Short sequence-paper

Structure of *Tetrahymena CCTθ* gene and its expression under colchicine treatment[1]

Célia Domingues [a], Helena Soares [a,b], Claudina Rodrigues-Pousada [a], Luisa Cyrne [a,c,*]

[a] *Instituto Gulbenkian de Ciência, Apartado 14, P-2781 Oeiras, Portugal*
[b] *Escola Superior de Tecnologia de Saúde de Lisboa, 1700 Lisbon, Portugal*
[c] *Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisbon, Portugal*

Abstract

We report here the cloning and the characterization of the *Tetrahymena pyriformis* chaperonin-containing-TCP1 theta gene (TpCCTθ), an orthologue of the mouse chaperonin gene CCTθ. TpCCTθ gene is interrupted by eight introns, ranging in size between 91 and 419 nucleotides, and encodes a protein consisting of 540 amino acid residues (59.1 kDa), with a putative pI of 5.73. The amino acid sequence of TpCCTθ reveals 39.4–46.0% identity with the sequences of *Candida albicans* and mouse CCTθ subunits and 28.0–32.6% identity with the other TpCCT subunits known so far. We have studied the expression of this gene in exponentially growing *Tetrahymena* cells and in cells treated with colchicine for different times. The steady-state levels of CCTθ mRNA rapidly decrease in the first 30 min of colchicine treatment. Interestingly, treatment for subsequent 60 min gives expression levels higher than those found in exponentially growing cells. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: CCTθ-chaperonin gene; Ciliated protozoan; Gene expression; Colchicine

Inside cells the majority of newly synthesized proteins require a class of proteins, generally designated as molecular chaperones, to achieve their correct three-dimensional structure [1]. One of the most studied classes of the molecular chaperones is the chaperonins, which recognize and bind unfolded polypeptides to prevent premature folding and aggregation [2]. Based on sequence homology chaperonins can be grouped in two subfamilies. The first, Hsp60 family, is represented by GroEL in the eubacterial cytoplasm, Rubisco binding protein in chloroplast and its mitochondrial homologue Hsp60 [3]. The second family is represented by TF55, thermosome and the CCT complex, being found in archaebacteria and eukaryotic cytoplasm, respectively [4–6]. The eukaryotic cytosolic chaperonin, named CCT (Chaperonin Containing TCP-1 (*t*-complex polypeptide 1)) is a large hetero-oligomeric complex of about 900 kDa, composed of eight different but related polypeptides, namely CCTα, CCTβ…CCTξ, of apparent molecular mass ranging from 52 to 65 kDa, arranged in two stacked rings. In mouse and yeast the genes encoding the eight CCT-subunit polypeptides have been isolated, whereas several CCT-subunit orthologues were already identified in a variety of organisms ranging from ciliates to human cells [7–9]. Together with co-factors and possibly other components still

* Corresponding author. Fax: +351-1-440-7970;
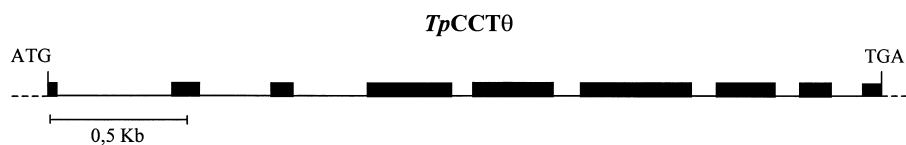E-mail: mcyrne@igc.gulbenkian.pt

*Tp*CCTθ

Fig. 1. Gene structure of the *Tetrahymena* CCTθ subunit. Schematic representation of the structure of the TpCCTθ gene. The boxes indicate exons and introns are shown by lines. ATG and TGA represent the first codon of the coding region and the stop codon, respectively.

