Cold Adaptation of Microtubule Assembly and Dynamics

STRUCTURAL INTERPRETATION OF PRIMARYSEQUENCE CHANGES PRESENT IN THE α- AND β-TUBULINS OF ANTARCTIC FISHES

The microtubules of Antarctic fishes, unlike those of homeotherms, assemble at very low temperatures (−1.8 °C). The adaptations that enhance assembly of these microtubules are intrinsic to the tubulin dimer and reduce its critical concentration for polymerization at 0 °C to ~0.9 mg/ml (Williams, R. C., Jr., Correa, J. J., and DeVries, A. L. (1985) Biochemistry 24, 2790–2798). Here we demonstrate that microtubules formed by pure brain tubulins of Antarctic fishes exhibit slow dynamics at both low (5 °C) and high (25 °C) temperatures; the rates of polymer growth and shortening and the frequencies of interconversion between these states are small relative to those observed for mammalian microtubules (37 °C). To investigate the contribution of tubulin primary sequence variation to the functional properties of the microtubules of Antarctic fishes, we have sequenced brain cDNAs that encode 9 α-tubulins and 4 β-tubulins from the yellowbelly rockcod Notothenia coriiceps and 4 α-tubulins and 2 β-tubulins from the oscellated icefish Chionodraco rastrosponis. The tubulins of these fishes were found to contain small sets of unique or rare residue substitutions that mapped to the lateral, interprotofilament surfaces or to the interiors of the α- and β-polypeptides; longitudinal interaction surfaces are not altered in the fish tubulins. Four changes (A278T and S287T in α; S280G and A285S in β) were present in the S7-H9 interprotofilament “M” loops of some monomers and would be expected to increase the hydrophobicity of the interprotofilament interaction. Two hydrophobic substitutions (α:S187A in helix H5 and β:Y202F in sheet S6) may act to stabilize the monomers in conformations favorable to polymerization. We propose that cold adaptation of microtubule assembly in Antarctic fishes has occurred in part by evolutionary restructuring of the lateral surfaces and the cores of the tubulin monomers.

The capacity of the cytoplasmic tubulins of Antarctic fishes to form microtubules at temperatures as low as ~−1.8 °C, the freezing point of the seawater habitat of these fish, is remarkable. The critical concentration for polymerization at 0 °C of purified, MAP1-free brain tubulin is ~0.9 mg/ml (1–3), which approximates the values observed for mammalian brain tubulins at 37 °C (1, 4). Unlike mammalian microtubules, however, the cytoplasmic microtubules of Antarctic fishes are very stable and exhibit slow dynamics at cold temperatures (5, 6). Evidently, both the capacity to polymerize at low critical concentration and the slow exchange of tubulin dimers into and out of microtubule polymers are properties intrinsic to the tubulin subunits of these fishes. These unusual functional characteristics must be explicable by structural alterations to the primary sequences and/or by changes in the posttranslational modifications of the tubulin chains that, alone or in concert, modify the quaternary interactions between dimers in a microtubule. Both the longitudinal, intraprotofilament and the lateral, interprotofilament contacts may be subject to structural remodeling, and adaptation might require coordinated changes located in the complementary contact domains of the tubulin chains.

Disentangling the contributions of isotype sequence variation and differential posttranslational modification to cold adaptation of microtubule assembly ideally requires comprehensive functional and structural analyses of tubulins that derive from comparable tissues of related cold- and warm-living taxa. Among vertebrates, the notothenioid fishes of the Southern Ocean and mammals experience body temperatures (−1.8 and +37 °C, respectively) that differ by almost 40 °C, and their brains provide abundant quantities of tubulins composed of multiple α and β isotypes (7). By using representative species from these taxonomic groups as models, we compare in this report the dynamics of their microtubules at cold and warm temperatures and determine via cDNA cloning the primary sequence variations that distinguish their tubulin isotypes. By mapping the residue substitutions found commonly in the Antarctic fish isotypes, but rarely in other vertebrates, onto the three-dimensional structures of the tubulin dimer and the microtubule (8, 9), we deduce potential structural interactions that predispose the fish tubulins to form stable, relatively non-dynamic polymers at cold temperatures. Our analysis of the role of posttranslational modification in cold adaptation of microtubule assembly will be presented elsewhere.2

1 The abbreviations used are: MAP, microtubule-associated protein; UTR, untranslated region; PIPES, 1,4-piperazinediethanesulfonic acid.
2 V. Redeker, A. Frankfurter, J. Rossier, S. Parker, and H. W. Detrich, III, manuscript in preparation.
The nucleotide sequences of the α- and β-tubulin cDNAs and the primary sequences of the encoded proteins were analyzed by use of the Clustal method provided by DNASTAR MegAlign. Comparisons of the sequences of the Antarctic fish tubulins to those of α- and β-tubulins from other organisms (GenBank™ and Swiss-Prot accession numbers provided in the text) were performed using the BLASTP program (National Center for Biotechnology Information).

