Polyglutamylation Is a Post-translational Modification with a Broad Range of Substrates

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Polyglutamylation is a post-translational modification that generates lateral acidic side chains on proteins by sequential addition of glutamate amino acids. This modification was first discovered on tubulins, and it is important for several microtubule functions. Besides tubulins, only the nucleosome assembly proteins NAP1 and NAP2 have been shown to be polyglutamylated. Here, using a proteomic approach, we identify a large number of putative substrates for polyglutamylation in HeLa cells. By analyzing a selection of these putative substrates, we show that several of them can serve as in vitro substrates for two of the recently discovered polyglutamylases, TTLL4 and TTLL5. We further show that TTLL4 is the main polyglutamylase enzyme present in HeLa cells and that new substrates of polyglutamylation are indeed modified by TTLL4 in a cellular context. No clear consensus polyglutamylation site could be defined from the primary sequence of the here-identified new substrates of polyglutamylation. However, we demonstrate that glutamate-rich stretches are important for a protein to become polyglutamylated. Most of the newly identified substrates of polyglutamylation are nucleocytoplasmic shuttling proteins, including many chromatin-binding proteins. Our work reveals that polyglutamylation is a much more widespread post-translational modification than initially thought and thus that it might be a regulator of many cellular processes.

One fundamental aspect of proteomic complexity comes from the various processing events that many proteins undergo following their synthesis. Post-translational modifications, such as phosphorylation, acetylation, and methylation, are reversible monomodifications and are known to function as switches for the activity of many proteins. Polyglutamylation is a reversible polymodification generated by sequential covalent attachment of glutamic acids (up to 20 in some cases) to an internal glutamate residue of the target protein (1). The length of the resulting side chain is regulated by the balance between the enzymes that catalyze glutamylation, recently identified as members of the tubulin-tyrosine ligase-like (TTLL)2 protein family (2–4), and yet unidentified deglutamylase enzymes (5). Thus, this modification does not only generate “on” and “off” states, but a range of signals that might allow for gradual regulation of protein functions.

The only known targets of polyglutamylation are α- and β-tubulins, the structural units of microtubules (MTs (1, 6)), and the nucleosome assembly proteins, NAP1 and NAP2 (7). Tubulins are modified in their acidic, glutamate-rich C terminus (1), which is the binding site for most MT-associated proteins (MAPs; reviewed in Ref. 8). Tubulin polyglutamylation was therefore proposed to generate functionally divergent MTs by regulating the affinity between MAPs and MTs (4, 9–11). It was also shown to be important for centriole stability (12), axonemal motility (2, 13, 14), and neurite outgrowth (15). The role of NAP polyglutamylation has not yet been addressed, but as for tubulins, it may regulate the affinity of NAPs for their binding partners.

Additional polyglutamylated proteins probably exist as the polyglutamylation-specific antibody GT335 (16) recognizes protein bands besides those of tubulins and NAPs on Western blots of HeLa cell extracts (4, 7). Here, using a proteomic approach, we identify new substrates for polyglutamylation and show that they are modified by two polyglutamylases from the TTLL protein family. No clear “glutamylation motif” could be defined in their primary sequences, but all the here-identified new substrates contain glutamate-rich stretches that are most likely the acceptor sites for the modification. Our study opens the door to further investigations of the role of polyglutamylation as a general regulatory event of a broad range of cellular functions.

**EXPERIMENTAL PROCEDURES**

**Purification of GT335-reactive Proteins from HeLa Cells by Immunoaffinity Chromatography**—10 mg of GT335 antibody was coupled to an N-hydroxysuccinimide-activated Hi-Trap column (1 ml, GE Healthcare) according to the manufacturer’s instructions. This work was supported in part by the CNRS, the Universities Montpellier 1 and 2, Association de la Recherche contre le Cancer Awards CR504/7817 and 3140 (to C.J.), French National Research Agency Award JC05_42022 (to C.J.), and the La Ligue contre le Cancer (to C.J. and B.L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1 and S2. To whom correspondence should be addressed: CRBM, CNRS, 1919 Route de Mende, 34293 Montpellier, France. Tel.: 33-4-67613335; Fax: 33-4-67521559; E-mail: carsten.janke@crbm.cnrs.fr.

