Polyglutamylation of Nucleosome Assembly Proteins*

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Catherine Regnard‡‡, Elisabeth Desbruyères‡, Jean-Claude Huet¶, Christian Beauvallet¶,
Jean-Claude Pernollet¶, and Bernard Eddé‡

From ‡Biochimie Cellulaire, Collège de France, 11 place Marcelin Berthelot, 75005 Paris and ¶Biochimie et Structure des Protéines, Institut National de la Recherche Agronomique, 78352 Jouy en Josas Cedex, France

Polyglutamylation is an original posttranslational modification, discovered on tubulin, consisting in side chains composed of several glutamyl units and leading to a very unusual protein structure. A monoclonal antibody directed against glutamylated tubulin (GT335) was found to react with other proteins present in HeLa cells. After immunopurification on a GT335 affinity column, two prominent proteins of ~50 kDa were observed. They were identified by microsequencing and mass spectrometry as NAP-1 and NAP-2, two members of the nucleosome assembly protein family that are implicated in the deposition of core histone complexes onto chromatin. Strikingly, NAP-1 and NAP-2 were found to be substrates of an ATP-dependent glutamyltransferase co-purifying on the same column. We took advantage of this property to specifically label and purify the polyglutamylated peptides. NAP-1 and NAP-2 are modified in their C-terminal domain by the addition of up to 9 and 10 glutamyl units, respectively. Two putative glutamyltransferase sites were localized for NAP-1 at Glu-356 and Glu-357 and, for NAP-2, at Glu-347 and Glu-348. These results demonstrate for the first time that proteins other than tubulin are polyglutamylated and open new perspectives for studying NAP function.

Tubulin, the main microtubule component, is subjected to several posttranslational modifications (for a recent review, see Ref. 1). Among them, polyglutamylation and polyglycylation, which occur on both α- and β-tubulin subunits, are very unusual (2, 3–6, 7). They are formally similar to each other, consisting of the addition of a variable number of glutamyl or glycyl units, in the case of polyglutamylation, or of glycyl units in the case of polyglycylation. In both cases, the first unit is linked by an amide bond between its ε-amino group and the γ-carboxylic group of a glutamate residue of the polypeptide chain. Additional units are linked together by isopeptidic bonds, thus leading to the formation of a linear side chain of amino acid residues, which extends from the main polypeptide chain and exhibits a second C terminus. In some cases, especially for polyglycylation, several glutamate residues of the polypeptide can be modified, leading to the formation of several side chains (8, 9). The total number of glutamyl and glycyl units present in each subunit can reach up to ~20 and ~34, respectively, but is more commonly in the range of 1–6 (10–12). The sites of modification are located in the C-terminal region of α- and β-tubulin, which are known to be exposed on the surface of the MT1 lattice and to interact with various microtubule associated proteins. Polyglutamylation and polyglycylation are thought to profoundly affect the tridimensional structure of these domains and to interfere with the binding of essential effectors involved in MT function.

Tubulin polyglutamylation is a very old modification, widely distributed among species, from protozoa to mammals. It is relatively abundant in centrioles, basal bodies, cilia, and flagella but is not restricted to these very particular structures. For instance, in neurons, ~90% of α-tubulin and ≥50% of β-tubulin are polyglutamylated (13). Modification of the α-tubulin subunit is triggered at the beginning of neurite extension, while the β subunit is modified much later, during a phase probably related to neuronal maturation (13, 14). The modification occurs also, but at much lower levels, in proliferative non-neuronal cells, such as HeLa or 3T3 cells (15). In these cells, interphasic microtubules are poorly glutamylated while other structures like mitotic spindle MTs, and especially MTs of the centrioles, are more highly glutamylated (15).

Consistent with its wide distribution, tubulin polyglutamylation is believed to be involved in multiple functions. In vitro blot overlay assays strongly suggest that the polyglutamyl side chain of tubulin can regulate differentially the binding of various microtubule-associated proteins, in a manner dependent on the length of the side chain (16, 17). These results are of particular interest because binding of microtubule-associated proteins strongly influences MT dynamics and is particularly important during neuronal differentiation, where plasticity and rigidity of the axodendritic processes must be precisely regulated in relation with environmental cues. Indications that polyglutamylation is involved in other biological processes come from ex vivo experiments using a mAb raised against glutamylated tubulin (GT335). For instance, mAb GT335 was shown to strongly inhibit the motility of demembranated, re-activated spermatozoa. Interestingly, the amplitude of the beating waves was strongly reduced while the frequency was unaffected (18). More recently, it was shown that introduction of mAb GT335 into HeLa cells by either microinjection or electroporation leads to the unexpected disappearance of the centrioles and the dispersion of the pericentriolar material (19).

