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ORIGINAL PAPER

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# Characterization of the cold-adapted α-tubulin from the psychrophilic ciliate *Euplotes focardii*

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**Abstract** Tubulin dimers of psychrophilic organisms can polymerize into microtubules at temperatures below 4°C, at which non-cold-adapted microtubules disassemble. This capacity requires specificities in the structure and/or in the posttranslational modifications of the tubulin subunits. A contribution to the knowledge of these specificities was provided by the finding that the amino acid sequence of the α-tubulin of the Antarctic ciliate *Euplotes focardii* contains substitutions that, in addition to conferring an increased hydrophobicity to the molecule, modify sites that are involved in α-/α-tubulin lateral contacts between protofilaments. At the level of the coding sequence, the α-tubulin gene of *E. focardii* revealed an A + T content appreciably higher than in its homologs in ciliates of temperate waters. This was interpreted as an adaptation to favor DNA strand separation in an environment which is energetically adverse.

**Key words** Psychrophilic protist · Cold-stable microtubules · Antarctic environment · Molecular cold-adaptation · Tubulin genes · Acetylation

## Introduction

The cooling of Antarctic waters has compelled organisms living in southern oceans to develop molecules with compensatory adaptive properties to withstand the negative effects of low temperatures on a variety of biochemical reactions and processes. Understanding the unique structural and functional properties of these cold-adapted molecules is arousing a tide of interest. This has implications for biotechnology (for a review, see Gerday et al. 2000).

Polymerization of tubulin dimers is strongly affected by thermal perturbation, and microtubules usually disassemble at a temperature below 4°C. In chronically cold habitats, the microtubule dynamics (homeothermic animals excluded) must thus reflect adaptive modifications of the microtubule molecular components. Analyses of in vitro polymerization of microtubules of cold-adapted fishes (see Wallin and Stromberg 1995, for a review; Detrich et al. 2000), and expression in human cells of cold-adapted tubulins from the Atlantic cod (Modig et al. 2000), have provided evidence that these modifications are mostly represented by amino acid changes in the tubulin subunits and/or occur posttranslationally, while an association of microtubules with stabilizing factors seems to be excluded.

To contribute to a better understanding of these modifications, we are studying the tubulins of *Euplotes focardii*, a psychrophilic endemic Antarctic ciliate which shows optimal survival and multiplication rates at 4°–5°C that decline sharply at temperatures greater than 8°–10°C (La Terza et al. 2001; Valbonesi and Luporini 1990, 1993). Previously, four different isotypes of β-tubulin have been characterized to varying extents (Miceli et al. 1994, 1996; Pucciarelli et al. 1997). Here we describe structural traits and posttranslational modifications of α-tubulin, with particular attention to amino acid substitutions in functionally relevant sites of the molecule, that have been identified on the basis of the resolution of the three-dimensional structure of the tubulin dimer and of the quaternary interactions between dimers within a microtubule (Nogales et al. 1998, 1999; Inclàn and Nogales 2001; Löwe et al. 2001).

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## Materials and methods

### Cell cultures, chemicals, and reagents

Cell cultures of the *E. focardii* strain TN1 (Valbonesi and Luporini 1990) were used. They were isolated from sedi-

ment and seawater samples collected from the coastal waters of Terra Nova Bay (temperature –1.8°C, salinity 35*f*, pH 8.1–8.2) and were grown in a cold room at 4°C, using the bacteria *Escherichia coli* (strain αDH5), as food.

DNA modifying and restriction enzymes, RNase A, [$^{32}$Pd]ATP, and nitrocellulose membranes were purchased from Amersham Pharmacia Biotech (Cologno Monzese, Milan, Italy); Taq polymerase from PE Applied Biosystems (Foster City, CA, USA). All routine chemicals, including protease inhibitors, were of analytical grade and were supplied by Sigma-Aldrich (Milan, Italy). Oligonucleotides were synthesized by Labtek Eurobio (Milan, Italy).

### Macronuclear DNA purification, rapid amplification of telomeric ends (RATE) by polymerase chain reaction (PCR), and subcloning

Macronuclear DNA was purified as described by Miceli et al. (1989). Before being used as template for RATE–PCR, DNA preparations were resuspended for 30 min in a solution containing 20% polyethylene glycol and 15 mM $MgCl_2$, and pelleted by centrifugation.

