Posttranslational Modification of Brain Tubulins from the Antarctic Fish *Notothenia coriiceps*: Reduced C-Terminal Glutamylation Correlates with Efficient Microtubule Assembly at Low Temperature†

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ABSTRACT: We have shown previously that the tubulins of Antarctic fish assemble into microtubules efficiently at low temperatures (−2 to +2 °C) due to adaptations intrinsic to the tubulin subunits. To determine whether changes in posttranslational glutamylation of the fish tubulins may contribute to cold adaptation of microtubule assembly, we have characterized C-terminal peptides from α- and β-tubulin chains from brains of adult specimens of the Antarctic rockcod *Notothenia coriiceps* by MALDI-TOF mass spectrometry and by Edman degradation amino acid sequencing. Of the four fish β-tubulin isotypes, nonglutamylated isoforms were more abundant than glutamylated isoforms. In addition, maximal glutamyl side-chain length was shorter than that observed for mammalian brain β tubulins. For the nine fish α-tubulin isotypes, nonglutamylated isoforms were also generally more abundant than glutamylated isoforms. When glutamylated, however, the maximal side-chain lengths of the fish α tubulins were generally longer than those of adult rat brain α chains. Thus, Antarctic fish adult brain tubulins are glutamylated differently than mammalian brain tubulins, resulting in a more heterogeneous population of α isoforms and a reduction in the number of β isoforms. By contrast, neonatal rat brain tubulin possesses low levels of glutamylation that are similar to that of the adult fish brain tubulins. We suggest that unique residue substitutions in the primary structures of Antarctic fish tubulin isotypes and quantitative changes in isoform glutamylation act synergistically to adapt microtubule assembly to low temperatures.

The ability of Antarctic fishes to form microtubules at temperatures as low as −1.8 °C, the freezing point of their seawater habitat, is both remarkable and intriguing. The critical concentration for polymerization of their brain tubulins (about 0.9 mg/mL) is comparable to that for pure brain tubulins from vertebrates with higher body temperatures (*2*−*4*), but unlike mammalian microtubules, those of Antarctic fishes are very stable and exhibit slow dynamics at cold and moderate temperatures (*1*, *5*, *6*). Both the conserved critical concentration of the Antarctic fish tubulins and the slow dynamics of their microtubule polymers are intrinsic to the tubulin subunits and, therefore, must be explicable by the structure imposed by their amino acid sequences, by their suite of posttranslational modifications, or by both. Previously, we identified seven novel changes in the primary structures of the α- and β-tubulin isotypes[1] of brain tubulins from the Antarctic rockcod *Notothenia coriiceps* and from the Antarctic icefish *Chionodraco rastrospinosus* that alone, or acting synergistically, may predispose the microtubules of these organisms to be stable and nondynamic (*1*). Some of these sequence variations are under investigation by site-directed mutagenesis (*7*). Here we examine the potential contribution of posttranslational modifications, in particular glutamylation of the C-termini of the tubulin chains, to the cold-assembling phenotype of the microtubules of Antarctic fishes.

Our prior characterization of brain tubulin from *N. coriiceps* suggested that structural changes (*8*) located within the C-termini of tubulin isotypes (*9*) might contribute to the efficiency of microtubule assembly at low temperature. For example, brain tubulins from Antarctic fishes and from the cow are highly heterogeneous in charge, with the fish α and β isoforms somewhat more basic (*10*). Furthermore, native tubulin dimers from *N. coriiceps* possess 1−2 fewer net negative charges than do bovine tubulins (*10*). Because the C-termini of tubulin polypeptides of both fish and mammals

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[1] Isotype refers to α- and β-tubulin polypeptides encoded by distinct genes, whereas isoform encompasses variants that differ in amino acid sequence (i.e., genetically), in posttranslational modification, or in both.

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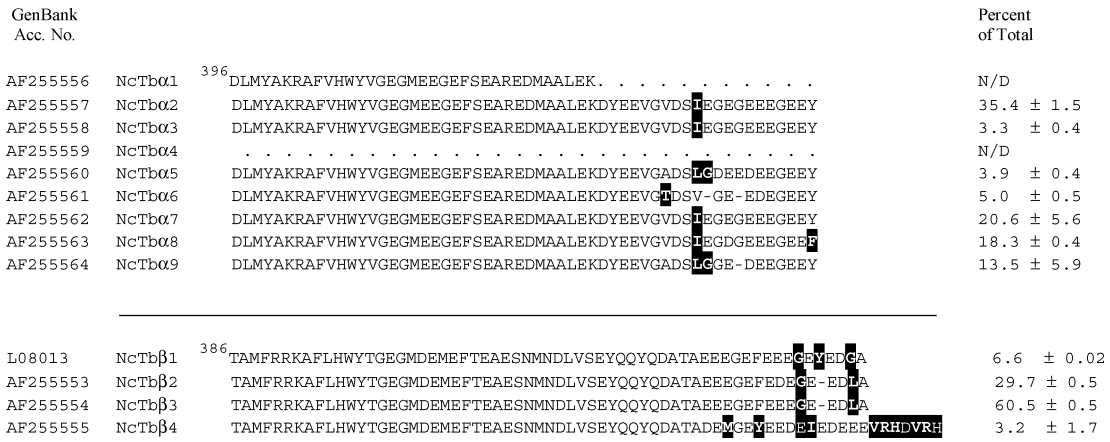

FIGURE 1: cDNA-derived C-terminal sequences of brain tubulin isotypes from *N. coriiceps* and relative abundances of the corresponding mRNAs. Isotype sequences are from Detrich et al. (*1*), and GenBank accession numbers are indicated. Abundance is given as mean percent ± SEM ($n = 2$). N/D, not determined. Residues highlighted in reverse text are those that differ from the vertebrate consensus sequences reported by Detrich et al. (*1*) for the α- and *β*-tubulin C-termini.

are rich in negative charge, we have hypothesized that cold adaptation of microtubule assembly may result, in part, through reduction of the acidic residue content of these regions, thus decreasing electrostatic repulsion between tubulin dimers as they interact (*8*). The C-terminal negative charge is contributed both by main-chain acidic residues and by posttranslational glutamylation, an unusual covalent modification that involves the formation of a chain of glutamate residues attached to the side chain of a glutamate in the polypeptide backbone (*11*−*13*). The glutamyl chains, which can vary in length from one to 10 or more residues (*14*) and are usually linked by α-carboxyl bonds (*15*), are attached to the main chain via an obligatory *γ*-carboxyl linkage.