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instructions. HeLa cells were extracted in PBS containing 0.1% Triton X-100 and protease inhibitors (aprotinin, leupeptin, and 4-(2-aminoethyl)-benzenesulfonyl fluoride, each at 10 μg/ml). The high speed supernatant (15 mg of protein) was loaded onto the column and after extensive washing with PBS, bound proteins were eluted with PBS containing 0.7 M NaCl and concentrated to 1 mg/ml on an Amicon ultrafiltration device (4 ml, cut-off 10 kDa, Millipore).

**Western Blotting**—Proteins fused to EYFP were immunodetected with rabbit anti-GFP antibody (1:5,000, Torrey Pines Biolabs) and horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare) followed by detection with enhanced chemiluminescence (GE Healthcare). GT335 antibody was biotinylated with NHS-LC-Biotin (Pierce) according to the manufacturer's instructions and incubated simultaneously with horseradish peroxidase-labeled streptavidin (GE Healthcare). For producing polyclonal anti-TTLL4 and anti-TTLL5 antibodies, bacterially expressed and purified full-length mouse TTLL4 or fragment 692–1152 of mouse TTLL5 were injected into rabbits. Anti-TTLL antibodies were then purified from antiserum against their respective targets.

**Mass Spectrometric Analysis**—To identify the protein content of the GT335 affinity-purified fraction, the Coomassie-stained protein bands were excised from the gel and submitted to in-gel trypsin digestion (17). Peptides were then concentrated on a C18 pre-column, separated on a reversed-phase capillary column (Pepmap C18, 75-μm inner diameter, 15-cm length, LC Packings) and analyzed by MS/MS on a Q-Tof II mass spectrometer (Micromass Ltd., Manchester, UK). Data analysis was performed with Mascot (Matrix Science Ltd., London, UK) against the NCBI (The National Center for Biotechnology Information) Data base. Double- and triple-charged peptides were used for the research in data base, and the peptide tolerance was set to 0.2 Da for MS and to 0.3 Da for MS/MS. One missed cleavage by trypsin was accepted and carbamidomethylated cysteine and oxidized methionine were set as variable modifications. All entries were selected for the taxonomy. The Mascot score cut-off value for a positive protein hit was set to 50, and peptides with a score below 40 were manually interpreted to validate or discard the identification. Identified proteins were submitted to a second research including polyglutamylation as variable modification. Additions of 1–8 glutamic acids were considered and a new research has been done each time.

**In Vitro Polyglutamylation of the New Substrates**—Putative substrates of polyglutamylation were cloned from mouse testis cDNA libraries, expressed as polychistidine tag fusion proteins in bacteria and purified by nickel affinity chromatography (except for EB1, which was a generous gift of S. Honnappa and M. O. Steinmetz). TTLL proteins were expressed as GST fusions in bacteria and purified on glutathione-Sepharose as previously described (4). Because only TTLL5 (CAM84325) was soluble and active when produced in bacteria as its full-length version, we produced truncated version of TTLL4 (TTLL4_C639, last 639 residues of CAM84324) and TTLL6 (TTLL6_N705, first 705 residues of CAM84326) and a shorter, naturally occurring version of TTLL7 (TTLL7S, CAM84327) (3, 4). These shorter enzymes have been previously demonstrated to possess similar activities as compared with the full-length TTLLs (4). The polyglutamylation assay was performed by incubating 1 μg of each purified TTLL enzyme with 4 μg of each putative substrate in 20 μl of 50 mM Tris–HCl, pH 9.0, 0.5 mM ATP, 2.4 mM MgCl₂, 0.5 mM dithiothreitol, and 7.4 μM L-[3H]glutamate (45–55 Ci/mmol, GE Healthcare) for 2 h at 30 °C. Alternatively, 4 μg of taxotere-stabilized MTs, prepared from adult mouse brains (18), were used as a substrate. Quantification of the radioactivity incorporated into the different putative substrate proteins was done by scintillation counting of the protein bands after SDS-PAGE and electrotransfer onto nitrocellulose. Each polyglutamylation test was performed at least three times in independent experiments. The overall count (dpm) varied between each set of experiments; however, the relative values of glutamate incorporated into different substrates tested with the same enzyme dilution were similar in all experiments. Each figure is one set of a given experiment performed at the same time with all substrates presented. Background levels (no incorporation) are below 100 dpm.

