Over-Expression Analysis of All Eight Subunits of the Molecular Chaperone CCT in Mammalian Cells Reveals a Novel Function for CCTdelta

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Abstract

Chaperonin containing tailless complex polypeptide 1 (CCT) forms a classical chaperonin barrel structure where two rings of subunits surround a central cavity. Each ring consists of eight distinct subunits, creating a complex binding interface that makes CCT unique among the chaperonins. In addition to acting as a multimeric chaperonin, there is increasing evidence indicating that the CCT subunits, when monomeric, possess additional functions. Here we assess the role of the CCT subunits individually, using a GFP (green fluorescent protein) tagging approach to express each of the subunits in their monomeric form in cultured mammalian cells. Over-expression of CCTdelta, but not the other seven CCT subunits, results in the appearance of numerous protrusions at the cell surface. Two point mutations, one in the apical domain and one in the ATP binding pocket of CCTdelta, that abolish protrusion formation have been identified, consistent with the apical domain containing a novel interaction site that is influenced by the ATPase activity in the equatorial domain. Structured illumination microscopy, together with sub-cellular fractionation, reveals that only the wild-type CCTdelta is associated with the plasma membrane, thus connecting spatial organization with surface protrusion formation. Expression of the equivalent subunit in yeast, GFP-Cct4, rescues growth of the temperature-sensitive strain cct4-1 at the non-permissive temperature, indicative of conserved subunit-specific activities for CCTdelta.

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mammalian fibroblasts and changes in monomeric CCT subunit levels influence cell shape [9,10]. Furthermore, CCTα, CCTγ, CCTδ and CCTθ have been shown to behave as microtubule associated proteins in vitro [11]. In yeast, over-expression of the Cct6 subunit can suppress growth phenotypes caused by both over-expression (Sit4p and Sap155p) and by mutations (e.g., tor2-21) [12]. These properties of Cct6 are subunit specific and require ATPase activity. Interestingly, in *Mycobacterium tuberculosis*, the chaperonin-60 appears to be present as lower oligomeric assemblies that possess ATPase-independent folding activities [13]. Chaperonin subunits have a conserved ATP binding sequence, GDGTT, where mutating D to E in the conserved ATP binding pocket that alter the spatial organization of CCT is situated directly within the ATP-binding motif sequence GDGTT in the equatorial domain. In order to visualize the cellular location of the CCT proteins, we performed structured illumination microscopy (SIM) to provide super-resolution images. GFP-CCTδ clearly localizes to the cell periphery, but GFP-CCTδG357Δ and GFP-CCTδD104E, which do not induce the phenotype, are localized throughout the cytosol (Fig. 3a). Expression of CCTδG357Δ-myc in B16F1 cells also fails to cause the protrusion phenotype (Fig. S1d), while expression of the apical substrate-binding domain alone, GFP-CCTδAD, induces protrusion formation (Fig. S1d). The positions of the conserved mutated residues are highlighted on the crystal structure of the α-subunit of the thermosome chaperonin (Fig. 3b). Further SIM analysis of cells expressing GFP-CCTδ co-stained with TRITC-phalloidin visualized actin bundles

The unique role of CCTδ in the induction of retraction fibers and filopodia-like protrusions

We developed a strategy for the over-expression of CCT subunits while maintaining their monomeric form, by fusing GFP (green fluorescent protein) to the N-termini of CCTα to CCTθ, thereby preventing incorporation into the chaperonin oligomer. This approach is confirmed by sucrose gradient fractionation of transfected BALB 3T3 cell lysates, where all fusion proteins migrate in the sucrose gradient only as monomeric species (Fig. 1a and Fig. S1b). This is in contrast to endogenous subunits that migrate both as components of the chaperonin oligomer and as a pool of monomeric subunits (Fig. 1a and Fig. S1b). Fluorescence microscopy analysis of individual transfections in B16F1 cells, where constructs are expressed at similar levels to one another, reveals that GFP-CCTδ over-expression induces a dramatic change in cell shape, characterized by cells displaying extensive protrusions (Fig. 1b). To determine the level of GFP-CCTδ over-expression, we probed a Western blot with an antibody to CCTδ and we show that, in lysates, the total levels of GFP-CCTδ fusion protein are 1.7-fold higher than endogenous CCTδ and are 6.2-fold higher in the monomer fractions (Fig. 1c). All cells transfected with the GFP-CCTδ fusion display the protrusion phenotype, while none of the other seven CCT subunit transfections show cells with protrusions. These observations were verified by transfection of the GFP-CCT fusion proteins into the fibroblast cell line BALB 3T3, where only GFP-CCTδ induces protrusion formation (Fig. S1c). Expression of CCTδ-myc, which despite being able to oligomerize will increase the CCTδ monomer pool [9], in B16F1 cells also induces the formation of protrusions (Fig. S1d). Proliferation was not affected by expression of GFP-CCTδ as compared to GFP alone, as determined by measuring EdU incorporation into the DNA during S phase (Fig. S2a).