Whether polyglutamylation and polyglycylation are specific for tubulin or are common to other proteins was not yet known. Recently, we examined polyglutamylation of tubulin in HeLa cells and its variations during the cell cycle (20). In the course

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‡ Recipient of a fellowship from the Association Française contre les Myopathies.

§ To whom correspondence should be addressed. Tel.: 33-1-44-27-13-07; Fax: 33-1-44-27-13-09; E-mail: edde@ext.jussieu.fr.
of that study, we observed a minor reactivity of mAb GT335 with proteins comigrating with tubulin on SDS-PAGE but not on two-dimensional PAGE. The possibility that this reactivity corresponded to a particularly high background seemed unlikely since other very abundant proteins were not labeled at all. In this report, we show indeed that proteins other than tubulin are subjected to polyglutamylation and identify two nucleosome assembly proteins as new targets for this modification. NAP-1 and NAP-2 are recognized by mAb GT335 in both their native and denatured states and are substrates of a polyglutamylase activity present in HeLa cells. The sites of modification have been localized in the very acidic C-terminal domains of both proteins, which bear up to 9–10 glutamyl units. Examination of other cell types and organisms indicates that polyglutamylation of NAPs appears as a general phenomenon. These results provide new insights for studying the regulation of NAP function. They furthermore suggest that polyglutamylation might be a more general posttranslational modification than previously believed.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, 100 IU penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO₂ atmosphere.

**Electrophoresis—**SDS-PAGE (21) was performed on 8% acrylamide, 0.11% bisacrylamide, slab gels (8 cm long) containing 0.1% (w/v) SDS (90% pure; Merck, Darmstadt, Germany). Isoelectric focusing in denaturing 9.5 × 5 mm cylindrical gels (12 cm long) was performed as described (22), with a mixture of pH 3.5–10 and 5–8 (1:4) ampholytes (Amersham Pharmacia Biotech, Saclay, France). Second dimension was performed on 24-cm-long slab gels as described above. Exposures of radioactive gels were performed at −80 °C, typically for 2–4 weeks, using XAR-5 films (Eastman Kodak Co.) after enhancement with Amplify (Amersham Pharmacia Biotech).

**Western Blotting—**Electrotransfer of polyacrylamide gels was performed onto nitrocellulose (Hybond C, Amersham Pharmacia Biotech) as described previously (23). mAb GT335 was biotinylated and incubated simultaneously with streptavidin conjugated to horseradish peroxidase, as described (24). α- and β-tubulin were detected by DM1A (1:5,000, Amersham Pharmacia Biotech) and Tub B 1:1 (1:2000, Sigma), respectively. Anti-NAP-1 hybridoma supernatant (mAb 4A8; Refs. 24 and 25) was a gift from Dr. Y. Ishimi (Mitsubishi-Kasei Institute of Life Sciences, Tokyo, Japan) and was dilated 1:20.

**Immunoprecipitation—**All steps were performed at 4 °C. HeLa cells were extracted in TBS (20 mM Tris, pH 7.2, 150 mM NaCl) 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 was obtained after centrifugation at 100,000 × g for 40 min. mAb GT335 (25 μg) was diluted in TBS and preincubated 3 h with 25 μl of a suspension ofagarose beads coupled to protein G (Amersham Pharmacia Biotech) on a rotary agitator. After washing with TBS, the beads were incubated overnight with the HeLa supernatant (400 μl, 10 mg/ml). After extensive washing, the bound proteins were eluted with 2 × 30 μl of extraction buffer containing 1% NaCl and analyzed by SDS-PAGE and Western blotting.

**Immunofinity Chromatography—**mAb GT335 (1 ml, 7–10 mg of Ig) was purified by affinity on a tubulin column and coupled to N-hydroxy-succinimide-activated Hi-Trap columns (1 ml, Amersham Pharmacia Biotech) according to the manufacturer's instructions. All subsequent steps were performed at 4 °C. High speed supernatant of HeLa cells, obtained as described in the previous paragraph, was diluted in TBS to a final protein concentration of 2 mg/ml and loaded on the column (50 mg/ml of gel) at a flow rate of 0.5 ml/min. After extensive washing with TBS containing 0.01% Triton X-100, bound proteins were eluted by increasing the NaCl concentration to 0.65 M. This was sufficient to break the ionic interactions involved in the antigen-antibody binding. The eluted fractions were detected by their absorbance at 280 nm, pooled, concentrated with protease inhibitors, and concentrated on an Amicon ultrafiltration device (2 ml, cut-off 10,000, Millipore). The yield of this last step was highly variable, ranging from 20% to 70%.