RATE–PCR is a strategy that is particularly useful for amplifying the macronuclear DNA molecules (minichromosomes) of *Euplotes* (Di Giuseppe et al. 2002), which are small, mostly gene-sized, and are always flanked by telomeres characterized by unique sequence repeats (Hoffman et al. 1995). DNA samples (0.5 µg) were first amplified through ten cycles of two independent and subsequent PCR reactions. One was run with a forward primer represented by the oligonucleotide 5′ ATGAGAGAAGTCATTTCA ATT 3′, corresponding to the sequence-spanning nucleotides 1–21 of the coding region of *Euplotes octocarinatus* α-tubulin (accession no X69466). The second was run with a reverse primer represented by the oligonucleotide 5′ CATG CAGACGGCTCTCATTAC 3′, which corresponds to the *E. focardii* α-tubulin sequence spanning nucleotides 1113–1131 and was obtained from the amplified and cloned product of the first reaction. Both these amplifications were completed through 20 additional cycles carried out in the presence of the oligonucleotide 5′-$(C_4A_4)_4$-3′, containing four repetitions of the motif $C_4A_4$ distinctive of the *E. focardii* telomere sequence, and hence was capable of acting as reverse primer in the former reaction and as forward primer in the latter. All cycles were performed in a Gene Amp 9700 thermal cycler under standard conditions as described for the amplification of other *Euplotes* macronuclear genes (Miceli et al. 1991).

Amplified products were purified by incubation in one volume of a solution containing 20% PEG (polyethylene glycol) 8000 and 2.5 mM NaCl, for 15 min, and centrifuged, for 10 min at 15,000 *g*, to remove reagents and primers. Purified DNA was cloned according to Miceli et al. (1994). Clones containing tubulin-recombinant plasmids were identified by colony blotting performed according to Sambrook et al. (1989), using as probes the α-tubulin oligonucleotides previously involved in RATE–PCR and labeled by incubation with [$\gamma^{32}$P]ATP.

### Sequencing and sequence analysis

Ten plasmid inserts were sequenced in both strands either by the Sanger method with T7 sequenase (United States Biochemical, Cleveland, OH) and [$\alpha^{32}$Pd]ATP, following the procedure suggested by the enzyme supplier, or using an automated ABI Prism sequence analyzer Model 373A (PE Applied Biosystems) using the big dye terminator methodology (PE Applied Biosystems). The complete nucleotide sequence of the α-tubulin gene was obtained by overlapping the sequences of the plasmid inserts and is now available on the GenBank database by accession number AF 408404.

Sequence alignments were based on the Clustal method of the Lasergene program (DNASTAR, Madison, WI). Accession numbers used for the evaluation of the A + T content of hypotrich tubulin coding regions are the following: X69466 (*E. octocarinatus*), Z11769 (*Euplotes vannus*), Z49851 (*Euplotes aediculatus*), U89692 (*Euplotes crassus*), Z11763 (*Oxytricha granulifera*), X01746 and X12365 (*Stylonychia lemnae*). Noncoding regions were excluded from the evaluation as they are characteristically rich in A/T in all ciliate genes (Ghosh et al. 1994).

### Cytoskeletal preparations, SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting

Cytoskeletal preparations were obtained from morphogenetically stable cells, arrested in the $G_0$–$G_1$ stage of the mitotic cycle after 2–3 days of starvation as described by Pucciarelli et al. (1997).

SDS-PAGE of cytoskeletal preparations was performed according to Laemmli (1970) with some modifications. To improve the resolution between α- and β-tubulin, samples incubated in SDS sample buffer and boiled for 3 min were treated with 40 mM iodoacetamide, for 1 h in the dark, before being loaded onto a 10% (w/v) polyacrylamide gel at pH 8.1.

