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Identification of β_{III}- and β_{IV}-Tubulin Isotypes in Cold-Adapted Microtubules From Atlantic Cod (*Gadus morhua*): Antibody Mapping and cDNA Sequencing

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Isolated microtubule proteins from the Atlantic cod (*Gadus morhua*) assemble at temperatures between 8 and 30°C. The cold-adaptation is an intrinsic property of the tubulin molecules, but the reason for it is unknown. To increase our knowledge of tubulin diversity and its role in cold-adaptation we have further characterized cod tubulins using α- and β-tubulin site-directed antibodies and antibodies towards posttranslationally modified tubulin. In addition, one cod brain β-tubulin isotype has been sequenced. In mammals there are five β-tubulins (β_I, β_{II}, β_{III}, β_{IVa} and β_{IVb}) expressed in brain. A cod β_{III}-tubulin was identified by its electrophoretic mobility after reduction and carboxymethylation. The β_{III}-like tubulin accounted for more than 30% of total brain β-tubulins, the highest yield yet observed in any animal. This tubulin corresponds most probably with an additional band, designated β_x, which was found between α- and β-tubulins on SDS-polyacrylamide gels. It was found to be phosphorylated and neurospecific, and constituted about 30% of total cod β-tubulin isoforms. The sequenced cod tubulin was identified as a β_{IV}-tubulin, and a β_{IV}-isotype was stained by a C-terminal specific antibody. The amount of staining indicates that this isotype, as in mammals, only accounts for a minor part of the total brain β-tubulin. Based on the estimated amounts of β_{III}- and β_{IV}-tubulins in cod brain, our results indicate that cod has at least one additional β-tubulin isotype and that β-tubulin diversity evolved early during fish evolution. The sequenced cod β_{IV}-tubulin had four unique amino acid substitutions when compared to β-tubulin sequences from other animals, while one substitution was in common with Antarctic rockcod β-tubulin. Residues 221, Thr to Ser, and 283, Ala to Ser, correspond in the bovine tubulin dimer structure to loops that most probably interact with other tubulin molecules within the microtubule, and might contribute to cold-adaptation of microtubules. Cell Motil. Cytoskeleton 42:315–330, 1999.

Key words: α-tubulin; posttranslational modifications; tubulin phosphorylation; fish

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INTRODUCTION

Microtubules from cold-adapted North Atlantic fish and Antarctic fish assemble at low temperatures in their physiological range [Wallin et al., 1993; Williams et al., 1985; Detrich et al., 1989], in contrast to microtubules from mammals which are unable to assemble below 20°C. We have previously shown that Atlantic cod (*Gadus morhua*) microtubules also differ in many other respects from mammalian microtubules, e.g. they show slow dynamics [Billger et al., 1994], their assembly has a temperature-dependent interaction with microtubule-associated proteins (MAPs) [Wallin et al., 1993], colchicine and Ca^{2+} are poor inhibitors of assembly [Strömberg et al., 1989; Billger et al., 1991; de Pereda et al., 1995], and their tubulin is highly posttranslationally modified by acetylation and detyrosination [Billger et al., 1991]. We have recently shown that neither acetylation nor detyrosination is involved in cold-adaptation of cod microtubules [Rutberg et al., 1995, 1996]. Even if the composition of MAPs differ between cod and bovine microtubule proteins, there is no evidence that MAPs are the cause of cold-adaptation. Cod MAPs cannot confer the ability to assemble at low temperatures to bovine microtubules [Billger et al., 1991]. It is therefore likely that adaptation to low temperature is caused either by changes in primary sequences or by an yet untested posttranslational modification of α- or β-tubulin, the subunit polypeptides of microtubules.

In higher vertebrates, α- and β-tubulins are encoded by multiple genes; there are up to six α- and seven β-tubulin isotypes in mammals [for a recent review, see Ludueña, 1998]. The α-tubulins are more conservative than the β-tubulins. There are five β-tubulins in mammalian brain ($\beta_{I, II, III, IVa, IVb}$) [Sullivan and Cleveland, 1986], of which β_{II} is expressed at high levels (58% of the total β-tubulin) [Banerjee et al., 1988]. The only neuron-specific β-tubulin in mammals is β_{III}. In the frog (*Xenopus leavis*) the β_{II}-tubulin homologue is neuron-specific and β_{III} was not detected with antibodies specific for the β_{III} C-terminal [Moody et al., 1996].

Little is known about the function and regulation of different tubulin isotypes, but a difference in localization and a temporal expression of the different tubulin isotypes exist. All α- and β-tubulins are able to co-assemble, but microtubules with different isotype composition have different dynamic properties [Panda et al., 1994; Lu and Ludueña, 1994]. It has therefore been speculated that different isotypes may have different functional properties, or be important for environmental challenges [reviewed by Ludueña, 1998]. One indication that specific isotype expression may be involved in cold-adaptation is that the plant *Arabidopsis* increases its expression of one β-tubulin gene (TUB9) at low temperature [Chu et al., 1993]. It is therefore possible that the cold-adapted cod have different isotypes/isoforms than do mammals. We have recently shown that two-dimensional electrophoresis of brain tubulins show striking differences between cod and mammalian tubulins. Cod brain contains large amounts of highly acidic β-tubulin isoforms [Billger et al., 1994]. Both cod and Antarctic rockcod β-tubulins separate into two groups with different electrophoretic mobility, and both fishes have α-tubulins that are more basic compared to mammals [Billger et al., 1994; Detrich and Overton, 1986]. One Antarctic fish β-tubulin has been sequenced so far. Its nucleotide sequence is most similar to that of mammalian β_{II}-tubulin, and its amino acid sequence is similar to both β_{II} and β_{IV} [Detrich and Parker, 1993]. A vertebrate β_{III}-like Antarctic fish tubulin has been identified by the use of the unusual electrophoretic mobility of β_{III}-tubulin [Detrich et al., 1987], making it probable that fish have at least two β-tubulin genes.

In this study we have used β-tubulin isotype specific antibodies and electrophoretic mobility to identify different cod β-tubulin isotypes, and antibodies towards posttranslationally modified tubulin to increase our understanding of tubulin diversity and evolution, and its role in cold-adaptation of microtubules. We have cloned and sequenced cod tubulin cDNAs to be able to determine the contribution of potentially important amino acid sequence differences, and report here the sequence of one cod β-tubulin. Site-directed antibodies raised against synthetic peptides from mammalian α- and β-tubulin sequences were used to determine whether changes in primary sequence can be one reason for changes in assembly properties of cold-adapted tubulins.

MATERIALS AND METHODS

Isolation of Cod and Bovine Brain Microtubule Proteins

Microtubule proteins, consisting of both tubulin and MAPs, were isolated from cod by one cycle of assembly-disassembly as described by Strömberg et al. [1986], modified by the addition of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.01 mM aprotinin and 0.01 mM leupeptin). Microtubule proteins were isolated from bovine brain in the absence of glycerol by two cycles of assembly-disassembly in the presence of 0.5 mM $MgSO_4$ as described by Borisy et al. [1974] and Larsson et al. [1976]. The final pellets of cod and bovine microtubule proteins were resuspended in PMG buffer (100 mM Pipes, 0.5 mM $MgSO_4$ and 1 mM GTP at pH 6.8), homogenized, kept at 4°C for 30 min, and centrifuged at 200,000*g* for 30 min at 4°C, drop-frozen and kept in liquid nitrogen.

Cod and bovine tubulins were separated from MAPs by phosphocellulose chromatography according to Weingarten et al. [1975] in the presence of 0.5 mM $MgSO_4$ [Williams and Detrich, 1979]. Cod tubulin, which can assemble without MAPs at 30°C, was further purified by one extra cycle of assembly at 30°C. The pelleted microtubules were redissolved in PMG buffer and drop-frozen.

Protein concentrations were determined according to Lowry et al. [1951] using bovine serum albumin (BSA) as standard.

Taxol-Dependent Purification of Microtubule Proteins

Microtubule proteins were isolated from cod brain, liver, heart and egg, and from brain of ray (*Raja clavata*), eel (*Anguilla anguilla*), viviparous eelpout (*Zoarces viviparus*), ballan wrasse (*Labrus berggylta*), rainbow trout (*Oncorhynchus mykiss*), and frog (*Rana temporaria*), by homogenization of the different tissues in ice-cold PMG buffer with 1 mM EGTA in the presence of 10 µM leupeptin, 10 µM aprotinin and 0.1 mM PMSF as protease inhibitors. Assembly of microtubules was performed at 30°C by addition of 20 µM taxol according to Vallee [1982].

The cod, ray, eel, eelpout, and wrasse were collected from the Swedish west coast and were maintained in large indoor aquaria supplied with aerated recirculated seawater at 10–12°C. The trout, obtained from a local hatchery, were maintained in fresh water aquaria at 10°C. Frogs, obtained from The Frog Farm (Kells, Ireland), were maintained in indoor terrarium at 4°C.

