Identification by Mass Spectrometry of a New α-Tubulin Isotype Expressed in Human Breast and Lung Carcinoma Cell Lines†

Srinivasa Rao,‡ Fredrik Åberg,‡ Edward Nieves,§ Susan Band Horwitz,‡ and George A. Orr*,‡

Departments of Molecular Pharmacology and Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461

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ABSTRACT: The extensive C-terminal molecular heterogeneity of α- and *β*-tubulin is a consequence of multiple isotypes, the products of distinct genes, that undergo several posttranslational modifications. These include polyglutamylation and polyglycylation of both subunits, reversible tyrosination and removal of the penultimate glutamate from α-tubulin, and phosphorylation of the *β*III isotype. A mass spectrometry-based method has been developed for the analysis of the C-terminal diversity of tubulin from human cell lines. Total cell extracts are resolved by SDS−PAGE and transferred to nitrocellulose, and the region of the blot corresponding to tubulin (∼50 kDa) was excised and digested with CNBr to release the highly divergent C-terminal tubulin fragments. The masses of the human α- and *β*-tubulin CNBr-derived C-terminal peptides are all in the 1500−4000 Da mass range and can be analyzed directly by MALDI-TOF mass spectrometry in the negative ion mode without significant interference from other released peptides. In this study, the tubulin isotype diversity in MDA-MB-231, a human breast carcinoma cell line, and A549, a human non-small lung cancer cell line, is reported. The major tubulin isotypes present in both cell lines are k-α1 and *β*1. Importantly, we report a previously unknown α isotype present at significant levels in both cell lines. Moreover, the degree of posttranslational modifications to all isotypes was limited. Glu-tubulin, in which the C-terminal tyrosine of α-tubulin is removed, was not detected. In contrast to mammalian neuronal tubulin which exhibits extensive polyglutamylation, only low-level monoglutamylation of the k-α1 and *β*1 isotypes was observed in these two human cell lines.

Microtubules, composed of α/*β*-tubulin heterodimers, are essential components of all eukaryotic cells and are involved in a diverse range of cellular functions, including motility, maintenance of cell shape, intracellular trafficking of macromolecules and organelles, and, most importantly, mitosis (*1*−*3*). One of the major difficulties encountered in the study of tubulin is its structural diversity, a consequence of the expression of both a minimum of six α- and *β*-tubulin isotypes, the products of distinct genes, and of numerous posttranslational modifications occurring in both subunits (*4*, *5*). The tubulin isotypes are differentially expressed in cells and tissues, and there is evidence that the isotypes may have different functions in vivo. The posttranslational modifications include polyglutamylation and polyglycylation of both subunits; in the case of α-tubulin, acetylation, reversible tyrosination, and excision of the C-terminal glutamate in nontyrosinable tubulin; and phosphorylation of the mammalian *β*III (*4*, *5*). Both polyglycylation and the reversible tyrosylation cycle appear to be tubulin-specific modifications. Polyglutamylation, once thought to be specific for tubulin, has recently been described for nucleosome assembly proteins (*6*). Significantly, the majority of the primary sequence divergence in the various tubulin isotypes and all of the posttranslational modifications, except acetylation of Lys_{40}, occur within the C-terminal 20 amino acids of α- and *β*-tubulin chains. This highly divergent region of α/*β*-tubulins is functionally important in in vitro microtubule assembly due to its interaction with known regulatory proteins, including MAP2 and tau, and also has been implicated in the binding of calcium (see ref *4*). It is suggested that these posttranslational modifications to tubulin are involved in regulating in vivo microtubule dynamics by modulating the ability of regulatory proteins to associate with the cytoskeleton.

Mass spectrometry has recently been used to characterize tubulins isolated from mammalian brain (*7*−*14*), avian erythrocytes (*15*), and several lower eukaryotes (*16*−*20*), including paramecium and sea urchin. However, no detailed structural information currently exists on C-terminal modifications to tubulins in human cell lines, tissues, and tumors. The goal of these current studies is to develop a mass spectrometry-based procedure for rapidly obtaining a global picture of the α/*β*-tubulin isotype composition, including posttranslational modifications, in human cell lines, tissue, and tumors. Since quantity is likely to be limiting in tissue-derived samples, we have developed procedures that avoid the need to purify tubulin by chromatographic techniques. Instead, total cell extracts were resolved by SDS−PAGE[1] and transferred to nitrocellulose, and the region of the blot corresponding to tubulin (∼50 kDa) was excised and digested with CNBr to release the highly divergent C-terminal tubulin

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* To whom correspondence should be addressed: Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Telephone: (718) 430-3742. Fax: (718) 430-8922. E-mail: orr@aecom.yu.edu.

‡ Department of Molecular Pharmacology.

§ Department of Biochemistry.

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fragments. The masses of the human α- and β-tubulin CNBr-derived C-terminal peptides are all in the 1500−4000 Da mass range and can be analyzed directly by MALDI-TOF mass spectrometry in the negative ion mode without significant interference from other released peptides. This is possible because the C-terminal tubulin peptides contain a high percentage of negatively charged amino acids, and selective detection in the negative ion mode is a characteristic feature of highly acidic peptides (*21*).

In this study, we report on the tubulin isotype diversity in MDA-MB-231, a human breast carcinoma cell line, and in A549, a human non-small lung cancer cell line. The major tubulin isotypes present in both cell lines were k-α1 and β1. The presence of a previously unknown α isotype at significant levels in both cell lines is reported. Moreover, the degree of posttranslational modifications to all the tubulin isotypes present in both cell lines was limited. Glu-tubulin, in which the C-terminal tyrosine of α-tubulin is removed, was not detected, and only low-level monoglutamylation of the k-α1 and β1 isotypes was observed. These studies represent the first detailed analysis of α/β-tubulin isotype diversity in human cell lines by mass spectrometry.

