Polyglutamylated α-Tubulin Can Enter the Tyrosination/Detyrosination Cycle†

Bernard Eddé,*,‡ Jean Rossier,§ Jean-Pierre Le Caer,§ Jean-Claude Promé,∥ Elisabeth Desbruyères,‡ François Gros,‡ and Philippe Denoulet‡

Laboratoire de Biochimie Cellulaire, Collège de France, 11 Place Marcelin Berthelot, 75231 Paris Cedex 05, France, Laboratoire de Physiologie Nerveuse, CNRS, 91198 Gif sur Yvette, France, and Centre de Recherche de Biochimie et Génétique Cellulaire, CNRS, 118 Route de Narbonne, 31062 Toulouse, France

Received July 16, 1991; Revised Manuscript Received October 10, 1991

ABSTRACT: We have previously identified a major modification of neuronal α-tubulin which consists of the posttranslational addition of a varying number of glutamyl units on the γ-carboxyl group of glutamate residue 445. This modification, called polyglutamylation, was initially found associated with detyrosinated α-tubulin [Eddé, B., Rossier, J., Le Caer, J. P., Desbruyères, E., Gros, F., & Denoulet, P. (1990) *Science 247*, 83–85]. In this report we show that a lateral chain of glutamyl units can also be present on tyrosinated α-tubulin. Incubation of cultured mouse brain neurons with radioactive tyrosine, in the presence of cycloheximide, resulted in a posttranslational labeling of six α-tubulin isoelectric variants. Because both tyrosination and polyglutamylation occur in the C-terminal region of α-tubulin, the structure of this region was investigated. [^{3}H]tyrosinated tubulin was mixed with a large excess of unlabeled mouse brain tubulin and digested with thermolysin. Five peptides, detected by their radioactivity, were purified by high-performance liquid chromatography. Amino acid sequencing and mass spectrometry showed that one of these peptides corresponds to the native C-terminal part of α-tubulin ^{440}VEGEGEEEGEEY^{451} and that the remainders bear a varying number of glutamyl units linked to glutamate residue 445, which explains the observed heterogeneity of tyrosinated α-tubulin. A quantitative analysis showed that the different tyrosinated forms of α-tubulin represent a minor (13%) fraction of the total α-tubulin present in the brain and that most (80%) of these tyrosinated forms are polyglutamylated. The different forms of α-tubulin were found to be equal substrates for tubulin tyrosine ligase and tubulin carboxypeptidase, which indicates that α-tubulin can enter the tyrosination/detyrosination cycle independently of its degree of glutamylation.

Tubulin heterogeneity is strikingly high in the brain, where it is essentially due to the contribution of neuronal cells (Gozes & Littauer, 1978; Dahl & Weibel, 1979; Gozes & Sweadner, 1981; George et al., 1981; Denoulet et al., 1982; Wolff et al., 1982; Moura-Neto et al., 1983; Field & Lee, 1988). This heterogeneity is generated at two levels: differential expression of several isotypes which are encoded by different isogenes (Lewis et al., 1985; Villasante et al., 1986; Sullivan & Cleveland, 1986) and extensive posttranslational modifications of some, if not all, primary gene products. Several modifications of α-tubulin have so far been characterized: removal and possible readdition of the C-terminal tyrosine (Barra et al., 1974), acetylation of K^{40} [1] (L'Hernault & Rosenbaum, 1985; Le Dizet & Piperno, 1987; Eddé et al., 1991), and polyglutamylation of E^{445} (Eddé et al., 1990). This latter modification consists of the progressive addition of a various number of glutamyl units forming a peptidic or pseudo-peptidic lateral chain extending from the main chain at a few residues from the C-terminus. Posttranslational modifications of β-tubulin are yet largely unknown, but phosphorylation (Eddé et al., 1981; Gard & Kirschner, 1985) and polyglutamylation (Alexander et al., 1991) of a class III β-tubulin isotype have been described.

High-resolution IEF analysis has shown that neuronal α-tubulin is composed of as many as eight isoelectric variants. Among these, the most basic variant, denoted α1, corresponds to the primary products of several isogenes [mα1, mα2, and mα4 in the mouse (Lewis et al., 1985; Villasante et al., 1986)], whereas the seven other more acidic forms are produced by posttranslational modifications (Denoulet et al., 1986, 1988; Eddé et al., 1989). This isoelectric heterogeneity could be accounted for by polyglutamylation, acetylation, and probably other yet unknown modifications. In contrast to glutamylation and acetylation, tyrosination/detyrosination does not affect the net charge of the polypeptide although it can also contribute to tubulin diversity. This modification takes place at a site located very close to the site of glutamylation, raising the possibility that the two modifications could interfere with each other. This question is of fundamental importance, especially as an abundant fraction of brain tubulin does not undergo the tyrosination/detyrosination cycle (Rodriguez & Borisy, 1978; Paturle et al., 1989).

In a previous study (Eddé et al., 1990), we posttranslationally labeled tubulin with a radioactive precursor of glutamate and isolated polyglutamylated C-terminal peptides which were mainly in the detyrosinated form, although some peptides were also detected which could correspond to tyrosinated forms. In this report, tyrosinated α-tubulin, posttranslationally labeled by incubating cultured neurons with

† This work was supported by grants from Centre National de la Recherche Scientifique (URA 1115 and UPR 2212), Institut National de la Santé et de la Recherche Médicale (CRE 89.6005), Commission of the European Communities (C1.1-0508-FR-H), and Fondation pour la Recherche Médicale Française.

* To whom correspondence should be addressed.
‡ Laboratoire de Biochimie Cellulaire, Collège de France.
§ Laboratoire de Physiologie Nerveuse, CNRS.
∥ Centre de Recherche de Biochimie et Génétique Cellulaire, CNRS.

[1] Abbreviations: *m/z*, mass/charge ratio; PTH, phenylthiohydantoin; TTL, tubulin tyrosine ligase; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing. Amino acids are indicated with the one-letter code: K, lysine; G, glycine; E, glutamate; Y, tyrosine; V, valine.