① Tubulin cDNA Designations and Their GenBank™ Accession Numbers—The extensive screens of the N. coriiceps libraries yielded nine cDNAs encoding distinct α-tubulins (designated NcTbα1-NcTbα9; from 13 tertiary clones sequenced), and four encoding different β-tubulins (CrTbβ1-CrTbβ4; this report; from 14 total tertiaries). The complete set of the cDNAs. The cDNAs were chosen at 0 °C, a sample was applied to a clean slide and covered with a coverslip, and the heater thermostat was adjusted to maintain the desired supra-ambient standard.

② Experimental Analysis of Microtubule Dynamic Instability—A videotape was recorded, and rates of dynamic instability were performed in PMD buffer (0.1 M PIPES-NaOH (pH 6.8 at 25 °C), 2 mM EGTA, 1 mM MgSO4, 2 mM dihydroethylenetriol) and 1 mM GTP. Videotapes were recorded, and rates of microtubule growth and shortening were analyzed by fitting, according to the method of Bradford (12) with bovine serum albumin as the standard.

③ Protein Determinations—Tubulin concentrations were measured by the method of Bradford (12) with bovine serum albumin as the standard.

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⑤ Collection of Fish Tissues—Specimens of the yellowbelly rockcod, Nototenia coriiceps, of the humped rockcod, Gobionotothen gibberifrons, and of the oscillated icefish, Chionodraco rastrospinosus, were collected by bottom trawling from the R/V Hero near Low and Brabant Islands 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 processing (RNA only).

⑥ Preparation of Tubulins—Tubulin from fresh brain tissues of G. gibberifrons or N. coriiceps was purified by DEAE ion-exchange chromatography and one cycle of microtubule assembly as described by Detry et al. (12). Bovine brain tubulin was prepared as described by Gildersleeve et al. (11).

⑦ Polymerization Buffer—Experimental analysis of microtubule dynamic instability was performed in 0.1 M PIPES-NaOH (pH 6.8 at 25 °C), 2 mM EGTA, 1 mM MgSO4, 2 mM dihydroethylenetriol and 1 mM GTP.

⑧ Video Microscopy of Microtubule Dynamics—Video-enhanced differential interference-contrast light microscopy was carried out by modifications of techniques described by Gildersleeve et al. (11). Samples were observed in closed chambers of approximately 20-μm thickness. Tubulin, sea urchin flagellar axonemal axonemes, and buffer were mixed at 0 °C, a sample was applied to a clean slide and covered with a coverslip, and the chamber was sealed with Valap. The slide was then mounted on the stage of the microscope for observation. Tubulin concentrations (0.1–1 mg/ml for bovine brain) were chosen at 0 °C, a sample was applied to a clean slide and covered with a coverslip, and the heater thermostat was adjusted to maintain the desired supra-ambient temperature. Videotapes of microtubules were recorded, and rates of microtubule growth and shortening were analyzed by fitting, according to the method of least squares, straight lines to moving windows of data points (covering length changes of approximately 0.5 mm and intervals of several minutes) as described by Gildersleeve et al. (11).

⑨ Procedure for Analysis of Microtubule Assembly—The production of two cDNA libraries from brain tissues of N. coriiceps (632,000 total recombinant phage used) was initiated 24 h in vivo by a modification (16) of the acid guanidinium thiocyanate/phenol/chloroform method (17). RNAs (5 μg/slot) were applied to nylon membranes (Magnagraph, MSI) by vacuum aspiration using a Bio-Rad Bio-Dot slot-blot apparatus. The RNA blots were hybridized to polymerase chain reaction-generated, 32P-labeled probes (φ[+p]CpC-ctGTP (3000 Ci/mmol) specific for the 3'-UTRs of seven N. coriiceps α-tubulin cDNAs (NcTbα2, NcTbα3, and NcTbα9) and of the four N. coriiceps β-tubulin cDNAs (NcTbβ1-NcTbβ4). To ensure accurate estimates of the relative steady-state abundances of the tubulin mRNAs, the probes were designed to be comparable in length (75–92 base pairs for β-tubulins and 68–87 base pairs for β-tubulins) and in GC composition (32–40% for α and 36–45% for β) so that their radiospecific activities were nearly constant. Probes were amplified using the following forward and reverse primers: NcTbα2, 5’TCTTTAAATACGAGAAGAG 3 and 5’TGTGAACTTGCATGATCTG 3; NcTbα3, 5’GGGACAAAGTTGAATACCTG 3 and 5’CATATGCCTGGTCATGTCC 3; NcTbα9, 5’GAGTCCTGAAATGGATTAC 3 and 5’CAATGCTGTCGACTGATTC 3; NcTbβ1, 5’GGCTTTGCTCTATGTTGCAGTCTG 3; NcTbβ4, 5’GGATTAGTGGATTTAGATA 3 and 5’TCTAGATGGTGGATGTATCTG 3; NcTbβ2, 5’GCAGGCAGAACACTGAG 3 and 5’ATCCATATGTTGATTCGTCC 3; NcTbβ3, 5’GGGCAGACATGCTACAC 3 and 5’AGAGAGAATACAAAAGTCG 3; NcTbβ4, 5’GAAAGGCAAACATTCCGC 3 and 5’CTTTGGTTGGTCGGTGGT 3. The nucleotide sequences of the cDNA inserts from the primary screens of the two N. coriiceps cDNA libraries (632,000 total recombinant phage used in three α-screens, 385,000 for three β-screens). Thirty primary isolates for α-tubulin cDNAs, and 30 for β, were picked from single screens of the C. rastrospinosus library (240,000 phage examined for α and 12,000 for β). For the cDNAs selected for subcloning and sequence analysis. Subcloning and DNA Sequence Analysis—cDNA inserts from N. coriiceps cDNA libraries (632,000 total recombinant phage used) were obtained from a total of six screens of the two N. coriiceps cDNA libraries (632,000 total recombinant phage used in three α-screens, 385,000 for three β-screens). Thirty primary isolates for α-tubulin cDNAs, and 30 for β, were picked from single screens of the C. rastrospinosus library (240,000 phage examined for α and 12,000 for β). For the cDNAs selected for subcloning and sequence analysis. Subcloning and DNA Sequence Analysis—cDNA inserts from N. coriiceps cDNA libraries (632,000 total recombinant phage used) were obtained from a total of six screens of the two N. coriiceps cDNA libraries (632,000 total recombinant phage used in three α-screens, 385,000 for three β-screens). Thirty primary isolates for α-tubulin cDNAs, and 30 for β, were picked from single screens of the C. rastrospinosus library (240,000 phage examined for α and 12,000 for β). For the cDNAs selected for subcloning and sequence analysis.