EXPERIMENTAL PROCEDURES

CNBr Release of C-Terminal Bovine Brain Tubulin Peptides. Bovine brain tubulin was obtained from Cytoskeleton, Inc. (Denver, CO). For digestion in solution, tubulin (5 μL, 10 mg/mL) was added to 1 mL of 70% formic acid containing 150 mg of CNBr (Pierce) and incubated for 3.5 h at room temperature. For digestion on nitrocellulose, tubulin (50 μg) was resolved by SDS−PAGE and electroblotted to nitrocellulose (*22*−*24*). The nitrocellulose was stained with Ponceau S and washed extensively with H_2O, and the region of the nitrocellulose blot containing tubulin was excised, cut into small pieces, and treated with CNBr (150 mg/mL) in 70% formic acid. After 3.5 h at room temperature, the supernatant was collected and the nitrocellulose was washed with 70% formic acid (2 × 100 μL). The combined washings were taken to dryness in a Speedvac to remove CNBr, and the residue was dissolved in 100 μL of 70% formic acid. To purify peptides corresponding to the individual isotypes, the formic acid solution was subjected to C18 reverse phase HPLC (Aquapore OD-300, 2.1 mm × 220 mm) on a HP 1090 chromatograph eluting with a linear gradient of a 0 to 50% acetonitrile/0.1% TFA mixture over the course of 30 min at a rate of 0.2 mL/min.

CNBr Release of C-Terminal Human Tubulin Peptides. The human breast carcinoma cell line, MDA-MB-231, and the human non-small cell lung cancer line, A549, were grown to 85% confluency in 100 mm plates. After washing, cell pellets from five plates were resuspended in 0.4 mL of 0.1 M MES (pH 6.8) containing 1 mM EGTA, 0.5 mM $MgCl_2$, and a cocktail of protease inhibitors. Samples were sonicated (3 × 30 s) and treated with DNase I (Boehringer Mannheim, 30 units) for 1 h at 37 °C, and then 4× SDS sample loading buffer was added to achieve a final concentration of 1×. Samples were boiled for 5 min prior to electrophoresis on a 9% SDS−PAGE gel. Bovine brain tubulin was used as a marker. After electrophoresis, proteins were transferred to nitrocellulose and visualized with Ponceau S. The region of the nitrocellulose blot containing tubulin was excised, washed extensively with H_2O, and digested with CNBr as described above. To isolate microtubule proteins, 20 μM Taxol in dimethyl sulfoxide was added to the DNase I-treated cell lysates to polymerize tubulin. After 30 min at 37 °C, microtubules were collected by centrifugation through a sucrose cushion (30% w/v). The microtubule pellet was digested in solution with CNBr as described for bovine tubulin. HPLC purification of individual peptides was performed as described above.

Carboxypeptidase Y and Trypsin Digestion of the Purified C-Terminal CNBr Peptides of Human Tubulins. CNBr-released peptides were resolved by C18 reverse phase HPLC (Aquapore OD-300, 2.1 mm × 220 mm) eluting with a linear gradient of a 20 to 40% acetonitrile/0.1% TFA mixture over the course of 60 min at a rate of 0.2 mL/min. The C-terminal CNBr tubulin peptides were detected by MALDI-TOF mass spectrometry. For carboxypeptidase Y (Boehringer) and trypsin (Promega) digestions, the appropriate fractions were taken to dryness and redissolved in 5 mM sodium citrate (pH 6.0) or 5 mM NH_4HCO_3 (pH 8.0). Carboxypeptidase Y or trypsin was added to a final concentration of 0.5 μg and allowed to incubate for 21 or 16 h, respectively. Aliquots of each digest were analyzed by MALDI-TOF mass spectrometry.

Chemical Synthesis of the Unmodified C-Terminal CNBr Peptides of Human Tubulins. Peptides were synthesized on an Applied Biosystems 431A peptide synthesizer using Fmoc-based chemistry. The purity and authenticity of each peptide were checked by reverse phase HPLC and MALDI-TOF mass spectrometry, respectively.

MALDI-TOF Mass Spectrometry. Unfractionated CNBr digests were dried in a Speedvac and resuspended in 100 μL of a 30% acetonitrile/0.1% TFA mixture, and 1.5 μL of each sample was diluted 1:10, 1:20, 1:30, 1:50, and 1:100 (v/v) in the same solution for mass analysis. Samples (1.5 μL) were mixed (1:1, v/v) with saturated sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, Aldrich) for negative ion mode analysis and in saturated α-cyano-4-hydroxycinnamic acid (Aldrich) for positive ion mode analysis. Both matrixes were prepared in 30% acetonitrile containing 0.1% TFA. Mass spectra were obtained using a Voyager-DE STR mass spectrometer (PerSeptive Biosystems, Foster City, CA) equipped with a nitrogen laser (337 nm) using an acceleration voltage of 15 kV. Negative ion mass spectra were recorded from 50 to 100 laser shots in the negative ion mode using delayed extraction. An ACTH clip (residues 18−39, Sigma) was used for internal calibration. Postsource decay (PSD) analysis was performed in the positive ion mode with or without in-source collision-activated dissociation (CAD) in which nitrogen was used as the collision gas at a source pressure of $\sim 1 \times 10^{-6}$ Torr.