The objective of this study was to determine the potential contribution of posttranslational glutamylation of Antarctic fish tubulins to cold adaptation of microtubule assembly. In particular, we compared the isotype and isoform composition of the fish brain tubulins to those of warm-polymerizing tubulins from neonatal and adult rat brain. To assist characterization of C-terminal peptides from brain tubulin chains of *N. coriiceps*, we draw upon the published C-terminal sequences of nine α tubulins and five *β* tubulins from *N. coriiceps* and *C. rastrospinosus* (*1*) as well as peptide sequences determined herein. Together, these sequences constitute a nearly complete description of the amino acid sequences of the tubulin C-termini of Antarctic fishes. The posttranslational modifications of these isotypes have been identified by purification of tubulin C-terminal peptides (*14*) and subsequent matrix-assisted-laser-desorption/ionization time-of-flight (MALDI-TOF)[2] mass spectrometry and/or Edman degradation. We present a comprehensive map of tubulin glutamylation from *N. coriiceps* brain, which indicates that the C-termini of most α and *β* isotypes are poorly glutamylated. Given the low glutamylation of neonatal rat brain tubulin relative to the high levels observed in the adult, we propose that the poorly glutamylated phenotype of Antarctic fish tubulins is an example of pedomorphism (retention of a larval or immature character in the adult). Furthermore, we hypothesize that the unique amino acid residues found in Antarctic fish tubulins (*1*) and the low levels of posttranslational glutamylation act synergistically to adapt the tubulin dimer for efficient assembly at cold temperatures. A preliminary report of some of this work has appeared (*16*).

EXPERIMENTAL PROCEDURES

Collection of Animals and Storage of Tissues. Specimens of the Antarctic yellowbelly rockcod, *N. coriiceps*, were collected by bottom trawling from the *R/V Polar Duke* near Low and Brabant Islands (Antarctic Specially Protected Areas 152 and 153, respectively) in the Palmer Archipelago. They were transported alive to Palmer Station, Antarctica, where they were maintained in seawater aquaria at −1.5 to +1 °C. Brain tissues were dissected and used immediately for preparation of microtubules or RNA. Some brains were frozen in liquid nitrogen and maintained at −70 °C for later isolation of RNA.

N. coriiceps Tubulin cDNAs. The isolation of nine cDNAs encoding distinct α-tubulin isotypes (NcTbα1−NcTbα9) and four encoding *β*-tubulin isotypes (NcTb*β*1−NcTb*β*4) from two *N. coriiceps* brain cDNA libraries has been reported previously (*1*, *9*, *17*). GenBank accession numbers are given in Figure 1.

Northern Analysis of α- and β-Tubulin Gene Expression. Slot blots of total brain RNA [5 *μ*g/slot; $n = 2$ preparations; 2 blots per preparation)] from *N. coriiceps* were hybridized to polymerase chain reaction generated, ^{32}P-labeled probes ([α-^{32}P]-5′-deoxycytidine triphosphate, 3000 Ci/mmol) specific for the 3′-UTRs of seven *N. coriiceps* α-tubulin cDNAs (NcTbα2, NcTbα3, and NcTbα5−NcTbα9) and of the four *N. coriiceps* *β*-tubulin cDNAs (NcTb*β*1−NcTb*β*4) (see ref *1* for primer pairs and other details). After exposure of the blots to Fuji RX X-ray film (−70 °C with intensification), the resulting autoradiograms were analyzed by densitometry using the program ImageJ 1.2 (available at http://

[2] Abbreviations: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis-(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]; HPLC, high-performance liquid chromatography; MAP, microtubule-associated protein; MES, 2-(*N*-morpholino)ethanesulfonic acid; *m*/*z*, mass-to-charge ratio; PIPES, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid); PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; UTR, untranslated region.

rsb.info.nih.gov/ij) to determine the relative abundance of the mRNA for each tubulin isotype.

Purification of Tubulins. Tubulin from fresh brain tissues of *N. coriiceps* was purified by DEAE ion-exchange chromatography and one cycle of microtubule assembly/disassembly as described by Detrich et al. (*3*, *10*). Neonatal and adult rat brain tubulins were purified from once-cycled microtubule preparations (*14*) by arginine-Sepharose chromatography (*18*).

Proteolytic Digestion of Tubulins. Purified, native tubulins (0.5−1.5 mg) were digested for 18 h at 37 °C by incubation with endoproteinase Lys-C (Roche Diagnostics GmbH, Mannheim, Germany) in digestion buffer [25 mM Tris-HCl (pH 8.5), 1 mM EDTA] at an enzyme-to-tubulin ratio of 1:100 (w/w). Digestion was terminated by heating the sample to 100 °C for 5 min. Prior to chromatography, the digest was sonicated briefly, and possible aggregates were removed from the solution by centrifugation at 12000*g* for 2 min.

Purification of the C-Terminal Peptides of Tubulin. Digestion of tubulin $\alpha\beta$ dimers by endoproteinase Lys-C yields C-terminal peptides of α tubulin that begin at Asp^{431} and β-chain peptides that start at Ala^{393}. Given these cleavage sites, the C-terminal peptides of α tubulin were expected to be small (masses between 2000 and 4000), whereas β tubulin should yield larger peptides (masses between 6000 and 8000). For mass spectrometric analysis of β peptides, the peptide digest was analyzed directly (see MALDI-TOF Mass Spectrometry). To analyze the complex mixture of α-tubulin peptides, additional purification was required. Typically, 400 μg of digested tubulin was loaded on an arginine-Sepharose column (1 mL) and the α-tubulin C-terminal peptides were isolated as described by Redeker et al. (*14*). Desalting of the peptide mixture was performed on a C18 Sep-Pak cartridge by washing the applied sample with solvent A (0.1% TFA) and eluting the peptides with solvent B (0.1% TFA in 80% acetonitrile). Separation of α peptides was accomplished by reversed-phase HPLC of the desalted peptide pool on a Vydac C18 column (5 μm particle size, 220 × 2.1 mm). C-Terminal α peptides were eluted by sequential application of 1% solvent B for 10 min, 1% to 15% solvent B for 1 min, and 15% to 40% solvent B for 100 min. The flow rate was 200 μL/min, and peptide elution was monitored at 215 nm.

Protein Microsequence Analysis. Selected C-terminal peptides were sequenced by automated Edman degradation using a Procise pulsed-liquid protein sequencer (model 494, Perkin Elmer Applied Biosystems Division, Foster City, CA).

Protein Determinations. Protein concentrations were estimated by the microprotein assay of Bradford (*19*) with bovine serum albumin as the standard.