⑩ RESULTS

① Dynamics of Microtubules from Antarctic Fishes at Near- and Supra-Physiological Temperatures—Fig. 1 presents temporal profiles of the length changes for typical individual microtubules composed of pure tubulins from Antarctic fish brain at 5 °C and 25 °C (B) and from bovine brain at 37 °C (C). Fig. 2 shows histograms, gathered by measuring many such microtubules composed of pure tubulins from Antarctic fish brain at 5 °C and 25 °C (B) and from bovine brain at 37 °C (C). For data from the 5 °C and 25 °C treatments, and the frequencies of transition between the two phases, which together govern the dynamics of the polymers.

⑫ From these data, it is apparent that the MAP-free brain microtubules of two Antarctic fishes are substantially less dy-
Different than MAP-free bovine microtubules. First, the rates of growth and shortening of fish brain microtubules at the near-physiological temperature of 5 °C were 1—2 orders of magnitude smaller than those observed for brain microtubules of the cow at its body temperature, 37 °C. (The dynamics of the microtubules from the two fish species were indistinguishable.) Second, the frequencies of catastrophe (transition from growth to shortening) and rescue (transition from shortening to growth) of the fish microtubules were more than an order of magnitude smaller than those observed for bovine microtubules. Third, the rates of growth and shortening of the fish microtubules remained small even at the supra-physiological temperature of 25 °C. Together, these results indicate that the sluggish dynamics and pronounced cold stability of brain microtubules from Antarctic fishes must derive from structural features intrinsic to their tubulin subunits.

**Primary Sequences of Brain α- and β-Tubulins from Two Antarctic Fishes**—To evaluate whether primary sequence variation contributes to cold adaptation of microtubule assembly, we have cloned cDNAs that encode most of the brain tubulin chains of the Antarctic nototheniid *N. coriiceps*. To date, nine distinct cDNAs encoding α-tubulins (NcTbα1—NcTbα9) and four encoding β isotypes (NcTbβ1—NcTbβ4) have been obtained. We have also sequenced a subset of tubulin cDNAs from the channichthyid *C. rastrosinus* (four α and two β) to determine if a common pattern of sequence changes characterizes the tubulins of fish species representing two different notothenioid families. Fig. 3 shows the α- and β-tubulin sequences of the two fishes, compared with consensus sequences for vertebrate α- and β-tubulin chains and positioned with respect to the secondary structural elements of the tubulin monomer (8). Residue substitutions that distinguished the Antarctic fish chains from most of the tubulins of other vertebrates are coded by color to indicate their effects on local polypeptide hydrophobicity (green, increased; blue, decreased; yellow, no change). Several of the *C. rastrosinus* α- and β-tubulins corresponded to isotypes from *N. coriiceps* and are numbered to indicate this identity (e.g. CrTbα9 and -β9 are identical to NcTbα7 and -β9). The discovery of *C. rastrosinus* tubulin cDNAs (CrTbα10 and -β11, CrTbβ5) that have not been found in *N. coriiceps* suggests that our screens of the latter fish were not exhaustive. Alternatively, some tubulin genes may have diverged slightly subsequent to the evolutionary separation of the rockcods and icefishes approximately 7—15 million years ago (21).
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Rates of growth and shortening of individual microtubules and frequencies of catastrophe and rescue were measured as described under “Experimental Procedures.” Mean rates are shown, together with their standard deviations where those could be determined. Most of the variation in the standard deviations was caused by actual differences in the rates of growth and shortening of the microtubules and not by error of measurement.