Electrophoresis

Cod and bovine microtubule proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a BioRad mini-PROTEAN II electrophoretic cell on a vertical slab gel, using 8% polyacrylamide gels according to Laemmli [1970]. The pH in the lower gel buffer was increased to pH 9.5 in order to increase the separation of α- and β-tubulins. Gels were stained with Coomassie Brilliant Blue R-250. SDS was from Sigma Chemical Co., St. Louis, MO.

Isoelectric focusing (IEF) of cod brain tubulin was performed by the method of Field et al. [1984] as modified by Detrich and Overton [1986]. IEF slab gels containing 4.2% polyacrylamide, 9.16 M urea, and 2% carrier ampholytes (0.5% servalyt 4–6, 1% servalyt 5–6, 0.5% servalyt 5–7, Serva, Germany), was prefocused (6 W, 1 h, 22°C), and thereafter tubulin was focused at 7 W for 5 h.

Two-dimensional electrophoresis was run by the method of Field et al. [1984] with some modifications [Detrich et al., 1992; Detrich and Overton, 1986, 1988] as described previously by Billger et al. [1994].

Western Blotting

Protein samples run on vertical slab gels were blotted onto nitrocelluose membranes, using BioRad or LKB electrophoretic transfer kit, according to the method of Towbin et al. [1979]. Membranes were blocked with either fat-free milk powder (5% in TBS; 20 mM Tris [hydroxymethyl]-aminomethan, 500 mM NaCl, pH 7.5), or fish gelatine from Amersham, England (2% in PBS; 136.9 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.3) and incubated with antibodies and developed as described by Billger et al. [1991]. Proteins were stained with amido black.

Preparation of Site-Directed Monoclonal Antibodies to Tubulin

Peptides from pig tubulin sequences were synthesized by Merriefield solid phase procedures and purified to HPLC homogeneity [Andreu et al., 1988]. These included: MREIVHIQAGQSGC [β(1–13)C] (prepared by Dr. D. Andreu, unpublished), ERLSVDYGKKSKLEC [α(155–168)C] [Arevalo et al., 1990], RRNLDIERPTYTN [α(214–226)], RYPGQLNADLRKLAVN [β(241–256)] [Andreu et al., 1988]. The peptides, α(214–226) and β(241–256), were conjugated to BSA with glutaraldehyde, and the other peptides were coupled to keyhole limpet haemocyanin (KLH) through their C-terminal cysteine employing *m*-maleimido-benzoyl-*N*-hydroxysuccinimide ester [Andreu et al., 1988; Barasoain et al., 1989; Arevalo et al, 1990].

Female Balb/c mice 8–12 weeks old were immunized intraperitoneally and subcutaneously with 20–100 µg of peptide-carrier conjugate in complete Freund adjuvant, and boosted with the same amount of conjugate in incomplete Freund adjuvant 2, 4 and 6 weeks after, as described previously [Stahli et al., 1980]. Spleen cells were fused with the Sp2/0-Ag14 myeloma line in the presence of 50% polyethylene glycol (PEG-1000) as described [Nowinsky et al., 1979]. Screening of positive hybridomas was performed by ELISA on 96 well plates (Costar 3590) coated with bovine brain tubulin (0.15 µg/well), Western blot of tubulin [Andreu et al., 1988] and indirect immunofluorescence as described previously [de Ines et al., 1994]. Hybridomas were cloned and injected into pristane-primed mice for ascites production [Barasoain et al., 1989]. Limited proteolysis and Western blotting [de Pereda and Andreu, 1996] were employed to further verify the sequence specificity of each antibody (not shown).

TABLE I. Monoclonal Mouse-Antibodies Against the C-Terminal of β-Tubulin Isotypes

Antibody to	Sequence[1]	Reference	% in bovine brain[2]
β_I (rat)-tubulin	CEEAEEEA	Roach et al. [1998]	3
β_{II} (chicken)-tubulin	CEGEEDEA	Banerjee et al. [1988]	58
β_{III} (human)-tubulin	CESESQGPK	Banerjee et al. [1990]	25
β_{IV} (human)-tubulin	CEAEEEVA	Banerjee et al. [1992]	13

[1]Sequence of peptide used as immunogen. C is not present in the tubulin, but is used to bind with the carrier protein in the immunization process.
[2]The amount of each isotypes correlated to total β-tubulin content in bovine cerebral tissue [Banejee et al., 1988].

Antibody Mapping of Cod and Bovine Tubulins

Cod and bovine microtubule proteins were run on 8% SDS-PAGE as described above, blotted to nitrocellulose membranes and incubated with the site-directed antibodies to tubulin.

The tubulin sequence-specific monoclonal antibodies employed in this work and their classes are the following (see also Fig. 1b): P3B7 [α(155–168), IgM]; P1F5 [α(214–226), Ig2b]; P12E11 [α(430–443), IgG1] [Chau et al., 1998; KDYEEVGVDSVEGE]; P11E12 [β(1–13), IgG1]; P14B4 [β(1–13), IgG1]; P4D6 [β(153–165), IgM]; P5C3 [β(153–165), IgG1] [Chau et al., 1998; SKIREEYPDRIMNC]; P2G8 [β(241–256), IgG1]; and DM1A α(415–443) IgG1] (Sigma Chemical Co., St. Louis, MO); DM1B [β(412–431) IgG1] (Amersham, England).

Posttranslational modifications of cod tubulins were determined with the use of a monoclonal antibody against acetylated α-tubulin (6–11B-1) from Sigma Chemical Co. (St. Louis, MO), polyclonal antibodies against Tyr- and Glu-α-tubulin were kind gifts from Drs. C. Bulinski and G. Gundersen (College of Physicians and Surgeons of Columbia, New York, USA). The anti-Tyr-tubulin and anti-Glu-tubulin antibodies were against short synthetic peptides corresponding to the carboxy terminus of α-tubulin, $^{+}H_3N$-Gly-Glu-Glu-Glu-Gly-Glu-Glu-COO^{-} and $^{+}H_3N$-Gly-Glu-Glu-Glu-Gly-Glu-Glu-Tyr-COO^{-} respectively, raised in rabbit [Gundersen et al., 1984]. Monoclonal antibodies against phosphorylated serine, threonone, and tyrosine were from Sigma Chemical Co. (St. Louis, MO).

Monoclonal antibodies recognizing the carboxy-termini of different β-tubulin isotypes present in mammalian and avian brains (Table I) were used to compare if cod microtubules were composed of the same β-tubulin isotypes as microtubules from higher vertebrates.

The secondary antibody were horse radish peroxidase conjugated goat anti-mouse IgGs and goat anti-rabbit IgGs (BioRad Laboratories, Richmond, CA).

Determination of the Amount of β_{III} in Cod and Bovine Tubulins

Tubulin was reduced and carboxymethylated with Na^{+}-iodoacetate by the method of Crestfield et al. [1963], modified by Ludueña and Woodward [1975]. Samples were analyzed on 5.5% polyacrylamide gels in the system of Laemmli [1970]. Gels were either slab gels and stained with Coomassie blue, or tube gels stained with fast green. The slab gel was photographed; the bands quantitated using a McIntosh II equipped with a video camera and frame grabber and Image 1.55bs software [O'Neill et al., 1989]. The tube gels were scanned at 640 nm in a Gilford system 2600 spectrophotometer equipped with a linear transport and a Hewlett-Packard 7225B plotter for automatic determination of the areas of the peaks.

Densitometry of untreated cod tubulin, separated by SDS-PAGE (8% acrylamide, pH 9.5) was performed using Ultroscan XL Laser Densitometer (LKB, Sweden) slab gels, stained with Coomassie blue, was scanned at 633 nm.

Construction and Screening of a Cod Brain cDNA Library

Total RNA was isolated from cod brain using RNeasy™ total RNA kit (Qiagen, Chatsworth, CA), and poly(A) RNA was thereafter isolated by Oligotex™ mRNA kit (Qiagen). The cDNA synthesis from poly(A) RNA, and ligation into the λ ZAP Express vector was performed using the ZAP Express™ cDNA synthesis kit (Stratagene, La Jolla, CA). The resulting cDNA library contained approximately 1.0×10^6 pfu. Prior to screening, the library was submitted to one round of amplification.

The library was plated out on 15 cm diameter Petri dishes at a concentration of 20,000 pfu/plate. Following overnight incubation the phages were transferred to duplicate nylon membranes (Hybond-N, Amersham, England). The library was screened for cod β-tubulin using a DIG-labeled β-tubulin DNA probe corresponding to the coding sequence of Antarctic yellowbelly rockcod (*Notothenia coriicepes neglecta*) β-tubulin (Ncnβ1) [Detrich and Parker, 1993]. After 16 h hybridization at 68°C in standard hybridization buffer (5 × SSC, 1% block, 0.1% N-lauroylsarcosine, 0.02% SDS), the nylon membranes were washed 2 × 5 min with 2 × SSC, 0.1% SDS at room temperature, and 2 × 15 min wash in 0.1 × SSC, 0.1% SDS at 68°C. Detection of positive plaques was performed by incubating the nylon membranes with anti-

body to DIG (Anti-digoxigenin conjugated to alkaline phosphatase; Boeringer Mannheim, Germany) followed by incubation with a chemiluminescent substrate, CSPD® (as described by Boehringer Mannheim). The luminescent light emission was detected by exposure of the nylon membranes to Kodak XAR-5 X-Omat film at room temperature at 1–5 h. Thirty candidate β-tubulin cDNA clones were obtained from 60,000 recombinant phages. Sequencing of 5'-end was used to select full length cDNAs.