Tandem Mass Spectrometry. Product ion analysis was obtained on a quadrupole ion trap mass spectrometer (Thermoquest LCQ, Finnigian Corp., San Jose, CA) using an ESI needle voltage of 4.5 kV. Direct infusion (5 μL/min) of the sample was used to introduce the sample into the mass spectrometer after diluting 1:1 (v/v) with a 50% acetonitrile/

[1] Abbreviations: CID, collision-induced dissociation; CNBr, cyanogen bromide; EST, expressed sequence tag; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MAPs, microtubule-associated proteins; *m*/*z*, mass-to-charge ratio; PSD, postsource decay; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

Table 1: Calculated Average Masses ([M − H]$^-$) of the Unmodified C-Terminal CNBr Peptides of the Characterized Human α-Tubulins

class/type (name)[a]	sequence	calculated average mass ([M − H]$^-$)
1 b-α1	$(M)_{425}$AALEKDYEEVGVHSVEGEGEEEGEEY	2882.9
k-α1	$(M)_{425}$AALEKDYEEVGVDSVEGEGEEEGEEY	2860.9
2 TBA4	$(M)_{425}$AALEKDYEEVGIDSYEDEDEGEE	2633.6
3 TBA2	$(M)_{413}$EEGEFSEAREDLAALEKDYEEVGVDSVEAEAEEGEEY	4152.2
4 HSATub 8	$(M)_{413}$EEGEFSEAREDLAALEKDYEEVGTDSFEEENEGEEF	4158.2

[a] See ref *26*.

Table 2: Calculated Average Mass ([M − H]$^-$) of the Unmodified C-Terminal CNBr Peptides of the Characterized Human β-Tubulins

class/type (name)[a]	sequence	calculated average mass ([M − H]$^-$)
I (TBB1)	(M_{416})NDLVSEYQQYQDATAEEEEDFGEEAEEEA	3367.3
II	(M_{416})NDLVSEYQQYQDATADEQGEFEEEEGEDEA	3467.4
III (TBB3)	(M_{437})YEDDEEESEAQGPK	1624.6
IVa (TBB5)	(M_{416})NDLVSEYQQYQDATAEQGEFEEEAEEEVA	3350.4
IVb (TBB2)	(M_{416})NDLVSEYQQYQDATAEEEGEFEEEAEEEVA	3480.5

[a] See ref *27*.

H_2O mixture containing 0.1% formic acid. The precursor ion of interest was selected with an isolation width of *m*/*z* ±2 and a relative collision energy set between 30 and 60%.

Bioinformatics. The fragmentation patterns from both the PSD and MS/MS analyses were used to search the protein (OWL and NCBInr) and EST databases using the Mascot search engine (Matrix Science).

RESULTS AND DISCUSSION

Our knowledge of the structural diversity of tubulin in human cell lines and tissues is limited. Northern blot analysis and PCR-based methods can be used to measure mRNA expression levels for specific tubulin isotypes. However, there is a need for the development of rapid and sensitive protein-based methods for determining the tubulin protein isotype composition, and characterizing posttranslational modifications to these isotypes, in human cell lines. The purpose of these studies was to develop procedures for investigating the heterogeneity of human tubulins, specifically the highly divergent C-termini, by mass spectrometric analysis. In recent studies of mammalian brain α-tubulin, both anion exchange and immunoaffinity chromatographic steps were utilized to purify the enzymatically released C-terminal peptides prior to mass analysis (*13*, *15*). Since quantities are likely to be limiting in human tissue-derived samples, we sought to develop a procedure which obviated the need to utilize conventional chromatographic steps. We decided, therefore, to achieve partial purification of tubulin by SDS−PAGE, and after transfer to nitrocellulose, to release the C-terminal peptides by CNBr digestion. We anticipated that MALDI-TOF mass spectrometry in the negative ion mode could be used for the selective detection of the highly acidic C-terminal tubulin peptides, even in the presence of other tubulin- or non-tubulin-derived peptides. For this method to be generally applicable for the analysis of tubulin isotype composition, it is important that the C-terminal tubulin peptides are not selectively lost during the CNBr release from nitrocellulose.

We have determined previously that the ^{32}P-labeled linker region (6.8 kDa) of the multidrug resistance transporter, P-glycoprotein, is released from nitrocellulose in greater than 95% yield under similar CNBr digestion conditions (*22*, *23*). Likewise, almost quantitative release of the 2.8 kDa [^{3}H]-7-benzophenone-Taxol-labeled CNBr fragment of bovine brain β-tubulin was obtained with this procedure (*24*). Even the hydrophobic transmembrane-spanning segments of P-glycoprotein, labeled with either the 3- or 7-BzDc analogues of Taxol, were liberated in high yield (60−65%) by this procedure (*25*). We expected that the smaller, and more hydrophilic, C-terminal α/β-tubulin peptides would be liberated in almost quantitative yield after CNBr digestion. To confirm these expectations, we have compared the MALDI-TOF mass spectra of the C-terminal peptides of bovine brain tubulin generated by CNBr digestion either in solution or on nitrocellulose.

Bovine brain expresses predominantly three α-tubulin isotypes, α1, α2, and α4, and four β-tubulin isotypes, βI, βII, βIII, and βIVa. The amino acid sequences of α1 and α2 in the C-terminal region are identical and, for this study, can be considered as a single protein. In addition to polyglutamylation, the mammalian α1/α2 isotypes can undergo three known modifications: reversible tyrosination and the removal of the penultimate glutamate in the detyrosinated α1/α2 species (*4*, *5*). Like the α-tubulins, the β-tubulin isotypes also undergo extensive polyglutamylation. The MALDI-TOF mass spectra of the CNBr-released brain tubulin C-terminal peptides from either solution or nitrocellulose digests, as expected, were complex with a very large number of ions occurring in the 1500−4000 Da mass range (data not shown). To reduce the complexity of the spectra, the C-terminal α/β-tubulin peptides were purified by reverse phase HPLC. In previous studies (*13*), arginine−Sepharose was used to purify the C-terminal peptides of rat brain α-tubulin prior to HPLC. In these current studies, this step was omitted.

