The eukaryote chaperonin CCT is a cold shock protein in *Saccharomyces cerevisiae*

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Abstract The eukaryotic Hsp60 cytoplasmic chaperonin CCT (chaperonin containing the T-complex polypeptide-1) is essential for growth in budding yeast, and mutations in individual CCT subunits have been shown to affect assembly of tubulin and actin. The present research focused mainly on the expression of the CCT subunits, CCT α and CCT β , in yeast (*Saccharomyces cerevisiae*). Previous studies showed that, unlike most other chaperones, CCT in yeast does not undergo induction following heat shock. In this study, messenger ribonucleic acid (mRNA) and protein levels of CCT subunits following exposure to low temperatures, were examined. The Northern blot analysis indicated a 3- to 4-fold increase in mRNA levels of CCT α and CCT β genes after cold shock at 4°C. Interestingly, Western blot analysis showed that cold shock induces an increase in the CCT α protein, which is expressed at 10°C, but not at 4°C. Transfer of 4°C cold-shocked cells to 10°C induced a 5-fold increase in the CCT α protein level. By means of fluorescent immunostaining and confocal microscopy, we found CCT α to be localized in the cortex and the cell cytoplasm of *S. cerevisiae*. Localization of CCT α was not affected at low temperatures. Co-localization of CCT and filaments of actin and tubulin was not observed by microscopy. The induction pattern of the CCT α protein suggests that expression of the chaperonin may be primarily important during the recovery from low temperatures and the transition to growth at higher temperatures, as found for other Hsps during the recovery phase from heat shock.

INTRODUCTION

Molecular chaperones are proteins, found in virtually all cells, that are responsible for correct folding and assembly of polypeptides by binding and stabilizing nonnative forms and mediating their folding and assembly (Mori-moto et al 1994; Hartl 1996; Fenton and Horwich 1997). Chaperones are classified into several families according to the size of their subunits. The Hsp60 family, also called chaperonins, are double-ring oligomeric protein complexes composed of 60 kDa subunits, with a central cavity that mediates adenosine triphosphate-dependent folding of polypeptides to their native form. The best-characterized chaperonins are the GroEL group present in bacterial cytosol, mitochondria, and chloroplasts (Fenton and Horwich 1997; Ranson et al 1998). The CCT group includes the archaea Hsp60 and the eukaryotic cytoplasmic chaperonin CCT (chaperonin containing the T-complex

polypeptide-1 [TCP1]) families (Gao et al 1992; Lewis et al 1992).

Unlike other chaperonins, which are composed of 1 (GroEL) or 2 different monomeric subunits (thermosomes, TF55), CCT is made up of 8 or 9 different subunits. Numerous studies have shown that tubulin and actin are the main cellular substrates of the eukaryotic CCT complex (Yaffe et al 1992; Sternlicht et al 1993; Ursic et al 1994; Vinh and Drubin 1994; Farr et al 1997). The CCT subunits are expressed constitutively under normal growth conditions in eukaryotes (Craig 1988). CCT is essential for growth in budding yeast (*Saccharomyces cerevisiae*), and mutations in CCT subunits adversely affect the assembly of tubulin and actin filaments (Ursic and Culbertson 1991; Chen et al 1994; Miklos et al 1994; Ursic et al 1994; Vinh and Drubin 1994; Stoldt et al 1996).

GroEL, the Hsp60 chaperonin of prokaryotes, and archaeobacterial Hsp60 are induced by heat and other stress factors. Initially, CCT was found not to be induced by heat shock or heavy metals in yeast (Ursic and Culbertson 1992). Recently, a number of studies provide evidence of

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induction of CCT by heat shock or chemical stress factors in mammalian cells and in the ciliate of *Oxytricha granulifera* (Shena et al 1996; Palmedo et al 1997; Yokota et al 2000). CCT promoters have also been shown to contain heat shock element sequences for binding heat shock factors in animal cells (Kubota et al 1999a). *Tetrahymena pyriformis* gives a higher level of expression of CCT θ after treatment with colchicine than those found in exponentially growing cells (Domingues et al 1999).

In response to alkylating agents, CCT8 was induced about 4.9-fold in *S. cerevisiae* (Jelinsky and Samson 1999). In cultured animal cells, CCT expression is strongly up-regulated during cell growth, especially from G1/S transition to early S phase, and is primarily controlled at the messenger ribonucleic acid (mRNA) level (Kubota et al 1999b; Yokota et al 1999). In the present study, we show that cold shock (4°C) can induce CCT transcription in *S. cerevisiae*.