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[^{3}H]tyrosine, was mixed with a large excess of unlabeled mouse brain tubulin and digested with thermolysin. The C-terminal peptides, detected by their radioactivity, were purified by HPLC. Peptide sequencing and mass spectrometry showed that these peptides contain, in addition to the C-terminal tyrosine, a varying number of glutamyl units linked to the residue E^{445}. Tyrosination and polyglutamylation can therefore occur on the same α-tubulin polypeptide. In addition, the rates of tyrosination and detyrosination were shown to be unaffected by the degree of glutamylation, indicating that this latter modification does not interfere with the entry of α-tubulin into the tyrosination/detyrosination cycle.

Experimental Procedures

Cell Culture and Labeling Conditions. Cultures of mouse brain neurons were performed as previously described (Berwald-Netter et al., 1981; Eddé et al., 1989). After six days of culture, the cells were transferred to phosphate-buffered saline containing 100 μg/mL cycloheximide, and, 30 min later, either L-[G-^{3}H]glutamate (20–40 Ci/mmol) or L-[3,5-^{3}H]-tyrosine (40–60 Ci/mmol) was added to a radioactive concentration of 100 and 50 μCi/mL, respectively. Incubation times were as indicated in the text.

Tubulin Purification and Proteolysis. Tubulin from cell cultures was purified with taxol (Vallee, 1982), using a miniscale procedure (Serrano et al., 1987). Tubulin from 1-month-old mouse brain was purified by two cycles of assembly/disassembly followed by phosphocellulose chromatography (Shelanski et al., 1973; Weingarten et al., 1974). Digestion of tubulin (4 mg/mL) with 1/20 (w/w) thermolysin (Boehringer-Mannheim) was performed for 5 h at 37 °C, in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM $CaCl_2$. Under these conditions, digestion was judged complete because further incubation or an increase in the enzyme concentration did not change the peptide profiles obtained by HPLC. Before analysis, the samples were centrifuged for 2 min at 10000*g*. The pellet, which contained less than 1% of the initial radioactivity, was discarded.

HPLC Procedures. The HPLC apparatus (Waters Millipore Inc.) was equipped with an in-line flow radioactivity detector (FLO-ONE, Radiomatic), which was placed immediately after the UV densitometer. HPLC eluates first entered the cell of the densitometer and then the cell of the detector where they were mixed with liquid scintillation cocktail. The radioactive signals were accumulated, usually for 6-s intervals. In preparative experiments, a splitter was placed before the radioactivity cell detector in order to recover 90% of the eluting material.

HPLC Systems. System A was used as a first step for purifying [^{3}H]tyrosinated peptides. A preparative anion-exchange column (DEAE, Protein Pack, 1 × 15 cm; Waters Millipore Inc.) was used at a flow rate of 1 mL/min. Solution A1 was 20 mM Tris-HCl (pH 8.0) containing 50 mM NaCl, and solution B1 was 20 mM Tris-HCl (pH 8) containing 500 mM NaCl. Samples were eluted with a linear gradient of 0–100% B1 in 40 min. System B used a reversed-phase C8-RP300 column (4.6 × 220 mm, Brownlee) at a flow rate of 1 mL/min. Solution A2 was 0.1% trifluoroacetic acid in water, and solution B2 was 0.09% trifluoroacetic acid and 70% acetonitrile in water. Samples were eluted with a linear gradient of 95% A2/5% B2 to 71% A2/29% B2 in 40 min. System C used a reversed-phase C18 column (4.6 × 220 mm, Brownlee) with conditions identical to those described for system B. In system D, salt-containing HPLC fractions were desalted using a reversed-phase C8-RP300 column (4.6 × 30 mm, Brownlee) at a flow rate of 1 mL/min. An initial isocratic wash of 95% A2/5% B2 was applied for 10 min, and the peptides were then eluted by applying a linear gradient to 0% A2/100% B2 in 5 min.

Peptide Sequencing. Peptides were sequenced using a 470A gas–liquid amino acid sequencer (Applied Biosystems Inc.) as described previously (Le Caer & Rossier, 1988). The initial sequencing efficiency was determined by using known amounts of synthetic peptides. For the peptide VEGEGEEEGEEY, the efficiency was 40%.

Gel Electrophoresis and Fluorography. One-dimensional SDS–PAGE (Laemmli, 1970) was performed on a 8% acrylamide/0.11% bisacrylamide slab gel (8 cm long) containing 0.1% (w/v) SDS (90% pure, Merck). Denaturating 9.5 M urea cylindrical IEF gels (O'Farrell, 1975) were performed as described by Wolff et al. (1982), except that pH 5.0–5.5 ampholytes (Serva) were used. This modification further increased the resolution of the tubulin isoelectric variants. After electrophoresis or electrofocusing, the gels were stained with R250 Coomassie blue, photographed, soaked for 20–30 min in Amplify (Amersham), and then dried and submitted to fluorography.

Mass Spectrometry. A ZAB-HS double-focusing mass spectrometer (VG Analytical, Manchester, U.K.) equipped with a fast atom bombardment gun (Iontech) was used. The spectra were generated by a xenon atom beam of 8 keV. The matrix was a 1/1 mixture of glycerol and thioglycerol (1 μL) acidified with 1 μL of 10% (w/v) TCA. Cesium iodide clusters were used for mass calibration. Because the underivatized forms of the peptides did not show any characteristic molecular ion, the peptides were methylated at their free carboxylic groups, as described previously (Eddé et al., 1990), before mass spectrometry analysis. About 100–200 pmol of each peptide was used in each experiment.

In Vitro Tyrosination. Eight-day neurons were incubated with [^{3}H]glutamate for 2 h, in the presence of cycloheximide. Microtubule proteins were purified by one cycle of assembly/disassembly and incubated with purified TTL [a kind gift from L. Paturle-Lafanechère and D. Job, prepared as described by Wehland and Weber (1987)] for 30 min at 30 °C. The incubation buffer was 0.1 M MES (pH 6.8) containing 1 mM EGTA, 25 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 5 mM ATP, and 0.1 mM tyrosine. The enzyme/substrate ratio was adjusted in order to yield a complete reaction in 20 min, as tested by measuring the time course of radioactive tyrosine incorporation into nonradioactive microtubule proteins under the same conditions. [^{3}H]Glutamylated tubulin, incubated or not with TTL, was then digested with thermolysin, as described above. The resulting peptides were analyzed by HPLC (system C).