**In Vivo Polyglutamylation of the New Substrates**—Putative substrates of polyglutamylation were cloned as EYFP fusion proteins in mammalian expression vectors and transfected into HeLa cells together with an active or an enzymatically inactive (mutation E901G in the ATP-binding site) version of TTLL4_C639-EYFP. After 24 h of transfection, cells were lysed in Laemmli buffer and subsequently analyzed by gel electrophoresis followed by Western blot. In Fig. 5C, NAPs and their chimeras were expressed alone and immunoprecipitated with anti-GFP antibody prior to analysis.

**Mutagenesis**—To exchange the C-terminal tails between the different NAPs, we introduced the restriction site BstBI into a highly conserved region of the NAPs. This was done by mutagenesis of NAPs (NAP1: T972C, NAP2: T951C, NAP4: T1170C T1171G T1172A, introducing a L391E amino acid change) using the QuikChange PCR method. The resulting mutants were expressed and shown to behave like the wild-type proteins in the polyglutamylation assay (data not shown). We then used BstBI in combination with other
enzymes to exchange the NAP domains. The resulting chimeras were cloned into bacterial and mammalian expression vectors.

RESULTS

Purification and Identification of Putative Substrates for Polyglutamylation—Because the GT335 antibody detects protein bands besides those corresponding to the glutamylated forms of tubulins and NAPs on Western blots of HeLa cell extracts, it is likely that this antibody specifically recognizes some other polyglutamylated proteins. We therefore performed a large scale purification of GT335-reactive proteins from HeLa cell extracts by chromatography on a GT335 column. The affinity-purified fraction was submitted to SDS-PAGE and stained with Coomassie Blue (Fig. 1, lane 1) or transferred onto nitrocellulose and probed with GT335 antibody (Fig. 1, lane 2). We visualized 40 protein bands on the gel after Coomassie staining, and about half of them were detected with GT335 antibody on Western blot. The remaining proteins might have been non-specifically retained on the column, for example by interacting with GT335-reactive proteins. However, it is also possible that some proteins are recognized by GT335 antibody only in their native form. We therefore excised all 40 protein bands from the gel and determined their protein content by nano-LC-MS/MS after trypsin digestion. This analysis gave rise to the identification of about 170 proteins (supplemental Table S1).

To identify true substrates of polyglutamylation, the masses obtained by nano-LC-MS/MS were re-analyzed to search for masses that could be attributed to polyglutamylated tryptic peptides. However, we found no masses corresponding to modified peptides (mass increments of \( n \) times 129 Da, the mass of a glutamyl unit, with \( n \) ranging from 1 to 8). It has been previously reported that the polyglutamylated tryptic peptides of tubulins and NAPs are undetectable with the classical mass spectrometry method used here (1, 7). The same seems to be true for the other substrates of polyglutamylation. Actually, because of their high acidity and/or branched structure, the modified peptides are lost during the reverse phase chromatography used to fractionate the peptides (7). An alternative procedure would be to fractionate the tryptic peptides by ion-exchange chromatography followed by a desalting step. This method has been used in the case of NAPs (7), but to follow the modified peptides during the purification steps, radioactive glutamate had to be incorporated into the proteins. Another problem, because of the acidic character of the modified peptides, is that their ionization in electrospray or MALDI is very hard even in the negative mode. Several procedures have been proposed to solve this problem (19–21), but the method remains far from straightforward. Taken together, we judged a mass spectrometry approach as not appropriate to identify new substrates of polyglutamylation.