MATERIALS AND METHODS

Yeast strains and growth medium

The yeast strain used was YPH499—MATa lys2-801 ade2-101 his-200 trp1-63 ura3-52 leu2-1 (Sikorski and Hieter 1989). Commercial baker's yeast was obtained from PACA (Bat Yam, Israel). The growth media were enriched medium yeast extract, pertone, dextrose (YPD) and synthetic minimal dextrose medium (SD) (Difco, USA).

Cold shock treatment

Cold-shocked cultures were prepared by transferring aliquots of exponentially growing cultures at 30°C to a temperature-controlled bath shaker at the indicated temperature.

Probes and oligonucleotides

The CCT α probe was made using the *PvuII* fragment from the coding sequence of the yeast TCP1 gene. The CCT β probe was excised from the coding sequence of the gene with *BglIII*. The actin probe was cut from the gene (ACT1) with *XhoI* and *HindIII*. The tubulin and 28S-rRNA probes were made by polymerase chain reaction with primers from the coding region of the genes.

Oligonucleotides used were as follows:

Tubulin: FFUb-809-5'-TCAAGGAGCTTTTCCATCCAG-3'
RTUb1-1580-5'-AGTGGACGAAAGCACGTT-
TGG-3'

28S rRNA: P51-5'-GGCAAAGCTCAAATTTGAA-3'
P463-5'-CCACCAAAGCTGATGCTGG-3'

Antibodies

Primary antibodies used were rat anti-mouse CCT α (CTA-191; Stressgen, Victoria, BC, Canada), mouse anti-TUB α (T-9026; Sigma, Rehovot, Israel), and goat anti-actin polyclonal IgG (I-19, SC-1616; Santa Cruz Biotechnology, Santa Cruz, CA).

Secondary antibodies used were horseradish peroxidase-conjugated donkey anti-goat IgG (705-035-147; Jackson ImmunoResearch Laboratories) and goat anti-rat IgG (112-035-062; Jackson ImmunoResearch Laboratories).

Secondary antibodies used for immunofluorescent staining were: FITC-conjugated donkey anti-rat IgG (712-095-153, Jackson ImmunoResearch Laboratories), and Texas red-conjugated goat anti-mouse IgG (115-015-146, Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Northern blotting and quantitation

Northern blotting was performed with total RNA isolated from 50 mL of yeast cell culture grown under specified conditions at indicated time points. The samples were harvested by centrifugation for 5 minutes at 3000 rpm, washed with water, and cell pellets were immediately frozen in liquid nitrogen. RNA was extracted by phenol-chloroform methods (Carlson and Botstein 1982). Fifteen micrograms of total RNA per lane were separated in 1% agarose-formaldehyde gel electrophoresis, transferred to nitrocellulose membranes, and hybridized overnight at 42°C with random-primer ³²P-labeled probe using the NEBlot™ kit (New England BioLabs, Beverly, MA, USA). After stringent washing (0.5% sodium dodecyl sulfate [SDS], 0.3× standard saline citrate, 30 minutes, 55°C), Northern blots were visualized and quantitated by PhosphorImager (Fuji, Edison, NJ, USA). Differences in the amount of RNA in the gels were normalized according to the hybridization intensity of the 28S-rRNA probe in each lane.

Western blotting and quantitation

Aliquots of 50 mL were removed from yeast cell cultures at the indicated times. The samples were immediately centrifuged for 5 minutes at 3000 rpm to pellet the cells. The cells were washed with water, and the cell pellets were quickly frozen in liquid nitrogen. The cells were lysed in ice-cold buffer containing 20 mM Tris buffer (pH 7.4), 20 mM KCl, 2 mM MgCl₂, and 1 mM ethylenediaminetetraacetic acid (Melki et al 1994), with glass beads (425–600 μ m; Sigma), by 4 cycles of 5-minute vortexing followed by 1 minute on ice. The supernatant was transferred to a new tube, and protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA, USA), using bovine serum albumin as a standard.