Redeker et al. (*13*) have reported previously that the extent of glutamylation did not affect the retention time of individual peptides within an isotype series. Figure 1 shows the MALDI-TOF mass spectra in the positive ion mode of the purified C-terminal peptides derived from the detyrosinated α1/α2-tubulins. Similar results were observed in the negative ion mode. Although the bovine brain tubulins have not been sequenced, on the basis of the sequences of the human α-tubulins, CNBr is predicted to cleave the bovine

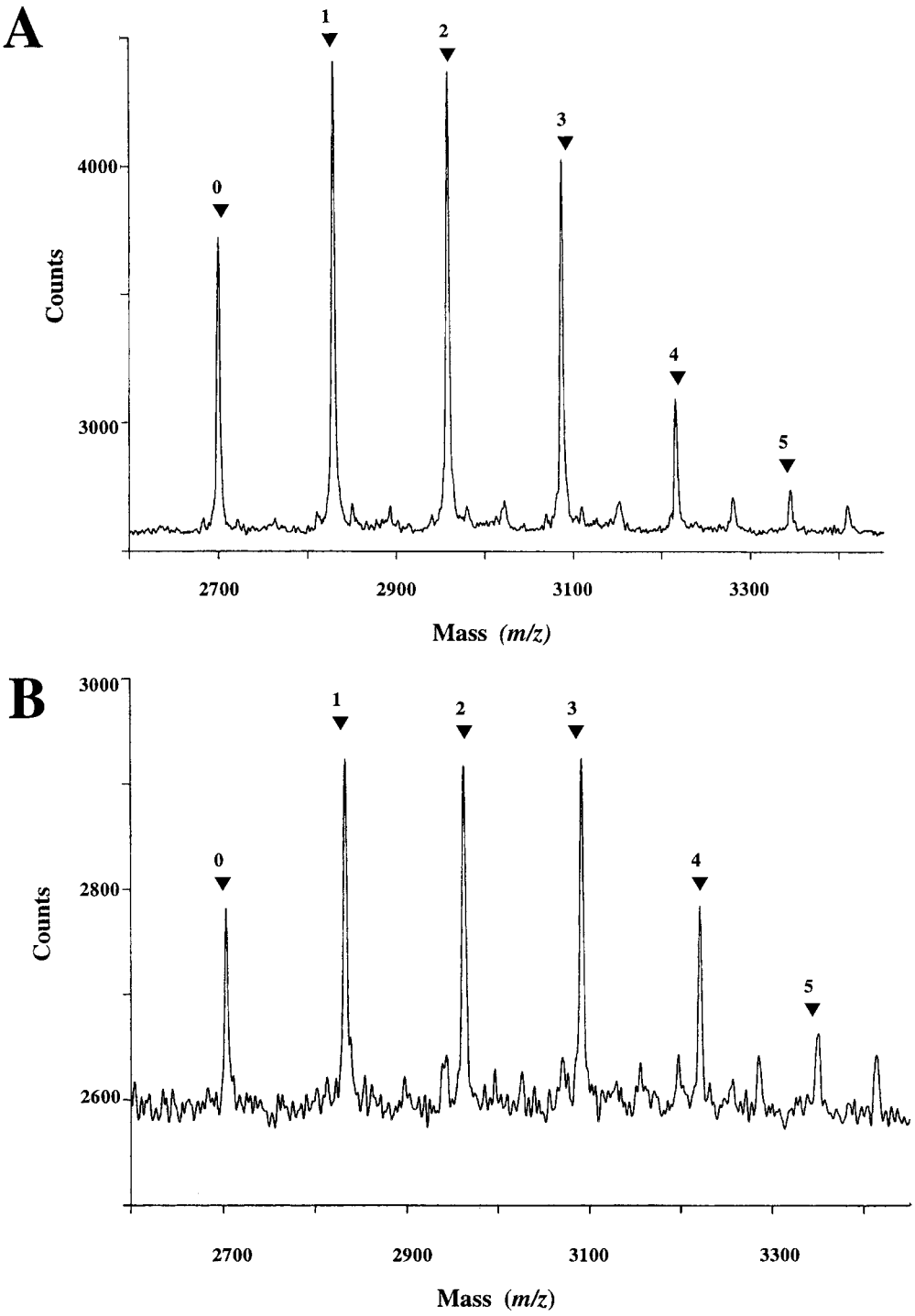

FIGURE 1: MALDI-MS of the CNBr-released C-terminal detyrosinated α1-tubulin peptides from bovine brain. (A) Tubulin digested in solution and (B) tubulin digested on nitrocellulose after SDS−PAGE and electroblotting (see Experimental Procedures for details). The numeral above the arrowheads refers to the number of attached glutamates.

detyrosinated α1/α2-tubulin at Met_{425}. It is apparent from Figure 1 that a similar constellation of molecular ions, each differing by the expected 129 Da increase for the addition of a glutamate residue, can be observed in both the solution (panel A) and nitrocellulose (panel B) digests. The mass of the parent, nonmodified, bovine peptide (i.e., 2699.7 Da) corresponds to the that of human k-α1. MALDI-TOF mass analysis of the other purified α/*β*-tubulin peptides also revealed that qualitatively similar spectra were obtained, irrespective of whether CNBr cleavage occurred in solution or on nitrocellulose (data not shown).