Chemical Synthesis of Peptides. Peptides, corresponding to the C-terminal region of α-tubulin (^{440}VEGEGEEEGEE^{450} and ^{440}VEGEGEEEGEEY^{451}), were synthesized using the standard t-Boc-amino acid procedure with an automatic synthesizer (430A, Applied Biosystems Inc.). One, two, or three glutamyl groups were added to the residue corresponding to E^{445}. The structures of the peptides were confirmed by peptide sequencing and mass spectrometry. The detailed method of synthesis has been recently published (Paturle-Lafanechère et al., 1991).

Results

Mouse brain neurons were cultured for six days and then incubated with either [^{3}H]glutamate or [^{3}H]tyrosine in the presence of cycloheximide, a strong inhibitor of protein synthesis. The resulting posttranslationally labeled proteins were analyzed by SDS–PAGE and fluorography. As shown in

Table I: Structure and Relative Abundance of the Tyrosinated Peptides

peak	sequence[a]	structure[b]	amount[c] (nmol)	% relative to total tubulin[d]	% relative to tyrosinated tubulin[e]
Tyr1	VEGEGEEEG...	VEGEGEEEGEEY	2.2	2.7	21
Tyr2	VEGEGXEEG...	VEGEGEEEGEEY E	3.1	3.9	30
Tyr3	VEGEGXEEG...	VEGEGEEEGEEY E-E	2.6	3.2	25
Tyr4	VEGEGXEEG...	VEGEGEEEGEEY E-E-E	1.5	1.9	15
Tyr5	VEGEGXEEG...	VEGEGEEEGEEY E-E-E-E	0.9	1.2	9
total			10.3	12.9	100

[a] The sequences of Tyr1 to Tyr5 peptides were determined by automatic Edman degradation. **X** denotes a cycle where no PTH-amino acid was released. The C-terminal EEY residues were not detected, probably due to problems of sequencing low amounts of C-terminal acidic residues. However, several considerations strongly suggest their presence: (1) It is not expected that thermolysin could cleave G–E or E–E bonds, and (2) these peptides are labeled with [^{3}H]tyrosine. [b] The characterization of the structure of these peptides was achieved by mass spectrometry. The position of the polyglutamyl lateral chain at residue E^{445} was confirmed by the absence of release of the corresponding PTH-amino acid. [c] The amount of peptide present in each fraction was determined by the amount of PTH-V released at the first cycle of the Edman degradation and was corrected for the sequencing efficiency (40%, see Experimental Procedures) and by the yield of the HPLC purification. This latter value was 67%, as determined by counting the radioactivity recovered with the five peaks Tyr1–Tyr5 (470 000 cpm) and dividing it by the radioactivity present in the initial tubulin preparation (700 000 cpm). [d] The percentage of each tyrosinated form, relative to total brain tubulin (brain tubulin represents >98%, and neuronal tubulin <2%, of the tubulin mixture submitted to proteolysis), was determined by dividing the amount of peptide present in each fraction by the total amount of tubulin (8 mg, 80 nmol) used for HPLC purification. [e] The percentage of each tyrosinated form, relative to total tyrosinated tubulin, was determined by dividing the amount of peptide present in each fraction by the total amount found in all fractions (10.3 nmol).

Figure 1, α-tubulin was strongly labeled after incubation of the cells with either amino acid, whereas β-tubulin was weakly labeled only after incubation with [^{3}H]glutamate. We have previously demonstrated that the strong labeling of α-tubulin observed with [^{3}H]glutamate is due to polyglutamylation of α-tubulin, that is, the posttranslational addition of a various number of glutamyl units to residue E^{445} (Eddé et al., 1990). As shown below, the strong label observed with [^{3}H]tyrosine is due to the already described C-terminal tyrosination of α-tubulin [for reviews, see Barra et al. (1988) and Greer and Rosenbaum (1989)].

Tubulin was then purified using taxol and analyzed by IEF (Figure 2). As previously reported (Eddé et al., 1989), eight isoelectric variants of α-tubulin were found. This important isoelectric heterogeneity is mainly due to polyglutamylation, but acetylation and probably other yet unknown modifications are also involved (Eddé et al., 1991). Among the observed variants, α3–α8 were glutamylated (Figure 2a) whereas α1–α6 were tyrosinated (Figure 2b). Thus, an important overlap of tyrosinated and glutamylated α-tubulin isoforms was observed. This raised the possibility that some tyrosinated α-tubulin isoforms could also be glutamylated. Because both modifications occur in the C-terminal region of α-tubulin, the structure of this region was investigated. For this purpose, a large excess (8 mg) of tubulin purified from mouse brain was mixed with [^{3}H]tyrosinated tubulin (100 μg; 700 000 cpm) purified from neurons, and the mixture was digested with thermolysin, a proteolytic enzyme which cleaves polypeptides at the N-side of hydrophobic residues (Matsubara, 1970). The C-terminal peptides, detected by their radioactivity, were purified by HPLC, starting with an anion-exchange column (system A). The elution profile (Figure 3a) showed the presence of six radioactive peaks (denoted 1–6), eluting between 17.50 and 26.70 min. Each peak, except peak 6, was desalted and purified further on a reversed-phase C8-RP300 column (system B). The corresponding elution profiles are shown in Figure 3b–f. In each case, a single radioactive peak was obtained (these peaks were denoted Tyr1–Tyr5); 10% aliquots of Tyr1–Tyr5 were sequenced by automatic Edman degradation. Tyr1 gave the sequence VEGEGEEEG, which matches that of mouse α-tubulin genes mα1 and mα2 from amino acids 440–448 (Villasante et al., 1986). Tyr2, Tyr3,

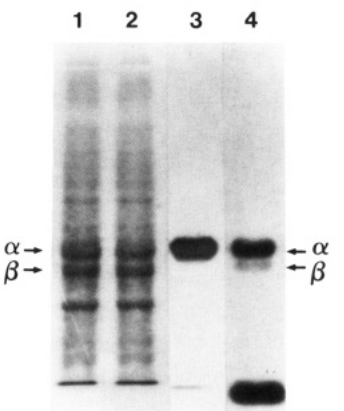

FIGURE 1: SDS–PAGE analysis of proteins posttranslationally labeled after cell incubation with [^{3}H]tyrosine or [^{3}H]glutamate. Neurons were cultured for six days and then incubated, in the presence of cycloheximide, with either [^{3}H]tyrosine for 2 h (lanes 1 and 3) or [^{3}H]glutamate for 1 h (lanes 2 and 4). Total protein extracts were analyzed. The gel was stained with Coomassie blue (lanes 1 and 2), dried, and submitted to fluorography (lanes 3 and 4). In lane 4, three bands corresponding to α-tubulin, to β-tubulin, and to an uncharacterized species migrating at the front of the gel were labeled. In lane 3, a single labeled band corresponding to α-tubulin was detected.