DNA Sequencing

One full length clone of cod β-tubulin was sequenced by the dideoxynucleotide chain termination method [Sanger et al., 1977] using Sequenase 2.0 and ^{35}SdATP (Amersham, Cleveland, OH). Sequencing was initially performed using T3 and T7 primers (Boehringer Mannheim, Germany). The obtained 5' and 3' sequences were used to design internal primers for further sequencing. In this manner the entire cDNA sequence, for the open reading frame, was obtained.

RESULTS

Cod and Bovine Tubulins Differ in Composition on SDS-PAGE

Cod and bovine microtubule proteins, isolated by a temperature dependent assembly-disassembly procedure, were run on 8% SDS-PAGE with pH 9.5 in the lower gel buffer. Bovine α- and β-tubulins were clearly separated in this system (Fig. 1a). A third protein band, in between the other two, was present in the cod sample. Western blotting with antibodies against α-tubulin (DM1A, directed against amino acids 415–430) and β-tubulin (DM1B, directed against amino acids 412–431) revealed that the upper cod and bovine protein bands were α-tubulin, and the lower protein bands β-tubulin. The middle band of cod tubulins was identified as a β-tubulin, and denoted β_x.

Densitometry of slab gels stained with Coomassie blue showed that β_x accounts for 32.3 ± 3.1% of total β-tubulin when the proteins were isolated by a temperature dependent assembly-disassembly method. In samples of taxol dependent isolated microtubule proteins, the amount of β_x was lower, constituting 19.0 ± 2.5% of total β-tubulin.

Antibody Mapping of Cod and Bovine Tubulin

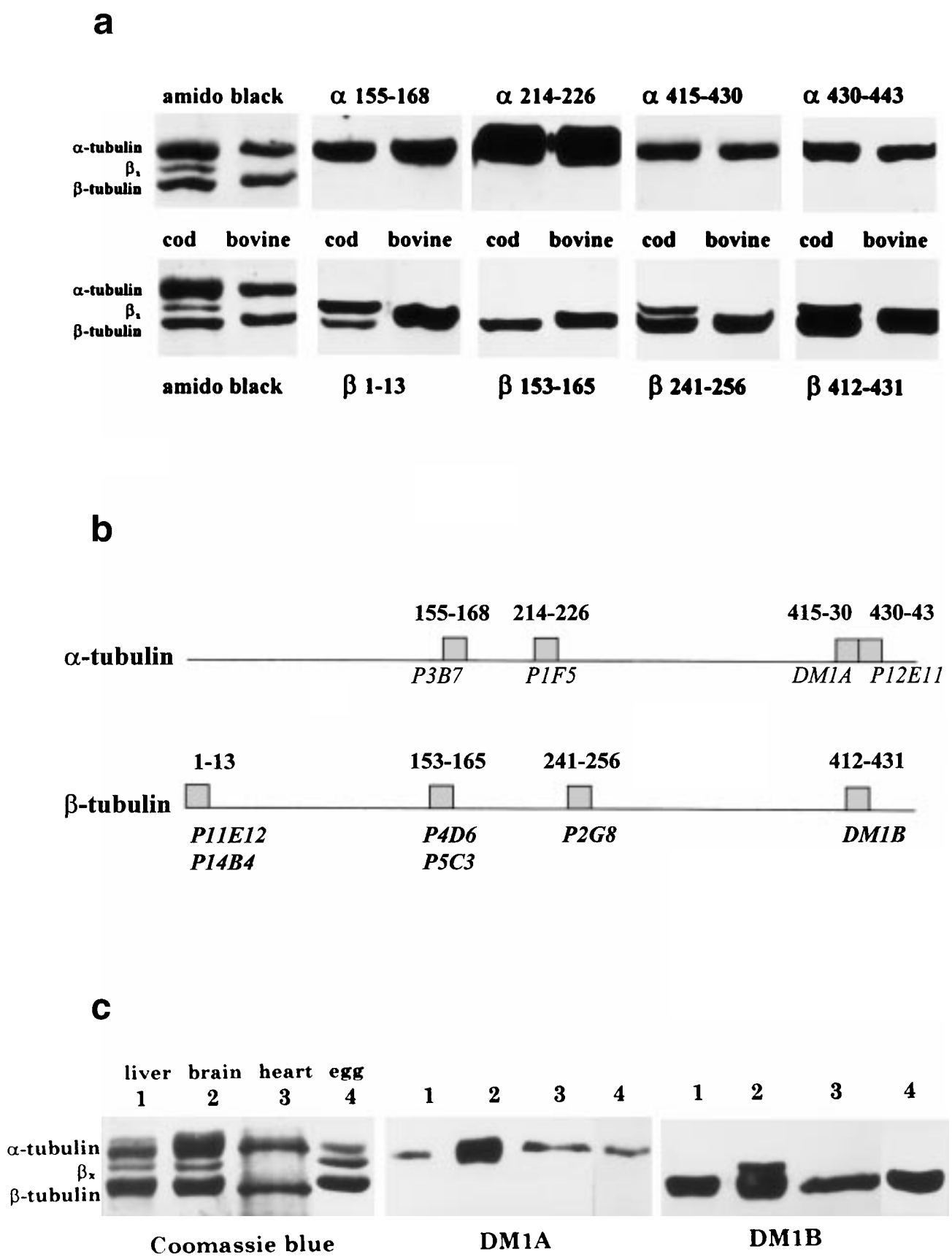
Site-specific monoclonal antibodies were used for further characterization of cod and bovine tubulins. Cod and bovine brain tubulins were run on 8% SDS-PAGE gels with pH 9.5 in the lower buffer to separate the different tubulins. They were thereafter blotted onto nitrocellulose paper and incubated with site-directed antibodies raised against pig brain tubulin sequences. The four tested anti α-tubulin antibodies reacted similarily with cod and bovine α-tubulins (Fig. 1a). The β-tubulin antibodies all stained bovine tubulin clearly, while they reacted differently with the cod. Two β-tubulin antibodies which are directed against β-tubulin residues 1–13, stained the cod β_x-tubulin band strongly, while the lower β-tubulin band staining was much fainter (only P11E12 is shown in Fig. 1a). The β-tubulin antibodies directed against residues 153–165 stained only the lower cod β-tubulin band (P4D6 is shown in Fig.1a). The antibodies against β-tubulin amino acids 241–256 and 412–431 did not differ in their staining of the two cod β-tubulins. Our results suggest that cod brain β-tubulin is composed of β-tubulin isotypes which are divergent from the more conserved mammalian N-terminal, and that the β_x isotype(s) differs in sequence in the region of amino acids 153–165.

SDS-PAGE and Western Blotting of Cod Microtubule Proteins From Brain, Liver, Heart and Egg

Microtubule proteins were isolated by a taxol-dependent procedure from brain, liver, heart and egg to determine whether the β_x-tubulin band was specific to the neuronal system, or a general feature of cod microtubule proteins. In heart, only one α- and one β-tubulin band were found, while in liver, egg and brain microtubule proteins, three protein bands were seen. The upper and lower bands were recognized by DM1A and DM1B tubulin antibodies. Brain β_x was stained by the β-tubulin antibody, but the middle bands in liver and egg microtubule protein samples were not stained, making its identity in these samples unclear (Fig. 1c). Antibody mapping of cod liver tubulins showed that none of the site-specific β-tubulin antibodies (Fig. 1b) stained the band between α- and β-tubulin in liver samples, and that anti-$\beta_{1–13}$, -$\beta_{153–165}$ and -$\beta_{241–256}$ stained the lower tubulin band with lower intensity than did DM1B (not shown). Our results therefore suggest that β_x-tubulin is brain tissue specific. The identity of the middle band in liver and egg is unclear; it might be a highly divergent β-tubulin, or a tubulin with an unusual posttranslational modification, but we cannot exclude the possibility that it represents a contaminant non-tubulin protein in these taxol preparations.

β_x-Tubulin Is Phosphorylated

In bovine brain, phosphorylation of tubulin preferentially occurs on β_{III}-tubulin, an isotype predominantly found in neurons [Khan and Ludueña, 1996]. We used antibodies to phosphorylated serine, threonine and tyrosine respectively, to determine whether cod tubulins were phosphorylated. Western blots showed that bovine β-tubu-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α 155-168
α 214-226
α 415-430
α 430-443
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β 1-13
β 153-165
β 241-256
β 412-431
b
155-168
214-226
415-30
430-43
α-tubulin
P3B7
P1F5
DM1A
P12E11
1-13
153-165
241-256
412-431
β-tubulin
P11E12
P14B4
P4D6
P5C3
P2G8
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Figure 1.