On the basis of phylogenetic reconstructions, the mammalian α- and *β*-tubulin isotypes have each been classified into several major groups (*26*, *27*). The predicted sequences, the classification, and the respective calculated average masses ($[M - H]^-$) of the unmodified C-terminal CNBr peptides of the known human α- and *β*-tubulins are listed in Tables 1 and 2. The MALDI-TOF mass spectra (negative ion mode) of the CNBr-released peptides from the MDA-MB-231 breast cell line and the A549 human non-small lung cancer cell line are shown in Figure 2. For these experiments, ∼6 and 9 mg of total cellular protein from the breast and

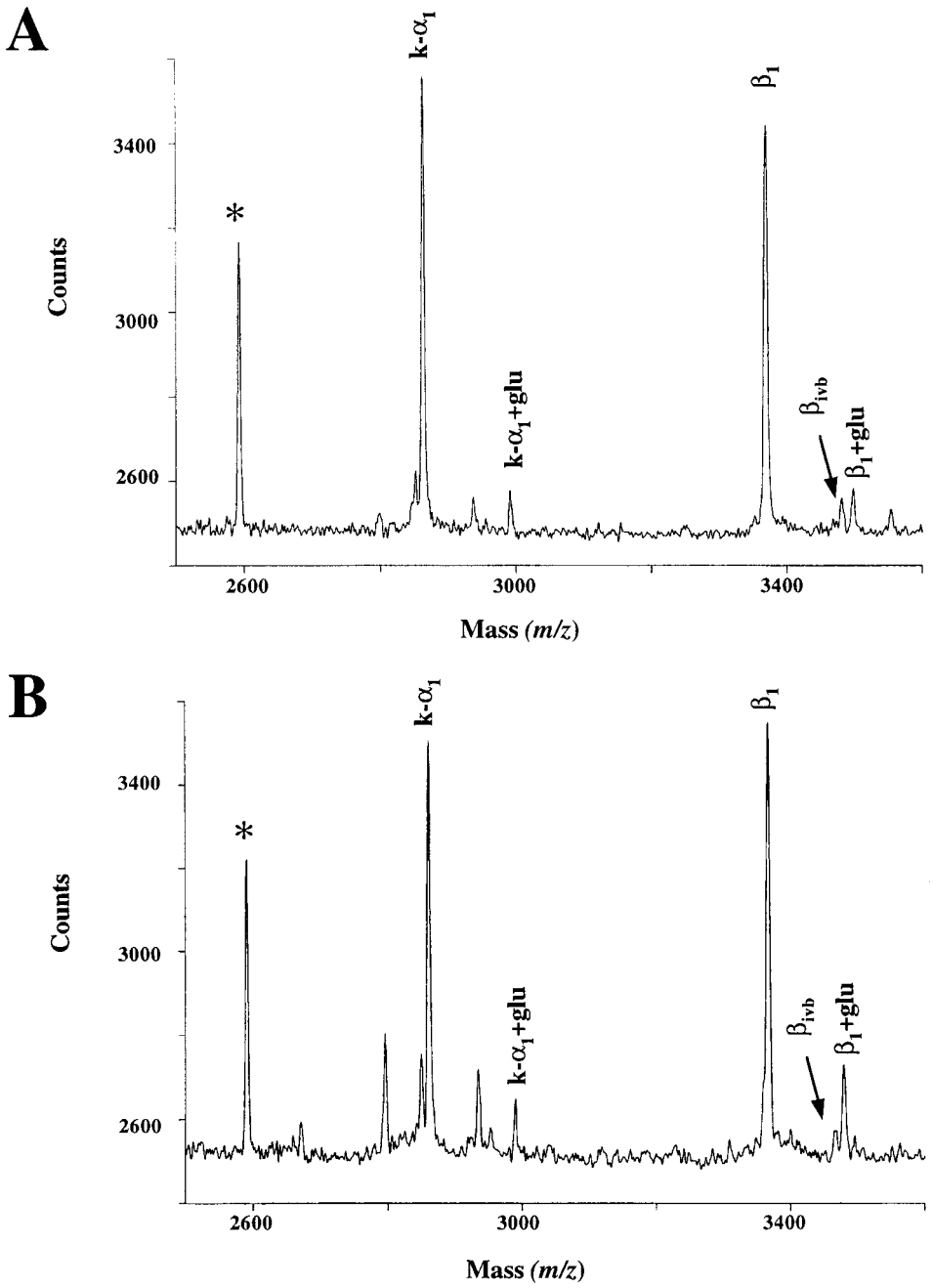

FIGURE 2: MALDI-MS of the CNBr-released C-terminal α/β-tubulin peptides from human cell lines. (A) MDA-MB-231, a human breast carcinoma cell line, and (B) A549, a human non-small lung cancer cell line. In each panel, total cell extracts were subjected to SDS−PAGE and transferred to nitrocellulose and the region of the nitrocellulose blot corresponding to tubulin was digested with CNBr. The released peptides were analyzed by MALDI-MS in the linear and negative ion mode as described in Experimental Procedures. The asterisks designate the ion corresponding to the C-terminal CNBr peptide of the newly identified α-tubulin.

lung cell lines, respectively, were resolved by SDS−PAGE and electroblotting to nitrocellulose. After CNBr digestion of the tubulin-containing region of the blot, the formic acid was removed and the released peptides were dissolved in 100 μL of a 30% acetonitrile/0.1% TFA mixture. Each sample (1.5 μL) was diluted 1:10, 1:20, 1:30, 1:50, and 1:100 (v/v) in the same solution for mass analysis. Three major, and several minor, ions were clearly resolved in the MALDI-TOF mass analysis of the undiluted sample. The ions with *m*/*z* values of 2860.5 and 3367 were close to the calculated average masses ($[M - H]^-$) for the C-terminal CNBr fragments of k-α1- and β1-tubulins, respectively. These precursor ions were subjected to PSD with or without CID. Protein database searching using Mascot and the masses of the b and y product ions, with a ±2 Da peptide and fragment mass tolerance, confirmed these assignments (data not shown). However, the ion with an *m*/*z* value of 2590.0 did not correspond to the mass of any known unmodified human α/β-tubulin C-terminal CNBr peptide. The minor ion with an *m*/*z* value of 3478.9 present in both cell lines has been tentatively identified as the βIVb isotype. Similar results were also obtained from samples diluted as much as 1:50 (v/v). On the basis of the sample amounts and dilutions used in these studies, we estimate that the tubulin isotype composition of these cell lines could be determined using less than 100 μg of total cell extract.