**Proteolysis—**Proteolytic fragments of proteins X and Y (see Fig. 3) were obtained according to the method of Aebersold et al. (26). Briefly, after one-dimensional or two-dimensional PAGE, proteins were electrotransferred on nitrocellulose. After Ponceau Red staining, the bands or spots were excised from the nitrocellulose sheets, saturated with 20 mg/ml PVP40 (Sigma), and incubated overnight at 30 °C, under constant agitation in digestion buffer (Tris 50 mM, pH 8) containing 2.5 μg/ml Trypsin (sequence grade, Promega).

**Chromatography—**In the experiment presented in Fig. 4, reversed phase chromatography was performed on an HPLC system (Waters) with a C18 column (Brownlee, 2.1 × 220 mm) at a flow rate of 0.2 ml/min. Solution A was 0.1% trifluoroacetic acid in water, and solution B was 0.09% trifluoroacetic acid, 70% acetonitrile in water. A gradient of 5–50% B in 50 min followed by 50–100% B in 20 min was developed.

In the experiments presented in Fig. 7, a DEAE column suitable for HPLC was used (AX300, Brownlee, 4.6 × 100 mm) at a flow rate of 0.4 ml/min. Solution A was 60 mM Tris-HCl, pH 8, and solution B was 50 mM Tris-HCl, pH 8, containing 1 M NaCl. A linear gradient of 0–100% B was developed. The main glutamylated peak eluted at ~0.75 ml at NaCl. In the experiment presented in Fig. 7A, an on-line flow radioactivity detector (Flo-One, Radiomatic, Packard Instruments) was placed immediately after the UV detector. The radioactive signals were accumulated for 9-s intervals.

In conclusion chromatography was carried out to desalt the radioactive eluates eluted from the DEAE chromatography. A column (1 × 50 cm) of Trisacryl gel GF50 (IBF, France) with a separating range of 300–2500 Da was used at a flow rate of 0.1 ml/min. Radioactive peptides were followed by counting an aliquot of each fraction and eluted just before the salt peak. Before being analyzed by microsequencing and mass spectrometry, the fractions were concentrated under speed vacuum.

**In Vitro Glutamylation of GT335 Affinity-purified Fraction—**The enzymatic assay was performed as described previously (27) in 50 mM Tris-HCl, pH 9.0 (final pH 8.7), 2 mM ATP (equilibrated to pH 7 with NaOH), 8 mM MgCl₂, 2.5 mM diithiothreitol, and 2 μCi/ml H[3]Hglutamate (45–55 Ci/mm, Amersham Pharmacia Biotech). To increase the radioactive signal, the [3H]glutamate solution was concentrated 10-fold under speed vacuum and added at a final concentration of 50 μM. Incubation was performed at 30 °C, usually for 1 h. For the assay presented in Fig. 6, concentrated affinity-purified fraction was used at a final protein concentration of 0.11 mg/ml, in the presence or absence of tubulin polyglutamylase partially purified from mouse brain (fraction IV of Ref. 27, at a final concentration of 0.04 μg/ml or from HeLa cells (phosphocellulose-purified fraction of Ref. 20, final concentration of 0.3 μg/ml). Quantitation of the radioactivity incorporated in the different proteins was performed by scintillation counting of the protein bands after one-dimensional PAGE and electrotransfer onto nitrocellulose sheets, as described previously (20). A duplicate sample (10 μl) of that presented in Fig. 6A (lane 3) gave ~1000, 500, and 800 dpm for the 52-, 48-, and 40-kDa proteins, respectively. If each of these species accounts for 10% of proteins present in the fraction, specific radioactivity could be grossly estimated to ~10,000, 5,000, and 8,000 dpm/μg protein, respectively.

For the preparative experiment presented in Fig. 7, the preparative ion-exchange separation of glutamylated peptides eluted from the DEAE chromatography. A column (1 × 50 cm) of Trisacryl gel GF50 (IBF, France) with a separating range of 300–2500 Da was used at a flow rate of 0.1 ml/min. Radioactive peptides were followed by counting an aliquot of each fraction and eluted just before the salt peak. Before being analyzed by microsequencing and mass spectrometry, the fractions were concentrated under speed vacuum.