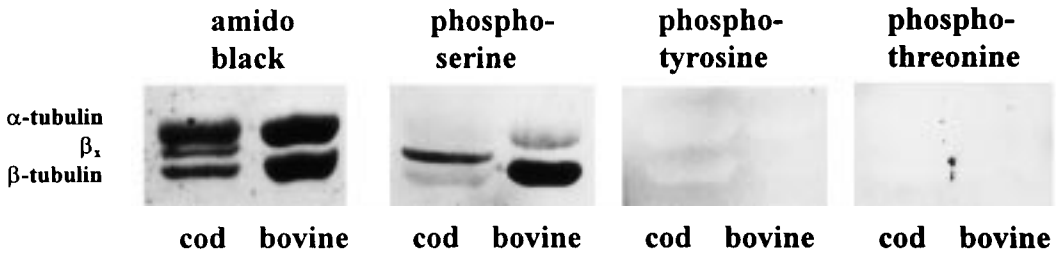

Fig. 2. Analysis of phosphorylated amino acids in cod β_x-tubulin. Cod tubulin (8 µg per lane) was separated by SDS-PAGE (8% polyacrylamide and pH 9.5 in the lower gel buffer), blotted to nitrocellulose membranes and stained with amido black, or incubated with antibodies to phosphorylated serine, threonine, or tyrosine.

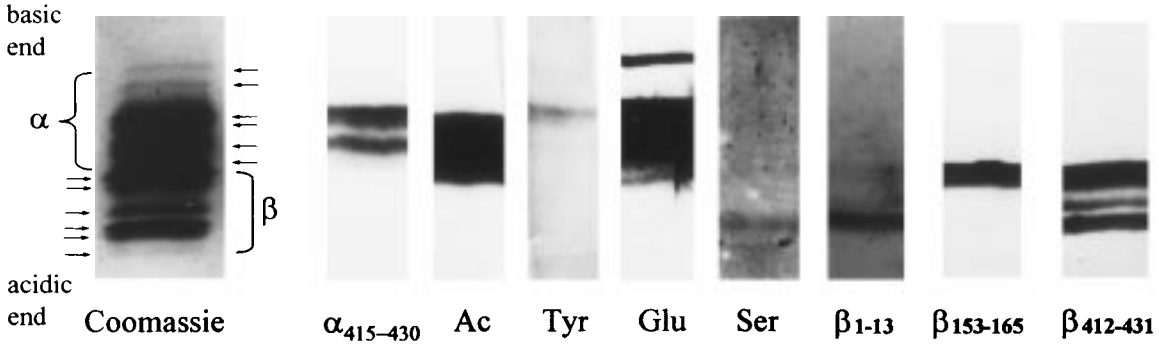

Fig. 3. Isoelectric focusing of cod brain tubulin. Tubulin (18 µg) was separated by isoelectric focusing and stained with Coomassie brilliant blue or blotted to nitrocellulose membrane and stained with antibodies to tubulin; $\alpha_{415–430}$ (DM1A), $\beta_{1–13}$ (P11E12), $\beta_{153–165}$ (P4D6), $\beta_{412–431}$ (DM1B), or to posttranslationally modified tubulin; acetylated α-tubulin (Ac), tyrosinated α-tubulin (Tyr), detyrosinated α-tubulin (Glu), and tubulin with phosphorylated serine (Ser). The basic and acidic ends of the pH gradient are indicated. Arrows indicate α- and β-tubulin isoforms.

lin and cod β_x-tubulin were serine phosphorylated (Fig. 2). Although a faint staining of both cod α- and β-tubulin could be seen, there was an intense anti-phosphoserine staining of β_x. Neither antibody against phosphotyrosine nor antibody against phosphothreonine stained cod or bovine brain tubulins. These antibodies gave a high background staining of the nitrocellulose membrane. Fish gelatine was used instead of milk powder to reduce the amount of unspecific staining of the membrane by these antibodies.

Fig. 1. Antibody mapping of cod and bovine tubulin. **a:** Cod and bovine brain microtubule proteins (6 µg per lane) were separated by SDS-PAGE, using 8% polyacrylamide and pH 9.5 in the lower gel buffer, and thereafter blotted to nitrocellulose and stained with amido black or site-directed antibodies against α- and β-tubulin. Cod brain tubulin separated into three bands, one α-tubulin and two β-tubulin bands, of which the middle band was denoted β_x. Two different antibodies were used for the experiments in panels a and c for regions 1–13 and 153–165 in β-tubulin (b), giving the same results. The results are presented with the use of P11E12 and P4D6 antibodies. **b:** A schematic representation of the regions to which the site-directed monoclonal antibodies bind. **c:** Taxol-purified microtubule proteins from cod liver, brain, heart and egg were separated on SDS-PAGE as described above, and transferred to nitrocellulose membrane. Gels were stained with Coomassie brilliant blue, and the Western blots with α- or β-tubulin antibodies (DM1A and DM1B respectively). DM1B recognized two β-tubulin bands in the cod brain microtubule samples, but only one in other tissues.

Isoelectric Focusing and Western Blotting of Cod Tubulin

We have previously shown that the isoelectric points for cod tubulins differ from mammalian tubulins [Billger et al., 1994]. Cod α-tubulins are more basic than bovine tubulins, and the β-tubulins separate into two spots of different mobility in two-dimensional gels. We used isoelectric focusing to investigate further the isotypes of cod brain α- and β-tubulin. About six α- and six β-tubulin bands could be seen (Fig. 3). These isoforms do not necessarily represent different gene products, but may also be products of posttranslational modifications. Three of the α-tubulin bands were highly acetylated, and with the exception of one α-tubulin band, they were all detyrosinated (Fig. 3). The two most acidic β-tubulin bands were both phosphorylated and stained by anti-$\beta_{1–13}$ (P11E12), while the most basic β's were stained with anti-$\beta_{153–165}$ (P4D6) (Fig. 3).