Fig 1. Levels of messenger ribonucleic acid (mRNA) transcripts of CCT α , CCT β , actin, and 28S ribosomal RNA (rRNA) in response to cold shock at 4°C. (A) Northern blots. (B) Densitometric quantification relative to 28S rRNA. YPH499 cells were grown at 30°C to early logarithmic phase in minimal medium, and then transferred to 4°C. Samples were taken before transfer to low temperature and after 6 hours at low temperature. The culture was then transferred back to 30°C. Samples were taken at intervals during the recovery phase. RNA was isolated from the samples, and 15 μ g RNA from each sample was run on agarose gel, and the Northern blot analyzed with the probes (a) CCT α , (b) CCT β , (c) ACT1, and (d) 28S rRNA. Lane 1: 0' after transfer from 30°C to 4°C; lane 2: 6 hours after transfer; lanes 3–7: 15', 30', 1 hour, 2 hours, and 3 hours following transfer from 4°C back to 30°C.

The extracted proteins were separated by SDS–polyacrylamide gel electrophoresis using 10% polyacrylamide gels, and blotted onto nitrocellulose membrane. After blocking with 5% skim milk in Tris-buffer saline with 0.1% Tween 20 (TBST) for 3 hours, the filters were incubated with the appropriate first antibody overnight. The filters were washed 3 times with TBST and incubated for 1.5 hours with horseradish peroxidase–conjugated secondary antibody, and washed twice with TBS. The proteins specifically recognized by the antibodies were visualized by the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK) and quantitated using RFLPscan software (Scanalytics, Massachusetts, USA). Differences in the amount of protein present in each lane of the gels were corrected by normalization to the amount of actin in each lane.

Immunofluorescent staining of yeast cells

Immunofluorescent staining and microscopy were performed as described in Kilmartin and Adams (1984), with

some modifications. Aliquots of 20-mL cell culture were removed at the indicated times during cold shock treatment and fixed with 5% formaldehyde for 30 minutes at room temperature. Spheroplasts were processed for 30–60 minutes with zymolyse and β -mercaptoethanol in 0.1 M phosphate, pH 6.5, and 1.2 M sorbitol. The slides were incubated overnight with primary antibodies at room temperature, followed by incubation with secondary antibodies for 2 hours at room temperature. The slides were examined using a Bio-Rad confocal microscope.

RESULTS

In order to study the response of CCT to low temperatures, the mRNA levels of CCT genes were analyzed in reaction to cold stress. Following the transfer of yeast cells from 30°C to 4°C, a gradual increase in the levels of CCT α and CCT β mRNA was observed, reaching a peak at 6 hours (data not shown). Figure 1A shows Northern blots of yeast CCT α , CCT β , ACT1, and 28S rRNA in response to cold shock—6 hours after transfer of the yeast culture from 30°C to 4°C. Both CCT α and CCT β showed a similar sharp increase in mRNA levels at 4°C. A gradual return to the previous low levels was observed during the 3-hour period following transfer back to 30°C. The mRNA levels of the control sequences, ACT1 and 28S rRNA, were stable over the entire period. Densitometric quantification of the blot relative to 28S rRNA is shown in Figure 1B, giving the ratio of CCT α and CCT β to 28S rRNA. A 3-fold and 2.5-fold increase in the levels of CCT α and CCT β mRNA, respectively, is apparent at 6 hours in cold shock. Induction of CCT α and CCT β was not observed in a cold shock experiment when yeast cells were transferred from 30°C to 15°C, but only in a transfer to 4°C (data not shown).

In order to examine the dependence of the induction response on strain and medium, cold shock induction of CCT α and CCT β was examined in a laboratory strain (YPH499) and in a commercial baking yeast grown in minimal (SD) and enriched (YPD) medium. All 4 strain-by-medium combinations showed induction of CCT α and CCT β at 6 hours after transfer from 30°C to 4°C. A comparable increase was not found for the control 28S-rRNA sequence (Fig 2A). Densitometric quantification of CCT α and CCT β hybridization blots relative to 28S rRNA is shown in Figure 2B. A 3- to 4-fold (CCT α) and 3- to 6-fold (CCT β) increase in RNA levels after 6 hours at 4°C is apparent.

In contrast to the induction observed upon transfer of yeast cells from 30°C to 4°C, the transfer from 30°C to 0°C did not result in the induction of CCT α and CCT β (Fig 3). This shows that the mRNA induction is limited to temperatures above freezing, and that the observed induction

Fig 2. Levels of messenger ribonucleic acid (mRNA) transcripts of CCT α , CCT β , and 28S ribosomal RNA (rRNA) in response to cold shock at 4°C, according to strain (YPH499 or baker's yeast) and medium (SD or YPD). (A) Northern blots. (B) Densitometric quantification for CCT α (I) and CCT β (II), relative to 28S rRNA. Cultures were grown at 30°C to early logarithmic phase in minimal medium, and then transferred to 4°C. Samples were taken before transfer to low temperature and after 6 hours at low temperature. RNA was isolated from the samples, and 15 μ g RNA from each sample was run on agarose gel, and the Northern blot analyzed with the probes (a) CCT α , (b) CCT β , and (c) 28S rRNA. Lanes 1–4: 30°C; lanes 5–8: 6 hours after transfer to 4°C; lanes 1 and 5: YPH499 in SD medium; lanes 2 and 6: YPH499 in YPD medium; lanes 3 and 7: baker's yeast in SD medium; lanes 4 and 8: baker's yeast in YPD medium.