After electrophoresis, gels were subjected to immunoblotting as previously described (Pucciarelli et al. 1997). Antibodies DM1A for α-tubulin and DM1B for β-tubulin (both purchased from Sigma) were used at a 1 : 300 dilution; "Anti-Ac" antibodies against acetylated α-tubulin (kindly provided by Prof. H.W. Detrich III) were used at a 1 : 1,000 dilution. Blots were incubated with the peroxidase-labeled secondary antibody (1 : 1,000 dilution), and immunorecognition was revealed by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

## Results and discussion

### Cloning and nucleotide composition of the α-tubulin gene

Only one α-tubulin gene of 1,532 base pairs (bp), telomeres excluded, was identified in macronuclear DNA preparations of *E. focardii* subjected to RATE–PCR. The coding region of 1,350 bp did not contain introns, predicts a

sequence of 449 amino acids, and is flanked by 91- and 80-bp noncoding regions at the 5′ and 3′ sides, respectively. The uniqueness of the cloned α-tubulin gene was supported by the following evidence: (1) preparations of macronuclear DNA and total RNA, hybridized at low stringencies in Southern and Northern blot analysis, respectively, with homologous and heterologous α-tubulin probes, always revealed a single band which was of 1,800 bp in the former case and of 1,500 bases in the latter (Miceli et al. 1994); (2) the 1,800-bp band was completely cut in Southern blot analysis carried out with macronuclear DNA preparations previously incubated with restriction enzymes KpnI, EcoRI, and Hind III, which recognize specific sequence sites of the cloned gene (data not shown); (3) only one product with a sequence equivalent to that determined for the α-tubulin gene was obtained by PCR run with primers specific of regions of α-tubulin which are universally conserved.

At level of nucleotide sequence, one salient feature of the cloned α-tubulin gene of *E. focardii* is an A + T content higher than in other α-tubulin genes of ciliates of temperate waters (see accession numbers in Materials and methods), i.e., 55.7% versus 50.97%–53.36%. Such a predilection for A/T has also been reported for α-globin, myoglobin, and tubulin genes of Antarctic fishes (Parker and Detrich 1998; Small et al. 1998; Zhao et al. 1998). This may reflect an adaptive strategy to chronically cold temperatures since, as originally proposed by Detrich et al. (2000), an A/T-rich genome composition can facilitate DNA strand separation and accession of the polymerases to their template, and hence favor DNA replication and transcription.

## Structure of α-tubulin

The α-tubulin amino acid sequence of *E. focardii*, with respect to that determined in the temperate-water species, *E. octocarinatus*, showed six substitutions (see Fig. 1A), denoted as D22E, A100V, I114F, S158R, S295A, and I437V (where each number refers to the sequence position that is involved in the substitution and the letters that precede and