remaining to be described, CCT mediates the folding or refolding of tubulins [10,11] and actins [12], and perhaps other unknown proteins. Studies of expression of some of the CCT-subunit genes show that the CCTα is highly expressed in testis [13], embryos of early stages and rapidly growing cells in tissue culture [9,14], suggesting that CCT is involved in cell-cycle regulated events. Several studies indicate a relationship between the chaperonin and the cytoskeleton. Analysis of temperature-sensitive mutants of yeast CCT subunits shows abnormal microtubular structures [15] and disruption of actin microfilaments [16]. In yeast, all the CCT-subunit genes are essential and none of them when overexpressed rescues the mutation in any of the other, suggesting that each of the CCT subunits has distinct function. The assumption that tubulin is one of the major substrates of CCT raises the hypothesis that this chaperonin could be a new factor involved in generating MT function diversity. Indeed, microtubules are dynamic polymers that participate in a wide variety of cellular functions, such as cytoskeleton organization, intracellular transport and cell division, as well as flagellar and ciliary movement [17]. MTs are composed of α- and β-tubulin heterodimers that constitute their basic structural unit. Tubulins have binding sites for a variety of antimitotic agents as, e.g., colchicine. This agent is known to affect the dynamics of microtubule polymerization both in vitro and in vivo [18,19]. It has been described that colchicine binds to the tubulin heterodimer changing its conformation and in consequence reduces the ability of the tubulin to dissociate from the microtubule end, hence impairing the capacity of the microtubule end to accept additional tubulin heterodimers [18].

In the ciliate *Tetrahymena* three members of the CCT-subunit gene family, namely TpCCTα [20], TpCCTγ [8] and TpCCTη [21], have already been cloned and characterized. These genes exhibit a co-ordinate response and are up-regulated with the tubulin genes during cilia regeneration and during active cell division, indicating a possible role of these subunits in these processes.

In the present work another member of the *Tetrahymena* CCT gene family, TpCCTθ, was characterized, and its expression was studied in reciliating cells and cells treated with colchicine at different times.

Degenerate primers were designed taking into account the sequence corresponding to the amino acid sequence conserved among all CCT subunits NGATI (CCTnd) and the specific sequence DDIERAV/IV (CCT8c) present in all CCTθ subunits so far known (CCTnd: 5′-GCCTCTAGAAAYGAYGGTGCYA-CYATY-3′ and CCT8c: 5′-CRAYRGCTCTTTC-RATRTCRTC-3′). These primers were used to amplify *Tetrahymena pyriformis* genomic DNA usingpolymerase chain reaction (PCR) cycling described previously [21]. The PCR products with 0.75 and 1.5 kb were cloned into pUC19 and sequenced. Given that the 1.5 kb product encodes the predicted amino acid sequence sharing a significant similarity with its mouse counterpart [22], the recombinant plasmid was named pCCTθ5. The fragment cloned in pCCTθ5 was labelled using the Megaprimer DNA labelling system and [α-^{32}P]dATP (3000 Ci/mmol) (Amersham) following the manufacturer's instructions, and then used to screen a *T. pyriformis* Sau 3AI genomic library and followed as essentially described in [8]. The DNA of one of the positive phage, named Tpλ6CCTθ, was analysed by restriction mapping following by subcloning the restriction fragments into pUC19. In order to sequence these fragments, sequential deletions were generated using a double-stranded Nested Detection kit (Pharmacia). Sequencing was performed on denatured plasmid DNA using a T7 sequencing kit (Pharmacia) according to the supplier's instructions. Sequence analysis was carried out using the GCG package

(1991) (Genetics Computer Group) and the program DNASIS 5.0 (Hitachi-LKB).