Tyr4, and Tyr5 all gave the same sequence, VEGEG**X**EEG (where **X** indicates a cycle in which no PTH-amino acid was released). As discussed in Table I, these peptides most probably extend to the C-terminal Y^{451}, which was confirmed by mass spectrometry analysis (see below). The sequences of Tyr2–Tyr5 were identical to that of Tyr1 except at position 445, where in the place of PTH-E no PTH-amino acid could be detected. A similar gap was observed during previous studies on α-tubulin glutamylation (Eddé et al., 1990) and supports the presence of a modified E^{445} residue.

The structure of these peptides was further characterized by fast atom bombardment mass spectrometry. To enhance the observed signals, it was necessary to methylate the peptides at their free carboxyl groups, prior to mass analysis. The mass/charge ratio (m/z) of Tyr1 was 1467.4 (Figure 4), corresponding to the protonated ($M + H^+$) form of the unmodified dodecapeptide ^{440}VEGEGEEEGEEY^{451} [1354.5 (calculated mass) + 112.1 (from the eight methyl groups) +

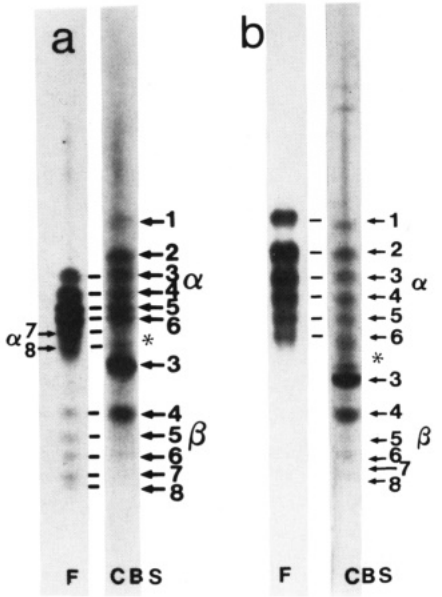

FIGURE 2: Isoelectric overlapping of tyrosinated and glutamylated α-tubulin isoforms. Tubulin was purified from six-day neurons incubated with either [^{3}H]glutamate (a) or [^{3}H]tyrosine (b), as in Figure 1, and was analyzed by IEF. The gels were stained with Coomassie blue (lanes labeled CBS) and submitted to fluorography (lanes labeled F). Isoelectric variants of α- and β-tubulin were identified as previously described (Eddé et al., 1989). The asterisk corresponds to a region of the gels where a comigration of very acidic α-tubulin isoforms (α7, α8) with very basic β-tubulin isoforms (β'1, β'2) was observed. The two most acidic and strongly glutamylated bands (α7 and α8) were attributed to α-tubulin after two-dimensional PAGE analysis (not shown).

1 (H^+) = 1467.6]. When compared to the mass of Tyr1, the masses of Tyr2, Tyr3, and Tyr4 show increments of 1 × 143 (mass of a methylated glutamyl group), 2 × 143, and 3 × 143 and, thus, correspond to the dodecapeptide bearing one, two, and three glutamyl units, respectively. No interpretable mass spectrum was obtained for Tyr5, probably because of the low amount of material contained in this fraction, but it was assumed that this peptide corresponds to the dodecapeptide bearing four glutamyl units (see Discussion). Table I schematizes the deduced structures of the different peptides and, in addition, presents a quantitative analysis of their relative occurrence. The results obtained indicate that the different tyrosinated forms represent altogether a minor fraction (13%) of the total α-tubulin present in mouse brain and that most (80%) of these tyrosinated forms were polyglutamylated (Table I).

The existence of polyglutamylated/tyrosinated forms of α-tubulin raises the question of whether polyglutamylated α-tubulin is an effective substrate for TTL. This was first tested in vitro by incubating [^{3}H]glutamylated tubulin with TTL in the presence of nonradioactive tyrosine (see Experimental Procedures). The addition of tyrosine on glutamylated α-tubulin was followed by a procedure which consisted of digesting the incubate with thermolysin and analyzing the resulting radioactive peptides by reversed-phase HPLC (system C). This system allowed the separation of the tyrosinated and detyrosinated C-terminal peptides, as tested with the synthetic peptides VEGEGEEEGEE and VEGEGEEEGEEY, bearing zero, one, two or three glutamyl units. The chromatographic profile shows that the presence of a C-terminal tyrosine delayed the elution times of the peptides by about 10 min (Figure 5a). In a control experiment, performed without incubation with TTL, the glutamate radioactivity was mostly (87%) associated with five peaks corresponding to the non-tyrosinated C-terminal peptides (Figure 5b). These peaks differ by their degree of glutamylation. Only 7.5% of the radioactivity was found to be associated with a group of poorly resolved peaks corresponding to the polyglutamylated and tyrosinated species, whereas the remainder (5.5%) eluted with an uncharacterized peak at 39.3 min. This result shows that most (92%) of the cellular [^{3}H]glutamylated tubulin was in the non-tyrosinated state. When [^{3}H]glutamylated tubulin was incubated with