**Polyglutamylation of NAP-1 and NAP-2**

**Protein Sequencing—**Edman sequencing was performed using a 494-HT Prowess apparatus (PE Biosystems, Foster City, CA). The reversed-phase samples were spotted on glass fiber disks treated with Biobrene (1.5 mg). The ion-exchange fractions were adsorbed onto polyacrylamide gel and subjected to Edman degradation using a blue capillary tube deprived of the 3-kDa ultrafiltration membrane, washed with 10% methanol, dried, and treated with 0.1 mg of Biobrene prior to loading onto the sequencer.

**MALDI-TOF Mass Spectrometry—**A Hewlett-Packard MALDI-TOF G2025 equipment was utilized for reversed-phase separated peptides using α-cyano-4-hydroxycinnamic acid as matrix (Hewlett Packard). For the analysis of the ion-exchange separated peptides, mass spectra...
RESULTS

Western blotting analysis of HeLa cell soluble extract with GT335, a mAb directed against the polyglutamylated motif of tubulin (28), revealed several reactive proteins of 48–52 kDa that fall within the region of migration of α- and β-tubulin. Two additional bands of ~66 and ~180 kDa were also detected after long exposure (Fig. 1, lanes 2 and 3). We showed previously by two-dimensional PAGE analysis that one of the reactive proteins indeed corresponds to glutamylated β-tubulin but that other ones of similar Mr are more acidic than α- and β-tubulin (Ref. 20, see also Fig. 3).

Whether the reactivity observed with non-tubulin components was an artifact related to denaturation by SDS or indicated the presence of polyglutamylation in these proteins was first tested by carrying out immunoprecipitation experiments in TBS buffer. A strong enrichment of the same reactive proteins was observed in the immunoprecipitate, indicating that they have been efficiently retained on the column. Some of the proteins present in the affinity-purified fraction, such as the 40- and 200-kDa species, did not react with mAb GT335 on immunoblots. These proteins were probably retained on the column via their interaction with GT335-reactive proteins. However, we cannot exclude that they are recognized by the mAb only in their native form (see “Discussion”).

When the affinity-purified fraction was analyzed by two-dimensional PAGE electrophoresis, two prominent spots, denoted X and Y, were observed in the 50-kDa mass range (Fig. 3A). Two other much less intense spots were also present in the same region (horizontal arrows). These four spots were all labeled by mAb GT335 proportionally to their relative abundance (Fig. 3B). An intense labeling corresponding to glutamylated β-tubulin was also observed although the protein was not detected by Coomassie Blue staining, which indicates a stronger reactivity of mAb GT335 toward glutamylated tubulin. None of the three ~40-kDa proteins resolved in these gels (arrowheads) was detected by mAb GT335.

We focused this study on the identification of the most abundant proteins in the affinity-purified fraction, proteins X and Y. Tryptic peptides were obtained after digestion of the excised two-dimensional spots and fractionated by reversed phase chromatography on a C18 column. Several well resolved peaks (Fig. 4) were analyzed by automated Edman degradation and mass spectrometry (Table I). All of the peptides analyzed resulted in amino acid sequences and mass values that allowed identification of proteins X and Y as members of the nucleo-
some associated protein family, NAP-2 and NAP-1, respectively (29, 30). However, two splicing variants have been described for NAP-2. Form I terminates at residue 375, while form II contains an additional C-terminal segment of 11 residues (31). Whether protein X corresponds to form I or II is not yet known.

This identification was supported by immunoblotting experiments with the anti-NAP mAb 4A8 (24, 25). As shown in Fig. 5A, this mAb reacted very strongly with the 48–52-kDa proteins enriched in the affinity-purified fraction. On two-dimensional gels, mAb 4A8 strongly labeled proteins X and Y, corresponding to NAP-2 and NAP-1, respectively, and faintly labeled the two minor species also detected by mAb GT335 (Fig. 5B). Although this antibody was obtained using NAP-1 as immunogen, it also recognized the spot corresponding to NAP-2. This is consistent with the suggested epitope of mAb 4A8 previously described to recognize the domain of NAP-1 from amino acids 290–305, and in particular the sequence FNF also present in NAP-2 (25). Immunodetection with mAb 4A8 indicated that ~50% of the total NAPs present in the extract were retained on the GT335 column. This result suggests the existence of two subpopulations of NAPs, one being glutamylated and recognized by mAb GT335 and the other one not.