Sequence analysis of the TpCCTθ subunit gene has shown that the coding region of this gene is interrupted by eight introns, as shown in Fig. 1. The introns are always flanked by the invariant dinucleotides GT and AG. TpCCTθ introns range in size between 91 and 419 nucleotides, which is similar to that found for the other TpCCT genes so far characterized. However, this gene contains one or two additional introns when compared to those of TpCCTη, TpCCTα and TpCCTγ genes, respectively [20]. The TpCCTθ gene encodes a protein consisting of 540 amino acid residues, with a predicted molecular mass of about 59.1 kDa and a putative pI of 5.73. The predicted TpCCTθ polypeptide has an identical size to CCTθ subunits from distinct organisms so far studied (*Tp* 540, *Sc* 568, *Sp* 546, *Ca* 546, *Mm* 548 and *Hh* 548) with the exception for *Saccharomyces cerevisiae* CCTθ subunit, which has 28 extra amino acid residues. The amino acid sequence comparison among CCTθ subunits from different species reveals a high degree of sequence similarity: 68.7% with *S. cerevisiae* (P47079), 68.8% with *Schizosaccharomyces pombe* (P78921), 63.8% with *Candida albicans* (P47828) and 69.9% with mouse and humans [22]; and a lower degree of sequence identity, with 41.9%, 42.3%, 39.4% and 46.0%, respectively. When this type of analysis is limited to *Tetrahymena* CCT subunits the degree of sequence identity and similarity decreases. Indeed a similarity of 56.9%, 55.3% and 53.2% was obtained when compared with the CCTα, CCTγ and CCTη subunits, whereas the identity values were 32.6%, 28.0% and 29.1%, respectively. These results support the idea that although the different CCT subunits are structurally related, they are more conserved interspecies than between intraspecies. Fig. 2 shows the alignment of the predicted amino acid sequence of *Tetrahymena* TpCCTθ and those of known CCTθ subunits from other organisms. This alignment reveals three conserved regions between all CCT subunits, as well as with other members of the chaperonin family [14]: the motifs TNDGATIL (**TSDAATIM**) (positions 64–71), GDGTTSV (**GDATNLV**) (positions 95–102) and V(P/A)GGG (**AAGAG**) (positions 408–412). Interestingly, a highly conserved region DDIER-AIDDGVN (positions 385–396) that is not shared

by the other CCT subunits and seems to be exclusively present in CCTθ subunits. It is possible that this sequence may be associated to a specific function of the CCTθ subunits inside the hetero-oligomeric complex of the chaperonins.

Previously we were able to show that *T. pyriformis* contains the hetero-oligomeric complex CCT and to assign in the two-dimensional (2-D) gel, using a set of polyclonal antibodies, the protein spots to the CCTα, CCTβ, CCTγ and CCTη [20]. In order to identify the CCTθ subunit in the 2-D gel, we have compared the putative pI values of the distinct TpCCT subunits so far known (see Table 1). Taking into account these results it is plausible to propose that spot P6 in the 2-D gel of *Tetrahymena* CCT (see Fig. 3) corresponds to TpCCTθ and CCTε is P5 as it has the most acidic pI. Moreover, Soares et al. [24] have observed that UM-1 antibody strongly recognize Hsp60, TpCCTβ, TpCCTγ, TpCCTη and P5, whereas TpCCTα, P6 and P7 spots were weakly recognized. In fact, UM-1 antibody was prepared against the chaperonin consensus motif which is highly conserved between all chaperonin sequences, that seems to be involved in ATP binding and hydrolysis [23]. When a comparison between the amino acid sequence used to produce the UM-1 antibody (**QDDEVGDGTTSVV**) and the corresponding sequence in TpCCTθ (**QENECGDATNLVI**) was done, we observe that these sequences only share six identical amino acids residues out of 13. This poor similarity can explain the weak signal for spot P6 (only visible after longer exposures) obtained when UM-1 antibody is used. It is worthwhile noting that CCTθ from mouse testis is also weakly recognized by UM-1 antibody [23], a fact that reinforces the idea that the UM1 antibody has different affinities for the distinct CCT subunits.

Total cytoplasmic RNAs from exponentially growing cells, cells recovering their cilia and cells treated with colchicine (4 mg/ml) at different times were ex-

Table 1
pI values predicted from the sequence analysis

	TpCCTα	TpCCTη	TpCCTγ	TpCCTθ	TpCCTε
pI	6.90	6.44	6.38	5.73	5.11

Data sources: TpCCTα [20], TpCCTη [22], TpCCTγ [22], TpCCTε (unpublished results).