MALDI-TOF Mass Spectrometry. Mass spectra were recorded in linear mode (except when indicated) on a MALDI-TOF mass spectrometer (Voyager Elite, PerSeptive Biosystems, Inc., Framingham, MA) equipped with a delayed extraction device (delay time set at 175 ns). Desorption was produced by a nitrogen laser beam (λ = 337 nm, 3 ns pulse at 20 Hz) focused on the target; laser power was set just above the desorption threshold. For each acquired spectrum, 100 to 256 shots were averaged. β-Tubulin peptides were analyzed in positive mode, whereas α-tubulin peptides were analyzed in negative mode. In positive mode, external calibration was performed with a mixture of insulin and apomyoglobin, whose mono- and biprotonated ions gave average *m*/*z* values of 5838.2 and 2867.75 (insulin) and 16952.5 and 8476.75 (apomyoglobin), respectively. In negative mode, external calibration was performed with a mixture of neurotensin, ACTH clip (18−39), and ACTH clip (7−38) with average *m*/*z* ratios corresponding to deprotonated ions of 1671.95, 2464.71, and 3658.17, respectively. In negative and reflectron modes, monoisotopic *m*/*z* ratios were used for calibration.

The samples were generally mixed 1:1 (v:v) with a saturated solution of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, Aldrich) in 30% acetonitrile and 0.1% aqueous TFA as matrix. β-Tubulin peptides were analyzed directly from the peptide mixture obtained after protein digestion. The sample in 25 mM Tris-HCl buffer at pH 8.5 was first mixed with an equal volume of 1% TFA followed by the addition of the matrix. The complete spectrum of α-tubulin peptides in the peptide mixture was analyzed after desalting by addition of one volume (0.5 to 1 μL) of sample to one volume of matrix. Individual α-tubulin peptides obtained by HPLC were examined in reflectron mode after addition of one volume of a saturated solution of 2,5-dihydroxybenzoic acid (Aldrich) in 0.1% aqueous TFA as matrix.

Peptide identity and level of glutamylation were determined by comparison of experimental masses to the theoretical masses of candidate peptides. Mass errors, which fell in the range 0.01−0.1%, were within the error range of the instrument in linear mode.

RESULTS

Tubulin Isotype Abundance. To appreciate the role of glutamylation, one must determine both the quantity of each tubulin isotype in the tubulin pool and the location(s) and length spectrum(a) of its polyglutamyl chain(s). Detrich et al. (*1*) reported that the Antarctic rockcod *N. coriiceps* expresses at least 9 α- and 4 β-tubulin genes in its brain tissues. Using probes specific to the 3′-UTRs of 7 α-tubulin genes and 4 β, we estimated the abundances of their transcripts on Northern slot blots containing total brain RNA. Figure 1 shows the C-terminal sequences and relative percentages of these isotypes. (Two α isotypes, α1 and α4, could not be examined because we lack 3′-UTRs for their cDNAs.) On the basis of the assumption that steady-state transcript levels are an approximate proxy for the relative quantities of the corresponding proteins in the tubulin pool, our results indicated that the NcTbα2, -α7, -α8, and -α9 isotypes were the predominate α-tubulin chains (total = 88%) and that NcTbβ2 and -β3 constituted approximately 90% of the β chains. As demonstrated previously at the protein level (*8*), expression of the NcTbβ4 chain, which corresponds to the vertebrate βIII isotype (*1*), was quite limited.

Comparative Proteomics of Tubulin Glutamylation. Preliminary immunoblot analyses of adult brain tubulins from *N. coriiceps* and from mammals (cow, rat) using a monoclonal antibody [GLU-1 (*20*)] that reacts with tubulin polyglutamyl side chains suggested that the C-termini of the former were depauperate for this posttranslational modification (A. Frankfurter and H. W. Detrich, III, unpublished results). To test this possibility, we employed MALDI-TOF mass spectrometry to characterize the polyglutamyl chains