In human, there are two closely related α1-like genes, k-α1 and b-α1, which differ at only three amino acid positions.

One of these substitutions involves residue 438, contained within the C-terminal CNBr peptide of each protein; the k-α1 and b-α1 isotypes have aspartate and histidine residues, respectively, at this position. As indicated above, the ion at *m*/*z* 2860.5 corresponds to the k-α1 isotype. However, the ion at *m*/*z* 2590.0 is close to the calculated average mass ([M − H]) for the C-terminal CNBr peptide of the b-α1 isotype with a histidine at residue 438 and the C-terminal tyrosine and penultimate glutamate removed. α-Tubulin lacking both of these residues is commonly termed Δ2-tubulin and has been found to be associated with long-lived microtubules (*28*). Carboxypeptidase Y digestion, in combination with MALDI-TOF mass spectrometry, was used to sequence the C-terminus of this peptide. The CNBr-released peptides were resolved by reverse phase HPLC, and the appropriate peptides were identified by mass spectrometry of individual fractions. The fraction containing the peptide with an ion at *m*/*z* 2590.0 was digested with carboxypeptidase Y (Figure 3A,B). MALDI-TOF mass spectrometry of the digest showed the appearance of four new ions generated by the sequential removal of tyrosine (−163 Da), glutamate (−129 Da), glutamate (−129 Da), and glycine (−57 Da) residues from the original peptide. The GEEY C-terminal sequence clearly eliminates the C-terminal CNBr peptide of Δ2 derived from b-α1-tubulin as a potential candidate for the 2590.0 Da molecular ion. Nevertheless, the C-terminal sequence that was obtained strongly suggested that this peptide was derived from an α-tubulin-like protein.

Although efforts to identify the *m*/*z* 2590.0 ion by MALDI-TOF PSD mass spectrometry and protein database searching were unsuccessful, searching of the human EST database identified four novel α-tubulin ESTs that had predicted C-terminal CNBr fragments with masses similar to that of the unknown ion (see Table 3). The presence of unique lysine residues in all four predicted sequences afforded us an opportunity to distinguish among these peptides. After complete tryptic digestion (incubation for 16 h) of the HPLC-purified peptide, MALDI-TOF mass spectrometry revealed that the parent ion at *m*/*z* 2590.0 disappeared and a new ion at *m*/*z* 2078.7 was generated. These data would suggest that the sequence of the newly identified α-tubulin C-terminal peptide corresponded to that of EST 4 (Figure 3C). For confirmation, a quadrupole ion trap mass spectrometer was used to obtain the N- and C-terminal fragment ions (i.e., the b and y ion series, respectively) of the HPLC-purified peptide. The product ion spectrum obtained from the doubly charged precursor ion at *m*/*z* 1296.3 is shown in Figure 4. Due to the *m*/*z* range limitation of the ion trap, only the *m*/*z* range from 350 to 2000 was scanned. Nevertheless, the masses of the b ions, b_5-b_{19}, and the y ions, y_5-y_{18}, clearly confirmed the sequence of the central region of this peptide, i.e., amino acid residues 6−20.

Accurate quantitation of the ratios of the various human tubulin isotypes from the MALDI-TOF mass spectra is unreliable due to the different ionization and desorption potentials of the peptides that are involved. However, it is possible using synthetic peptides to estimate their relative desorption efficiencies. For example, it has been shown that the desorption of a tyrosinated α-chain peptide is approximately twice that of its detyrosinated counterpart (*13*). In contrast, the level of glutamylation within a specific C-terminal isotype peptide had little effect on desorption.