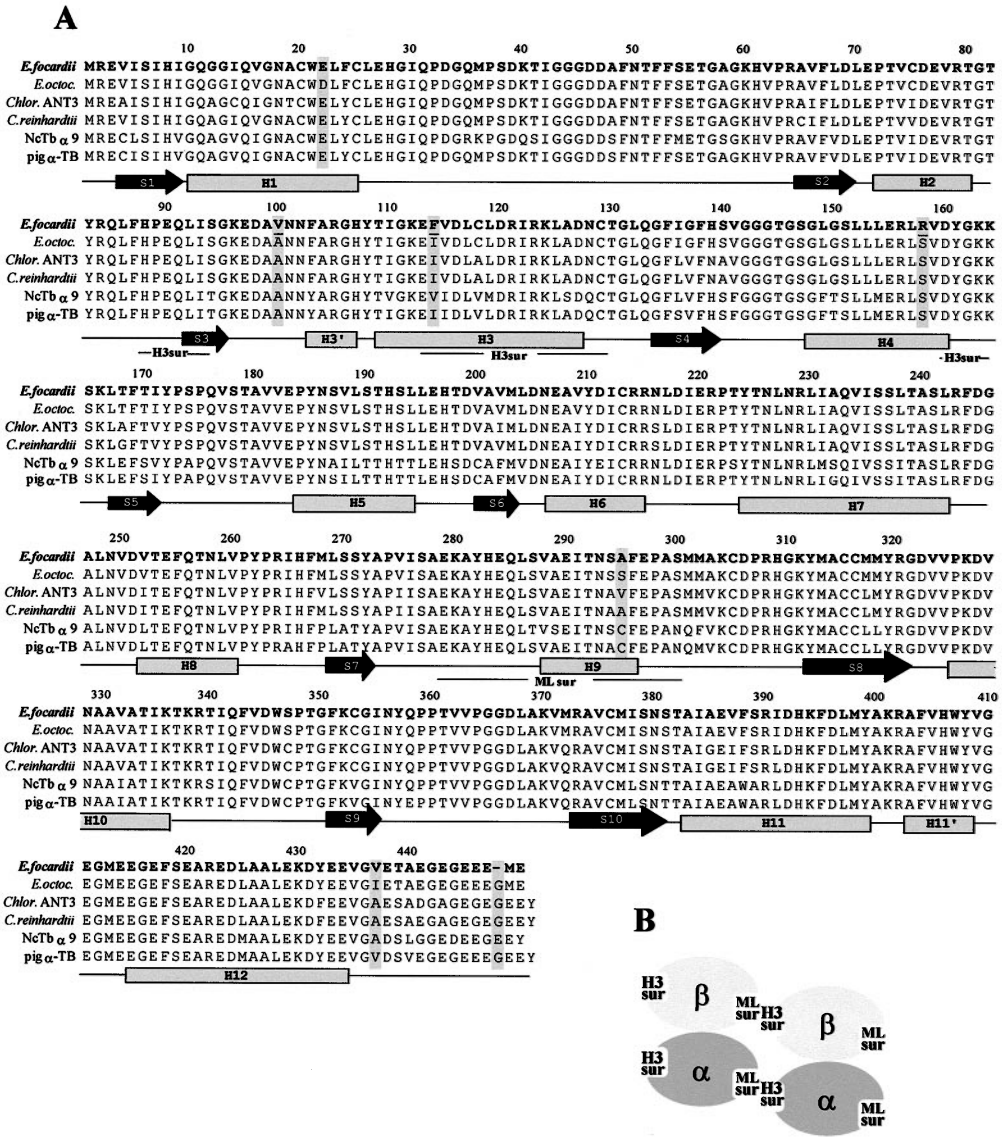


**Fig. 1. A** Comparison of the *Euplotes focardii* α-tubulin sequence with that of *Euplotes octocarinatus* (*E. octoc*., acc. no. X69466), of the psychrophilic alga *Chloromonas* (*Chlor*. ANT3, acc. no. AAB86648), of the evolutionary close mesophilic alga *Chlamydomonas reinhardtii* (acc. no. A53298), of the Antarctic fish *Notothenia coriiceps* (isotype NcTbα9, acc. no. AAG15326) and of the pig (pig α-TB, Inclän and Nogales 2001). *Gray shaded* amino acids highlight sequence positions in which substitutions of the α-tubulin from *E. focardii* with respect to *E. octocarinatus* were found; substitutions unique to *E. focardii* are *underlined*. The secondary structure determined for the pig α-tubulin, and the positions of the H3- and ML-lateral surfaces (indicated as *H3 sur* and *ML sur*, respectively) involved in lateral contacts between protofilaments, are reported according to Löwe et al. (2001) and Inclän and Nogales (2001). Helices are indicated by *gray boxes* and β-sheets by *black arrows*. **B** Schematic representation of the quaternary interactions between tubulin dimers with the localization of the *H3 sur* and *ML sur* according to the model of Inclän and Nogales (2001)

follow the number stand for amino acids present in *E. octocarinatus* and *E. focardii*, respectively).

At least four of these substitutions, i.e., A100V, I114F, S158R, and S295A, may be related to cold-adaptation, as they map in functionally important sites of the molecule. In addition, they (the S295A substitution excepted) are unique to the α-tubulin of *E. focardii* with respect to all the sequences available in databases. Substitutions A100V, I114F, and S295A appear to be located close to, or inside, tubulin regions which have been indicated as H3- and ML-lateral surfaces and shown to be directly involved in establishing interprotofilament α-/α- and β-/β-lateral contacts (Nogales et al. 1999; Inclàn and Nogales 2001) (Fig. 1A, B). These regions play a key role in the conversion from the "straight" (growing) conformation to the "curved" (shortening) state of a microtubule during phases of tubulin polymerization/depolymerization (a phenomenon known as microtubule dynamic instability) (Tran et al. 1997). Furthermore, the replacements of an alanine with a valine at position 100 and of a serine with an alanine at position 295 determine an increased hydrophobicity of *E. focardii* α-tubulin. A similar increase has been shown to occur, through specific amino acid substitutions, also in the β-tubulins of *E. focardii* (Miceli et al. 1994, 1996; and personal observation), as well as in tubulins of Antarctic fishes (Detrich et al. 2000) and psychrophilic species of the alga *Chloromonas* (Willem et al. 1999). In these species, the introduction of a valine at residue 295 (in the place of the alanine present in the evolutionarily close alga *Chlamydomonas reinhardtii*) increases tubulin hydrophobicity exactly at the same position where an alanine replaces a serine in *E. focardii* α-tubulin (see Fig. 1A). The substitution S158R maps at the end of helix 4, which, according to Nogales et al. (1999), is involved in ensuring stability of the intradimer interactions. With regard to substitutions D22E and I437V, these are probably not significant in this context, as they are shared by several α-tubulins of temperate-water species of ciliates and vertebrates. However, it is worth noting that I437V produces a decrease in hydrophobicity at the same position where, in several isotypes of Antarctic fishes (see Fig. 1A), an alanine substitutes the valine present in the α-tubulin consensus for fish and vertebrates (Detrich et al. 2000). This decrease in hydrophobicity may favor the flexibility of the C-terminal region, which is involved in the reversible interaction with microtubule-associated proteins (MAPs).