### Table I

| Microtubules | Temperature °C | Concentration mg/ml | Growth rate μm min⁻¹ | Shortening rate μm min⁻¹ | Catastrophe frequency min⁻¹ | Rescue frequency min⁻¹ |
|--------------|----------------|---------------------|-----------------------|--------------------------|-----------------------------|-----------------------|
| Antarctic fish* | 5 | 0.6–1.1 | 0.27 ± 0.15 | −0.18 ± 0.5 | 0.008³ | <0.0004 |
| G. giberifrons | 25 | 0.12–0.22 | 0.26 ± 0.14 | 0 to −0.4³ | <0.01³ | None observed |
| B. taurus (cow) | 37 | 1.4 | 2.18 ± 0.54 | −61.2 ± 30 | 0.52 | 3.1 |

* Pooled measurements for N. coriiceps and G. giberifrons microtubules.

β-Tubulin isotypes are strongly conserved among advanced vertebrate taxa (birds and mammals), with distinct classes largely defined by shared, carboxyl-terminal signature sequences (residues 431 to end) (7, 22, 23). (α-Tubulin chains, by contrast, tend to show greater interspecific variability.) Is the strict conservation of β-chains also characteristic of fishes, the most phylogenetically primitive of vertebrates? Previously, we identified NcTbβ3 as a possible class II isotype but noted that assignment as a class IVb type was nearly as plausible (13). With a nearly complete set of β-tubulin cDNAs from two Antarctic fishes in hand, we can now address the question more definitively. NcTbβ4 was classified as a neuron-specific βIII isotype based on its long carboxyl terminus that ends in a basic motif (VRHDVRI) and on several conserved internal substitutions (Ser³⁰⁵ and Tyr⁴³⁶). With the ambiguous exception of NcTbβ1, the remaining β-tubulins (NcTbβ2 and -β3, Gtβ2 and -β5), based on their carboxyl-terminal sequences, were most closely related to the vertebrate class IVb isotype (Table II); consideration of conserved, coordinated substitutions at isotypic “hot spots” (7) failed to differentiate these chains into other β-classes. Furthermore, each of the β-tubulins, excluding NcTbβ4, contained the axonemal signal sequence EGEFXXX (positions 433–439 of vertebrate class IVb; X = acidic residue). Thus, the interspecific conservation of β-chain isotypes that typifies higher vertebrates appears not to hold for the more distantly related fishes.

Three brain β-tubulin sequences (β1–β3; GenBank™ accessions numbers AF102890, AAD56401, and AF184595, respectively) from the Atlantic cod (Gadus morhua), a northern marine fish that typically lives at 8–15°C but is able to acclimatize to near-freezing temperatures in winter, have been described (24). Although the southern notothenioids and northern gadoids diverged long before their respective oceans cooled (25, 26), some of the unique β-chain substitutions of the Antarctic fishes were present in one or more of the gadid chains (e.g. β-Y202F of cod β2; Table III). The significance of the conserved notothenioid/gadid changes will be considered below (see “Mapping Residue Substitutions to the Structure of the Tubulin Dimer” and “Discussion”).

### Steady-state Expression of Brain α- and β-Tubulin Genes—

The neural tissues of vertebrates typically express four or more α-tubulin genes and a comparable number of β (7, 22, 23). Our results indicate that Antarctic fishes express 10–11 α- and 4–5 β-tubulin genes in their brain tissues. To estimate the relative, steady-state abundances of the corresponding tubulin isotypes, we hybridized Northern slot blots of total brain RNA from N. coriiceps to probes specific to the 3′-UTRs of 7 α- and 4 β-tubulin cDNAs. Fig. 4 shows that NcTbα2, -α7, -α8, and -α9 mRNAs were prominent components of the α-tubulin transcript pool, whereas NcTbα3, -α5, and -α6 were considerably less prevalent. Among β-chain transcripts, mRNAs for NcTbβ2 and -β3 were abundant and roughly equivalent in amount, the NcTbβ1 message was less prominent, and the NcTbβ4 transcript, corresponding to the βIII isotype, constituted a minor component. Under the assumption that steady-state transcript levels mirror the relative quantities of the polypeptides generated by translation and disregarding the contribution of post-translational modification, we suggest that the brain tubulins isolated from Antarctic fishes contain four major α- and two major β-tubulin chains. It is noteworthy that the more abundant tubulin chains almost universally contained the novel sequence substitutions shown in Fig. 3.

### Mapping Residue Substitutions to the Structure of the Tubulin Dimer—

The solution of the tertiary structure of the tubulin dimer (8) and of the quaternary interactions between dimers within a microtubule (9) provides the framework for interpretation of the unusual primary sequence variations of the brain tubulins of Antarctic fishes. We consider these changes first in the context of potential remodeling of the longitudinal and lateral contact surfaces of the dimer and second with respect to their alteration of the internal structure of the tubulin monomers.