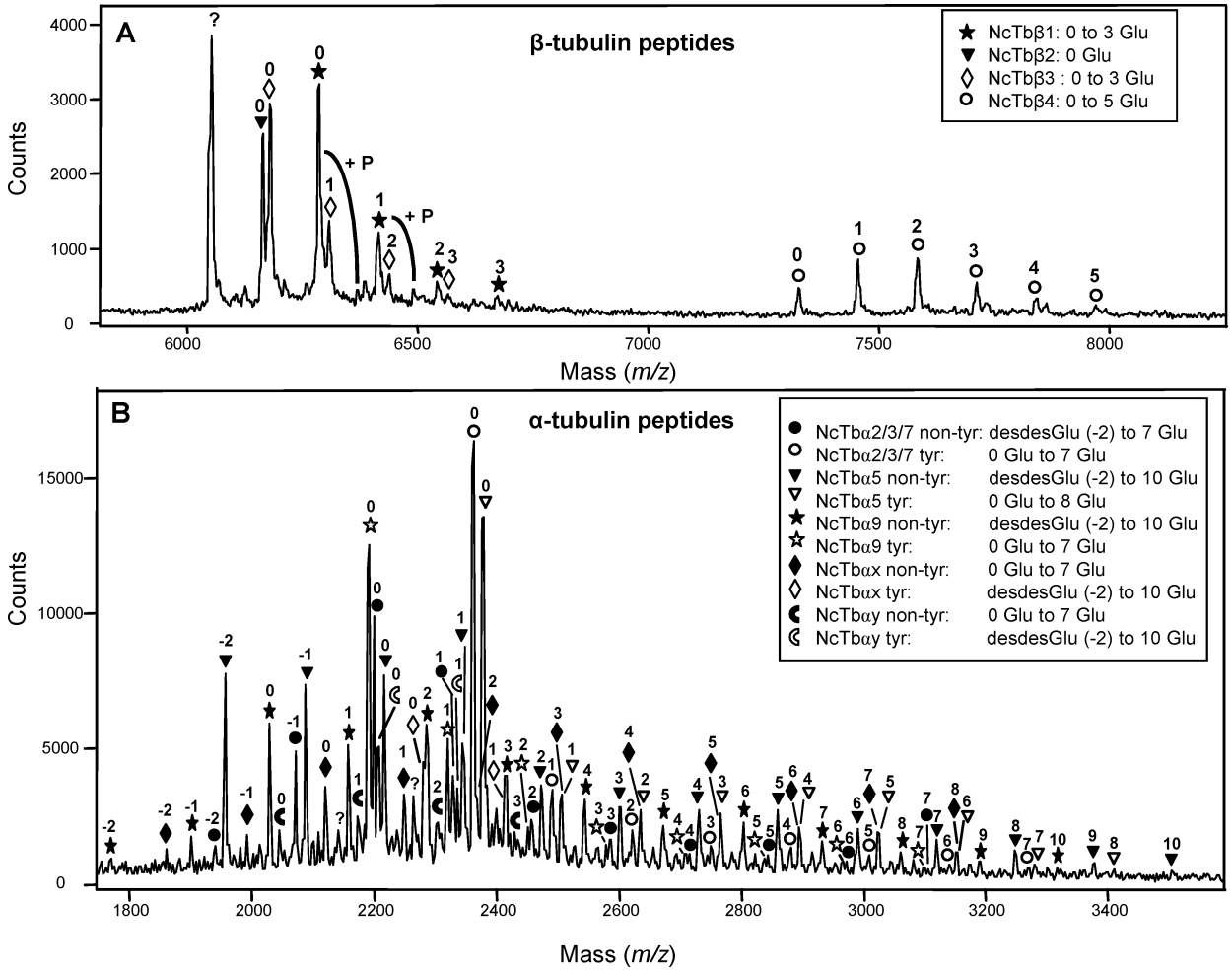

FIGURE 2: Mass spectra of the C-terminal peptides of α and *β* tubulins from adult brain tissues of *N. coriiceps*. Peptide digests were prepared using endoproteinase Lys-C and spectra were recorded in linear mode as described under Experimental Procedures. (A) *β*- and (B) α-tubulin peptides from *N. coriiceps*. The C-terminal peptides of *β* tubulin were analyzed directly after digestion in positive mode. The C-terminal peptides of α tubulin were analyzed in negative mode after purification by chromatography on arginine-Sepharose. Three nonidentified peaks (?), one in the *β* series and two in the α series, may represent internal peptides of the α- or *β*-tubulin chains.

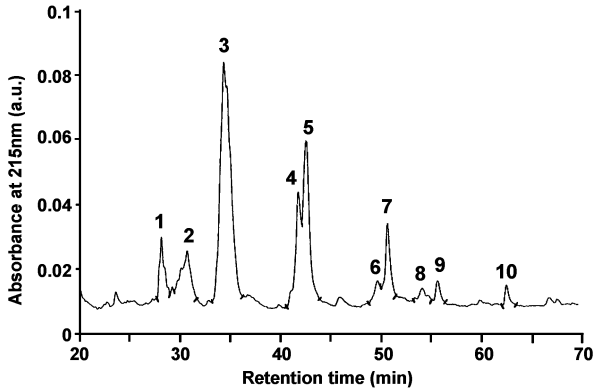

FIGURE 3: Reversed-phase HPLC separation of arginine-Sepharose-purified α-tubulin peptides from *N. coriiceps*. Fractions 1−10 were chosen for sequence analysis and MALDI-TOF mass spectrometry.

present on C-terminal peptides obtained from adult brain tubulins of *N. coriiceps* and the rat by endoproteinase Lys-C digestion. Figure 2 shows the mass spectra of the *β* (A) and α (B) C-termini of *N. coriiceps* tubulins. (Comparable data for adult rat are presented in Figure 5A,B.) Given the relative simplicity of the *β* spectra, we describe these first.

Figure 2A shows that *N. coriiceps* tubulin gave four clearly resolved series of protonated ions that derived from the C-termini of the NcTb*β*1−NcTb*β*4 chains (closed stars, closed triangles, open diamonds, open circles). Each series contained one ion with an experimental mass corresponding to the calculated mass of the unmodified peptide and additional ions whose masses increased by 129 Da, the mass of a single glutamate residue. The predominant molecular ion found in the first three series, which corresponded to NcTb*β*2, -*β*3, and -*β*1, was the unmodified form (experimental average m/z = 6162.1, 6175.0, and 6284.8, respectively). Small quantities of ions that contained 1 to 3 additional glutamates completed the *β*1 and *β*3 series. Two minor peaks (curved lines, +P) corresponding to mass increments of 80, the mass of one phosphate group, represented phosphorylated variants of the unmodified and monoglutamylated NcTb*β*1 peptides. The fourth series (open circles), corresponding to the longer C-terminal peptide of the least abundant isotype, NcTb*β*4, began with a small quantity of the unmodified peptide (average m/z = 7332.0), continued with larger amounts of the mono-, di-, and triglutamylated forms, and ended with tetra- and pentamodified variants. Thus, our results demonstrate that three of the four *β*-tubulin isotypes of *N. coriiceps* adult brain (NcTb*β*1−NcTb*β*3, 97% of the *β* pool) contained low levels of glutamylation, the sole exception being the low-abundance *β*4 chain. The