In order to characterize the nature of these cellular protrusions, we utilized time-lapse microscopy to visualize their formation (Fig. 2 and Videos 1 and 2). Both newly formed filopodia, which are actively extending out from the cell periphery (Fig. 2a and Video 1, arrow heads), and retraction fibers (Fig. 2b and Videos 1 and 2) contribute to the protrusion phenotype. The retraction fibers, many of which form branched structures, are formed from retracting lamelipodia and are thus distinct from de novo filopodia.

Treatment of cells expressing GFP-CCTδ with the drug Latrunculin B, which results in actin depolymerization, causes cells to round up, and following removal of the drug, the protrusion phenotype reforms (Fig. 2c). The co-transfection of the dominant-negative Rho GTPase Cdc42 T17N [16] with GFP-CCTδ prevents the formation of the protrusion phenotype (Fig. 2d). These data strongly indicate that actin remodeling is required for the GFP-CCTδ-induced protrusion formation.

Protrusion formation is abrogated by mutations situated in the apical domain and the ATP binding pocket that alter the spatial organization of CCTδ

To evaluate the cct4-1 mutation and the CCTδ ATPase activity in the context of our CCT monomer over-expression approach, we generated GFP-CCTδG357Δ and GFP-CCTδD104E. D104 is situated directly within the ATP-binding motif sequence GDGTT in the equatorial domain. In order to visualize the cellular location of the GFP-CCTδ proteins, we performed structured illumination microscopy (SIM) to provide super-resolution images. GFP-CCTδ clearly localizes to the cell periphery, but GFP-CCTδG357Δ and GFP-CCTδD104E, which do not induce the phenotype, are localized throughout the cytosol (Fig. 3a). Expression of CCTδG357Δ-myc in B16F1 cells also fails to cause the protrusion phenotype (Fig. S1d), while expression of the apical substrate-binding domain alone, GFP-CCTδAD, induces protrusion formation (Fig. S1d). The positions of the conserved mutated residues are highlighted on the crystal structure of the α-subunit of the thermosome chaperonin (Fig. 3b). Further SIM analysis of cells expressing GFP-CCTδ co-stained with TRITC-phalloidin visualized actin bundles
Over-expression analysis of CCT in mammalian cells. (a) Over-expression strategy for mouse CCT subunits in cultured mammalian cells using pEGFP-C1 constructs where positioning of the GFP tag inhibits incorporation into the CCT oligomer. BALB 3T3 cells were transiently transfected using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, cells were detached from dishes by incubation in PSB containing 1 mM ethylenediaminetetraacetic acid, collected by centrifugation at low speed then rinsed once in ice-cold PBS. Cells were lysed by resuspension in ice-cold 50 mM Hepes (pH 7.2) (NaOH), 90 mM KCl, 0.5% (w/v) Igepal and protease inhibitor cocktail (Sigma-Aldrich), then clarified by centrifugation at 7000 rpm in a benchtop micro-centrifuge for 5 min at 4 °C. Lysates (150–250 μl) were loaded onto 5-ml continuous gradients of 10–40% (w/v) sucrose in 50 mM Hepes (pH 7.2) (NaOH). 90 mM KCl and protease inhibitor cocktail (Sigma-Aldrich). Centrifugation was performed using a Beckman ultracentrifuge with a SW55Ti rotor at 85,000g for 18 h at 4 °C. Gradients were then fractionated into 12 equal volume fractions for analysis by SDS-PAGE and Western blotting using anti-GFP (Roche 11814460001), anti-CCTδ clone 8g [6] or anti-CCTη clone η81a [23]. (b) Wide-field fluorescence microscopy of B16F1 cells expressing the GFP-CCT subunit fusions for 24 h reveals that only GFP-CCTδ induces numerous surface protrusions. Cells were grown on glass coverslips (#1.5), which were pre-coated with laminin (Sigma-Aldrich). Cells were rinsed twice in 37 °C PBS then fixed by addition of 37 °C PBS containing 4% (v/v) formaldehyde and incubated for 10 min at room temperature. After washing three times with PBS and once with water, coverslips were mounted using ProlongGold mounting media (Invitrogen). Fluorescence was visualized with a Zeiss Axioplan 2 Imaging microscope using Axiovision acquisition software. The scale bar corresponds to 10 μm. (c) B16F1 cells were transiently transfected with GFP-CCTδ and the lysate was analyzed by sucrose gradient fractionation as in (a). Western blot analysis of the lysate (L) and the fractions from the sucrose gradient using the antibody to CCTδ reveals the relative levels of endogenous and GFP-tagged CCTδ.
running along the centre of some protrusions (Fig. 3c). Analysis of plasma membrane preparations shows that the wild-type (wt) GFP-CCTδ is present both in the soluble fraction and in the plasma membrane pellet, while the two mutants are not detectable in the pellet by Western blotting (Fig. 3d). Localization at the plasma membrane places CCTδ at sites of extensive actin remodeling and signaling, both of which are essential components for changes in morphology at the cell surface, reviewed in Ref. [17]. That the two mutants of CCTδ (G357D and D104E), which do not induce the protrusion phenotype, fail to localize to the cell periphery supports the hypothesis that the accumulation of CCTδ at the plasma membrane is essential for induction of the protrusion phenotype. None of the GFP-CCTδ fusion proteins appeared to be toxic as cells remained attached to the dish 4 days post-transfection (Supplementary 2b).