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          1                                                              60
TpCCTθ    ..MSLQSAYG INSLLKEGHR HFSGMEEALL KNINACKEIS NMTKTSLGPN GMKKMVINHL
ScCCTθ    MSLR PQNPN .AG F Q YN SY NADGQII  S A IR LH Q CL  M  C  RN IIV
SpCCTθ    MALRVPK S .PQ FR  Y  IMQ V D VI R C  IR L  EI R        KN I V
CaCCTθ    MSLK PQ PN .SG F Q YS S  NADG II R VE VR  A SILL  M  S  RN IIV K
MmCCTθ    MALHVPK P  FAQM  D AK    L   VY R  Q    LA QT R AY     N      R
HsCCTθ    MALHVPK P  FAQM    AK    L   VY R  Q    LA QT R AY K   N

          61                                                            120
TpCCTθ    DKIFVTSDAA TIMQEMEVQH PAAKMIVMAS KMQENECGDA TNLVIALAGE LLSQAESLIK
ScCCTθ    G  II N    MLR LDIV   V  VL    T EQ KIDM  G     MI     NVS K  S
SpCCTθ    QQT L N     IR L  I      LV D T QQ     L   A F  VFS A  LK  NM R
CaCCTθ    G  K I N   MLN L IV  VV ILIQ   Q  F M  N     I   F NV  K LT
MmCCTθ    E  L  N    LR L         H   Q V  G   F LVF  A   EL  E LR
HsCCTθ    E  L  N    LR L         H   Q V  G   F LVF  A   EL  E LR

          121                                                           180
TpCCTθ    MGLHPSQIIT GYEQALKAAI ELLNTLSVFT VEDPTNLEQV SKALKASLSS KLIHHADFFA
ScCCTθ         SAVE Q    NM R FTL KE DEMV GE IT KNDKNEL L MI PVI    KYGSE ILS
SpCCTθ         T LE AK    M  SHTM  V EEICADK I TVESEKEL I   IRTCI   QYGNE  LS
CaCCTθ    L  NV E  Q   FNL N FVM KT DE V EK    SFE..TDL L  V PVIAA  QYGVE TI
MmCCTθ    I  SV EV S     I C K H  I PE VCCS AKNLRDVDE   SL RT IM    QYGSET L
HsCCTθ    I  SV EV E     I CRK H  I PN VCCS AKNLRDIDE   SL RT IM    QYGNEV L

          181                                                           240
TpCCTθ    QIVAQACINS KPENDDT... ..FDLEFVRV AKILGASIED SYVQQGLIIT RNAEGSITRV
ScCCTθ    EL SE VSHV L VAQQAGEI PY NVDSI  V  M G LSN  T IK MVFN  EP  HVKSL
SpCCTθ    DL  K ILTV L KDPSK... .. NVDNI  V  M S LYN  Q VK MVFP  EP  TV  S
CaCCTθ    KL VD VALV MKNGS..... .. NVDNI  V VM   LSQ  Q VK MVFP  EP  TVK..
MmCCTθ    KLI    VSI F .DSGN... .. NVDNI  C    SG YS  S LH MVFK  KET  DV S
HsCCTθ    KLI    VSI F .DSGH... .. NVDNI  C    SG SS  S LH MVFK  KET  DV S

          241                                                           300
TpCCTθ    SNPK...VAV YSCPLDTQQA ETKGTVLIQN AKELLNYTKS EESHAESIVK KIADSGINLI
ScCCTθ     ED  HK    FT   IANT         LH  Q M DFS G    KQIDAMM  E   M VEC
SpCCTθ    KEA ...    F    IS T         LH  Q M DFS G    NLI  HI  E Y A VRVV
CaCCTθ    NADQIQSCR  QPHRYFHHR NQRYSCSSTM PRKC ISPRA KNNSWT CAR   STIQ LRWL
MmCCTθ    KDA ...I      F GMIT        KT  E  M FS G    NLMDAQ  A  GT A V
HsCCTθ    KDA ...I      F GMIT        KT  E  M FS G    NLMDAQ  A   T A VV

          301                                                           360
TpCCTθ    IAGGSISEIV LHYVEKYKMM IVKCQSKFEL KRICKALGAC PVARLDAPNP EEIGFCDSAS
ScCCTθ    V  AGVG LA    LNR GIL VL VP    R L RVC  T  LP  G  T   L LVETVK
SpCCTθ    VTS NVNDL    LNRFEIL VIRVP    R L RVV  T  L  MGV M   M SV VVE
CaCCTθ    LP S VG LA    LN  GIL VLRVP    D R  QVC  T  LP  G  T D M EI IIE
MmCCTθ    VT  KVAD A    AN  NI  L RLN  WD R L  TV  T ALPK TP VQ   M H   VY
HsCCTθ    VT  KVADMA    AN  NI  L RLN  WD R L  TV  T ALP  TP VL   M H   VY