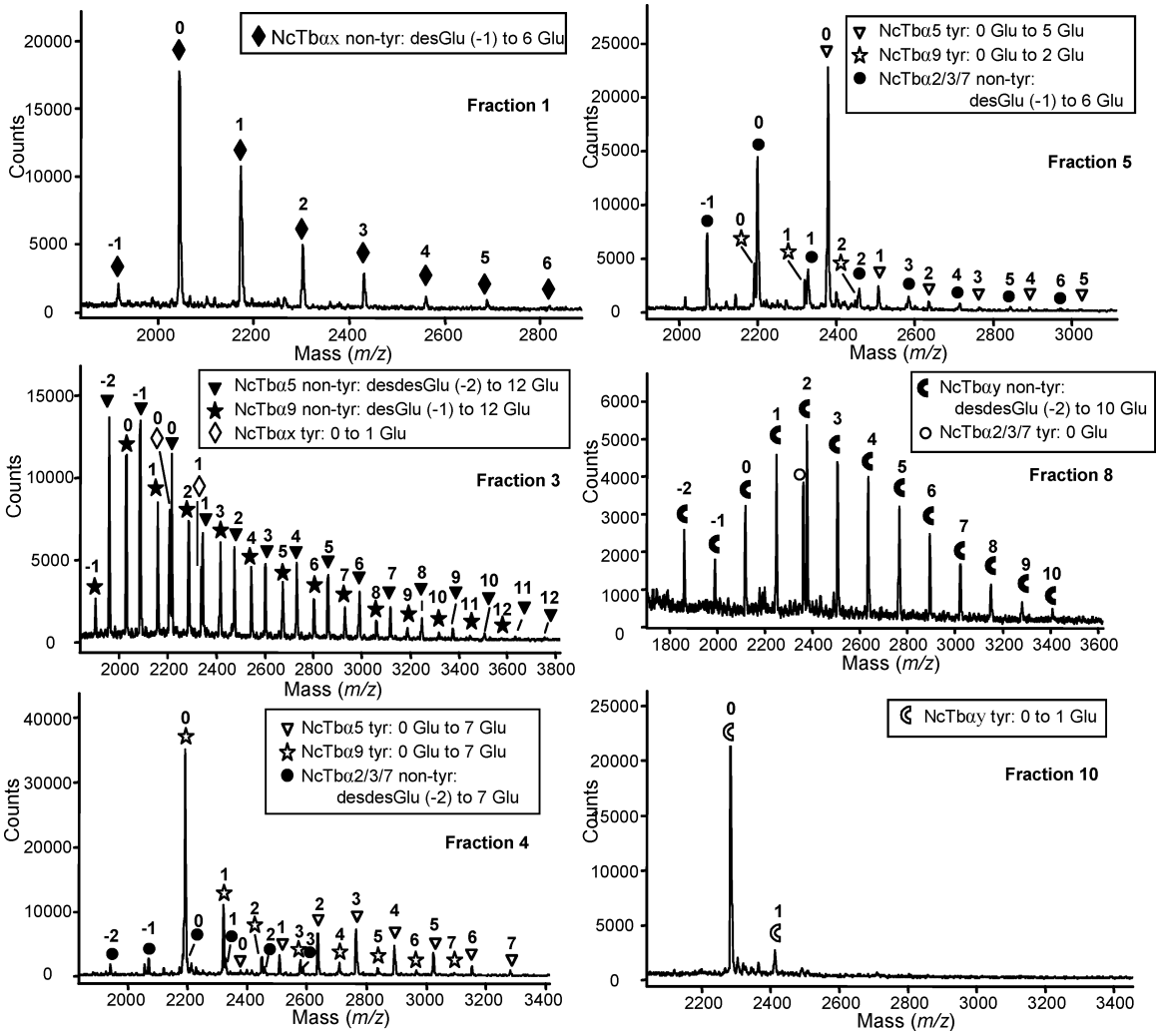

FIGURE 4: Mass spectra of selected fractions of HPLC-purified α-tubulin peptides from *N. coriiceps*. For details, see Experimental Procedures.

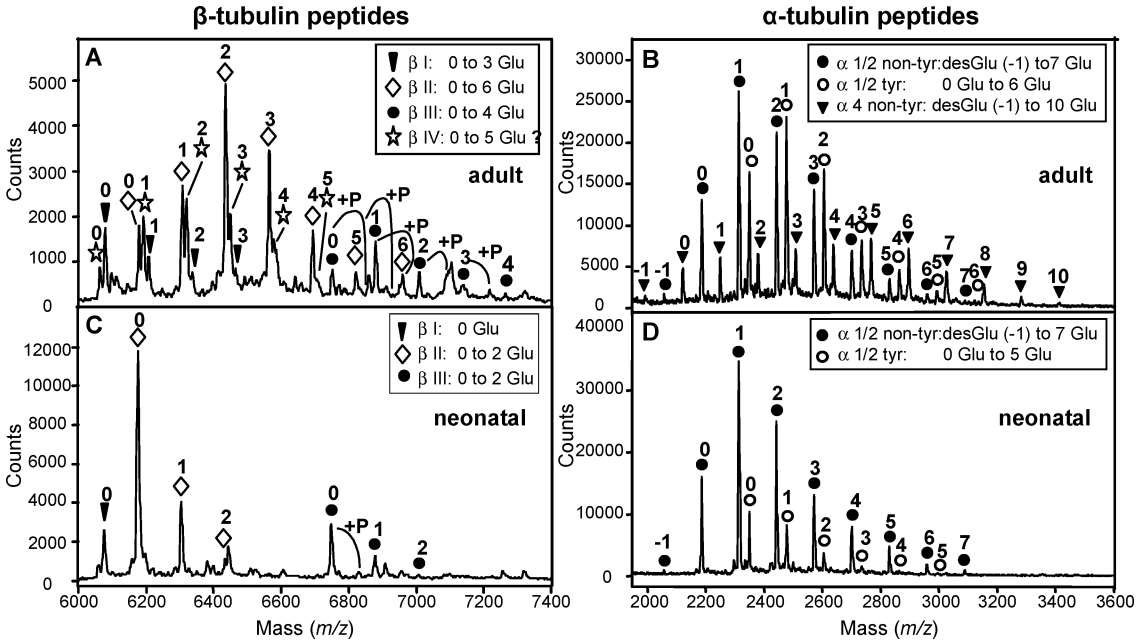

FIGURE 5: Mass spectra of the C-terminal peptides of α and *β* tubulins from adult and neonatal rat brain. Generation and purification of peptides and MALDI-TOF analytical modes are described under Experimental Procedures.

glutamylation pattern of the fish *β* chains contrasted sharply with that observed for adult rat brain *β* tubulin, whose four isotypes were all extensively glutamylated (Figure 5A).

The mass spectrum in negative and linear mode of the pool of purified α peptides from *N. coriiceps* showed a striking level of diversity (Figure 2B). Dissection of this

Table 1: Identification of α-Tubulin Peptides Purified by HPLC

fraction no.	sequence(s) identified (%)[a]	peptide	no. of additional Glu
1	DYEEVGTDSVGDEGEEGEE	αx non-Tyr	0 to 6 Glu (0 major)
2	minimum of 4 unidentified peptides	nd[b]	
3	DYEEVGADSLGDEEDEEGEE (60−80%)	α5 non-Tyr	−2 to 12 Glu (−2 to 0 major)
	DYEEVGADSLGGEDEEEGEE (16−36%)	α9 non-Tyr	−2 to 12 Glu (0 major)
	DYEEVGTDSVGDEGEEGEEY (5−9%)	αx Tyr	0 to 1 Glu
4−5	DYEEVGADSLGDEEDEEGEEY (20%)	α5 Tyr	0 to 7−8 Glu (0 major)
	DYEEVGADSLGGEDEEEGEEY (35%)	α9 Tyr	0 to 7 Glu (0 major)
	DYEEVGVDSIEGEGEEEGEE (45%)	α2/3/7 non-Tyr	−2 to 7 Glu (0 major)
6−7	DYEEVGVDSIEGEGEEEGEEY (80%)	α2/3/7 Tyr	0 to 6 Glu (0 major)
	4 unidentified peptides	nd	
8−9	DYEEVGIDSFEEDEEGEE	αy non-Tyr	−2 to 10 Glu (2 major)
	DYEEVGVDSIEGEGEEEGEEY (traces)	α2/3/7 Tyr	0 to 1 (0 major)
10	DYEEVGIDSFEEDEEDEEY	αy Tyr	0 to 1 Glu (0 major)

[a] % relative abundance estimated by amino acid sequence analysis. [b] Not determined.

complex spectrum revealed at least 10 series of deprotonated ions, with each series increasing in mass steps equivalent to a glutamate residue. Six of the series were identified tentatively as nontyrosinated or tyrosinated C-terminal peptides of α-tubulin isotypes 2/3/7 (identical C-terminal sequences: closed and open circles), 5 (closed and open triangles) and 9 (closed and open stars).