To preselect proteins with a high probability of being polyglutamylated, we took advantage of the fact that a polyglutamylase activity co-purifies with the GT335-reactive proteins (7). Incubation of the GT335 affinity-purified fraction with \(^{3} \text{H}\) glutamate and MgATP resulted in the radioactive labeling of 13 protein bands (Fig. 1, lane 3). Because the new substrates of polyglutamylation might, as tubulins and NAPs, be modified within a glutamate-rich sequence we searched for proteins containing such motifs among the proteins identified within the 13 \(^{3} \text{H}\)-labeled bands. We found at least one protein with a glutamate-rich stretch in each of these bands (Table 1) and thus
New Substrates of Polyglutamylation

TABLE 1
Preselection of very likely targets of polyglutamylation

Among the proteins identified within the 13 protein bands that were labeled with [3H]glutamate (Fig. 1 and Supplemental Table S1), we selected those that possess long glutamate-rich stretches within their primary sequence and that thus are very likely targets of polyglutamylation. The band numbers correspond to those indicated in Fig. 1. In vitro polyglutamylation tests were performed on the proteins in bold and all of them did incorporate glutamate.

| Band | Protein | Accession | Mass | pI |
|------|---------|-----------|------|----|
| 1    | Nucleoporin | gi 857368 | 357993 | 5.86 |
| 5    | Proline-, glutamic acid-, leucine-rich protein 1 (PELP1) | gi 24415383 | 119548 | 4.30 |
| 19   | α-Tubulin | gi 32015 | 49761 | 4.95 |
| 20   | Nucleosom assembly protein 1- like 4 (NAP2) | gi 5174613 | 42797 | 4.60 |
| 21   | β-Tubulin | gi 18088719 | 49640 | 4.75 |
| 22   | Nucleosom assembly protein 1- like 1 (NAP1) | gi 4758756 | 45346 | 4.36 |
| 26   | NF45 protein | gi 532313 | 44669 | 8.26 |
| 27   | Protein SET | gi 46397790 | 33469 | 4.23 |
| 32   | PHAPII | gi 403009 | 32084 | 4.12 |
| 32   | Acidic nuclear phosphoprotein 32 family, member E (ANP32E) | gi 13569879 | 30674 | 3.77 |
| 34   | Acidic nuclear phosphoprotein 32 family, member A (ANP32A) | gi 5453880 | 28568 | 3.99 |
| 35   | Acidic nuclear phosphoprotein 32 family, member B (ANP32B) | gi 1498227 | 22263 | 4.19 |

expect that these proteins are very likely targets of polyglutamylation.

TTLL4 and TTLL5 Modify New Substrates in Vitro—To confirm whether the very likely targets of polyglutamylation, preselected in Table 1, can indeed be polyglutamylated, a selection of them was produced in bacteria and purified for analysis in an in vitro polyglutamylation assay. Not all proteins were tested because some could not be produced in bacteria due to their large size (nucleoporin, PELP1, Myb-binding protein 1A) and others shared a high homology with some of the tested proteins (PHAPII is a shorter version of SET). The expected size on Western blot (not shown), most probably because of the absence or low concentration of the enzymes. Because both antibodies have been demonstrated to precipitate the expected size on Western blot (not shown), most probably because of the absence or low concentration of the enzymes.

To show that the proteins that are polyglutamylated in vitro can also be modified by TTLL4 under in vivo conditions, we expressed them as EYFP-tagged versions in HeLa cells. In untransfected cells (not shown) or cells overexpressing an inactive (ATPase-dead) TTLL4_C639 (Fig. 4A), the two other enzymes, TTLL6_N705 and TTLL7S, which are both highly active on MTs, did not modify the new substrates (Fig. 2A). Thus, in vivo, non-tubulin substrates are specifically polyglutamylated by TTLL4 and TTLL5. Moreover, because all preselected proteins tested are modified, it is likely that the presence of glutamate-rich sequences is indeed, as proposed earlier, a signature of polyglutamylated proteins.