To our surprise and contrary to our prior predictions (13, 27), none of the residue changes that characterize the Antarctic fish tubulins mapped to the longitudinal, interdimer interfaces. Rather, surface substitutions were restricted to loops of the lateral, interprotofilament contacts and the luminal sides of the monomers. Fig. 5 presents a polarity difference map of the lateral contacts, with residue changes coded by color as before (green, increased hydrophobicity; blue, decreased; yellow, no change). Four of the five substitutions (α-chain: A278T and S287T; β-chain: S280G and A285S) at the lateral contact faces were found in the S7–H9 “M” (microtubule) loops of some, but not all, of the α and β isotypes (cf. Fig. 5). Relative to their structure-forming tendencies (28), most of these changes would be expected to introduce greater flexibility into the “hinge points” of the M loops. Because the M loop of the β-chain is thought to interact with the nucleotide-sensitive H3 helix of the neighboring β-subunit to control the strength of the lateral interaction between dimers (9), increased flexibility in this region may strengthen interprotofilament contacts and slow the conversion of the “straight” dimers of a growing microtubule end to the “curved” dimers of the shortening state (29, 30).

Previously, we suggested that evolution of their tubulins to form greater numbers of hydrophobic interactions at sites of interdimer contact may cause the greater entropic control of microtubule assembly in Antarctic fishes (2, 3). The fifth, α-specific lateral substitution, M302L/F, was distinctly hydrophobic in character (cf. Ref. 31) and was present in most of the fish...
subunits (Fig. 3). Found adjacent to Val303 (and near Val204) at the base of a hydrophobic pocket formed by the H9–S8 loop, M302L,F may contribute to immobilization of additional water molecules by the dimer. Displacement of this water upon lateral association provides a plausible rationale for the unexpected energetics (1–3) of polymerization of Antarctic fish tubulins. If operative, the mode of displacement (perhaps local conformational changes in the α-chain induced by contact with the H3 helix of the α monomer in the adjacent protofilament) remains uncertain.

Fig. 6 shows a ribbon diagram of two dimers interacting laterally. Highlighted are both the residue changes of the lateral contact loops and substitutions within buried elements of secondary structure. The latter changes, some of which occur consistently in many of the fish isotypes (α:S187A in helix H5, β:Y202F in sheet S6; see Fig. 3), would be expected to increase the hydrophobicities of the monomeric cores. We postulate that β:Y202F may be particularly important for strengthening the lateral contacts between microtubule protofilaments. This residue, which is shared by one of three Atlantic cod β isotypes (β2; GenBank™ accession number AAD56401), resides near the interface between the amino-terminal GTP-binding domain.

Note that side chains shown belong to the vertebrate consensus but have been coded per Fig. 3 to indicate the polarity change introduced by the fish substitutions.
and the central taxol-binding domain of the β monomer. It has been proposed, based on the comparison of the electron crystalllographic structure of tubulin and the x-ray crystallographic structure of FtsZ (32), that GTP hydrolysis in β-tubulin could result in a change in the relative orientation of these domains (33). The βY202F substitution may rigidify the structure such that the activation energy barrier for the hydrolysis-induced conformational change is elevated substantially. In effect,
Steady-state transcript levels of individual interactions, even at low temperatures. GTP-like structure of tubulin that results in strong lateral probes derived from the 3′applied to nylon membranes by vacuum aspiration) to gene-specific sequence similarity. The longer class III carboxyl termini (cβ4, NcTbβ4) are shown unaligned and in reduced point size.

### DISCUSSION

Microtubules assembled from pure brain tubulins of cold-living fishes, unlike those of homeotherms, are stable at temperatures between 0 and 25 °C and exhibit limited dynamic instability (see Refs. 5 and 6 and this report). Slow dynamics must therefore result from structural changes intrinsic to the tubulins of these organisms that modify the quaternary interactions between dimers in a microtubule. To investigate the contribution of tubulin primary sequence variation to the unique functional properties of the microtubules of Antarctic fishes, we have sequenced a total of 11 α- and 5 β-tubulin cDNAs from two representative species of Antarctic notothenioid fishes. We find that these tubulins contain small sets of unusual residue substitutions that map to the lateral, interprotofilament surfaces or to the interiors of the α- and β-polypeptides. By contrast, the longitudinal, intraprotofilament interaction surfaces appear not to be altered.

### Figure 4

**Expression of brain tubulin isotypes in N. coriiceps.** Steady-state transcript levels of individual *N. coriiceps* α- and β-tubulin isotypes were evaluated by hybridization of total brain RNA (5 µg/slot applied to nylon membranes by vacuum aspiration) to gene-specific probes derived from the 3′-UTRs of seven α-tubulin cDNAs (NcTbα2, NcTbα3, and NcTbα5-NcTbα9) and of four β-tubulin cDNAs (NcTbβ1-NcTbβ4) (see “Experimental Procedures”).

β-Y202F may be acting as a surrogate taxol to stabilize a GTP-like structure of tubulin that results in strong lateral interactions, even at low temperatures.