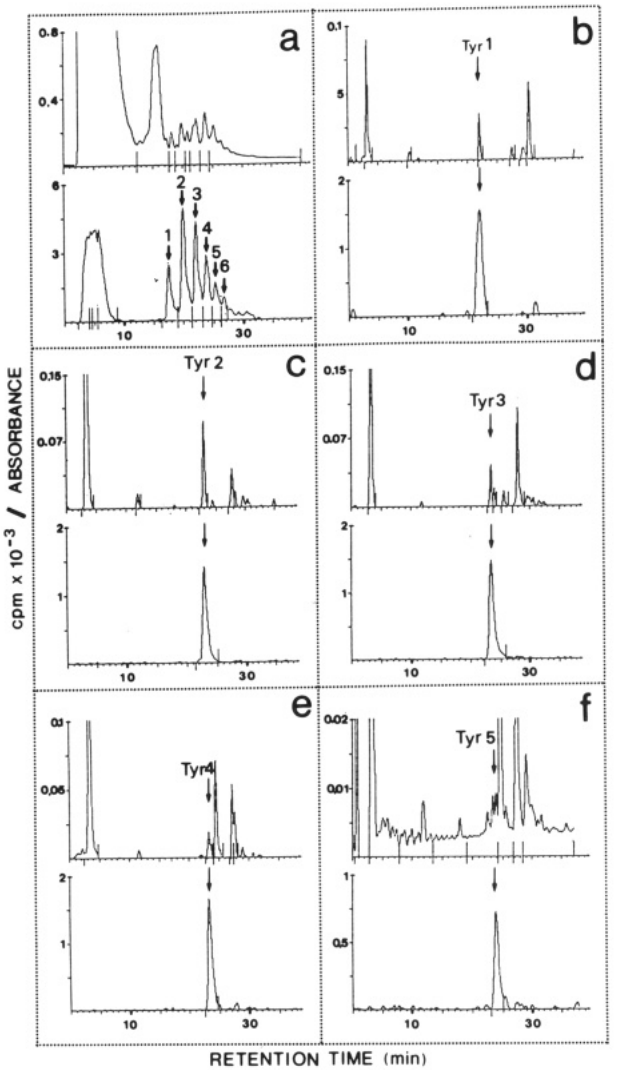

FIGURE 3: HPLC purification of tyrosinated tubulin peptides. [^{3}H]Tyrosinated tubulin (100 μg, 700 000 cpm) purified from neurons was mixed with unlabeled tubulin (8 mg) purified from one-month-old mouse brains, and the mixture was digested with thermolysin. (a) The proteolytic products were first separated on an anion-exchange column (system A). Four successive injections of 0.5-mL aliquots of the sample were performed with 1-min intervals, and the elution gradient was then initiated. The first radioactive fraction eluted between 2 and 7 min corresponds to free [^{3}H]tyrosine, which was not retained on the column. The incorporated radioactivity was associated with six peaks eluting at 17.5, 20.0, 22.1, 23.8, 25.3, and 26.70 min (denoted 1–6, respectively). (b–f) Peaks 1–5 were desalted (system D) and injected separately on a C8-RP300 reversed-phase column (system B). In each case, a single radioactive peak was observed and collected. These peaks were denoted Tyr1–Tyr5 and had retention times of 22.0, 22.9, 23.3, 23.4, and 23.9 min, respectively. Note that the different radioactive peaks were very well resolved by anion-exchange chromatography, which indicates that they differed by their net charge content, but they had very close retention times on the C8-RP300 column. This latter column, however, allowed each radioactive peak to be separated from a bulk of contaminating, nonradioactive material. The peptides were detected by their absorbance at 215 nm (top profiles, OD units) and their radioactivity (bottom profiles, ^{3}H cpm).

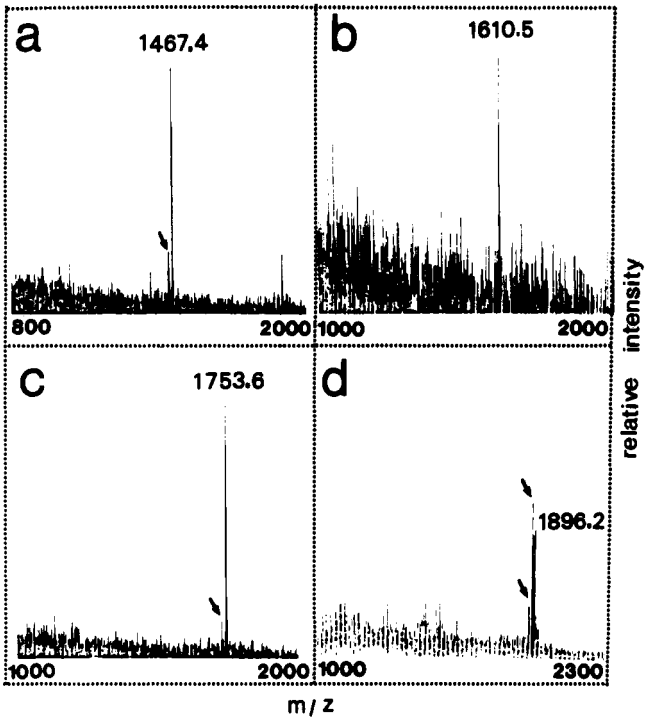

FIGURE 4: Mass spectrometry analysis of Tyr1–Tyr4 peptides. The peptides Tyr1–Tyr4 were carboxyl-methylated and analyzed by fast atom bombardment mass spectrometry (panels a–d, respectively). Masses of the protonated molecular ions (M + H^+) are indicated. Mass values at m/z = 1467.4 (panel a), 1610.5 (panel b), 1753.6 (panel c), and 1896.2 (panel d) correspond to fully methylated VEGEGEEEGEEY peptides bearing zero, one, two, and three methylated glutamyl units, respectively. The mass signals indicated by arrows correspond to uncompletely methylated species. Mass ranges are indicated in each panel.

TTL prior to the digestion with thermolysin, 56% of the radioactivity shifted to an elution time corresponding to the polyglutamylated/tyrosinated peptides (Figure 5c), showing that polyglutamylated α-tubulin is an effective substrate for TTL. However, a significant fraction has not been tyrosinated, suggesting that polyglutamylated α-tubulin contains also a non-substrate subpopulation. The degree of polyglutamylation of this fraction is not significantly different from that of the original preparation (compare the distribution of the different glutamylated non-tyrosinated peptides before and after incubation with TTL in Figure 5b,c), which indicates that the different polyglutamylated forms have been equally tyrosinated.