## Posttranslational modifications of α-tubulin

Three α-tubulin posttranslational modifications, i.e., reversible tyrosination, polyglutamylation, and acetylation, have so far been investigated for cold adaptation and all have been shown to occur in the Atlantic cod (Wallin and Stromberg 1995; Klotz et al. 1999). With regard to reversible tyrosination, its occurrence in *E. focardii* seems to be very unlikely. In fact, the consensus motif GEE at the α-tubulin C-terminus, which is strictly conserved in most of the known α-tubulin sequences (see databases and Alfa and Hyams 1991) and required for the recognition by the tubulin tyrosine ligase (MacRae 1997), is modified due to the deletion of the glycine at position 448 that is included in this motif.

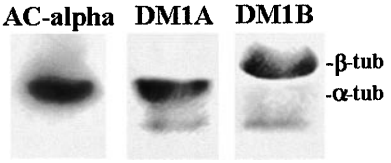


**Fig. 2.** Immunorecognition of acetylated α-tubulin. Three identical samples (10 μg each) of *E. focardii* cytoskeleton, were analyzed by immunoblotting with anti-acetylated α-tubulin antibodies (*Ac-alpha*) and with the anti α- and β-tubulin antibodies (*DM1A* and *DM1B*, respectively) to identify the relative position of the two tubulin subunits

Polyglutamylation has previously been revealed by immunorecognition with GT335 antibodies applied to purified preparations of *E. focardii* cytoskeleton (Pucciarelli et al. 1997) and is now confirmed by the finding that the site of polyglutamylation (Bré et al. 1994) represented by the glutamic acid at position 438 is preserved in *E. focardii* α-tubulin. Instead, polyglutamylation was not detected in β-tubulin, which is phosphorylated and shows aspartic acid in the place of glutamic acid at the glutamylation site (Pucciarelli et al. 1997).

Acetylation is a typical modification of the lysine at position 40 of α-tubulin. It has been observed in a subset of morphologically distinct microtubules of a number of cell types (Piperno et al. 1987) and correlated to microtubule stability although a direct role in cold adaptation has not been proved (Wallin and Stromberg 1995). The availability of antibodies specific for acetylated α-tubulin has permitted us to determine whether this modification could be detected in *E. focardii* cytoskeletal samples. As shown in Fig. 2, these antibodies recognized acetylated α-tubulin. The role of acetylation on microtubule cold adaptation is still a debatable question. It has been shown that the amount of acetylated α-tubulin increases along with an increase in microtubule population stabilized by other factors, such as microtubule-associated proteins MAP1B and MAP2 (Takemura et al. 1992). MAP-free microtubules from Antarctic fishes have been demonstrated to be able to polymerize and stay stable in the cold, and their dynamic is one or two orders slower than that of non-cold-adapted organisms (Detrich et al. 2000). Similar properties have been shown also for *E. focardii* microtubules (Miceli et al. 2000). Therefore, acetylation may target microtubules that have already reached cold stability by other compensatory modifications of tubulin dimers.

In conclusion, the results reported here suggest that even evolutionarily distant organisms may have adopted convergent solutions to preserve microtubule activities at cold temperatures. These solutions rely on variations of the tubulin amino acid sequences mainly concentrated in sites involved in lateral contacts between protofilaments that can increase the hydrophobic interactions during microtubule assembly. A decreased hydrophobicity is found at sites in

which flexibility can be necessary to facilitate reversible microtubule interactions with associated proteins.

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