It was not possible to directly identify the fractions corresponding to the glutamylated peptides in the bulk of the eluted peptides. We thus searched for a way to specifically label glutamylated peptides and allow their efficient detection. For this purpose, the affinity-purified fraction was incubated, in the presence of [3H]glutamate and MgATP, with partially purified preparations of mouse brain or HeLa cell tubulin polyglutamylase, as described previously (20, 27). Glutamate incorporation into proteins was followed by SDS-PAGE and fluorography. Proteins of 180, 48–52, and 40 kDa were labeled similarly with either enzyme preparation (Fig. 6A, lanes 1 and 2). Surprisingly, when a control experiment was performed in the absence of any added glutamylase fraction, a similar labeling pattern and intensity was observed (Fig. 6A, lane 3). However, no labeling was observed when ATP was omitted from the incubation mixture (data not shown). This was described previously for tubulin polyglutamylation (27) and is indicative of an enzymatic process. Thus, several proteins in the affinity-purified fraction are substrates of glutamylating enzyme(s), which co-puriy on the GT335 column. Two-dimensional PAGE analysis showed a prominent labeling of NAP-1 and NAP-2, as well as of other minor species, including the two stretched spots in the 40-kDa range (Fig. 6, B and C).

In vitro glutamylation could thus be used to label NAP proteins in order to identify glutamylated peptides. This was performed at very low concentrations of glutamate with a very high specific radioactivity in order to leave the overall level of glutamylation unchanged. Indeed, we determined that a maximum of 0.5 pmol of glutamate was incorporated into ≥100 pmol of NAP proteins in our conditions. Labeled NAP proteins were submitted to one-dimensional PAGE, which avoided the particularly high loss of material occurring on two-dimensional fractions.

### Table I

Amino acid sequencing and mass spectrometry analysis of trypsinized proteins X and Y

| Peptide* | Mass** | Sequence*** | Identification†† |
|----------|--------|-------------|------------------|
| X-23     | 901    | VLAALQER    | NAP-2 (37–44)    |
|          | 899    | VLAALQER    |                  |
| X-27     | 1338   | FYEEVHDLER  | NAP-2 (84–93) or NAP-1 (95–104) |
|          | 1336   | FYEEVHDLER  |                  |
| X-35     | 1916   | LDNVPHTPSYIETLPK |                  |
|          | 1911   | LDNVPHTPSYIETLPK |                  |
| X-51     | 2499   | -----AEEPDKGIPEF-FT--R | NAP-2 (45–61) |
|          | 2494   | AAAATAEEPDKGIPEFWFTIFR |                  |
| X-57     | 4800   | QVPNESFFNFNFPLKAS |                  |
|          | 4789   | QVPNESFFNFNFPLKAS | NAP-2 (283–324) |
| Y-43     | 3986   | -EIINAIYEPEEEEE | NAP-1 (118–149) |
|          | 3989   | -EIINAIYEPEEEEE |                  |
| Y-51     | 4522   | -VSNDSFFNFNPFAPEVPESGDDL |                  |
|          | 4517   | TVSNDSFFNFNPFAPEVPESGDDL | NAP-1 (291–331) |

* The peptides were named after the peak number in Fig. 4. X and Y indicate peptides derived from proteins X and Y, respectively.

** For each peptide, the first line corresponds to experimental data (partial sequences) and the second one to whole tryptic peptide sequences deduced from the known sequences of human NAP-1 or NAP-2. Dashes indicate undetermined residues.

†† The position of each peptide in the sequence of NAP-1 or NAP-2 is indicated in parentheses.

‡‡ The mass increment of 71 is explained by alkylation of the cysteine residue by acrylamide, as observed during protein sequencing.
and C lane 3 added enzymatic fraction (two-dimensional PAGE. The gel was stained with Coomassie Blue (from HeLa cells (tamylation in the presence of partially purified tubulin polyglutamylase in vitro and fluorography of affinity-purified fraction submitted to human PAGE analysis of HeLa fractions purified on the GT335 column and submitted to fluorography (same as in Fig. 2). High speed supernatant (lane 1), flow-through (lane 2), and affinity-purified fraction (lane 3) were immunodetected by mAb 4A8. Spots are labeled as in Fig. 3. Only part of the two-dimensional gel is presented.