The first series of peptides (closed circles) contained a molecular ion (average $m/z = 2198.7$) that matched the C-terminus of the nontyrosinated, nonglutamylated α2/3/7 C-terminal peptide (calculated $m/z = 2199.1$). Two ions of lower masses ($m/z = 1940.7$ and 2069.9) in the series represented, respectively, (1) a nontyrosinated α2/3/7 peptide desdesglutamylated in its C-terminus (i.e., removal of two C-terminal Glu residues that are encoded by the respective tubulin gene); and (2) either a nontyrosinated desglutamylated peptide or a nontyrosinated desdesglutamylated peptide that was monoglutamylated on its lateral chain. The latter probably corresponded to a mixture of both peptide species (*15*), which cannot be distinguished by virtue of their identical *m*/*z* ratios. (This possibility must be considered for all desdesglutamylated and desglutamylated series observed.) The other seven molecular ions in this series were more extensively polyglutamylated forms (mono- to hepta-) of these nontyrosinated peptides.

The second series (open circles) began with a deprotonated molecular ion (average $m/z = 2362.0$) corresponding to the C-terminal peptide of the tyrosinated nonglutamylated α2/3/7 isotype (calculated $m/z = 2362.3$). The next ion in the series presented a mass ($m/z = 2491.0$) that was consistent with a tyrosinated monoglutamylated α2/3/7 C-terminal peptide. The other ions in this series corresponded to the addition of 2 to 7 glutamate residues.

A third series of peptides (closed triangles) began with a deprotonated molecular ion at a *m*/*z* of 1956.4, which matched a nontyrosinated desdesglutamylated peptide of the α5 tubulin isotype (calculated $m/z = 1956.4$). As previously noted for the α2/3/7 isotypes, the next ions of this series corresponded to more extensively polyglutamylated nontyrosinated peptides (up to 10 glutamates). The last ion of this series ($m/z = 3505.7$) was the nontyrosinated α5 peptide with 10 additional Glu residues.

A fourth series of peptides (open triangles) started with a deprotonated molecular ion at *m*/*z* of 2378.7 and ended with an ion at *m*/*z* of 3411.0. The first ion of this series probably corresponded to the tyrosinated nonglutamylated C-terminal peptide of the α5 tubulin isotype ($m/z = 2378.3$), and the last to the tyrosinated peptide bearing eight glutamate residues ($m/z = 3411.2$).

The fifth series of ions (closed stars) was assigned as the nontyrosinated form of the α9 isotype. It began with a peptide of mass 1769.3, which matched a nontyrosinated desdesglutamylated α9 peptide ($m/z = 1769.7$) and ended with a peptide of mass 3189.3, which corresponded to a nontyrosinated α9 peptide with 10 additional glutamate residues.

The sixth series of ions (open stars) began with a deprotonated ion of molecular mass 2190.8 and ended with an ion of mass 2965.7. These ions matched the tyrosinated, nonglutamylated α9 peptide ($m/z = 2191.1$) and the peptide bearing up to seven additional glutamate residues.

Despite the high complexity of the α-tubulin C-terminal peptides, five of the seven isotypes whose C-termini are known (Figure 1) were provisionally identified: α2/3/7, α5, and α9. These peptides represent 59.3%, 3.9%, and 13.5%, respectively, of the α-tubulin isotypes identified at the genetic level (Figure 1). In these spectra, the most abundant species was usually the nonglutamylated peptide. Furthermore, two low-abundance isotypes not previously identified in *N. coriiceps*, NcTbαx (open and closed diamonds) and -αy (open C, closed C), were also recognized. These will be described more completely in the next section.

High-Resolution Analysis of Purified α-Tubulin C-Terminal Peptides. To verify the identity of the six α-tubulin peptide series that were tentatively identified in the complex pool (Figure 2B) and to characterize the unidentified series, we separated the mixture of arginine-Sepharose-purified α-tubulin C-terminal peptides by reverse-phase HPLC. The 10 major fractions obtained by C18 reverse-phase HPLC (Figure 3) were analyzed separately by MALDI-TOF mass spectrometry (2,5-dihydroxybenzoic acid as matrix, reflectron mode for greater mass accuracy) and by Edman degradation for peptide sequence analysis. Figure 4 shows the spectra of six fractions, and Table 1 presents the sequences of the peptides identified in each of the 10 fractions. Several points can be made about these results. First, several of the peaks contained multiple peptides (e.g., fractions 3, 4, and 5); we conjecture that their coelution during HPLC resulted from comparable peptide polarities. Second, the patterns of glutamyl modification of many of the purified peptides matched closely those deduced for the peptides of the α-peptide pool. For example, the nontyrosinated α5 and α9

peptides (fraction 3; closed triangles and closed stars) ranged from the des or desdes variants (−1 or −2 Glu) to +12 Glu, in good agreement with the −2 to +10 peptides identified in the pool, whereas their tyrosinated variants (fraction 4; open triangles and open stars) contained 0 to 7 Glu residues, almost exactly as shown in Figure 2B. Third, nonglutamylated peptides were generally the predominant forms in each series, although the α5 peptide mixture contained even larger quantities of the des and desdes forms (Figure 2B, Figure 4, fraction 3). Finally, the location of the posttranslationally modified residue(s) could not be identified by Edman degradation because the predominant peptide in each fraction was generally nonglutamylated.

The unknown *N. coriiceps* α-tubulin C-terminal peptides, αx and αy, were recovered, in most cases in discrete fractions, via HPLC purification (spectra in Figure 4, fractions 1, 3, 8, and 10). The amino acid sequence of the αy peptide (Table 1, fractions 8 and 10) corresponded exactly to the same C-terminal segment of the CrTbα10 isotype[3] found in the icefish *C. rastrospinosus* (*1*), which suggested that *N. coriiceps* might also possess the α10 isotype as a tyrosinated isoform with essentially no glutamylation (Figure 4, fraction 10) and as nontryosinated, desdes to multiglutamylated (−2 to +10) peptides (Figure 4, fraction 8). The sequence of the αx peptide (Table 1, fraction 1) was similar to the nontyrosinated C-terminus of the α11 isotype of *C. rastrospinosus* but contained a serine at position 439 in place of the threonine residue of the icefish. Nontyrosinated αx (Figure 4, fraction 1) was present in desglutamylated form through +6 Glu modified forms, whereas tyrosinated αx (Figure 4, fraction 3) was minimally modified (0−1Glu). If the assignments of the αx and αy C-termini to CrTbα11 and -α10 are correct, then our HPLC purification and Edman sequence analysis would harmonize the α-tubulin pools of both *N. coriiceps* and *C. rastrospinosus*, and the mass spectrometric analysis of the peptide subsets (Figures 2 and 4, Table 1) probably establishes the patterns of glutamylation for almost all of the α-isotypic variants. If, alternatively, the αx and αy C-termini belong to NcTbα1 and -α4, then it is possible that the two fish species share overlapping, but not identical, populations of brain α isotypes. Table 1 also contains a number of unidentified C-terminal sequences that are candidates to complete the α1 and α4 isotypes. Given the close evolutionary relationships of *N. coriiceps* and *C. rastrospinosus*, we consider it plausible that both species produce 9 to 11 or more orthologous brain α-tubulin isotypes.