Tyrosination of polyglutamylated α-tubulin was also studied under culture conditions by incubating neurons with [^{3}H]-tyrosine for various times between 10 and 60 min, in the presence of cycloheximide. Tubulin was purified at each time point, and its specific radioactivity was found to reach a plateau at around 30 min (half-life 16 min). The rates of tyrosination of the different α-tubulin isoforms were first compared by IEF. Incorporation of radioactive tyrosine appeared very similar in all the labeled α-tubulin isoforms, but a precise quantification could not be performed due to the relatively high background observed between two adjacent IEF bands (see, for instance, the fluorography presented in Figure 2b). To avoid this problem, another analytical method was used which consisted of digesting the tubulin preparations obtained at each time point with thermolysin and quantifying the radioactivity present in the C-terminal peptides after anion-exchange HPLC (system A). The HPLC conditions used, in this case, were similar to those described for Figure 3 and allowed the resolution of the tyrosinated peptides according to their degree of

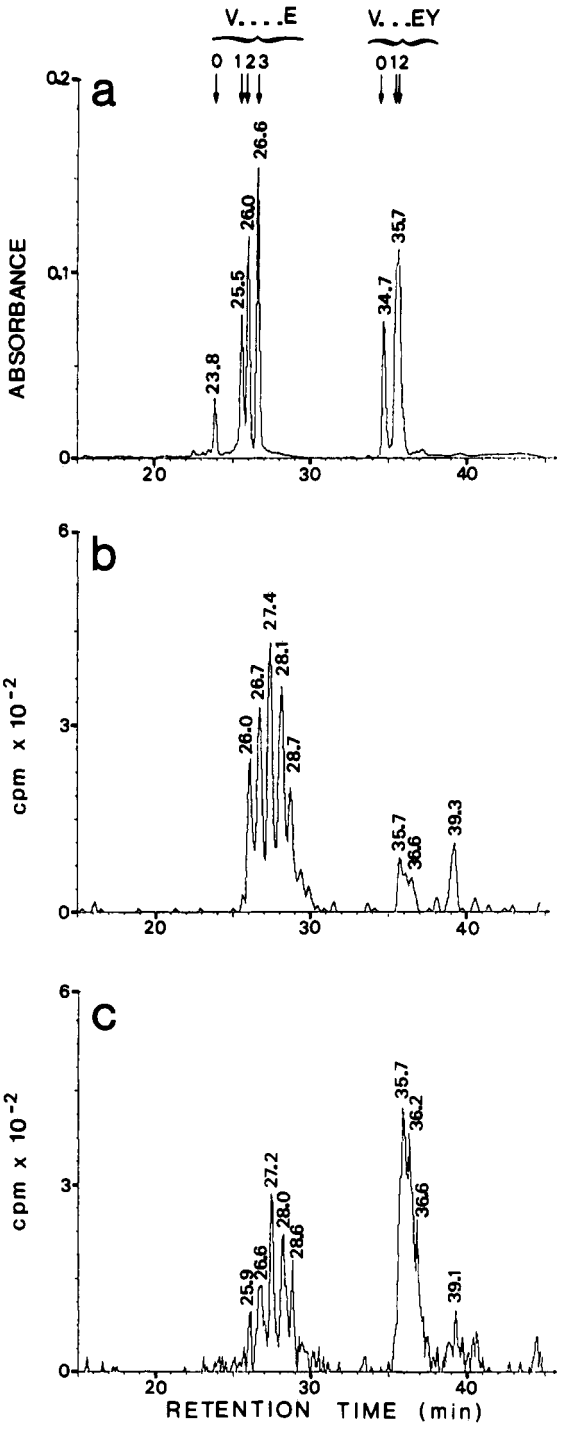

FIGURE 5: In vitro tyrosination of [^{3}H]glutamylated α-tubulin. A reversed-phase C18 column was used (system C). (a) Elution profile of two groups of synthetic peptides corresponding to ^{440}VEGEGEEEGEE^{450} (indicated by V...E) bearing zero, one, two, or three glutamyl units (arrows) and ^{440}VEGEGEEEGEEY^{451} (indicated by V...EY) bearing zero, one, or two glutamyl units (arrows). In both cases, glutamyl units were bound at the E^{445} site. The presence of a C-terminal tyrosine delayed the elution of the peptides by about 10 min. In addition, detyrosinated peptides, but not tyrosinated ones, were resolved according to their degree of glutamylation. (b and c) Radioactivity profiles of thermolysin digests of [^{3}H]glutamylated tubulin before (b) and after (c) incubation with TTL. A major part of the radioactivity shifted from a position corresponding to non-tyrosinated peptides to a position corresponding to tyrosinated peptides. The radioactivity contained in each peak was quantified using the in-line detector of radioactivity.

glutamylation. The radioactive profiles are presented in Figure 6 (left-hand side; note that the radioactivity scales have been adjusted to yield similarly sized curves). Six radioactive peaks