Among the protein bands that were not labeled with [3H]glutamate by the polyglutamylase activity co-purified on the GT335 column, we identified several other proteins containing glutamate-rich stretches (supplemental Table S1, proteins in bold). These proteins might be polyglutamylated either to a lower extent or by a different enzyme, which was not co-purified on the GT335-column. To investigate this possibility, we repeated the in vitro polyglutamylation assays with the same set of TTLL enzymes on 6 of these proteins (GRP78, NASP, RANGAP, RNPK, NCT, and EB1; Fig. 2B). As before, TTLL4_C639 was the most efficient enzyme. It incorporated [3H]glutamate into RNPK and EB1, to a lesser extent into RANGAP and NCT, but poorly into GRP78 and NASP. Note that TTLL6_N705 and TTLL7S, which were inactive on the previously tested non-tubulin substrates, slightly modified EB1. This might be due to the close structural homology between EB1 and tubulins (23). Overall, the proteins modified in this test incorporated much less glutamate than the previously identified substrates, which might explain why they were not seen in the autoradiography (Fig. 1, lane 3). However, they have to be considered as potential substrates of polyglutamylation and their modification might be important in vivo.

TTLL4 Is the Major Polyglutamylase Co-purified with the New Substrates—Because the GT335-affinity-purified fraction possesses a polyglutamylase activity able to modify the new substrates (Fig. 1, lane 3), we wanted to determine if it contains either TTLL4 or TTLL5, the two enzymes that modify the substrates in vitro. We therefore raised polyclonal antibodies against these TTLLs. The epitopes of both, anti-TTLL4 and anti-TTLL5 antibodies are located outside the domains that are conserved between the different TTLLs (supplemental Fig. S1). Accordingly, when we probed extracts of HeLa cells overexpressing the different full-length TTLL polyglutamylases, anti-TTLL4, and anti-TTLL5 antibodies specifically recognized their targets and exhibited no cross-reactivity with any other TTLL polyglutamylase (Fig. 3A). We then used these antibodies to probe the GT335-affinity-purified fraction, but none of the antibodies detected a protein of the expected size on Western blot (not shown), most probably because of the absence or low concentration of the enzymes.
in Western blot. Accordingly, in the absence of exogenous polyglutamylase activity, only substrates that were strongly modified in vitro (NAP1, NAP2, SET, and the ANP32 proteins) could be detected by GT335 antibody following their overexpression (Fig. 4, lower panel, “D”). We then expressed the selected proteins together with an active version of TTLL4_C639 (Fig. 4, “A”). A significant increase in GT335 reactivity was observed on a range of endogenous proteins (Fig. 4, compare first and second lane), demonstrating that GT335 specifically detects polyglutamylation on a large range of proteins. Moreover, the GT335 reactivity of NAP1, NAP2, SET, and the ANP32 proteins increased further, and other putative substrates (RANGAP and nucleolin) also became labeled with GT335 antibody (Fig. 4, lower panel, “A”). The expression levels of the substrates, as visualized with anti-GFP antibody, are similar in cells co-transfected with ATPase-dead or active TTLL4_C639 (Fig. 4, upper panel, “D” and “A”). Thus, increase of GT335 detection levels clearly correlates with increased polyglutamylation of the respective proteins by the active TTLL4_C639. Moreover, a shift in migration was observed for most of the modified proteins, suggesting that the charge and/or size of these proteins have been changed (Fig. 4, upper panel, compare “D” with “A” for the modified proteins).

**Requirements for Polyglutamylation**—We identified a number of proteins that are subjected to polyglutamylation. All these proteins possess glutamate-rich stretches within their primary sequence, but no obvious polyglutamylation consensus site could be defined. To better understand the determinants of polyglutamylation, we analyzed the primary sequences of several members of the NAP family (Fig. 5A and supplemental Fig. S2A) and their ability to be modified in vitro by TTLL4_C639 (Fig. 5B) or in HeLa cells by endogenous polyglutamylases (Fig. 5C). NAP1 and NAP2 are, in contrast to NAP3 and NAP4, strongly polyglutamylated in vitro and in vivo (Fig. 5). NAP1 was previously shown to possess a major polyglutamylation site in its C terminus and a minor site in its N terminus (7). Both
sites consist of glutamate-rich stretches that are not well conserved among the members of the NAP family (supplemental Fig. S2A and Fig. 5A). NAP2 diverges from NAP1 in the N-terminal site. Because this is a minor polyglutamylation site, NAP2 is still strongly modified. On the contrary, the sequence of NAP4 diverges in the major C-terminal site and thus NAP4 is only weakly modified. NAP3 diverges in both sites and accordingly, it is not modified. Thus, we have shown a correlation between the presence of the two previously identified polyglutamylation sites and the extent of modification. In addition to these two sites, all NAPs possess a central domain which is rich in glutamates (supplemental Fig. S2A). This domain does not seem to be targeted by the polyglutamylation, as NAP3 is not modified although it has the most extended glutamate-rich central domain (Fig. 5A). The fact that this domain is not modified, although it is located at the outer surface of the protein according to the structure of NAP1 (24), suggests that the presence of a glutamate-rich sequence is not the only prerequisite for its polyglutamylation.