Fig 3. Northern blots showing levels of messenger ribonucleic acid (mRNA) transcripts of CCT α , CCT β , ACT1, tubulin, and 28S ribosomal RNA (rRNA) in response to cold shock at 0°C. YPH499 cells were grown at 30°C to early logarithmic phase in enriched medium, and then transferred to 0°C. Samples were taken before transfer to low temperature and after 6 hours at low temperature. The culture was then transferred back to 30°C. Samples were taken at intervals during the recovery phase. RNA was isolated from the samples, and 15 μ g RNA from each sample was run on agarose gel, and the Northern blot analyzed with the probes (a) CCT α , (b) CCT β , (c) ACT1, (d) TUB1, and (e) 28S rRNA. Lane 1: 0 minute before transfer from 30°C to 0°C; lane 2: 6 hours after transfer to 0°C; lanes 3–5, 15 minutes, 30 minutes, and 1 hour after transfer from 0°C back to 30°C.

response is not because of the technical reasons associated with the low-temperature extraction of mRNA.

In order to determine whether the cold shock induction of CCT α is expressed at the protein level, Western blots were analyzed for protein levels 6 hours after transfer

from 30°C to 4°C. Interestingly, CCT α protein levels did not show any increase (data not shown, but see Fig 4). Because it was possible that at 4°C, translation in general was greatly reduced, we examined CCT α protein levels by transferring the cultures to 10°C or 30°C after 6 hours at 4°C. Indeed, higher levels of the CCT α subunit were clearly observed after this treatment (Fig 4A). This figure also shows low protein expression after 6 hours at 4°C. After transferring to 10°C, maximal protein levels were reached at 15–20 minutes, about 5-fold the level at 4°C or the uninduced level at 30°C. At 30°C, high levels (5 times the uninduced levels) were reached 5 minutes after transfer from 4°C (Fig 4B). Interestingly, when CCT α protein levels were examined after direct transfer from 30°C to 10°C, an increase of almost 3-fold was observed after 6 hours (Fig 5). This may represent a direct effect on translation, CCT α subunit stability, or a slight induction of CCT α mRNA that was not detected by Northern analysis in our system.

Because CCT α was induced at 4°C, it was of interest to compare the cellular distribution of the CCT α protein complex at the studied temperatures. Yeast cells were analyzed in immunofluorescent microscopy for cellular localization of CCT α and TUB α at 30°C, 10°C, or 4°C. Whereas TUB α shows the expected dispersed distribution at 4°C as compared with the filamentous appearance at 30°C and 10°C, CCT α appears to retain the same cortical location pattern at all 3 temperatures (data not shown).

Fig 4. Levels of CCT α protein in response to cold shock at 4°C. (A) Western blots, CCT α . (B) Densitometric quantification relative to actin. YPH499 cells were grown at 30°C to early logarithmic phase in enriched medium, and then transferred to 4°C. Samples were taken before transfer to low temperature and after 6 hours at 4°C. Cultures were then transferred to 10°C and 30°C, and samples taken at intervals during the recovery phase. Proteins were extracted from the samples, and equal protein quantities of each sample were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by Western blot procedures with anti-CCT α and actin antibodies.

Fig 5. Levels of CCT α protein in response to cold shock at 10°C. (A) Western blots. (B) Densitometric quantification relative to actin. YPH499 cells were grown at 30°C to early logarithmic phase in enriched medium, and then transferred to 10°C. Samples were taken immediately after transfer to low temperature and at intervals as shown in the figure. Proteins were extracted from the samples, and equal protein quantities of each sample were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by Western blot procedures with anti-CCT α and actin antibodies.