FIG. 6. *In vitro* glutamylation of NAPs. A, one-dimensional PAGE and fluorography of affinity-purified fraction submitted to *in vitro* glutamylation in the presence of partially purified tubulin polyglutamylase from HeLa cells (lane 1) or from brain (lane 2), or in the absence of any added enzymatic fraction (lane 3). The fluorographic film is shown. B and C, a sample similar to that loaded in lane 3 was analyzed by two-dimensional PAGE. The gel was stained with Coomassie Blue (B) and submitted to fluorography (C). Spots are labeled as in Fig. 3.

gels, likely due to the partial precipitation of the protein upon high loading. Tryptic peptides were obtained as above and fractionated on a DEAE column by HPLC. Fig. 7 shows the radioactive profile obtained in an analytical experiment in which the HPLC column outlet was connected directly to an on-line radioactivity detector. Two series of peaks eluting at 24–28 and 32–38 min were detected, each containing ~7–8 individual peaks (Fig. 7A). By analogy with the results previously obtained for tubulin glutamylation (10), these data suggest the existence of different peptides bearing a variable number of glutamyl units.

The same experiment was then performed at a preparative scale. Affinity-purified fractions obtained from five independent experiments, each from 5 × 10⁸ HeLa cells, were pooled, submitted to one-dimensional PAGE and processed as described above. Two broad peaks were observed both on the UV profile and by radioactivity counting (Fig. 7B). The main peak eluting at a concentration of ~0.75 M NaCl was analyzed. Our initial plan was to further fractionate and desalt this fraction by reversed phase chromatography. Surprisingly, the peptides were irreversibly adsorbed on C4, C8, or C18 columns. This problem was not related to the silica phase because the DEAE column we used contained the same phase. This was unexpected for such acidic peptides and suggests the existence of an unusual structure. Therefore, we used gel exclusion chromatography against water to desalt the different fractions for subsequent analysis. The main peak was divided into three fractions called A, B, and C (see Fig. 7B), which were loaded separately on the desalting column. The eluted peptides were detected by their radioactivity content, concentrated by centrifugal evaporation under vacuum, and analyzed. Fractions A and C each contained two peptide sequences derived from the C-terminal domains of NAP-1 and NAP-2, beginning at Ser-338 and Ala-331, respectively.

NAP-1: SVLTYFGEAIEDDDDYD−G...
NAP-2: AVLYFTGEAIEDDDNF−G...

**Sequences 1 and 2**

The only difference between fractions A and C was the relative proportion of the NAP-1 and NAP-2 peptides. In fraction A, 4 pmol of Ser (corresponding to amino acid 338 of NAP-1) and 20 pmol of Ala (corresponding to amino acid 331 of NAP-2) were obtained at the first cycle of the Edman degradation whereas, in fraction C, there were 6 pmol of Ser and 1 pmol of Ala.

The sequence of NAP-2 was followed up to the 19th cycle in fraction A. Strikingly, the glutamate residues encoded at positions 347 and 348 were not detected at the corresponding Edman degradation cycles (cycles 17 and 18; these gaps are indicated by dashes in the sequences presented above). The sequence of NAP-1 was followed up to the 21st cycle in fraction C, and gaps, also corresponding to Glu residues, were observed at positions 356 and 357. Similar gaps were evidenced during previous studies on tubulin glutamylation and led to the identification of glutamylation sites (see Refs. 2–5 and 7). This suggested that Glu-356 and Glu-357 in NAP-1 and Glu-347 and Glu-348 in NAP-2 likely represent glutamic acid residues.
Glutamyl units formed on fractions A, B, and C. This analysis revealed two ring on NAP peptides, mass spectrometry analysis was performed in this region (see Fig. 9). Could not be determined, due to the high number of Glu present downstream in the sequences. Glu-348 in NAP-2 were modified residues. Whether other modified Glu residues were present upstream in the sequences is not known.

Fractions A, B, and C. The deduced numbers of glutamyl units (E) are also indicated (I-3E to 1-9E). See text for further details.

**Fig. 8. MALDI-TOF analysis of polyglutamylated peptides.** Fractions A (upper panel), B (middle panel), and C (lower panel) were analyzed in the linear mode. m/z values are indicated. The two series of prominent signals were labeled as I, for NAP-1, and II, for NAP-2. The deduced numbers of glutamyl units (E) are also indicated (I-3E to I-9E, II-6E to II-10E). See text for further details.