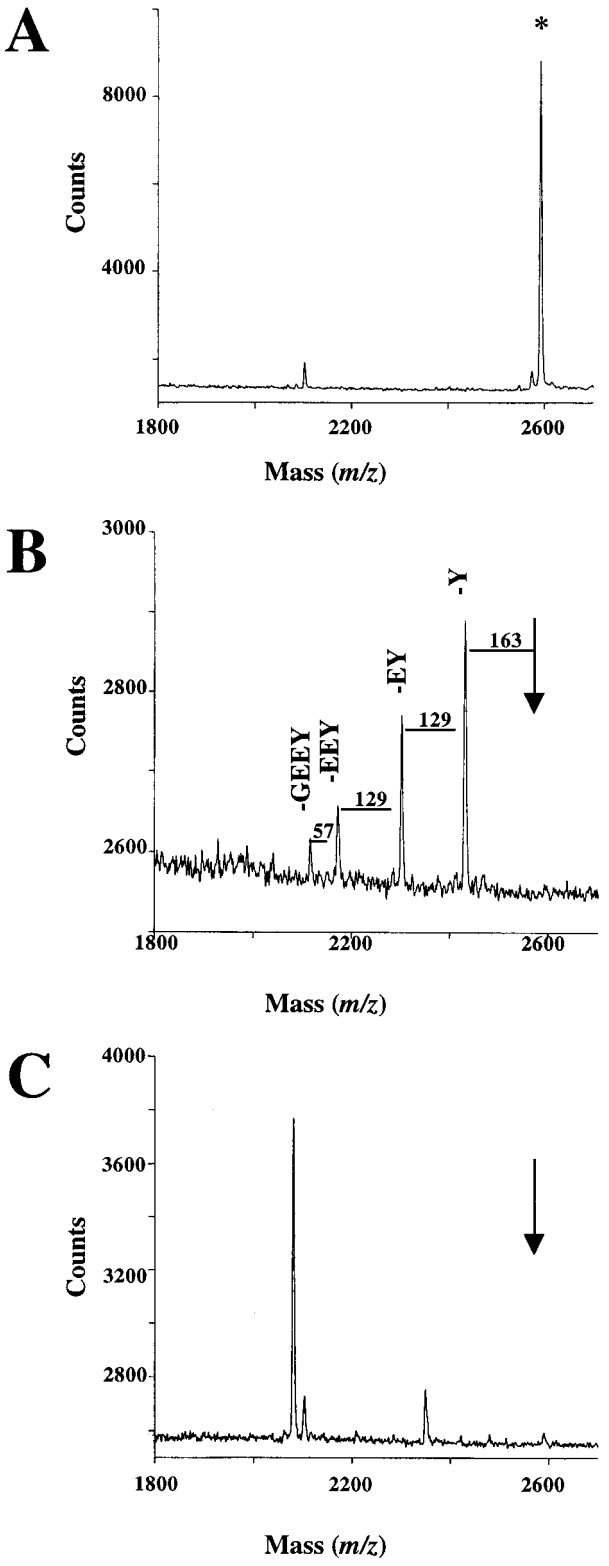

FIGURE 3: MALDI-MS of the purified unknown α-tubulin C-terminal CNBr peptide after carboxypetidase and trypsin treatments. (A) HPLC-purified peptide, (B) the peptide after carboxypeptidase Y treatment, and (C) the peptide after trypsin treatment (see Experimental Procedures for details). In panels B and C, the arrow indicates the position of the parent ion (asterisk), shown in panel A, prior to proteolytic digestion.

In this study, we have synthesized the C-terminal CNBr peptides of k-α1 and the newly described α-tubulin. MALDI-TOF mass analysis of the purified peptides revealed that both peptides desorbed with similar efficiencies (data not shown).

Table 3: Calculated Average Mass ([M − H]$^-$) of the Unmodified C-Terminal CNBr Peptides of the Uncharacterized Human α-Tubulins Present in the EST Databases

predicted C-terminal sequence of α-tubulin EST (accession number)	calculated average mass ([M − H]$^-$) of the CNBR fragment	calculated average mass ([M − H]$^-$) of the major tryptic fragment (underlined)
EST 1 (AALE**K**DYE**K**VGADSADGEDEGEEY) (14898815)	2589.6	1541.4
EST 2 (AALE**K**DYEEVGADSADG**K**DEGEEY) (14486883)	2589.6	1354.3
EST 3 (AALE**K**DYEEVGADSADGED**K**GEEY) (17540986)	2589.6	1598.5
EST 4 (AALE**K**DYEEVGADSADGEDEGEEY) (17539507)	2590.6	2077.9