Glutamylation of Adult and Neonatal Rat Brain Tubulins. The paucity of glutamylation of brain tubulins from the Antarctic fishes is reminiscent of the low levels of glutamyl modification observed for tubulins from neonatal rat brain (*15*). Figure 5 shows that the heterogeneity of tubulin isotype expression and of posttranslational glutamylation both increased during development. Neonatal rat brain expressed the α1/2 and βI, βII, and βIII tubulin chains, whereas the α4 and βIV isotypes were expressed later in the adult. Furthermore, the extent of glutamylation of the β isotypes increased strikingly with development, from a maximum of two for βII and βIII in the neonate to three for βI, six for βII, four for βIII, and five for βIV in the adult. The most abundant neonatal β isoforms were nonglutamylated, whereas mono-, bi-, and triglutamylated forms predominated in the adult β chains. Thus, the spectrum of neonatal (but not adult) rat β isotype modification resembled that of the adult fish β chains (Figure 2). Phosphorylation of the βIII isotype also increased with developmental stage. By contrast, the extent of glutamlylation of the α isotypes increased modestly during development in the rat (from a significant basal level in neonatal α1/2), and no shifts in the relative abundances of the modified α peptides were evident. Adult Antarctic fish brain expressed many more α isotypes than either developmental stage of the rat, and maximum glutamyl chain lengths were greater, but, with rare exceptions, nonglutamlyated C-terminal peptides predominated in the fish. We conclude that glutamyl modification of Antarctic fish brain α and β chains (i.e., predominant glutamylated isoforms) is less extensive than in neonatal and adult rat brain, dramatically so in the case of the fish β-tubulin isoforms.

DISCUSSION

Toward a Comprehensive Hypothesis for Thermal Adaptation of Microtubule Assembly by Antarctic Fishes. Almost twenty years ago, Williams et al. (*2*) determined that the critical concentration for assembly of MAP-free brain tubulins from Antarctic fishes and from mammals was conserved, despite a difference of nearly 40 Centigrade degrees in their body temperatures. Having determined the suite of residue substitutions that distinguish the primary structures of the tubulins of Antarctic fishes and other vertebrates (*1*) and characterized the major differences in posttranslational modification of fish and rat brain tubulins (this work), we are now able to advance a comprehensive hypothesis to explain the adaptation of vertebrate tubulins to psychrophilic conditions. *We propose that the differences in primary structure and posttranslational modification exhibited by the Antarctic fish tubulins combine, either additively or perhaps cooperatively, to conserve the critical concentration at a vertebrate norm while maintaining microtubule dynamics at a level compatible with normal cellular function at low temperature.* Below, we discuss the genetic and posttranslational contributions in turn.

The principal regions of the α and β tubulins of Antarctic fishes that contain novel residue substitutions are their lateral, interprotofilament contact surfaces and their hydrophobic cores (*1*). We have proposed that four substitutions that map to the "hinge points" of the "M" (microtubule) loops of some of the α and β isotypes may, by increasing loop flexibility, permit stronger interprotofilament contacts to form. (For details, see ref *1*.) Such polymer-stabilizing substitutions need not be present in the M loops of each isotype, since a small mole fraction of a cold-assembling tubulin can confer significantly greater thermal stability on microtubules containing a majority of cold-sensitive tubulin (*21*). The function(s) of these M-loop substitutions should be amenable to dissection by site-directed mutagenesis (cf. ref *7*).

Paluh et al. (*7*) recently clarified the role of the conserved β-isotype residue, Phe^{200}, of Antarctic fishes. Also found in the β tubulins of fungi, which are able to form microtubules at temperatures as low as 4 °C, Phe^{200} is located at the

[3] The CrTbα10 and -α11 isotypes, which contain sequence substitutions elsewhere in their protein chains that differentiate them from NcTbα1−α9, have not yet been found in *N. coriiceps* (*1*).

Table 2: Summary of the Glutamylation of α and β Tubulins from Antarctic Fish Brain[a]

isotype	number of Glu	most abundant
	α-Tubulins	
NcTbα2/3/7 non-Tyr	desdes-Glu to 7 Glu	0 Glu
NcTbα2/3/7 Tyr	0 to 6 Glu	0 Glu
NcTbα5 non-Tyr	desdes-Glu to 12 Glu	$-2 \approx -1 \approx 0$ Glu
NcTbα5 Tyr	0 to 8 Glu	0 Glu
NcTbα9 non-Tyr	0 to12 Glu	0 > 1 > 2 Glu
NcTbα9 Tyr	0 to 7 Glu	0 Glu
NcTbαx non-Tyr	0 to 5 Glu	$-1 < 0 > 1 > 2$ Glu
NcTbαx Tyr	0 to 1 Glu	0 Glu
NcTbαy non-Tyr	desdes-Glu to 10 Glu	$0 < 1 < 2 > 3 > 4$ Glu
NcTbαy Tyr	0 to 1 Glu	0 Glu
	β-Tubulins	
NcTbβ1	0 to 3 Glu[b]	0 Glu
NcTbβ2	0 Glu	0 Glu
NcTbβ3	0 to 3 Glu	0 Glu
NcTbβ4	0 to 5 Glu	1, 2 Glu

[a] Composite interpretation of data from Figures 2 and 4. [b] One phosphate residue added to the nonglutamylated and monoglutamylated forms.

internal interface between the nucleotide-binding and intermediate domains of the β chain. Essentially all mesophilic animals have a tyrosine residue at this position. Motion between these two domains is thought to be necessary for the conversion of the "straight" dimers of a growing microtubule end to the "curved" dimers of the shortening state (*7*, *22*, *23*). Detrich et al. (*1*) proposed that the Phe^{200} substitution would resist the conformational change normally induced by nucleotide hydrolysis and phosphate release, thus stabilizing the linear, GTP-like structure that forms strong lateral interactions. When Phe^{200} was mutated to a tyrosine in the β tubulin of *Schizosaccharomyces pombe*, the temperature stability of the yeast microtubules was not changed, whereas the depolymerization rate decreased strikingly such that several microtubule-dependent functions (exit from mitosis, normal completion of meiosis II) were compromised. Because Tyr^{200} of most animal tubulins probably forms a hydrogen bond with Asn^{165}, it is now clear that the Phe^{200} of Antarctic fishes would eliminate this bond and probably facilitate disassembly (*7*). In essence, Phe^{200} provides the "grease" that enables the microtubules of Antarctic fishes and fungi to maintain dynamic instability at low temperature.