### Table II

| Class | Organism | Tubulin | Accession no. | Carboxyl-terminal sequence | % Sequence identity |
|-------|----------|---------|---------------|---------------------------|--------------------|
| I     | Mouse    | Mβ5     | P05218        | 1EEEDDFGEEAE*E*EA         | 56 53 60 26 53 53  |
|       | Chicken  | cβ7     | P09244        | 1EEEDDFGEEAE*E*EA         | 56 53 60 26 53 53  |
| II    | Mouse    | Mβ2     | B25437        | 2DEQGEFEEEEE*EDEA         | 62 60 67 43 60 53  |
|       | Chicken  | cβ1/2   | AAA49124      | 2DEQGEFEEEEE*EDEA         | 75 73 80 39 73 67  |
|       |          |         | AAA49125      |                           |                    |
|       | N. coriiceps | Ncβ1  | L08013        | 3EDEGFEEGEGEYEDGA         | 81 87 35 81 75     |
|       |          | Ncβ2    | AF255553      | 3EDEGFEEGEGEYEDGA         | 93 26 100 93       |
|       |          | Ncβ3    | AF255554      | 3EDEGFEEGEGEYEDGA         | 35 93 87           |
|       |          | Ncβ4    | AF255555      | 3DEMGYEDEIDEKEVHRDVRH     | 39 39              |
|       | C. rastrospinus | Crβ2  | AF255954      | 3EDEGFEEGEGEYEDGA         | 93                 |
|       |          | Crβ5    | AF255955      | 3EDEGFEEGEGEYEDGA         |                    |
| IVb   | Mouse    | Mβ3     | C25437        | 4EDEGFEEGEEA*EEVA         | 75 73 80 43 73 80  |
|       | Chicken  | cβ3     | P09206        | 4EDEGFEEGEEA*EEVE         | 69 67 73 30 67 73  |
| IVa   | Mouse    | Mβ4     | D25437        | 5EE*GEFEYDEEESEQGAK       | 42 53 47 39 53 53  |

### Table III

| Chain | Residue position |
|-------|------------------|
|       | 37 128 202 269 280 285 335 | Carboxyl terminus (residue 431-end) |
| NcTbβ1 | H G F I G S A V | EEEEQEEDDEEESEQGAK |
| NcTbβ2 | Q S F M S S V | EE*GEFEEEAE*EEVA |
| NcTbβ3 | H S F M S S V | EE*GEFEEEAE*EEVA |
| NcTbβ4 | Q N F M S S V | EE*GEFEEEAE*EEVA |
| CrTbβ2 | Q S F M S S V | EE*GEFEEEAE*EEVA |
| CrTbβ5 | ?* ? F M S S V | EE*GEFEEEAE*EEVA |
| Cod β1 | H S Y M S S S V | EEEEQEEDDEEESEQGAK |
| Cod β2 | N S F M S S A V | DEEGEFDEEAE-ED-G |
| Cod β3 | V N Y M S S A V | DEEENFDEEAD-EEIA |

*Question marks indicate residues that are unknown because the CrTbβ5 cDNA lacked some 5′-coding sequence.
Furthermore, two of three Atlantic cod non-permissive for assembly of the mammalian tubulin alone. Bulins from the Antarctic yellowbelly rockcod, *N. coriiceps* from the cow, *Bos taurus* a polymers. Assembling and slowly dynamic phenotype of their microtubule brain tubulins of Antarctic fishes may contribute to the cold-tolerance on hybrid fish/human microtubules (39), when expressed in human HepG2 cells, confer partial affinity chromatography polymerizes more readily than does un- fractionated tubulin (41), consistent with an inhibitory effect of inhibitory effect of Leu or Phe for Met at position 302 of the Antarctic fish microtubules composed of αβ dimers on assembly of the brain tubulin ensemble. Furthermore, microtubules composed of αβ dimers are considerably more dynamic than microtubules containing other specific β isotypes (42). Hence, the paucity of the βIII isotype in the brain tubulins of Antarctic fishes may contribute to the cold-assembling and slowly dynamic phenotype of their microtubule polymers.

**Cold Adaptation of Microtubule Assembly Via Small Numbers of Primary Sequence Changes to the α- and β-Tubulins: Potential Mechanisms**—We envision that the residue substitutions present in the α- and β-tubulin subunits of Antarctic fishes predispose the dimer to form microtubules by three potentially synergistic mechanisms. First, the lateral interactions between adjacent protofilaments may be strengthened by the flexibility introduced into the α- and β-tubulin M loops. Nogales et al. (9) have proposed that the amino- and carboxyl-terminal parts of the M loops function as hinges that can accommodate variation in microtubule protofilament number. Although not universally present in the α and β monomers of the Antarctic fishes, four of the five substitutions that we have detected at the lateral interfaces of the tubulin dimer (two in α and two in β) occur at or near the potential M loop hinge points. Relaxation of the M loop conformation, particularly in the β-subunit, may enable the ββ′ lateral interaction to accommodate to the probable hydrolysis-dependent conformational change in H3, thus mitigating its destabilizing effect. It is interesting that many cold-sensitive, charged-to-alanine mutants of yeast α- and β-tubulins map to their respective M loops, H3 helices, and H1-S2 loops, the major contributors to lateral, protofilament contact (43, 44). Second, provision of additional hydrophobic interactions at the lateral surface of the α-chain would strengthen protofilament interaction at low temperature and explain the greater entropic control of the polymerization of the fish tubulins. The near universal substitution of Leu or Phe for Met at position 302 of the Antarctic fish α-chains may create a pocket of greater hydrophobicity that constrains additional water molecules whose release on subunit association would increase the entropy of polymerization. Similarly, α-tubulins from two psychrophilic algae of the genus *Chloromonas* contain lateral hydrophobic substitutions (M268V and A295V relative to the α-tubulins of the temperate alga *Chlamydomonas reinhardtii*) that are likely to increase
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Distantly Related, Cold-living Fish—The southern notothenioids and northern gadoids diverged well before the establishment of glacial conditions in their respective polar oceans (25, 26), and their cold-adapted phenotypes evolved independently during different geologic epochs (46–49). Nevertheless, the Antarctic rockcod *N. coriiceps* and the temperate/cold-living Atlantic cod *G. morhua* have evolved β-tubulins that share some M loop and internal residue substitutions (Table III), and their microtubules demonstrate similar dynamic properties (6).