We then produced six chimeras of the NAP1, 2, and 4 proteins by swapping their C-terminal tails (Fig. 5A) and tested their ability to be modified by TTLL4_C639 in vitro (Fig. 5B) or by endogenous polyglutamylase activity in HeLa cells (Fig. 5C). Chimeras that possess the non-acidic C-terminal tail of NAP4 (NAP1_4 and NAP2_4), were poorly polyglutamylated both in vitro and in vivo, in good agreement with the lack of the major polyglutamylation site. On the other hand, chimeras that bear the highly acidic tail of NAP2 (NAP1_2 and NAP4_2) are, as NAP2, strongly modified. Concerning the chimeras that have the highly acidic tail of NAP1 (NAP2_1 and NAP4_1), NAP2_1 is modified to the same extent as NAP1 but NAP4_1 is less modified.

FIGURE 3. Identification of the polyglutamylase activity co-purified with the GT335-reactive proteins. The specificity of polyclonal anti-TTLL4 and anti-TTLL5 was demonstrated on extracts from HeLa cells overexpressing the different TTLL polyglutamylases in fusion with EYFP (A). These antibodies were then used to immunodeplete the GT335 affinity-purified fraction (B). As a control, depletion was also performed with rabbit IgG. The depleted fractions were incubated with [3H]glutamate for 2 h, run on an SDS-PAGE and stained with Coomassie Blue. [3H]Glutamate incorporation was visualized by autoradiography. Only the depletion with anti-TTLL4 antibody reduced the polyglutamylase activity in the GT335 affinity-purified fraction. Molecular mass markers are indicated.

FIGURE 4. In vivo polyglutamylation of selected putative substrates. The previously tested substrates of polyglutamylation (Fig. 2), as well as nucleolin which was not tested in vitro because it was hard to produce in bacteria, were co-transfected into HeLa cells with an active or inactive (ATPase-dead) version of TTLL4_C639 in fusion with EYFP. After 24 h of transfection, the cells were lysed in Laemmli buffer, run on SDS-PAGE, transferred onto nitrocellulose and probed with anti-GFP antibody to check the expression level of the overexpressed proteins (upper panel) and with GT335 antibody to visualize the extent of polyglutamylation of the substrates (lower panel). Orange arrows point to the different overexpressed substrates visualized with anti-GFP antibody. In the lower panel, green arrows indicate the substrates that have been polyglutamylated in vivo, while red arrows point to proteins that were not modified. In general, we considered a substrate as being modified, when: (i) a band of the size of the protein (as compared with the anti-GFP signal) is recognized by GT335, and (ii) its GT335 reactivity increases upon co-expression of active TTLL4_C639, and (iii) this band does not correspond to endogenous GT335-reactive proteins (compare with cells not expressing the given substrate). Note that cells co-overexpressing B23 and active TTLL4_C639 were dying, explaining the decreased overall GT335 reactivity in the corresponding lane. Molecular mass markers are indicated.
than one would expect according to the presence of the tail of NAP1. This again suggests that other, yet unknown determinants of polyglutamylation contribute to the modification.