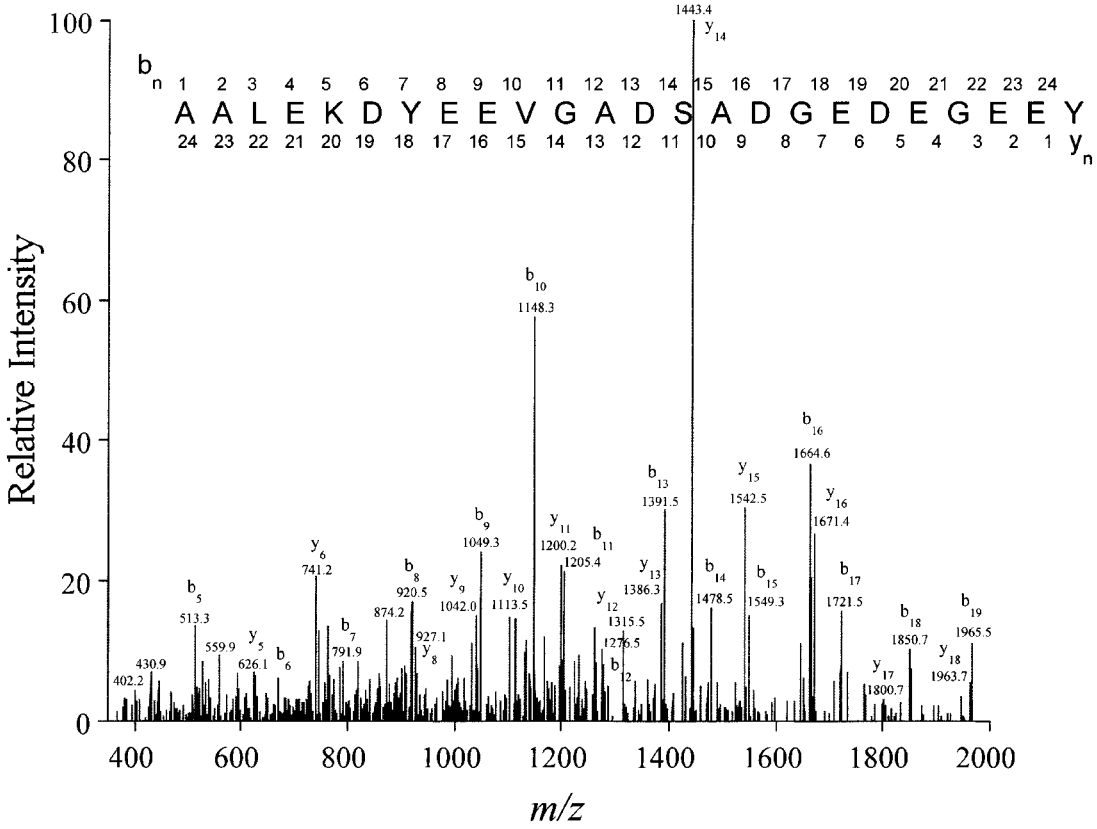

FIGURE 4: Tandem mass spectrometry of the purified unknown α-tubulin C-terminal CNBr peptide. The amino acid sequence of the peptide is displayed above the mass spectrum. Type b and y ion fragments are listed above and below the sequence, respectively.

This would suggest that the observed intensities of these two ions in the mass spectra shown in Figure 2 may be a reasonable estimate of the actual protein levels in these two human cell lines. In a separate series of experiments, paclitaxel-stabilized microtubules, rather than total cell extracts, were subjected to the CNBr digestion procedure and then analyzed by MALDI-TOF mass spectrometry. The mass spectra obtained from both the Taxol-stabilized microtubule preparation and total cell extracts were similar, suggesting that the newly identified α-tubulin isotype was functionally incorporated into microtubules (data not shown). The amino acid sequence of the C-terminal peptide of this human α-tubulin isotype shares similarities with the corresponding region of the mouse α-6 isotype, but is clearly distinct. Residues Glu_{440} and Asp_{442} of mouse α-6 tubulin are Asp and Glu, respectively, in the newly described human α-tubulin. Moreover, immunoblot analysis, using an antibody raised against the C-terminal 13 amino acids of mouse α-6 tubulin, revealed that this isotype was present at very low levels in several mouse tissues, including heart, kidney, spleen, lung, ovary, and kidney (*4*, *29*). Since the current human α-tubulin nomenclature is confusing, we have refrained from giving a name to this new isotype until the full sequence is known. Nevertheless, on the basis of its similarities with mouse α-6 tubulin, we believe that this new isotype will be placed in the class 1 family.

The "expected" common modification to α- and *β*-tubulin is polyglutamylation, which will produce a pattern of 129 Da increments, the mass of a glutamate residue, to each isotype. The sites of polyglutamylation of many of the α- and *β*-tubulin isotypes have been established from studies on mammalian brain tubulin (*9*−*11*, *13*). Modifications specific to α-tubulin include reversible tyrosination and removal of the penultimate glutamate (Δ-Glu) from the detyrosinated α-chains. Ser_{444} of *β*III-tubulin is a known site of phosphorylation in brain tubulin (*8*). However, it has been variously reported that other tubulins may be phosphorylated on serine and tyrosine residues. The latter phosphorylation sites have not been characterized. It is apparent that the C-terminal diversity of these breast and lung cell-derived tubulins are minimal compared to their brain counterparts. For example, the detyrosinated and Δ2 forms of α-tubulin were below the levels of detection in both cell lines. The ions corresponding to the addition of a single glutamate residue to α1 and *β*1 tubulins have been tentatively assigned, but are present at a low abundance (Figure 2). In contrast,

approximately 70% of the α-tubulin in rat brain is lacking the C-terminal tyrosine, and all of the mammalian neuronal isotypes, both α and *β*, are extensively polyglutamylated. Our mass spectrometry data showing limited polyglutamylation of tubulin in the human breast- and lung-derived cell lines are in general agreement with previous immunological-based approaches using monoclonal antibodies specific for polyglutamylated tubulin (*30*, *31*). In these studies, glutamylation was restricted mainly to tubulin associated with the centriole and was minimal on cytoplasmic microtubules. Significantly, the extent of tubulin glutamylation was shown to be cell cycle-dependent (*31*). The functional relevance of the various modifications to the C-terminal domain of α- and *β*-tubulins is unclear. However, since this region of α- and *β*-tubulins is believed to be an important determinant in the binding of several structural and motor MAPs, reversible modification within this domain could have significant effects on these interactions. For example, it is known that the ability of tau, MAP-2, and kinesin to interact with tubulin under in vitro conditions is regulated by the level of polyglutamylation (*32*, *33*).

There is evidence to suggest that the tubulin isotype profile of mammalian cells may influence the sensitivity to tubulin-binding drugs. For example, the dynamics of microtubules derived from purified α/*β*-tubulin heterodimers have been reported to be differentially suppressed by Taxol (*34*). Also, enhanced Taxol-induced assembly was observed in microtubules depleted of class III *β*-tubulin compared with their nondepleted counterpart (*35*). In addition, changes in *β*-tubulin isotype expression have been correlated with drug resistance in a number of human tumor cell lines (see ref *36*). The data described in this report represent the first mass spectrometric analysis of tubulin isotype composition in human cell lines. Of special significance was our ability to identify and characterize a previously unrecognized major α-tubulin isotype in the MDA-MB-231 breast carcinoma cell line and the A549 human non-small lung cancer cell line. Our studies also document that CNBr digestion of tubulin immobilized on nitrocellulose, followed by MALDI-MS analysis, is a powerful and direct method for producing a fingerprint of the tubulin isotype diversity in human cell lines. An important advantage of the procedure is that the need to purify tubulin by either chromatographic or assembly and/or dissassembly procedures is avoided. In addition, the small sample amounts required suggest that this procedure will be applicable to the characterization of human tubulins in normal and malignant tissue-derived samples.

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