Available online at www.sciencedirect.com
ScienceDirect
Protein Expression and Purification 49 (2006) 196–202
Protein Expression & Purification
www.elsevier.com/locate/yprep

Native tubulin-folding cofactor E purified from baculovirus-infected Sf9 cells dissociates tubulin dimers

D. Kortazar [1], G. Carranza, J. Bellido, J.C. Villegas, M.L. Fanarraga, J.C. Zabala *

Departamentos de Biología Molecular—Unidad Asociada al Centro de Investigaciones (CSIC) and Anatomía y Biología Celular, Universidad de Cantabria, Cardenal Herrera Oria s/n. 39011, Santander, Spain

Received 15 February 2006, and in revised form 7 March 2006
Available online 30 March 2006

Abstract

Tubulin-folding cofactor E (TBCE) is an α-tubulin-binding protein involved in the formation of the tubulin dimer and in microtubule dynamics, through the regulation of tubulin heterodimer dissociation. TBCE has also been implicated in two important related human disorders, the Kenny–Caffey and Sanjad–Sakati syndromes. The expression of TBCE as a recombinant protein in bacteria results in the formation of insoluble inclusion bodies in the absence of denaturing agents. Although the active protein can be obtained from mammalian tissues, biochemical studies of TBCE present a special challenge. To express and purify native TBCE, a recombinant baculovirus expression system was used. Native wild-type TBCE purified from Sf9 extracts was sequentially purified chromatographically through cation exchange, hydrophobic interaction, and high-resolution gel-filtration columns. Mass spectrometric analysis identified 30% of the sequence of human TBCE. A stoichiometric excess of purified TBCE dissociated tubulin heterodimers. This reaction produced a highly unstable TBCE–α-tubulin complex, which formed aggregates. To distinguish between the aggregation of tubulin dimers induced by TBCE and tubulin dissociation, TBCE and tubulin were incubated with tubulin-folding cofactor A (TBCA). This cofactor captures the β-tubulin released from the heterodimer with a stoichiometry of 1:1, as previously demonstrated. The β-tubulin polypeptide was recovered as TBCA–β-tubulin complexes, as demonstrated by non-denaturing gel electrophoresis and specific antibodies directed against β-tubulin and TBCA.

Keywords: TBCE purification; Baculovirus expression; Sf9; Tubulin dissociation

Protein misfolding is thought to be responsible for up to 50% of all human diseases. Many a priori unrelated diseases share similar pathological features. Recently, it has become clear that mutations in genes encoding molecular chaperones are responsible for some human diseases with complex phenotypes. These diseases are caused by the loss of function of one or more of the substrates of the altered chaperones [1].

The correct folding and association of newly synthesized α- and β-tubulin polypeptides to generate functional αβ-tubulin heterodimers requires the assistance of a series of recently described proteins known as tubulin-folding cofactors (TBCs[2] A–D) [2–4]. These folding proteins seem to be necessary to preserve α- and β-tubulin monomers from unwanted hydrophobic interactions, because tubulin molecules have many "interacting surfaces" that must be protected during folding. Single α- and β-tubulin polypeptides interact to form the αβ-heterodimer.

Tubulin-folding cofactor E was discovered during the analysis of the release of αβ-tubulin heterodimers from intermediate complexes in cell extracts in vitro. These

* Corresponding author. Fax: +34 942 201945.
E-mail address: zabalajc@unican.es (J.C. Zabala).
[1] Present address: Unidad de Metabolómica, CICbioGUNE, Parque Tecnológico de Bizkaia, 48160 Derio, Spain.

[2] *Abbreviations used:* CAP-Gly, cytoskeletal associated protein-glycine-rich domain; TBCA, tubulin folding cofactor A; TBCE, tubulin folding cofactor E; TBCs, tubulin folding cofactors; Ubl, ubiquitin-like domain.

1046-5928/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2006.03.005

experiments identified a protein, designated TBCE, with an apparent molecular mass of 150 kDa on a gel-filtration column and about 60 kDa on denaturing gels [5]. TBCE binds to α-tubulin after it is released from the cytosolic chaperonin CCT. Homologs of TBCE have been identified in *Saccharomyces cerevisiae* (Pac2p, perish in the absence of CIN8) [6] and in *Schizosaccharomyces pombe* (Alp21, altered polarity [7], also known as Sto1p, suppressor of tsm one [8]). Pac2p, which has 26% identity to human TBCE, is required for normal microtubule stability and, when overexpressed in *S. cerevisiae*, is detected in a complex with Tub1p (α-tubulin). Alp21/Sto1p is an essential protein in *S. pombe* involved in the generation of normal microtubules. The TBCE ortholog of *Arabidopsis thaliana*, which has been identified and named PFI, has 31% identity to human TBCE. Mutants of the gene encoding PFI exhibit severe microtubular defects, as seen in other tubulin-folding cofactor mutants [9]. Although the exact mechanism involved in the binding of TBCE to α-tubulin is still unknown, all TBCE homologs (except Alp21, which does not localize with microtubules) contain an N-terminal CLIP170 (CAP–Gly) domain. The structure of the *Caenorhabditis elegans* CAP–Gly domain of TBCB has recently been resolved [10].