Since the discovery of polyglutamylation, there has been a question as to whether this posttranslational modification is restricted to tubulin. The investigations reported here bring the first evidence that proteins other than tubulin can be polyglutamylated. Among these proteins, we identified two members of the nucleosome assembly protein family. We used GT335, a mAb raised against the biglutamylated motif of tubulin, to detect and purify immunoreactive proteins from HeLa cells. We showed that some of these proteins are substrates of glutamylating enzyme(s), which copurified on the GT335 affinity column. The proteins retained on the column can be divided into four groups. The first group is composed of proteins that do not react with GT335 on Western blots and are not labeled in the glutamylation assay (the 66-kDa protein for instance). The second group comprises proteins that react with GT335 on Western blots but are not labeled in the glutamylation assay (the 66-kDa protein for instance). The second group comprises proteins such as the 40-kDa protein, which is labeled in the glutamylation assay but does not react on Western blots. Finally, the fourth group is composed of proteins that both react on Western blots and are labeled in the glutamylation assay. Except for the proteins of the first group, which could have been retained on the immunoaffinity column through the interaction with GT335-reactive proteins, all other proteins considered here likely bear a polyglutamylated motif.

Copurification of polyglutamylase(s) on the GT335 column was rather unexpected. One can speculate that this enzyme possesses an autocatalytic activity leading to the formation of an internal polyglutamyl side chain recognized by the mAb. Another possibility is that the enzyme strongly binds to its substrate and forms a complex that is retained on the column. In this case, however, the polyglutamyl side chain of the substrate must be accessible to interact with the mAb. Polyglutamylation of the affinity-purified proteins and, in particular, those identified as NAPs was not stimulated by the addition of partially purified tubulin polyglutamylase from brain or HeLa cells, which are active on MTs. It thus appears that tubulin polyglutamylase was unable to glutamylate NAPs in the conditions used, suggesting that tubulin and NAPs are glutamylated by distinct enzymes. Nevertheless, other experiments, such as competition assays with MTs and NAPs or biochemical separation of the two enzymatic activities, are required to further document this point.
The two major proteins of the fourth group have been identified as members of the NAP family, namely NAP-1 and NAP-2 (29–31). Two minor proteins of the same group (Figs. 3 and 6, horizontal arrows), also detected by anti-NAP mAb A48 (Fig. 5), likely represent other glutamylated members of the NAP family. NAP-1 and NAP-2 bear very acidic domains rich in Glu and Asp residues, a feature shared with the C-terminal domains of tubulin subunits around the glutamylation sites. Demonstration that the C-terminal acidic domains of NAPs indeed carry polyglutamyl side chains was obtained by protein sequencing and mass spectrometry. The number of glutamyl units found in NAP peptides is high, ranging from 3 to 9 units for NAP-1 and from 6 to 10 units for NAP-2. This can be compared with the 1–6 units found for most tubulin isotypes in mammalian brain (2–5, 7). Another level of complexity is related to the number of glutamylation sites. The presence of multiple sites of glutamylation is rather rare for tubulin, where, in most cases, a single side chain of variable length occurs. However, one isoform expressed in brain, called a4, bears up to 11 glutamyl units distributed on two distinct sites (8). Similarly, a- and b-tubulin from Tritrichomonas mobilensis bear up to 21 and 17 units distributed in four and two adjacent side chains, respectively (12). We identified here two adjacent putative sites of glutamylation for both NAP-1 and NAP-2, which appear as gaps in the amino acid sequence. The radioactivity initially associated with the peptides was retained on the filter of the sequencer so that we could not measure any released radioactivity at the cycles corresponding to the gaps. As reported previously (2–5, 7, 8, 10, 11), glutamylated residues of tubulin could not be directly identified by Edman degradation because the corresponding phenylthiohydantoin-Glu (Glu)n is not released from the filter of the sequencer and thus produces a gap in the sequence. Several attempts to elute this phenylthiohydantoin after completion of the sequencing were unsuccessful (3). It is not yet known whether additional sites are present downstream in the sequence, which is very rich in Glu repeats (Fig. 9). Fragmentation techniques (mass spectrometry—mass spectrometry) could be very useful to determine the precise structure of the C-terminal domain. Moreover, preliminary data obtained from the analysis of the minor peak eluting from the DEAE column (Fig. 7B) indicated that another acidic peptide located in the N-terminal domain of NAP-1 from amino acids 8 to 32 was also glutamylated, being substituted by up to 5 glutamyl units. Thus, NAP-1 exhibits two distinct polyglutamylation domains very distant along its primary sequence. It is not known whether the other acidic domain present in the middle region of both NAP-1 and NAP-2 is also glutamylated (Fig. 9).