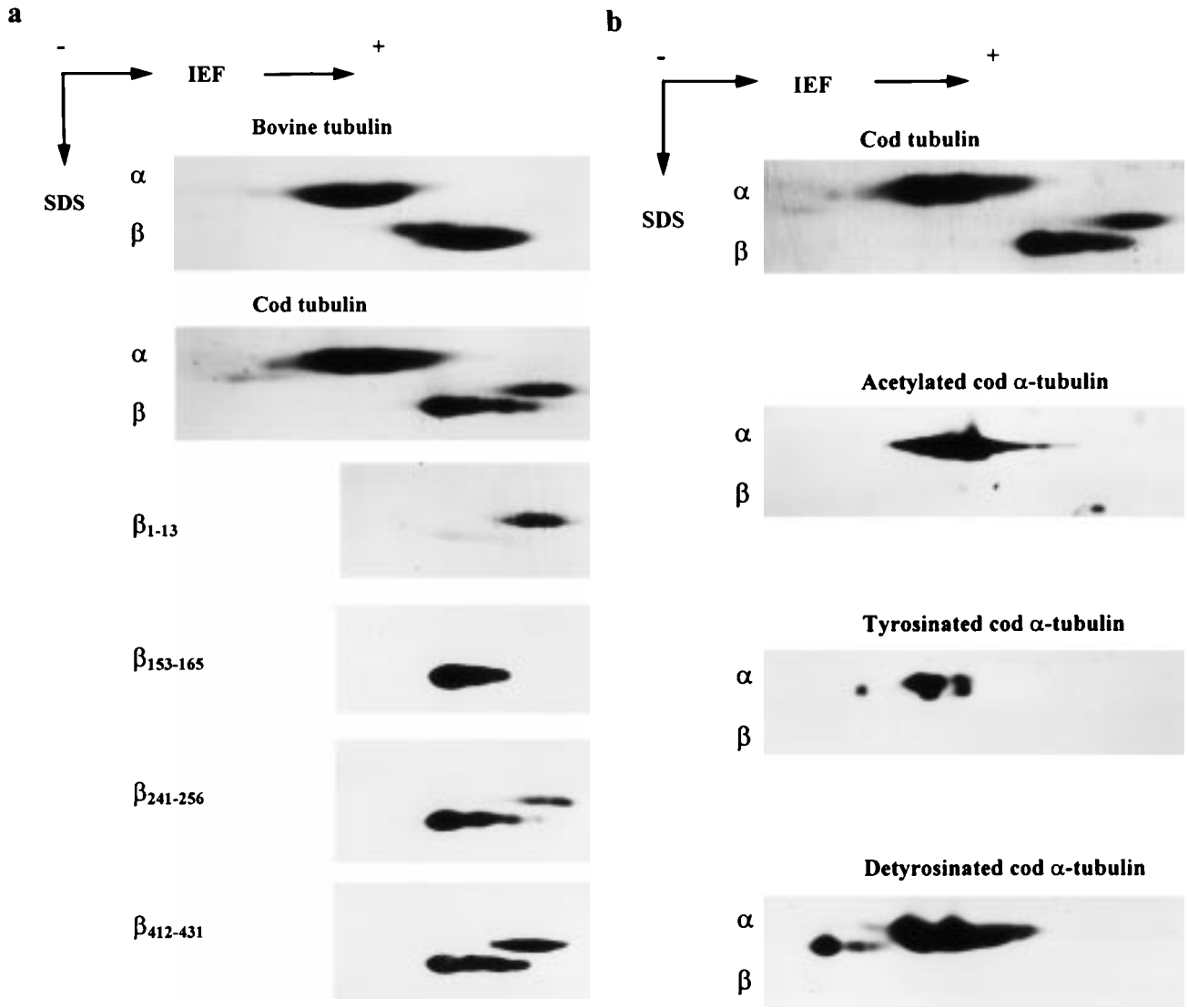

Fig. 4. Two-dimensional electrophoresis and immunoblots of cod brain tubulin. Tubulin (10 μg) was separated by isoelectric focusing in the first dimension followed by SDS-PAGE using gradient slab gels with 2–8% urea and 3–15% acrylamide. The second dimension gels were stained with Coomassie brilliant blue, or blotted to nitrocellulose membranes and stained with tubulin antibodies against amino acid region 1–13 (P11E12), 153–165 (P4D6), 241–256 (P2G8) and 412–431 (DM1B) in β-tubulin **(a)** or acetylated, tyrosinated, and detyrosinated α-tubulin **(b)**. The focusing (IEF) and electrophoretic (SDS) dimensions are indicated. The basic end of the first dimension gel is at left on the second-dimension gel. Bovine tubulins are shown for comparison.

Site-specific antibody mapping of cod tubulin separated on two-dimensional gels showed that the acidic β-tubulin isoforms in the upper spot did not react with the antibody against pig β-tubulin residues 153–165 (P4D6) (Fig. 4a). This antibody also did not recognize the most acidic isoforms of β-tubulin in the lower β-tubulin spot. The antibody against amino acid 1–13 (P11E12) stained the β-tubulin isoforms in the upper spot, but the β-tubulins in the lower spot were only stained very faintly (Fig. 4a). The two β-tubulin antibodies P2G8 and DM1B stained all cod β-tubulin isoforms. The results show that the β_x-tubulin band found on SDS-PAGE is composed of highly acidic β-tubulin isoforms found on IEF gels and two-dimensional gels. It is also clear that these isoforms are divergent in the sequence region 153–165, since this antibody was unable to bind to the upper spot with acidic β-tubulins. Cod brain microtubules are composed of several β-tubulin isoforms; β_x and the major β-band which according to Coomassie blue and antibody staining are composed of two and at least three forms respectively. Cod brain α-tubulins are a mixture of tyrosinated and detyrosinated α-tubulin, most of the α's are acetylated, but there is

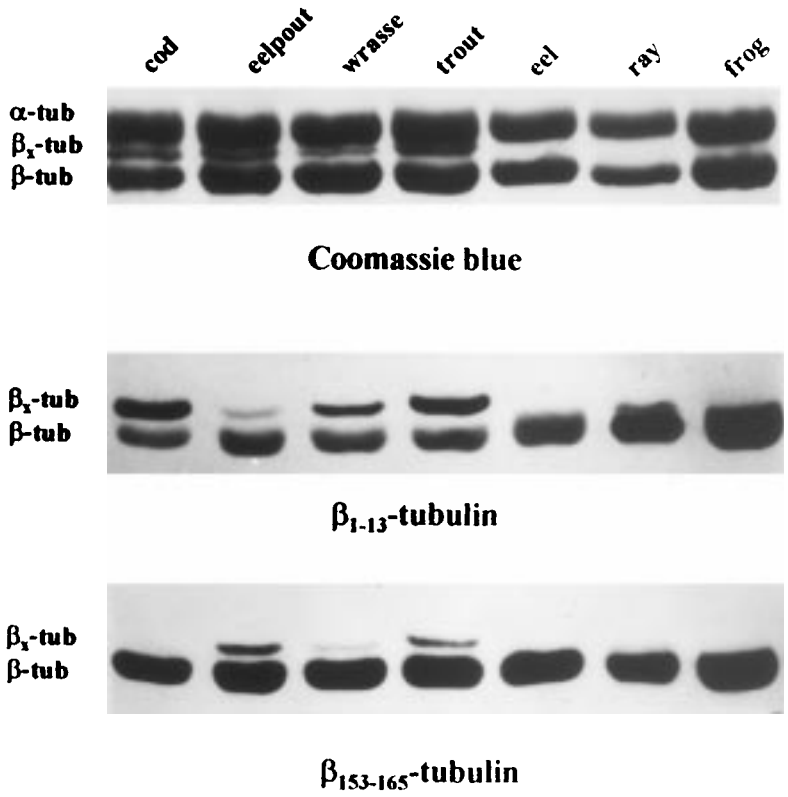

Fig. 5. Characterization of α- and β-tubulins in several fish species and frog. Higher bony fish expose a tubulin pattern which was different from other fishes, frog and mammals. Brain microtubule proteins from fish and frog were separated by SDS-PAGE (8% acrylamide gel, pH 9.5), and stained with Coomassie brilliant blue, or transferred to nitrocellulose membrane and stained with β_{1-13}- and $\beta_{153-165}$-tubulin antibodies. From left to right: cod, viviparous eelpout, ballan wrasse, rainbow trout, eel, ray, and frog. The tubulin containing part of gel and membranes are shown, α-, β_x-, and β-tubulin are indicated.

also a subgroup, the most basic α's, which are detyrosinated but not acetylated (Fig. 4b). Whether there are different cod α-tubulin isotypes have to await cloning of these proteins.

β_x-Tubulin Is Present in Higher Bony Fishes

To determine whether β_x-tubulin is cod-specific or present in other lower vertebrates, brain tubulin from several fishes and frog were run on SDS-PAGE and immunostained as described for cod tubulin. Three categories of fish were examined: Chondrichthyes (cartilaginous fish), the more primitive Osteichtyes (teleost), and the more advanced Osteichthyes (euteleosts; higher bony fish). The chondrichthyid ray, and the teleost eel showed, as did frog and bovine tubulins, only a single β-tubulin band, while the advanced osteichtyids (cod, eelpout, wrasse, and trout) contained the β_x-band as well as the major β-tubulin band. When the samples were blotted onto nitrocellulose and stained with the site-specific antibody β_{1-13}, β_x were stained in both cod and rainbow trout intensely, while a lower staining intensity was seen of β_x in eelpout and ballan wrasse (Fig. 5). A middle band was surprisingly also found in the ray sample. This band was of higher mobility than the euteleostei β_x-tubulin, and was almost invisible when the original gel was stained with Coomassie brilliant blue, indicating that it represents only a very minor tubulin isoform in ray, an isoform which seem to be different from β_x regarding electrophoretic mobility. The antibody against $\beta_{153-165}$, which did not stain cod β_x-tubulin recognized the β_x band in the other three bony fish, even if the staining was at relatively low intensity in the ballan wrasse sample (Fig. 5). DM1B stained β-tubulin in all species (not shown). The site-specific antibodies against α-tubulins were also staining α-tubulins in all species (not shown).

β-Tubulin Isotypes in Cod Brain

Isotype-specific antibodies have been prepared for mammalian tubulins. These antibodies have been raised against the highly divergent C-terminals of β-tubulins. The isotypes are expressed at different ratios in mammalian brain (see Table I). These antibodies were used for further characterization of cod β-tubulins.

Cod β-tubulin was not recognized by β_I, β_{II}, or β_{III} antibodies, while β_{IV} stained the cod β-tubulin (Fig. 6a). No staining of β_x was seen, which also was verified by blot of a 2D-gel, where the upper highly acidic β-tubulin band (identified as β_x) was unstained in contrast to the lower β-tubulin band (Fig. 6b). The β_{IV}-antibody stained although only the most basic cod β-tubulin isoforms. The staining was weak, suggesting that most cod β-tubulin isotypes are divergent in their C-terminals compared to avian and mammalian β-tubulins.