Mutations in this domain of human TBCE lead to Kenny–Caffey and Sanjad–Sakati syndromes [11]. Isolated cells from patients display abnormalities related to microtubule defects, which is consistent with possible defects in the tubulin-folding and dimerization pathways. These two related syndromes are produced by the same founder mutation. A set of Belgian twins with related symptoms have been shown to express a truncated form of the TBCE protein. Furthermore, a missense mutation in the gene encoding TBCE causes progressive motor neuropathy in mice [12,13]. The basis for these differences is not yet understood. However, some of the defects observed in these mice are consistent with changes in the folding and dimerization pathways of tubulins.

Tubulin-folding cofactors D and E also participate in microtubule dynamics, dissociating the tubulin heterodimer by sequestering β- and α-tubulin, respectively [14,15]. It has recently been shown that gigaxonin, which is mutated in human giant axonal neuropathy, controls the degradation of tubulin-folding cofactor B. In this disease, TBCB accumulates and the number of microtubules decreases substantially [16]. The biochemical mechanism that explains how the accumulation of TBCB causes a decrease in the number of microtubules involves the ability of TBCB to interact with TBCE to induce an efficient tubulin heterodimer dissociation effect (unpublished results). In this work, we have cloned the human gene for TBCE and produced the active protein using a baculovirus system with Sf9 insect cells. Recombinant TBCE was purified through three chromatographic columns and its biological activity studied in reactions with purified tubulin and cofactor A (TBCA), which were analyzed by non-denaturing electrophoresis.

Materials and methods

Materials

All reagents, unless otherwise stated, were purchased from Sigma (Sigma–Aldrich, Madrid, Spain). Milli-Q water (Millipore, Bedford, MA, USA) was used in the preparation of all buffers and solvents. Formic acid (FA) and trifluoroacetic acid (TFA) were purchased from Merck (Germany) and acetonitrile (ACN) from Romil (Cambridge, UK).

Quantification of protein concentrations and Western blot analysis

Western blots were performed following standard procedures. Total cell protein extracts were prepared in a buffer containing 20 mM Tris (pH 7.3), 20 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and standard protease and phosphatase inhibitors. The Bradford (Bio-Rad Laboratories, Madrid, Spain), bicinchoninic acid (Pierce Biotechnology Inc, Rockford, USA), and micro Lowry (Peterson's modification; Sigma–Aldrich) methods measured total protein contents. Antibodies recognizing TBCA have been previously characterized [17]. The anti-β-tubulin antibody used in this work, anti-β-tubulin I+II, was commercially purchased (Sigma–Aldrich). The secondary antibodies used in western blotting were donkey anti-rabbit (GE Healthcare, Europe GmbH) and anti-mouse horseradish peroxidase conjugates (Invitrogen). Immunoreactions were visualized with enhanced chemiluminescence (Perkin-Elmer, Wellesley, USA).

Tubulin and TBCA/p14 protein purification

Microtubule proteins (tubulin and microtubule-associated proteins) were prepared by repeated cycles of assembly/disassembly using a temperature-dependent method [18,19], following previously described procedures [20]. Purified brain tubulin was obtained by phosphocellulose FPLC chromatography (GE Healthcare, Europe GmbH). Microtubule proteins were loaded onto an XK50 column equilibrated with buffer MME (0.1 M MES [pH 6.7], 1 mM $MgCl_2$, and 1 mM EGTA) supplemented with 0.2 mM GTP. Tubulin fractions collected in the breakthrough were eluted at 0.5 mL/min.

TBCA/p14 was purified from a 500 mL culture of *Escherichia coli* BL21:DE3 cells carrying human p14 cDNA cloned into an overexpressing plasmid using a modification of a procedure already described [17,21]. Expression was induced with the addition of 300 μL of 1 M IPTG for 4 h. Cells were pelleted by centrifugation, washed, and stored frozen at −70 °C. Pellets were resuspended in 10 mL of CelLytic BII (Sigma–Aldrich), and incubated with shaking for 10 min at room temperature. After ultracentrifugation at 110,000*g* in a 70.1Ti rotor for 35 min at 4 °C, the supernatant was removed and heated at 75 °C for 1 h. After 10 min