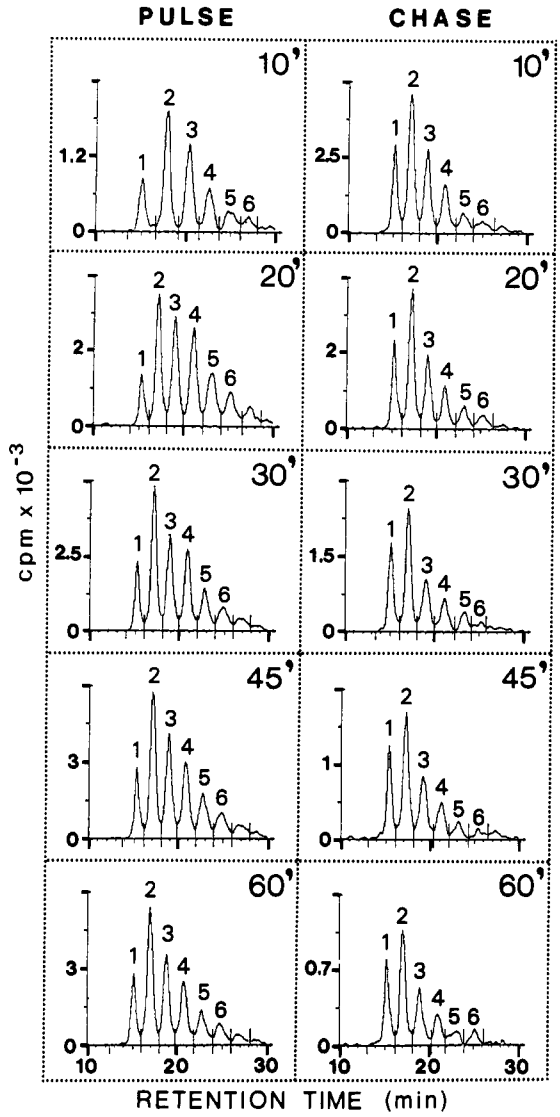

FIGURE 6: Pulse–chase experiment with [^{3}H]tyrosine. Six-day neurons were incubated with [^{3}H]tyrosine for 10, 20, 30, 45, and 60 min (pulse). Duplicate plates were incubated for 1 h and then allowed to chase in a nonradioactive medium for 10, 20, 30, 45, and 60 min (chase). At each time point, tubulin was purified and digested with thermolysin, and the resulting peptides were chromatographed on an analytical anion-exchange column (system A). Peptides were detected by their radioactivity. The C-terminal tyrosinated peptides were resolved into six peaks (numbered 1, 2, 3, 4, 5, and 6). Except for the time point Pulse 10 min, the retention times of peaks 1–6 were 15.2 ± 0.1, 17.1 ± 0.1, 18.9 ± 0.1, 20.9 ± 0.1, 23.0 ± 0.15, and 25.1 ± 0.2 min, respectively. Retention times of the corresponding peaks at Pulse 10 min were 15.1, 17.9, 20.3, 22.6, 24.7, and 27.0 min. This difference is likely to be due to an uncontrolled change of the column temperature during this chromatography. The radioactivity scales have been adjusted in order to yield similarly sized curves. Only parts of the radioactivity profiles (between retention times 10 and 30 min) are shown. No radioactive peaks were detected in the other regions.

were detected at each time point and were quantified. The percentage of radioactivity present in each peak, relative to the total radioactivity present in the six peaks, at a given time point were determined and found to remain constant with time (the values for peaks 1–6, respectively, were, in percentages, 12.0 ± 2.3, 28.2 ± 2.9, 22.3 ± 1.4, 18.3 ± 3.0, 11.6 ± 2.3, and 7.6 ± 0.9). This result means that the rates of tyrosination of the different α-tubulin forms were similar and, thus, independent of the degree of glutamylation.

A similar method was used to analyze the rates of detyrosination. Neurons were incubated with [^{3}H]tyrosine for 60 min, and a chase was then initiated for 10–60 min. The half-life of the detyrosination reaction was found to be 24 min. HPLC analysis (Figure 6, right-hand side; again the radioactivity scales have been adjusted to yield similarly sized curves), again, showed no change in the relative proportion of the different peaks during the time of chase (the values for peaks 1–6, respectively, were, in percentages, 21.4 ± 2.2, 33.7 ± 0.6, 20.1 ± 1.3, 12.5 ± 1.5, 7.0 ± 0.6, and 5.3 ± 1.1). These data show that detyrosination, as well as tyrosination, is not affected by the degree of glutamylation.

Discussion

Mouse brain neurons in primary culture develop very long axodendritic processes and form functional synapses of very characteristic morphologies (Berwald-Netter et al., 1981). They express and accumulate a number of neuronal specific proteins and, in particular, have a very high level of tubulin heterogeneity (Eddé et al., 1989). This heterogeneity increases during the culture with a time course remarkably similar to that observed for the whole brain during development in vivo (Wolff et al, 1982; Denoulet et al., 1988). Advantage was taken of this model of neuronal differentiation and maturation to further analyze the molecular basis of brain α-tubulin heterogeneity. Incubating the cells with [^{3}H]tyrosine, in the presence of cycloheximide, led to the specific labeling of tyrosinated α-tubulin. IEF analysis revealed the presence of six labeled bands, indicating a high charge heterogeneity.

We have recently described that a varying number of glutamyl units can be added onto residue E^{445} of α-tubulin (Eddé et al., 1990). This modification, which can be specifically followed by incubating cells with [^{3}H]glutamate in the presence of cycloheximide, produces a short peptidic or pseudopeptidic polyglutamyl chain of variable length extending laterally from the main chain at a few residues from the C-terminus. In the experiments reported here, an important overlapping between [^{3}H]glutamylated and [^{3}H]tyrosinated α-tubulin isoforms was observed, which suggested that the heterogeneity of tyrosinated α-tubulin could be related to polyglutamylation.

This hypothesis was confirmed by analyzing the C-terminal region of α-tubulin, which was generated by complete digestion with thermolysin. It has to be noted that the tubulin preparation used for this study contained a large excess of unlabeled mouse brain tubulin (>98%), which allowed the purification of enough material for peptide sequencing and mass spectrometry, and [^{3}H]tyrosinated neuronal tubulin, for the detection of the C-terminal tyrosinated peptides by their radioactivity. Anion-exchange HPLC revealed the presence of six radioactive peptides, differing by their net charge. Among these, the five most abundant were purified further by reversed-phase HPLC to give five peptides, denoted Tyr1–Tyr5. These peptides were characterized by peptide sequencing and mass spectrometry. The results showed that Tyr1 corresponds to the C-terminal part, ^{440}VEGEGEEEGEEY^{451}, of the most abundant mouse brain isotypes (mα1 and mα2) (Lewis et al., 1985) and that Tyr2, Tyr3, and Tyr4, were similar to Tyr1 except that they beared one, two, and three glutamyl units, respectively, linked to Glu^{445}. Although no definitive data are available for Tyr5 and Tyr6, their chromatographic behavior on an anion-exchange column (Figure 3a), as well as their labeling with [^{3}H]tyrosine and their amino acid sequence (obtained only for Tyr5), suggests that they correspond to the tetra- and pentaglutamylated species, respectively. These data can explain the observed charge heterogeneity of tyrosinated α-tubulin: a nonglutamylated form corresponding to α1, and a series of polyglutamylated forms differing by the number of glutamyl units and corresponding to the acidic variants

α2–α6. The reason why α2, which according to the data presented above must correspond to the monoglutamylated species, was not labeled after cell incubation with [^{3}H]-glutamate (Figure 2) could be related to a particular feature of the glutamylation reaction. Indeed, preliminary results indicate that the glutamylated forms, except the monoglutamylated one, can turn over. Consequently, these results suggest the existence of an irreversible step for the addition of the first glutamyl unit which could not then, under steady-state conditions, be labeled by [^{3}H]glutamate.

The presence of a lateral chain of glutamyl units produces an additional available carboxy-terminal glutamate residue and, thus, raises the theoretical possibility of a second site of tyrosination. Mass spectrometry data, however, clearly indicated that the isolated C-terminal peptides did not contain two tyrosine residues.