DISCUSSION

In the present study, we show by Northern blot analysis that 2 genes of the CCT complex, CCT α and CCT β , are induced by cold shock in *S. cerevisiae*. This result was obtained in both a laboratory strain and a commercial baking yeast, and under growth in either a minimal or an enriched medium. We further show that cold shock induces an increase in the CCT α protein, which is expressed at 10°C, but not at 4°C. The levels of induction (3- to 5-fold at the level of the gene, 5- to 6-fold at the level of the protein) are comparable to those found for other cold shock proteins in yeast (Kondo and Inouye 1991; Kondo et al 1992; Kowalski et al 1995). This is in strong contrast to the very high levels (up to 100-fold or more) induced by heat shock in typical Hsps. Other proteins of the CCT complex, particularly as they have different promoters, might show a different behavior pattern than that shown here for CCT α and CCT β .

Previous studies applying moderate temperature shift

(from 30°C to 18°C) using expression arrays of the complete *S. cerevisiae* genome showed cold shock induction of a number of Hsps, including Hsp70, Hsp30, Hsp82, and others (Lashkari et al 1997). In agreement with our results, these studies did not find an increase in the expression of CCT complex mRNA as a consequence of transfer from 30°C to 18°C. It is possible that the expression array analysis of the cold shock effects of transfer from 30°C to 4°C would have uncovered CCT induction.

The CCT complex is rather unique in that a cold shock of 4°C is required for its induction, compared with the more moderate cold shock of 15°C to 18°C required for induction of other cold shock proteins in *S. cerevisiae*. A known factor that changes in a nonlinear manner when temperatures approach 4°C is water activity, which affects the higher-level structure of membranes, proteins, and other macromolecules. Thus, one possibility is that these changes are the specific signals for CCT induction. In any event, the unique behavior of CCT complex mRNAs at 4°C suggests that this temperature should be investigated more widely as an inducing factor in *S. cerevisiae*.

As noted, the cold shock-induced increase in CCT α protein level was found at 10°C, but not at 4°C. Although this may be a result of temperature dependence stability of the protein, we interpret it as a consequence of the very limited translation in *S. cerevisiae* at 4°C (Stapulionis et al 1997). Our hypothesis is that CCT α mRNA transcription is induced at 4°C, and mRNA accumulates in the cell at this temperature, but is expressed as increased protein synthesis only at higher temperatures. This seems plausible when taken in the light of the natural ecology of *S. cerevisiae*, where cold shock at 4°C would tend to be followed by warmer weather. In this case, the induction pattern of the CCT α protein suggests that expression of the chaperonin may be important during the recovery from low temperatures and the transition to growth at higher temperatures, as found for other Hsps during the recovery phase from heat shock (Lindquist 1986).

A large number of studies have shown that there is a close relationship between the CCT complex and tubulin-actin. Mutations in the CCT subunits in *S. cerevisiae* affect the formation of tubulin and actin filaments (Ursic and Culbertson 1991; Ursic et al 1994; Miklos et al 1994). Similarly, screening for mutations affecting filament formation by tubulin and actin uncovered mutants in the CCT proteins (Welch et al 1993; Chen et al 1994; Vinh and Drubin 1994). In vitro studies uncovered the ability of the CCT proteins to induce filament formation of tubulin and actin. Moreover, specific attachment sites for CCT proteins on the tubulin and actin molecules have been identified (Llorca et al 1999; Rommelaere et al 1999). It is striking that both tubulin and actin filaments undergo depolymerization to monomers at 3°C (Joshi et al 1986; Upadhyaya and Strasberg 1999), exposing the sites for CCT

attachment. Because monomers of tubulin and actin are the major substrate for CCT, it is possible that induction of CCT at 4°C is related to the depolymerization of tubulin and actin, and the consequent appearance of their monomers. CCT mRNA would be prepared in anticipation of the recovery phase when temperatures increase and re-formation of tubulin and actin filaments is needed to renew growth. This hypothesis is supported by the finding that treatment of *T. pyriformis* with colchicine induces the expression level of CCT θ (Domingues et al 1999).

Trent et al (1997) raised the provocative hypothesis that in archaeobacteria, CCT filaments may have substituted for tubulin and actin filaments. In the present study, fluorescent visualization of CCT α distribution at 30°C and 10°C, or even at 4°C (at which temperature tubulin and actin filaments undergo depolymerization), did not show clear filament arrangement of the CCT proteins (data not shown). Similar results were obtained by Ursic et al (1994), who studied the overexpression of CCT and showed that it was localized to the cortex. Nevertheless, there was a noticeable granular nature to the CCT immunofluorescent distribution, which may indicate some polymeric structure.

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