detectable glycylation) and strongly glycylated MTs of ciliary axonemes of *Tetrahymena thermophila*. Both TTLL3 and 8 promoted incorporation of [^{3}H]-glycine into brain MTs but had very low activity on axonemal MTs. In contrast, TTLL10 was almost inactive on brain MTs but promoted efficient [^{3}H]-glycine incorporation into axonemal MTs (Figure 1A). These observations suggest that TTLL3 and 8 are initiating glycylases, whereas TTLL10 is an elongating glycylase (polyglycylase) for tubulin. A similar functional specialization has been previously observed among polyglutamylases (van Dijk et al., 2007; Wloga et al., 2008).

We also examined the selectivity of the enzymes for α- and β-tubulin. TTLL3 and 8 modified both α- and β-tubulin of brain MTs, with TTLL8 showing preference for α-tubulin (Figure 1A). For TTLL10, which modified only axonemal MTs, two-dimensional electrophoresis was used to separate axonemal α- and β-tubulin. Using anti-α- or β-tubulin antibodies as well as TAP952 (an antibody that recognizes monoglycylated sites on tubulins; Bré et al., 1998), we found that both tubulin subunits of *Tetrahymena* axonemes are monoglycylated and thus are potential substrates for the elongation reaction. However, TTLL10 incorporated [^{3}H]-glycine mainly into α-tubulin (Figure 1B).

Next, we expressed TTLL3, 8, and 10 in U2OS cells and labeled the cells with antibodies that are specific to either mono- or polyglycylation: TAP952 and polyG (polyG specifically recognizes polyglycine chains; Duan and Gorovsky, 2002), respectively (Figure 1C). Nontransfected U2OS cells show no reactivity with either of these two antibodies (arrowheads). After expression of TTLL3 or 8, we detected specific labeling of MTs with TAP952 but not with polyG. Thus, TTLL3 and 8 mediate initiation of glycine side chains on MTs in vivo but not side chain elongation. In contrast, cells expressing TTLL10 alone showed no labeling with TAP952 but had a diffuse labeling with polyG that did not correspond to MTs. This suggests that TTLL10 adds polyglycine chains directly onto proteins that are not associated with MTs, while it cannot modify MTs without prior monoglycylation.

Two-Step Glycylation Mechanism in Mammals

To test whether the two types of mammalian glycylases can function in a cooperative manner to create long side chains on MTs, we co-overexpressed either TTLL3 or 8 together with TTLL10 in U2OS cells. In both cases, we observed a strong labeling of MTs with the polyG antibody (Figure 2A). These results indicate that MTs have acquired polyglycine side chains, which was not the case when TTLL3, 8, or TTLL10 were expressed alone (Figure 1C).

On immunoblots, the monoglycylation-specific antibody TAP952 detected strong signals in the tubulin region upon expression of TTLL3 or 8 alone. Additional protein bands were labeled after TTLL8 expression, suggesting that TTLL8 can mediate glycylation of other non-tubulin proteins (Figure 2B). TTLL3 increased glycylation of mostly β-tubulin in U2OS cells, while it promoted modification of both α- and β-tubulin in the in vitro assay (Figures 2B and 1A). Thus, it appears that the substrate preferences of the glycylating enzymes change depending on additional factors. Neither TTLL3 nor TTLL8 alone generated polyG-reactive epitopes, indicating that they are indeed restricted to reactions that generate monoglycine side chains (Figure 2B). In contrast, no TAP952 labeling was detected in extracts of cells expressing TTLL10. However, a strong polyG signal was induced on two distinct protein bands, one of them the size of α-tubulin (Figure 2B). We demonstrate that the substrate in the tubulin region corresponds to NAPs. First, two-dimensional immunoblot analysis shows that the polyglycylated 50 kDa protein band superimposes with NAPs but not with tubulins (Figure 2C), and second, ectopically expressed NAP1-EYFP and NAP2-EYFP were strongly polyglycylated by TTLL10 (Figure S1 available online). This is in agreement with the results of Ikegami et al. (2008) and indicates that in contrast to tubulins, NAPs can be polyglycylated directly by TTLL10.

After coexpression of TTLL3 or 8 with TTLL10, no TAP952 labeling was detected, whereas several protein bands were labeled with the polyG antibody. Virtually all protein bands labeled with TAP952 after expression of TTLL3 or 8 alone became labeled with polyG when TTLL10 was coexpressed (Figure 2B), suggesting that following monoglycylation by TTLL3 or 8, TTLL10 elongates glycine side chains of all major substrates of the initiating enzymes. The efficiency of this process is illustrated by the reduction of monoglycylated epitopes below detectable levels. This further indicates that the substrate specificity of polyglycylation in mammals is predetermined by the specificity of the initiating