The inhibitory role of the acidic C-terminal domains of mammalian tubulins on microtubule assembly has been well documented. Removal of these regions by limited proteolysis has been shown to reduce the isothermal critical concentration for tubulin polymerization by 10−50-fold (*24*−*26*), probably by reducing electrostatic repulsion between dimers. We (*4*, *10*, *27*, *28*) have hypothesized that the cold-assembling phenotype of Antarctic fish tubulins might have evolved, in part, via mechanisms that reduce the acidity of the C-terminal tails of the α- and β-tubulin chains. Although the amino acid sequences of the C-termini of Antarctic fish tubulins, deduced from tubulin cDNAs, show no evidence of reduced acidic residue content (*1*, *9*), the data presented here demonstrate that the fish isotypes are, in general, poorly glutamylated, the major exception being the rare β4 (βIII) chain (Table 2). Maximal polyglutamyl chain length can, however, be quite long, as shown for several α isotypes. Further support for the posttranslational hypothesis comes from comparative analysis of the assembly and structure of egg and brain tubulins from *N. coriiceps*. Detrich et al. (*4*) found that unfertilized fish egg tubulin polymerized at a critical concentration that was 4-fold smaller than for adult fish brain tubulin at the near-physiological temperature of 3 °C, that the isoforms of the former were fewer in number and more basic, and that the former lacked several acidic, probably C-terminal, peptides that were prevalent in the fish brain α chains. Finally, Klotz et al. (*29*) have reported that brain tubulins of the cold-living Atlantic cod *Gadus morhua* contain smaller amounts of glutamylation than does bovine brain tubulin, with α-isotype glutamylation being moderately reduced and the β chains nearly devoid of the modification. We conclude that reduction of posttranslational glutamylation is an adaptive feature of the microtubule systems of cold-living fishes.

A second, potentially adaptive, feature of the α-chain C-termini is the frequent and substantial occurrence of nontyrosinated isoforms that have been further trimmed by one or two glutamates of the primary structure (cf. Figures 1, 2, and 4). By contrast, the β-tubulin C-termini, which like those of the α isoforms are exposed on the surface of the microtubule (*30*, *31*), are not shortened in a similar fashion. The presumed enzymatic mechanism of this α-specific trimming remains to be determined.

Why, then, do the neonatal brain tubulins of mammals not assemble at 0 °C? The most likely explanation is that they lack the isotypic residue changes possessed by the Antarctic fishes. Analysis of the temperature dependence of the critical concentrations of the brain tubulins of neonatal and adult rats, together with the extant data for Antarctic fishes (*2*−*4*), should enable the determination of the relative energetic contributions of the genetic and posttranslational components of thermal adaptation of microtubule assembly.

Tubulin Glutamylation in Antarctic Fishes: A Molecular Pedomorphism? Pedomorphosis is the retention by adults of a taxon of traits that characterize the juveniles of outgroups [for a discussion of pedomorphism in Antarctic fishes, see Eastman (*32*)]. We propose that the reduced polyglutamylation of Antarctic fish tubulins may constitute an adaptive, pedomorphic character at the molecular level. The observation that the adult brain tubulins of Antarctic fishes resemble neonatal, but not adult, rat brain tubulins in their levels of glutamylation is consistent with this hypothesis, although the taxonomic separation of these groups is considerable. Whether the tubulins of Antarctic fishes are truly pedomorphic can best be answered by comparison of tubulin glutamylation as a function of developmental stage and through appropriate comparisons to cool-temperate, non-Antarctic notothenioid fishes (e.g., the New Zealand black cod, *Notothenia angustata*).

Complexity of α-Tubulin Isotypes. Our analyses of the amino acid sequences of the C-termini of α tubulins support the conclusion that 9 to 11 or more genes are expressed by brain tissues of two species of Antarctic fishes. The apparent 2:1 preponderance of α isotypes over β (9−11 vs 5, respectively) is puzzling, but may indicate that the promoters of the α-tubulin genes are not as strong as those for the β genes. It is intriguing to note that the α2, α7, and α8 isotypes, the three most abundantly expressed mRNAs of *N. coriiceps* (Figure 1), are encoded by the multigene complex that we described previously (*17*). We suggest that this complex evolved by duplication to provide the gene templates, and

perhaps stronger promoters, necessary to support efficient low-temperture synthesis of α-tubulin polypeptides at levels to match the β-tubulin pool.

We were not able to identify C-terminal peptides corresponding to NcTbα6 and -α8. Considering that the message level for α6 is low, the corresponding tubulin polypeptide may comprise a very small fraction of the α-tubulin protein pool, below that detectable by our instrumentation. In contrast, the α8 mRNA, which encodes an isotype ending in phenylalanine, is quite abundant (18.3%). For many reasons relating, perhaps, to peptide polarity and/or the presence of other posttranslational modifications, our purification strategy may not have recovered these C-terminal peptides. Furthermore, high peptide complexity can lead to competition in desorption of peptides during the ionization process in mass spectromery, thus leading to the nondetection of some peptides. Finally, our data may support the growing realization that message levels are not always appropriate proxies for the abundance of the encoded polypeptides.

Glutamylation and MAP/Motor Binding. Recent evidence indicates that C-terminal posttranslational modifications of tubulins, including glutamylation, glycylation, and detyrosination, affect the binding of MAPs and microtubule motors to the microtubule wall (*33*). The association of MAP1A, MAP1B, tau, kinesin, and dynein with microtubules is modulated by polyglutamyl chain length, with increasing affinities observed for 1 to 3 glutamyl residues and decreasing affinities observed for longer chains (*34*−*37*). The reduced levels of glutamylation of fish tubulins, including those of the Antarctic fishes studied here, may explain the observation that the teleost-specific high molecular weight MAP0 binds to microtubules at low affinity (relative to mammalian MAPs) through weak, electrostatic interactions (*38*, *39*). Thus, the rare but robustly glutamylated β4 (βIII) isotype, with peptides containing 1 to 2 residues predominating, may be particularly important for MAP and motor binding to *N. coriiceps* microtubules.

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