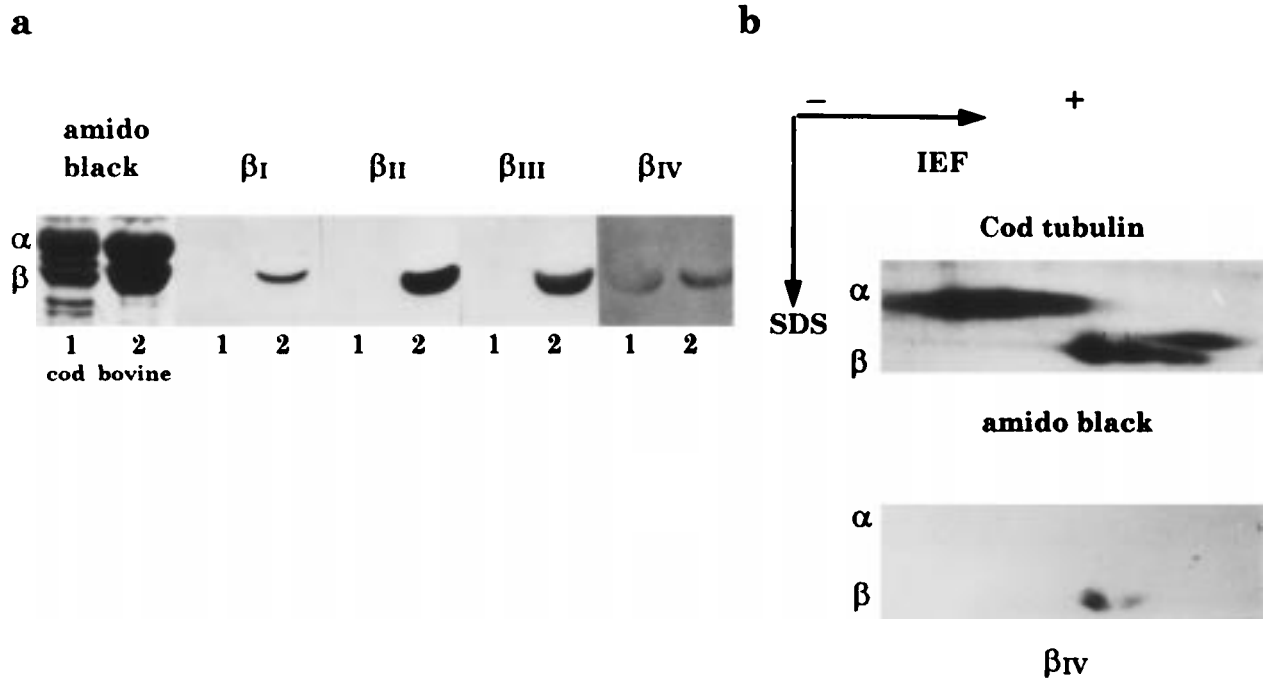

Fig. 6. **a:** Characterization of β-tubulin isotypes with C-terminal specific antibodies. Cod and bovine brain tubulins (6 µg per lane) were separated by 8% SDS-PAGE (pH 9.5) and transferred to nitrocellulose membrane and stained for immunoreactivity with antibodies against chicken and mammalian β-tubulin isotypes. The major cod β-tubulin band reacted with the β_{IV}-antibody, while β_x-tubulin did not. **b:** Blot of cod tubulin (10 µg), separated by two-dimension-gel electophoresis, and stained with amido black or antibody to β_{IV}-tubulin.

Determination of the Amount of β_{III}-like Tubulin in Cod and Bovine Tubulins

Cod and bovine tubulins were reduced and carboxymethylated with Na^+-iodoacetate, and run on 5.5% SDS-PAGE. Under these conditions cod brain tubulin clearly contained β_{III}-like tubulin (Fig. 7a). This band has been named β_2-tubulin but consists in mammals only of the β_{III}-tubulin isotype [Banerjee et al., 1990]. The β_{III}-like tubulin (β_2 band) was not found in cod egg or liver tubulin (Fig. 7b). Cod tubulin also contained a small amount of an α isotype moving slightly ahead of α. This band is designated as α' in Figure 7.

The amount of β_{III}-like tubulin was 35.4 ± 1.1% of total β-tubulins for cod tubulin and 28.9 ± 2.0 for bovine tubulin, the former significantly higher ($P < 0.002$) than the latter.

Sequence of a Cod β Tubulin

Figure 8 show the cDNA sequence and corresponding amino acid sequence of a β-tubulin from cod. Since this is the first cod β-tubulin sequence it is named cod β1, and the sequence has been registered in the GenBank database (accession number AF1023890). The cod β1 cDNA has a open reading frame of 1338 nucleotides including the stop codon. This correspond to a translation product of 445 amino acids. Although there are some unique amino acid differences, (Val^{84}, Ser^{221}, Ser^{283},

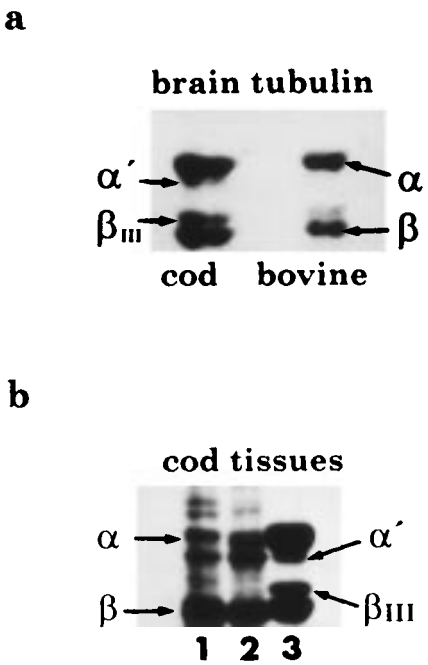

Fig. 7. Analysis of cod brain β_{III}-like tubulin. Cod and bovine tubulins were reduced and carboxymethylated with Na^+-iodoacetate followed by separation on 5.5% SDS-PAGE. α-, β-tubulin, the β_{III} isotype (this band is also called β_2), and a band of an isotype moving ahead of α-tubulin (α') are indicated in the figures. **a:** Cod brain tubulin (10 µg) are compared with bovine brain tubulin. **b:** Cod microtubule proteins from liver, **lane 1;** egg, **lane 2;** and brain, **lane 3.** The tubulin containing part of the gel are shown.