We propose that the two taxa have converged independently on a comparable evolutionary strategy to preserve microtubule assembly and function at cold temperatures. However, the eurythermal Atlantic cod, unlike the stenothermal notothenioids of the Southern Ocean, must acclimatize to seasonal variations in habitat temperature. One likely adjustment to seasonal homeostasis of microtubule function would be the differential synthesis of β-chain isotypes containing Ser236 and Ala238 (e.g. cod β1 versus β3) and/or Phe202 and Tyr202 (e.g. cod β2 versus β3) under winter and summer conditions, respectively.

### β-Tubulin Isotypes and Microtubule Dynamics—Microtubules polymerized from the tubulins of chicken erythrocytes or of the budding yeast *Saccharomyces cerevisiae*, like those formed by Antarctic fish brain tubulins, demonstrate slow dynamics in vitro (50–52). Yeast microtubules, for example, display rates of growth and shortening that are approximately a tenth those shown by bovine tubulin (51, 52). Dynamics can be further suppressed in yeast tubulins by a mutation (T107K) introduced into the β-tubulin chain (52). Although the β-tubulins from Antarctic fish brain, chicken erythrocytes, and yeast cells are quite divergent, their primary sequences share a phenylalanine residue at position 202 in an otherwise conserved context (DETFCIDN). Thus, we propose that the repressed dynamics of the microtubules formed by these tubulins may be caused by a shared β-chain substitution whose effect is to strengthen lateral interactions by resisting conversion of the tubulin dimer to the curved, depolymerizing conformation. The potential importance of lateral interactions in governing dynamic instability has been emphasized in the “lateral cap” scheme (53).

In that model, which successfully mimics many of the aspects of microtubule dynamics, it changes in lateral affinity, more than changes in longitudinal interactions, which cause the microtubule to switch between growing and shortening phases.

#### Multifunctionality of Antarctic Fish β-Tubulins—The similarity of most Antarctic fish β-tubulins (excluding NcTbβ4) to the vertebrate class IV β-chain (Table II) suggests that β<sub>PvB</sub> may represent an ancestral, multifunctional isotype. This hypothesis is supported further by the presence of the “axonomal” signal sequence EGFXXX (X = acidic residue (7)) in each of the non-class III β-chains. Perhaps β-tubulin isotypes, with the exception of class III, had not evolved specialized functions prior to separation of the chordates from other metazoans. Fishes may subsequently have retained multifunctionality of their β-tubulins, whereas more advanced vertebrates and advanced invertebrate taxa evolved β isotypes with distinct cellular functions (reviewed by Ludueña (7)).