          361                                                           420
TpCCTθ    VEEIGSQKVT IIKKEN.QDC KLNTIILRGS TINFLDDIER AIDDGVNVYR CLLK..DGKF
ScCCTθ    TM   GDR   VF Q QGEIS RTS       A Q N          AAVK G M PSG  L
SpCCTθ    TI   GDR   VFRQVE.DIT RTA  V    A KTY           IVK A V .. NRL
CaCCTθ    TK   GDR   FRQDESS.S RTA  V    A Q N           SIK G I .. NRL
MmCCTθ    LS V DTQ V VF H K.E G AIS  V      D LM     V    TFK V TR.. KRL
HsCCTθ    LS V DTQ V VF H K.E G AIS  V      D LM     V    TFK V TR.. KRL

          421                                                           480
TpCCTθ    AAGAGATEAV LSHKLQQQAK SLEDLSQYAF NRFALSFEII PRILSDNAGL NSNEIIPKLN
ScCCTθ    LP      IE ISRITKYGE RTPG L L I KQ  VA  VV    T AET    DV  VL N Y
SpCCTθ    IF      SDMQ CIR ISVGE KTPGIY H I KQYGEA  VV    TI E    DPTDV S  Y
CaCCTθ    LP     V IE MKRITAY.Q  TPG L L I KS  KA  V     V AETS H D S  LS  H
MmCCTθ    VP G    IE AKQITSYGE TCPG E    I KK  EA  A     A AE S V KA  V S  Y
HsCCTθ    VP G    IE AKQITSYGE TCPG E    I KK  EA  A     A AE S V KA  V S  Y
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         481                                                         540
TpCCTθ   TA.....NKE E.......SY GIDIENG.TI RKSSELAVYD HLQSKIWAVR LAADAAITIL
ScCCTθ   A HNVTEPGA VKTDHLYKGV D  G SDEGV KDIR ENI   M AT KF IN V TE   T V
SpCCTθ   A HHKENGES I.......GV DVEC  D.GT LDAK AGIF  V LA KS I     TETVL V
CaCCTθ   A H..AEDTG LRA.....GI D  SG..... .EVADT  L  I AT KS ID    V  TN
MmCCTθ   SVHQ.EG N V.......GL D EA VP.AV  KDML ASIL  TYLG Y  IK    TN   V V
HsCCTθ   AVHQ.EG N V.......GL D EA VP.AV  KDML AGIL  TYLG Y  IK    TN   V V

         541                                   575
TpCCTθ   KVDQIVIAKQ AGGPKMNQQ. .QGHWDEE.. .....
ScCCTθ   SI    IM K      RAP GP RP N  Q D. .....
SpCCTθ   N   V MS P        PPGP. .NP  DD.. .....
CaCCTθ   SI    IM R      V P  P RP N  QTTE RVPFV
MmCCTθ   R     IM P        PPSG. .KKD DDQN D....
HsCCTθ   R     IM P        PPSG. .KKD DDQN D....
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Fig. 2. Alignment and comparison of *Tetrahymena* TpCCTθ deduced amino acid sequence with CCTθ from other species. The deduced amino acid sequence of the *T. pyriformis* CCTθ polypeptide was aligned and compared with those of CCTθ from other organisms (accession numbers in the EMBL database are indicated): *Saccharomyces cerevisiae* (ScCCTθ – P47079), *Schizosaccharomyces pombe* (SpCCTθ – P78921), *Candida albicans* (CaCCTθ – P47828), *Mus musculus* (MmCCCθ – P42932) and *Homo sapiens* (HsCCTθ – P50990). Gaps have been inserted when required to maximize the alignment and are represented by dots. Sequences conserved between almost all chaperonins, including the traditional chaperonins, are underlined. Sequence exclusively conserved between CCTθ subunit is double-underlined.