-37				CGGCACGAGCATCGAGACGTCCTTACTGCCAGACAAA
1	ATG AGG GAA ATT GTC CAT CTG CAG GCC GGC	CAG TGT GGA AAC CAA ATC GGT GCC AAG TTC	TGG GAA GTG ATC AGC GAT GAG CAT GGA ATC	
1	Met Arg Glu Ile Val His Leu Gln Ala Gly	Gln Cys Gly Asn Gln Ile Gly Ala Lys Phe	Trp Glu Val Ile Ser Asp Glu His Gly Ile	
91	GAC CCA ACT GGC ACA TAC CAC GGA GAC AGC	GAC CTG CAG CTG GAC AGG ATC AAC GTC TAC	TAC AAT GAG GCC TCG GGT GGC AAA TAC GTC	
31	Asp Pro Thr Gly Thr Tyr His Gly Asp Ser	Asp Leu Gln Leu Asp Arg Ile Asn Val Tyr	Tyr Asn Glu Ala Ser Gly Gly Lys Tyr Val	
181	CCC CGT GCT GTT CTG GTC GAT CTT GAG CCC	GGC ACC ATG GAC TCT GTG AGG TCC GGT GCT	TTC GGT CAG GTC TTC AGG CCG GAC AAC TTC	
61	Pro Arg Ala Val Leu Val Asp Leu Glu Pro	Gly Thr Met Asp Ser Val Arg Ser Gly Ala	Phe Gly Gln ***Val*** Phe Arg Pro Asp Asn Phe	
271	GTT TTC GGC CAG AGT GGT GCT GGC AAC AAC	TGG GCC AAG GGT CAC TAC ACG GAA GGT GCC	GAG CTG GTG GAC TCT GTC CTC GAC GTG GTG	
91	Val Phe Gly Gln Ser Gly Ala Gly Asn Asn	Trp Ala Lys Gly His Tyr Thr Glu Gly Ala	Glu Leu Val Asp Ser Val Leu Asp Val Val	
361	AGG AAA GAG GCG GAG AGC TGC GAC TGC CTG	CAG GGC TTC CAG CTC ACA CAC TCG CTT GGT	GGC GGC ACC GGT TCC GGC ATG GGT ACC CTC	
121	Arg Lys Glu Ala Glu Ser Cys Asp Cys Leu	Gln Gly Phe Gln Leu Thr His Ser Leu Gly	Gly Gly Thr Gly Ser Gly Met Gly Thr Leu	
451	CTC ATT AGC AAG ATC CGT GAG GAG TAC CCC	GAC CGC ATC ATG AAC ACC TTC AGC GTG GTG	CCC TCG CCC AAA GTG TCG GAC ACA GTG GTC	
151	Leu Ile Ser Lys Ile Arg Glu Glu Tyr Pro	Asp Arg Ile Met Asn Thr Phe Ser Val Val	Pro Ser Pro Lys Val Ser Asp Thr Val Val	
541	GAG CCC TAC AAC GCC ACC CTC TCC GTC CAC	CAG CTG GTC GAG AAC ACA GAC GAG ACC TAC	TGC ATC GAC AAT GAG GCT CTG TAC GAC ATC	
181	Glu Pro Tyr Asn Ala Thr Leu Ser Val His	Gln Leu Val Glu Asn Thr Asp Glu Thr Tyr	Cys Ile Asp Asn Glu Ala Leu Tyr Asp Ile	
631	TGC TTC CGC ACC CTC AAG CTC ACC ACG CCC	TCG TAC GGC GAC CTC AAC CAC CTG GTC TCG	GCC ACC ATG AGC GGC GTC ACC ACC TGC CTC	
211	Cys Phe Arg Asn Leu Lys Leu Thr Thr Pro	***Ser*** Tyr Gly Asp Leu Asn His Leu Val Ser	Ala Thr Met Ser Gly Val Thr Thr Cys Leu	
721	CGC TTC CCC GGA CAG CTC AAC GCC GAC CTC	CGC AAG CTG GCC GTC AAC ATG GTG CCT TTC	CCA CGT CTG CAC TTC TTC ATG CCC GGG TTC	
241	Arg Phe Pro Gly Gln Leu Asn Ala Asp Leu	Arg Lys Leu Ala Val Asn Met Val Pro Phe	Pro Arg Leu His Phe Phe Met Pro Gly Phe	
811	GCC CCG CTC ACC AGC CGC GGC AGC CAG CAG	TAC CGC TCG CTC ACC GTG CCC GAG CTC ACC	CAG CAG ATG TTC GAC GGC AAG AAC ATG ATG	
271	Ala Pro Leu Thr Ser Arg Gly Ser Gln Gln	Tyr Arg ***Ser*** Leu Thr Val Pro Glu Leu Thr	Gln Gln Met Phe Asp ***Gly*** Lys Asn Met Met	
901	GCG GCG TGC GAC CCC CGC CAC GGG CGC TAC	CTC ACG GTG GCG GCC ATC TTC CGC GGA CGC	ATG TCC ATG AAG GAG GTG GAC GAG CAG ATG	
301	Ala Ala Cys Asp Pro Arg His Gly Arg Tyr	Leu Thr Val Ala Ala Ile Phe Arg Gly Arg	Met Ser Met Lys Glu Val Asp Glu Gln Met	
991	CTC AAC GTG CAG AAC AAG AAC AGC AGC TAC	TTC GTG GAA TGG ATC CCC AAC AAC GTG AAG	ACG GCC GTG TGC GAC ATC CCT CCC CGT GGG	
331	Leu Asn Val Gln Asn Lys Asn Ser Ser Tyr	Phe Val Glu Trp Ile Pro Asn Asn Val Lys	Thr Ala Val Cys Asp Ile Pro Pro Arg Gly	
1081	CTC AAA ATG GCC GCC ACC TTC ATC GGC AAC	AGC ACC GCC ATC CAG GAG CTG TTC AAA CGC	ATC TCC GAG CAG TTC ACC GCC ATG TTC CGC	
361	Leu Lys Met Ala Ala Thr Phe Ile Gly Asn	Ser Thr Ala Ile Gln Glu Leu Phe Lys Arg	Ile Ser Glu Gln Phe Thr Ala Met Phe Arg	
1171	CGC AAG GCC TTC CTC CAT TGG TAC ACC GGC	GAG GGC ATG GAC GAG ATG GAG TTC ACC GAG	GCG GAG AGC AAC ATG AAC GAC CTG GTG TCC	
391	Arg Lys Ala Phe Leu His Trp Tyr Thr Gly	Glu Gly Met Asp Glu Met Glu Phe Thr Glu	Ala Glu Ser Asn Met Asn Asp Leu Val Ser	
1261	GAG TAC CAG CAG TAC CAG GAC GCC ACC GCA	GAG GAG GAG GGT GAG TTC GAG GAG GAA GGC	GAA GAA GAG CTT GCC	TAA GAAGTGTCTGT
421	Glu Tyr Gln Gln Tyr Gln Asp Ala Thr Ala	Glu Glu Glu Gly Glu Phe Glu Glu Glu Gly	Glu Glu Glu ***Leu*** Ala	Stop

1350 GTAACTTTGTTTTCTCGTTCTAAGATGTATAATTTCCTGACCTACTCGAGTCGGTCTTTCCAGTGTTCTTGTTTGTTTTTTTCTGTCCCTTGTCGTCCTGTTTGTACAGAAACTACATGTTAA
1473 TAAAAACGTTCTTTTACAAAAAAAAAAAAAAAAAAAA

Fig. 8. cDNA sequence and corresponding amino acid sequence of cod brain β1-tubulin. Nucleotide and amino acid positions are numbered on the left. The polyadenylation signal is underlined. The GenBank database accession number for cod β1 sequence is No.AF1023890. Five amino acids (Val^{84}, Ser^{221}, Ser^{283}, Gly^{296}, Leu^{444}), shown in bold italic type, differ from avian and mammalian β-tubulins (isotypes I–IV).

TABLE II. Divergent Amino Acid Residues of Different β-Tubulin Isotypes Are Listed

			β isotypes from higher vertebrates						
Position	Cod β1	Ncnβ1	I	II	III	IV_a	IV_b	V	VI
7	L	L	I	I	I	L	L	I	L
35	T	S	T	S	N	T	T	G	N
45	D	D	D	E	E	E	E	E	E
48	N	N	S	N	S	N	N	N	N
55	S	S	T	A/T	S	T	T	S	Y
57	G	S	G	N	H	G	G	Q	H
64	V	V	I	I	I	V	V	V	I
80	A	P	P	P	A	P	P	P	K
84	V	I	I	I	L	I	I	L	L
115	S	S	S	S	S	A	S	S	N
124	A	A	A	S	C	A	A	C	C
159	Y	Y	Y	Y	Y	F/Y	Y	Y	Y
170	V	V	V	M	V	V	V	M	V
221	S	T	T	T	T	T	T	T	T
283	S	S	A	A	A	A/G	A	A	A
293	M	M	V	M	M	M	M	M	M
296	G	S	A	S	A	A	A	A	A
316	I	I	V	I	V	V	V	V	I
332	N	N	N	N	A	S	N	A	S
335	N	N	N	N	S	S	N	N	T
364	A	A	A	S	S	A	S	A	A
365	A	A	V	A	S	A/V	A	S	A

The cod β1-tubulin are compared to Antarctic fish and higher vertebrates β-tubulin isotypes. C-terminal comparison are in Table III. The Antarctic rockcod (Ncnβ1), chicken, mouse and human β-tubulin isotype sequences were from GenBank database.

Gly[296], and Leu[444]), the cod β1-tubulin shows high homology with β-tubulins from higher vertebrates (database comparison is not shown). Analysis of so-called hot spots, regions in the β-tubulin sequence that differ between isotypes [Burns and Surridge, 1990; Ludueña, 1993], show that cod β1 is similar to mammalian and avian β_I and β_{IV}. Divergent amino acids and the C-terminal zones are shown in Tables II and III, cod β1-tubulin is compared with an Antarctic fish and with β-tubulin isotypes from higher vertebrates. Based on the criteria for typing of mammalian tubulins, the cod β1-tubulin sequenced in this study is characterized as a β_{IV}-tubulin isotype.

Three of the residues which differ from mammalian tubulin sequences, namely Ser[221], Ser[283], and Gly[296], are according to the crystallographic model structure of mammalian tubulin [Nogales et al., 1998] located at the surface of the tubulin dimer, not involved in α-helices or β-sheets formation. Ser[221] is at a loop making longitudinal contact with the next tubulin dimer along the protofilament. Ser[283] is at a zone probably involved in lateral protofilament interactions. Ser[283] is the only substitution common to both the cod β_{IV}-tubulin and the Antarctic rockcod β_{II}-tubulin sequence [Detrich and Parker, 1993], differing from higher vertebrate β-tubulin sequences.

Comparison of the sequenced cod β1-tubulin with the peptide sequences recognized by the site-specific tubulin antibodies (see Fig. 1b), shows two amino acid substitutions in region 1–13 (Ile[7], in peptide, → Leu, in cod, and Ser[12] → Cys) and one substitution in region 241–256 (Tyr → Phe), and complete identity in region 153–165. These substitutions could explain the weak staining by anti-β_{1-13} of the major cod β-tubulin band (Fig. 1a); clearly, however, the sequenced β1-tubulin is not β_x.

DISCUSSION

In the present study we have shown that cod tubulin separates into three bands (α, β_x and β) on SDS-PAGE under conditions where bovine tubulin separates into two bands (α and β). The β_x-tubulin was found to be highly acidic, phosphorylated on serine, neurospecific, and to constitute about 30% of total cod β-tubulin isoforms. Is β_x identical with the mammalian β_{III} isotype? In mammals β_{III}-tubulin is neuron-specific, often phosphorylated on residues Ser[444] and Tyr[437] [Sullivan and Cleveland, 1986; Diaz-Nido et al., 1990; Alexander et al., 1991; Khan and Ludueña, 1996], and can be identified by its unusual electrophoretic mobility after reduction and methylation, as well as by the use of C-terminal specific antibodies. However, bovine β_{III}-tubulin, when not reduced and carboxymethylated, does not migrate on SDS-PAGE as an extra band like does β_x. β_{III} is also not as acidic as cod β_x-tubulin. In spite of these differences it is reasonable to speculate that cod β_x is a β_{III}-tubulin; it is phosphorylated, localized to brain, and the amount of β_x on SDS-PAGE corresponded to the amount in the cod β2 band in the reduced and carboxymethylated samples; the β2 band is known to contain the β_{III} isotype [Banerjee et al., 1990].