To further demonstrate that the presence of a glutamate-rich stretch is not the only prerequisite for a protein to become polyglutamylated, we analyzed different members of the nucleophosmin/nucleoplasmin family. Nucleophosmin B23 has two putative acidic polyglutamylation sites (supplemental Fig. S2B). NPN2, an oocyte-specific homologue of B23, is more acidic in the first site but less in the second (supplemental Fig. S2B). Although, taken together, the total number of glutamate residues in the two potential polyglutamylation sites is similar, NPN2 is modified to a lower extent than nucleophosmin B23 by TTLL4\_C639 (Fig. 6). This suggests that the second site contributes more to the degree of polyglutamylation than the first one. Consistently, NPN3, which completely lacks the second site, is poorly modified by TTLL4\_C639 (Fig. 6). Again, these results reveal that not all glutamate-rich stretches present in proteins are equally subjected to polyglutamylation.

We further analyzed the highly homologous proteins EB1 and EB3. Although both proteins carry a glutamate-rich C terminus, EB3 is modified by TTLL4\_C639 to a lower extent than nucleophosmin B23 by TTLL4\_C639 (Fig. 6). This suggests that the second site contributes more to the degree of polyglutamylation than the first one. Consistently, NPN3, which completely lacks the second site, is poorly modified by TTLL4\_C639 (Fig. 6). Again, these results reveal that not all glutamate-rich stretches present in proteins are equally subjected to polyglutamylation.

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We further analyzed the highly homologous proteins EB1 and EB3. Although both proteins carry a glutamate-rich C terminus, EB3 is modified by TTLL4\_C639 to a lower extent than nucleophosmin B23 by TTLL4\_C639 (Fig. 6). This suggests that the second site contributes more to the degree of polyglutamylation than the first one. Consistently, NPN3, which completely lacks the second site, is poorly modified by TTLL4\_C639 (Fig. 6). Again, these results reveal that not all glutamate-rich stretches present in proteins are equally subjected to polyglutamylation.
nism. Solving the crystal structure of the polyglutamylase enzymes and thus knowing the properties of the surface residues of the enzyme that are in contact with the substrate, will certainly facilitate understanding of the prerequisites for the polyglutamylation reaction. Taken together, we suggest that proteins with glutamate-rich sequences can be generally considered as potential substrates for polyglutamylation, although this needs to be tested for each single case.

**Possible New Roles of Polyglutamylation**—Polyglutamylation generates acidic side chains of variable lengths on proteins. Such a modification might act as a fine-regulator of many cellular functions, for example by controlling the affinity of the here-identified target proteins for their binding partners, as earlier proposed for tubulins (reviewed in Ref. 25). Many of the new substrates of polyglutamylation (ANP32 proteins, SET, nucleophosmin B23, nucleolin, and RNP-K), as well as the NAP families, shuttle between the cytosol and the nucleus (22, 26, 27). Because nucleoporin and RANGAP, proteins involved in nucleocytoplasmic transport, are also modified, polyglutamylation could play an important regulatory role in nucleocytoplasmic shuttling. Moreover, most of these shuttling proteins are histone chaperones (NAP1, NAP2, B23, SET/PHAPII, nucleolin) and/or regulators of transcription (ANP32 proteins, SET/PHAPII, NF45, PELP1, RNP-K), suggesting that polyglutamylation might also regulate access to chromatin. Finally, EB1 and RANGAP are regulators of MT dynamics, suggesting that not only the polyglutamylation of tubulin, but also of its associated proteins could be important for the dynamics and functions of MTs. In cycling cells, the maximum yield of tubulin polyglutamylation is achieved during mitosis (18). It is thus tempting to speculate that, by targeting simultaneously MTs and chromatin-binding proteins, polyglutamylation allows for a cross-talk between spindle dynamics and changes in chromatin structure accompanying mitosis.

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**FIGURE 6.** In vitro polyglutamylation of proteins related to the new substrates. In vitro polyglutamylation assays were performed using recombinant TTTL4_C639 on proteins from the nucleophosmin/nucleoplasmin and EB families as well as on HMG-B1 (see sequences in supplemental Fig. S2A). The amount of [3H]glutamate incorporated in the different substrates was determined by scintillation counting.