The relative abundance of the different α-tubulin isoforms was estimated by calculating the amounts of modified peptides released after digestion with thermolysin. These amounts were determined from amino acid sequencing data, as described in Table I. The different tyrosinated α-tubulin isoforms were shown to represent only a minor fraction (about 13%) of the total brain tubulin present in our preparation. This value is similar to those (15–18%) reported by Ponstingl et al. (1981) and Barra et al. (1980) but lower than the value of 32% obtained by Rodriguez and Borisy (1979). Our data also show that, when considering tyrosinated α-tubulin, the non-glutamylated form (corresponding to α1) is minor (20%) whereas the different glutamylated forms are the most abundant (80%). Moreover, when compared to our previous results which estimated the relative abundance of polyglutamylated/detyrosinated α-tubulin to about 50% of total brain tubulin (Eddé et al., 1990), these data show that polyglutamylated α-tubulin is mainly in the detyrosinated form.

The existence of tyrosinated α-tubulin, polyglutamylated or not, and of polyglutamylated α-tubulin, tyrosinated or not, suggests that the two modifications are completely independent. We have shown, using an in vitro assay, that a major part of polyglutamylated α-tubulin is a substrate for tubulin tyrosine ligase, independently of its degree of glutamylation. This absence of effect of glutamylation on the entry of α-tubulin in the tyrosination/detyrosination cycle was also observed in pulse–chase experiments with [^{3}H]tyrosine, under culture conditions (Figure 6). However, whether tyrosination/detyrosination affects glutamylation could not be tested, but this question appeared irrelevant. Indeed, the turnover of this latter modification was low (half-life > 6 h, unpublished results), indicating that, within neurons, tubulin can be tyrosine-cycled several times, statistically, before being glutamylated or deglutamylated.

The in vitro assay for tyrosination has also revealed that a substantial proportion of glutamylated α-tubulin could not be tyrosinated. Our results clearly indicate that glutamylation by itself does not affect the tyrosination competence of tubulin (Rodriguez & Borisy, 1978; Paturle et al., 1989), which suggests that glutamylated α-tubulin can be modified in other ways which make it resistant to tyrosination. This is supported by very recent results showing that some polyglutamylated α-tubulin polypeptides end at residue E^{449}. The lack of E^{450} could directly account for the tyrosination incompetence of this tubulin species (Paturle-Lafanechère et al., 1991).

The functional role of tyrosination/detyrosination, although extensively studied [for recent reviews, see Barra et al. (1988) and Greer and Rosenbaum (1989)], is not completely understood. Preferential association of detyrosinated α-tubulin with long-life polymers has been reported in most cases, although it was proposed that detyrosination is a consequence rather than a cause of microtubule stability (Schulze & Kirschner, 1987; Khawaja et al., 1988). Whether the function of tyrosination in neuronal cells could be influenced by the presence of a lateral chain of glutamyl units located at a very few residues of the C-terminus remains to be determined.

Acknowledgments

We thank C. Gruszcynski, A. Koulakoff, and Y. Berwald-Netter for providing cultures of mouse brain neurons; H. Mazarguil for providing synthetic peptides; L. Paturle-Lafanechère and D. Job for their gift of purified tubulin tyrosine ligase; and R. Guénard for providing taxol.

Registry No. TTL, 60321-03-1; tubulin carboxypeptidase, 73050-23-4.

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Isolation from Opossum Serum of a Metalloproteinase Inhibitor Homologous to Human α1B-Glycoprotein[†,‡]

Joseph J. Catanese and Lawrence F. Kress*
Molecular and Cellular Biology Department, Roswell Park Cancer Institute, Buffalo, New York 14263
Received July 24, 1991; Revised Manuscript Received September 25, 1991

ABSTRACT: Fractionation of opossum (*Didelphis virginiana*) serum with $(NH_4)_2SO_4$, followed by chromatography on DEAE-Sepharose, phenyl-Sepharose, and Mono Q HR 5/5, has resulted in the isolation in homogeneous condition of a metalloproteinase inhibitor designated oprin (opossum proteinase inhibitor). Oprin is a single-chain glycoprotein (26% carbohydrate) with an estimated M_r = 52000, pI = 3.5, and E(1%/1 cm) = 11. Oprin inhibited snake venom metalloproteinases, but showed no activity on venom serine proteinases or on bacterial metalloproteinases. Incubation of *Crotalus atrox* α-proteinase (EC 3.4.24.1) with oprin, and analysis of the reaction products by chromatography on Mono Q HR 5/5 and by electrophoresis under nondenaturing conditions, indicated formation of an inactive enzyme/inhibitor complex. The complex dissociated during SDS/polyacrylamide gel electrophoresis. An opossum liver cDNA library was immunoscreened, and clones containing cDNA encoding for part of the open reading frame for oprin were isolated. The cDNA inserts contained nucleotide sequences corresponding to two internal amino acid sequences of oprin which had been separately determined by protein sequence analysis. Protein database screening using a 211 amino acid sequence deduced from one of the cDNA inserts showed no significant homology to known proteinase inhibitors. There was, however, a 36% identity with human α1B-glycoprotein, a plasma protein of unknown function related to the immunoglobulin supergene family. In addition, the amino-terminal sequence of oprin showed 46% identity with human α1B-glycoprotein in a 26 amino acid residue overlap. Comparisons of sequence, molecular weight, and disulfide content of the two proteins suggest that oprin contains 4 of the 5 domains found in human α1B-G. The presence of oprin in opossum serum may partially account for the resistance of this marsupial to those localized effects of rattlesnake envenomation which are caused by venom metalloproteinases.

The major manifestations of rattlesnake envenomation in most mammalian victims are localized hemorrhage, tissue necrosis, edema, and systemic coagulation defects, all of which result in part from the direct or indirect action of metalloproteinases and serine proteinases in the venom, (Ohsaka, 1979). However, some mammals exhibit a resistance or decreased sensitivity to the localized and lethal effects of rattlesnake and other poisonous snake venoms (Ovadia & Kochva, 1977; De Wit, 1982; Tomihara et al., 1987), and this phenomenon has been studied extensively in the North American opossum (*Didelphis virginiana*).

Opossums resisted the lethal effects of rattlesnake venom and showed little or no localized hemorrhage, edema, or tissue

[†] This research was supported by National Institutes of Health Grant HL22996.
[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J05356.

0006-2960/92/0431-410$03.00/0