It is striking that cod brain tubulin has the highest yield yet observed of the β_{III}-like tubulin (the β_2 band), accounting for 35% of the total β-tubulins. No β_{III}-like tubulin was found in cod liver or eggs, showing that it is not a ubiquitous β-tubulin. In mammalian brains, the yield of this band is 20–26%; in chicken it is about 15% [Ludueña et al., 1982], in shark 17%, and in three Antarctic fish, it is 4–12% [Detrich et al., 1987]. The β_x-tubulin is most probably not involved in cold-adaptation of cod microtubules, since β_x-tubulin was not present in all tissues, and it was absent in some of the other cold-adapted animals which we isolated microtubules from. Cod β_{III}-tubulin seems to be divergent in its C-terminal, since the β_{III}-tubulin antibody raised against mammalian β_{III}-tubulin was unable to stain cod β-tubulin. Moody et al. [1996] were also unable to detect β_{III}-tubulin in frogs or fish (the latter unpublished) with the use of antibodies to this isotype-defining domain,

TABLE III. Comparison of the Carboxy-Terminal Sequence of Cod Brain β1-Tubulin With Antarctic Rockcod (Ncnβ1) and β-Tubulin Isotypes From Higher Vertebrates

Isotype	Species	431	% Identity
(IV)	Cod β1	E E E G E F E E E G E E E L A	
(II[a])	Ncnβ1	· · · · · · · · · · · · · Y · D G A	75
I	Human β1	· · · E D · G · · A · · · - ·	66
II	Chicken β-1	D · Q · · · · · · · · · · D E ·	73
III	Chicken β-4	· · · · · M Y · D D · · · S E Q G A K	47
IV	Chicken β-3	· · · · · · · · · · A · · · A E	80
IVa	Mouse β-4	- · · · · · · · · · A · · · V ·	80
IVb	Mouse β-3	· · · · · · · · · · A · · · V ·	86

Single amino acid gaps introduced for maximal sequence homology are indicated with a line. Sequence homology between the last 15 amino acids in cod β1-tubulin and different β isotypes was calculated as percentage amino acid identity with respect to the longer sequence. Tubulin sequences are from GenBank database.
[a]Classified as isotype II by Detrich and Parker [1993].

making it difficult to classify different β-tubulins from lower vertebrates and invertebrates and to discuss their phylogeny and distributions in cells and tissues by available immunologic criteria.

Many different cod tubulin isoforms were identified with IEF, but of the four antibodies used against the C-terminal of mammalian and chicken β-tubulin isotypes only one, anti-β_{IV}, recognized cod tubulin, staining the major cod β-tubulin band. Based on sequence homology with β-tubulin from higher vertebrates, we suggest that the β-tubulin which we have sequenced is a β_{IV}-tubulin. The whole amino acid sequence is not identical to higher vertebrate β_{IV}, but comparison of the three hot spots [see Burns and Surridge, 1990] on amino acids 35, 55–57 and 124, and the C-terminals shows a high homology. It is likely that, in lower vertebrates there may be only one β_{IV}-isotype, which in mammals diverged into two; β_{IVa} is found only in the brain, and β_{IVb} is expressed in high levels in testis and lower in other tissues [reviewed by Ludueña, 1998]. The intensity of the C-terminal specific β_{IV}-staining in cod samples was comparable to that of bovine tubulin, and β_{IV}-staining was only prominent among the most basic β-tubulin isoforms. This isotype accounts for 13% of the total β-tubulin in bovine brain [Banerjee et al., 1988], and based on anti-β_{IV}-staining, it is likely that cod β_{IV}-tubulin, like bovine β_{IV}-tubulin, only comprises a small fraction of the total β-tubulin isotypes in the brain. However, we cannot exclude the possibility that this antibody does not stain the sequenced cod β_{IV}, because there are two amino acid substitutions in this region. Further studies are needed to determine whether two different β_{IV}-tubulins exist in cod brain. The site-specific antibody against $\beta_{1–13}$ stained the major cod β-tubulin band weakly. This antibody is raised against a sequence with isoleucine at residue 7. The sequenced cod β_{IV}-tubulin has a leucine at this position, which is common to some other β_{IV}-tubulins. One amino acid difference might be enough to decrease the binding of the site-specific antibody to this β-tubulin. The remaining β-tubulin isotypes may therefore be β_I- and/or β_{II}-tubulins, which have isoleucine at residue 7. Cod β_x-tubulin, which we have suggested to be a β_{III}-tubulin, was markedly stained with the $\beta_{1–13}$ antibody, a result which fits well with the knowledge that β_{III}-tubulins have isoleucine at residue 7. Further, β_x-tubulin was not stained by anti-$\beta_{153–165}$. In this region there is one amino acid difference between the peptide and chicken β_{III}-tubulin sequence, Ile^{155} in peptide → Val in chicken, [Sullivan and Cleveland, 1984].

The substitutions in the cod β_{IV}-tubulin sequence are located at loops in the crystallographic model structure of the mammalian αβ tubulin dimer [Nogales et al., 1998]. The interesting substitutions are Ile to Val 84, Thr to Ser 221, Ala to Ser 283, and Ala to Gly 296, at each position the last named residue is from the cod sequence. Residue 84 is at the loop between helix H2 and beta strand B3 and residue 221 is at the loop between helices H6 and H7 (a loop making longitudinal contact with the next tubulin dimer). Residue 283 is at the main lateral loop between beta strand B7 and helix H9, probably making contact with a molecule in the next protofilament. The latter is the only substitution which is common to the sequence of the Antarctic rockcod isotype II β-tubulin [Detrich and Parker, 1993] and the Atlantic cod isotype IV β-tubulin (this work), in comparison with the higher vertebrate sequences. Given the probable location of this unique Ser OH-group at the lateral contact interface it is tempting to speculate that it may have an important role (such as hydrogen bonding to the next protofilament) in increasing the cold-adaptation of these microtubules, by offsetting the energetics of the protein-protein interaction at this interface. However, this substitution was not found in β-tubulins from an Antarctic ciliate [Miceli et al., 1994; Pucciarelli et al., 1997]. Finally, residue 296 is at the loop between H9 and B8, and Leu 444 is at the flexible C-terminal end, which is lost from the structure.

Pucciarelli et al. [1997] have suggested that microtubule cold-adaptation could be a function of tubulin phosphorylation, since both the only sequenced Antarctic fish β-tubulin [Detrich and Parker, 1993] and three β-tubulins from an Antarctic protozoa have C-terminal sequences with possible phosphorylation sites. The cod tubulin sequence reported here has no phosphorylation site at its C-terminal. However, one must keep in mind that animals living in Antarctic water diverged from North Atlantic species a long time ago and cold-adaptation can therefore have arisen independently.

Recent results have suggested that to make a microtubule cold-adapted, it is not necessary that all isotypes within the microtubules be adapted for assembly at low temperatures. When bovine microtubule proteins co-assemble with as little as 33% cod microtubule proteins, these hybrid microtubules are able to assemble at low temperatures [Wallin and Billger, 1997]. Similar results have been found when tubulins from the cold-tolerant plant *Mimosa pudica* co-assemble with cold-labile goat tubulin [Chaudhuri and Biswas, 1993]. How much cold-adapted tubulin is necessary to make a microtubule cold-adapted is not known. Even if 33% cod microtubule proteins (composed of about 25% tubulins) can induce assembly of non-adapted tubulins at low temperature, perhaps only one or a few of the α- or β-tubulins are involved. It will therefore be very important to sequence all α- and β-tubulin isotypes within cold-adapted species to be able to predict important substitutions.

We have found six α- and six β-tubulin isoforms on one-dimensional isoelectric focusing. Some of these may be explained by posttranslational modifications, which alter their isoelectrical points. We have shown here that most α-tubulins are highly acetylated and detyrosinated, but only a subpopulation of β-tubulins was found to be phosphorylated. Cod β_x- and α-tubulins have recently shown to be highly polyglutamylated [Klotz et al., submitted for publication]. We have no indications that cod tubulins are polyglycylated (not shown), but other so far unknown posttranslational modifications might exist. The significance of the posttranslational modifications are unclear, but they may affect stability and interaction with other proteins, and may also generate functional differences of microtubules in the cell [reviewed by Ludueña, 1998].

Ludueña [1998] has recently proposed that fish have two β-tubulin isotypes, class 1 (β_{IV}) and class 3 (β_{III}), which most probably have evolved from a β_{IV}. Our results fit well with that hypothesis. However, Detrich and Parker [1993] found a β_{II}-like tubulin in Antarctic fish, and based on antibody-staining and estimated amounts of two cod brain β-tubulin isotypes (β_{IV} and β_{III}), we believe that cod must have at least one additional β-tubulin isotype, and that the divergence to more than two β-tubulins could have started already in fish.

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