tracted from *T. pyriformis* cells as reported by Soares et al. [24]. RNAs were subjected to Northern blot analysis as described by Sambrook et al. [25] and the membranes were incubated with pCCTθ5 1.5 kb fragment as probe in RNA hybridization buffer at 35°C [21]. The results show that TpCCTθ is expressed coordinately with the other TpCCT subunit genes (TpCCTα, TpCCTγ and TpCCTη) as well as

with tubulin genes during cilia regeneration (results not shown) [8,20,21]. These results indicate that at least these four subunits are required for the function of the chaperonin during the biogenesis of the new cilia. We have also studied the expression of TpCCTθ in cells treated with a microtubule depolymerizing agent, colchicine (4 mg/ml). Results in Fig. 4a illustrate the hybridization pattern of the mRNA levels from exponentially growing cells and cells treated for different times with colchicine. As one can observe, TpCCTθ produces a unique transcript with about 2.1 kb during the whole period of colchi-
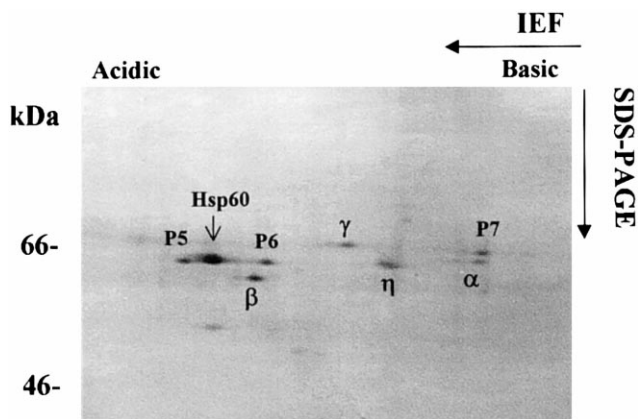

Fig. 3. Two-dimensional isoelectric focusing (IEF) and SDS–PAGE analysis of *Tetrahymena* CCT from exponentially growing cells. Protein extracts containing CCT particles isolated from exponentially growing *Tetrahymena* cells by sucrose-gradient fractionation followed by ATP-affinity column chromatography were resolved by two-dimensional electrophoresis and detected by silver staining. The subunits TpCCTα, TpCCTη, TpCCTγ and TpCCTβ where identified as described in [20].
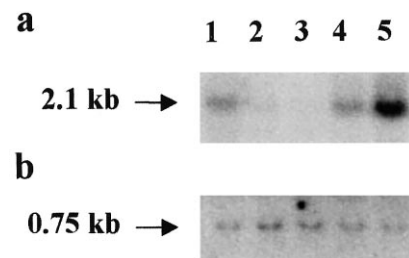

Fig. 4. TpCCTθ mRNA levels in *Tetrahymena* cells treated with colchicine. Total cytoplasmic RNA (30 μg) from exponentially growing cells (lane 1) and from cells treated with colchicine (final concentration 4 mg/ml) after 15 min (lane 2), 30 min (lane 3), 60 min (lane 4) and 90 min (lane 5), was analysed in 1.5% agarose formaldehyde gels, transferred onto nitrocellulose filters and hybridized (a) with pCCTθ5 1.5 kb fragment as probe and (b) with the 239 bp *Pst*I–*Sph*I DNA fragment from pTU11 [26].

cine treatment, as well as in control cells. The amount of the steady-state population of the CCTθ mRNA decreases rapidly until 30 min of colchicine treatment as compared to control cells. However, the amount of the steady-state population of this transcript significantly increases after 60 min, reaching levels higher than those found in exponentially growing cells for up to 90 min of drug treatment. Densitometric quantification of the relevant bands indicates a 1.3- and 3.5-fold increase of the levels at 60 and 90 min of treated cells, respectively, compared to exponentially growing cells. As a loading and hybridization control we used the 239 bp *Pst*I–*Sph*I DNA fragment isolated from the ubiquitin fusion gene as probe in the same RNA blot [26]. The results obtained (Fig. 4b) show that the respective mRNA levels do not change during colchicine treatment.

The fact that colchicine binds specifically to the tubulin heterodimer, modifying the exchange rate of tubulin at microtubule ends, suggests that the TpCCTθ gene is able to change its pattern of expression in response to an alteration of microtubule polymerization. Experiments are in progress with the aim of elucidating the potential meaning of the colchicine effect.

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