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### REFERENCES

1. Williams, R. C., Jr., Correia, J. J., and DeVries, A. L. (1985) Biochemistry 24, 2790–2798
2. Detrich, H. W., III, Johnson, K. A., and Marchese-Ragona, S. P. (1989) Biochemistry 28, 10085–10093
3. Detrich, H. W., III, Fitzgerald, T. J., Dinsmore, J. H., and Marchese-Ragona, S. P. (1992) J. Biol. Chem. 267, 18766–18775
4. Herzog, W., and Weber, K. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 1860–1864
5. Himes, R. H., and Detrich, H. W., III (1989) Biochemistry 28, 5099–5095
6. Billiger, M., Wallin, M., Williams, R. C. Jr., and Detrich, H. W., III (1984) Cell Motil. Cytoskeleton 24, 327–332
7. Ludueña, R. F. (1998) Int. Rev. Cytol. 178, 207–275
8. Nogales, E., Wolf, S. G., and Downing, R. K. (1998) Nature 391, 199–203
9. Nogales, E., Whittaker, M., Milligan, R. A., and Downing, R. K. (1999) Cell 96, 79–88
10. Detrich, H. W., III, and Overton, S. A. (1986) J. Biol. Chem. 261, 10922–10930
11. Gildersleeve, R. F., Cross, A. R., Cullen, K. E., Fagen, A. P., and Williams, R. C. Jr. (1992) J. Biol. Chem. 267, 1995–2000
12. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
13. Ludueña, R. F., III, and Parker, S. P. (1993) Cell Motil. Cytoskeleton 24, 156–166
14. Parker, S. K., Detrich, H. W., III (1988) J. Biol. Chem. 273, 34358–34369
15. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
16. Puisant, C., and Houdebine, L.-M. (1990) BioTechniques 8, 148–149
17. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 166, 156–159
18. Denhardt, D. T. (1966) Biochem. Biophys. Res. Commun. 23, 641–646
19. Nicholls, A. (1993) GRASP: Graphical Representation and Analysis of Surface Properties, Columbia University, New York
20. Kraulis, P. J. (1991) C. R. Acad. Sci. Paris, Sér. III, 313, 687–716
21. Ludueña, R. F. (1993) Mol. Biol. Cell 4, 445–457
22. Morgan, C., Olson, P. F., Buraescu, I., de Ines, C., Andrew, J. M., Roach, M. C., Ludueña, R. F., and Wallin, M. (1999) Cell Motil. Cytoskeleton 42, 315–330
23. Greenwood, P. H., Rosen, D. E., Weitzman, S. H., and Myers, G. S. (1966) Bull. Am. Mus. Nat. Hist. 131, 339–456
24. Eastman, J. T., and Grande, L. (1989) in Origins and Evolution of the Antarctic Biota (Crame, J. A., ed) pp. 241–252, Geological Society Special Publication 47, London
25. Detrich, H. W., III (1997) Comp. Biochem. Physiol. A. Comp. Physiol. 118, 501–513
26. Chou, P. Y., and Fasman, G. D. (1978) Annu. Rev. Biochem. 47, 251–276
27. Nicholls, A., Mandelkow, E., and Mandelkow, E. (1991) J. Cell Biol. 114, 977–991
28. Tran, P. T., Joshi, P., and Salmon, E. D. (1997) J. Struct. Biol. 118, 107–118
29. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
30. Nogales, E., Downing, K. H., Amos, L. A., and Lowe, J. (1996) Nat. Struct. Biol. 3, 451–458
31. Amos, L. A., and Lowe, J. (1999) Chem. Biol. 6, 655–669
32. Kollman, J. P. (1977) J. Geophys. Res. 82, 3843–3860
33. Eastman, J. T. (1993) Antarctic Fish Biology: Evolution in a Unique Environment, 322 pp., Academic Press, San Diego
34. DeWitt, H. H. (1971) in Antarctic Map Folio Series (Bushnell, V. C., ed) pp. 1–10, American Geographical Society, New York
35. Wallin, M., and Billiger, M. (1997) Cell Motil. Cytoskeleton 38, 297–307
36. Mogev, C., Wallin, M., and Olson, P.-E. (2000) Biochem. Biophys. Res. Commun. 269, 787–791
37. Fields, P. A., and Somero, G. N. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11476–11481
38. Detrich, H. W., III, Prasad, V., and Ludueña, R. F. (1997) J. Biol. Chem. 262, 8360–8366
39. Banerjee, A., Roach, M. C., Treka, P., and Ludueña, R. F. (1990) J. Biol. Chem. 265, 1794–1799

* A. S. Davis, personal communication.
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42. Panda, D., Miller, H. P., Banerjee, A., Luduena, R. F., and Wilson, L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11358–11362
43. Reijo, R. A., Cooper, E. M., Beagle, G. J., and Huffaker, T. C. (1994) Mol. Biol. Cell 5, 29–43
44. Richards, K. L., Anders, K. R., Nogales, E., Schwartz, K., Downing, K. H., and Botstein, D. (2000) Mol. Biol. Cell 11, 1887–1963
45. Willem, S., Stahns, M., Devos, N., Gerlay, C., Loppes, R., and Matagne, R. F. (1999) Extremophiles 3, 221–226
46. Shackleton, N. J., and Kennett, J. P. (1976) Init. Rep. Deep Sea Drilling Project 29, 743–755
47. Kennett, J. P., and Shackleton, N. J. (1975) Nature 250, 513–515
48. Shackleton, N. J., Backman, J., Zimmerman, H., Kent, D. V., Hall, M. A., Roberts, D. G., Schnitker, D., Baldauf, J. G., Desprairies, A., Homrighausen, R., Huddleston, P., Keene, J. B., Kaltenback, A. J., Krumsk, K. A. O., Morton, A. C., Murray, J. W., and Westberg-Smith, J. (1984) Nature 307, 620–623
49. Clarke, A., and Crane, J. A. (1989) in Origins and Evolution of the Antarctic Biota (Crane, J. A., ed) pp. 253–268, Geographical Society Special Publication 47, London
50. Trinecek, B., Marx, A., Mandelkow, E., Murphy, D. B., and Mandelkow, E. (1993) Mol. Biol. Cell 4, 323–335
51. Davis, A., Sage, C. R., Dougherty, C. A., and Farrell, K. W. (1994) Science 264, 839–842
52. Sage, C. R., Davis, A. S., Dougherty, C. A., Sullivan, K., and Farrell, K. W. (1995) Cell Motil. Cytoskeleton 30, 285–300
53. Martin, S. R., Schliistra, M. J., and Bayley, P. M. (1993) Biophys. J. 65, 578–596
54. Binder, L. I., Dentler, W. L., and Rosenbaum, J. L. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 1122–1126
55. Gamblin, T. C., and Williams, R. C., Jr. (1995) Anal. Biochem. 232, 43–46