Comparison of NAP and tubulin polyglutamylation in HeLa cells revealed interesting differences. In these cells, cytoplasmic a- and b-tubulin contain very low levels of glutamylated forms, which bear only 1–2 glutamyl units (~4% of b-tubulin is glutamylated and even less for a-tubulin) (20). In contrast, a substantial proportion of NAPs, estimated to ~50%, appears to be modified and bears a much higher number of glutamyl units. These differences indicate that glutamylation levels and behavior of NAPs and tubulin are very different within the same cell. Obviously, our results show that mAb GT335 is not only specific for polyglutamylated tubulin but more generally appears to recognize polyglutamylated motifs. However, the strength of mAb GT335 binding likely depends on the nature of the glutamylated protein. For instance, the specific reactivity with NAPs was much weaker than that observed with tubulin (compare GT335 reactivity with Coomassie staining in Fig. 3). As reported previously, this is likely due to primary sequence differences around the site of glutamylation (18, 28). Thus, mAb GT335 can be considered primarily as an anti-glutamylated tubulin antibody but must be used with caution to follow either tubulin or NAPs. It is important to verify its reactivity by Western blotting, not only by one-dimensional PAGE, which in most cases does not resolve tubulin from NAPs, but also by two-dimensional PAGE with broad range pH gradients. Special attention must be taken in the interpretation of immunocytochemical data. In our hands, reactivity against non-microtubule components was usually very faint or absent. However, in certain cases, misinterpretations of immunofluorescence data could be made and one should verify the nature of the labeling by manipulating the polymerization state of tubulin with microtubular drugs, such as paclitaxel or nocodazole.

The presence of glutamylated NAPs (i.e., labeled by mAb GT335) is not restricted to HeLa cells but is also observed in other tissues and cell types, such as mouse liver and spleen (data not shown), and in other organisms: Drosophila\(^2\) and Xenopus.\(^3\) These results suggest that polyglutamylation could be of general importance for NAP function.

NAP-1 has been implicated in nucleosome assembly (for a recent review, see Ref. 32). In vitro assays of nucleosome assembly revealed that NAP-1 alone can facilitate the ATP-independent and random deposition of histones onto DNA (24, 33). A similar role has been proposed for the much more recently discovered NAP-2 (31). In the presence of other factors such as chromatin assembly factor-1 (CAF-1), NAP-1 functions as part of a complex machinery to mediate ATP-facilitated assembly of regularly spaced nucleosomal arrays, similar to those observed in vivo (34). NAP-1 likely acts as a chaperone involved in the addition of histones H2a and H2b to form with H3 and H4 the core nucleosome. In addition, NAP-1 has been implicated in

\(^2\) C. Clapier, P. Becker, and B. Eddé, unpublished results.

\(^3\) G. Almouzni and B. Eddé, unpublished results.
nucleosome disruption accompanying the binding of transcription factors by carrying out the first step, i.e. removal of H2a/H2b dimers (35). Assembly and disassembly of nucleosomes are known to be regulated by histone acetylation/deacetylation (for a recent review, see Ref. 36). The very high number of lysines involved in this regulation allows considerable modification of the net charge borne by specific regions of histones. Polyglutamylation produces similar changes involving negatively charged residues. It is thus possible that the two modifications could cooperate to regulate the binding of NAPs to histones. The very particular structure of polyglutamylation with its side chain extending away from the main polypeptide could allow strong stabilization of the interactions with positively charged partners.

A striking feature of NAP-1 is its distribution within the cell. Contrary to what is expected for a protein solely involved in chromatin structure, a cytoplasmic localization is very frequently observed, in particular in yeast (37) and in HeLa cells (38). NAPs were shown to interact with newly synthesized histones and might be involved in their transport to the nucleus (39). Unexpectedly, it was shown that yeast and Xenopus NAP-1 interacts specifically with B-type cyclins (37, 40), and seems to be required for normal progression through mitosis (41). This role was supported by the observation that mutant strains of *Saccharomyces cerevisiae* deleted for the NAP-1 gene are not able to shift from polar to isotropic bud growth and form large clumps of interconnected cells that have an unusual elongated morphology. Moreover, NAP-1 was proposed to play a role in controlling microtubule stability. In particular, deletion of the NAP-1 gene allowed cell growth at concentrations of the microtubule depolymerizing drug benomyl that almost completely block growth of wild-type cells (37). This suggests that NAP-1 is required for destabilization of microtubules at the onset of mitosis. Whether such a role of NAP-1 is related to polyglutamylation is not yet known. However, it is relevant to note that glutamylation of tubulin is very precisely regulated during the cell cycle, exhibiting a peak of enzymatic activity in G2 phase (20). Further experiments will be required to determine if polyglutamylation of NAPs and tubulin are related.

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