Although there are no obvious regions of sequence within CCTδ that would provide direct attachment to the lipid bilayer of the plasma membrane, it has not been possible to identify a membrane-associated protein binding partner for CCTδ by protein:protein interaction assays that would confer attachment.
Therefore, the exact manner in which CCTδ accumulation at the plasma membrane occurs is yet to be determined. However, the CCTδ apical domain alone is capable of inducing the protrusion phenotype (Fig. S1d), consistent with attachment being via the apical domain. It is possible therefore that the G357D mutation directly disrupts an interaction site or, alternatively, induces long-range conformational changes.
changes within the apical domain. The G345D mutation in yeast disrupts the allostery of the CCT ATPase, indicating that communications between the ATP binding site in the equatorial domain and the apical substrate-binding domain are affected in the context of the assembled CCT oligomer [8]. GFP-Cct4G345D does not induce the protrusion phenotype, indicating that, in the context of the full-length monomeric CCT subunit, additional contributions that are conferred by the nucleotide state are required. This suggests that the nucleotide cycle of the CCT subunit may regulate its binding activities when monomeric and that the apical domain alone is in an active conformation.

**Conservation of Cct4 monomer activity in yeast**

We then chose *Saccharomyces cerevisiae* as a model system to explore the conservation of CCT monomer function. Plasmids encoding GFP-Cct4, GFP-Cct4G345D and GFP-Cct4D91E (equivalent to D104E in mouse) were generated and their cellular localization was analyzed by fluorescence microscopy (Fig. 4a). The GFP-Cct4 is enriched at the cell periphery and within internal structures, while GFP-Cct4D91E and GFP-Cct4G345D are located throughout the cells, which is consistent with the localization patterns seen in mammalian cells (Fig. 3a). Sucrose gradient fractionation revealed that the GFP-Cct4 and GFP-Cct4D91E were present predominantly in the oligomer fraction, while GFP-Cct4G345D was present over several fractions and did not elute as a distinct peak (Fig. 4b). To determine if the GFP-Cct4 subunits were incorporated into endogenous oligomer and not forming homooligomers as described in Ref. [18], we performed co-immunoprecipitation assays (Fig. 4c). This confirmed that the GFP-Cct4 and GFP-Cct4D91E had been incorporated into the endogenous oligomer but that GFP-Cct4G345D had not. It is important to note that several studies using CCT oligomer purified from yeast use high concentrations of glycerol to maintain the assembly state [1,8,19]. Thus, CCT in yeast may be more dynamic than CCT in mammalian cells and this may enable the GFP-CCT fusion proteins to be sampled for oligomerization by the endogenous subunits more frequently than in mammalian cells.

To address the question of why no monomer peak was seen for the GFP-Cct4 fusion proteins, we used differential centrifugation to compare levels of GFP-Cct4 to endogenous Cct4 in total cell extract and pellets. This revealed that much of the GFP-Cct4 protein is present in the membrane enriched pellets and only very low amounts are present in the lysate that was loaded on the sucrose gradient (Fig. 4d).

It has been reported previously that CCT oligomers harboring the Cct4G345D mutation fold actin in *in vitro* folding assays only 2-fold slower than wt CCT at the non-permissive temperature [20]. This raises the possibility of additional functions of Cct4 contributing to the cct4-1 phenotype. We therefore asked if the GFP-Cct4 proteins could rescue growth of the temperature-sensitive cct4-1 strain at 37 °C. GFP-Cct4 rescues the growth of the cct4-1 strain at the non-permissive temperature (Fig. 4e) whereas over-expression of GFP-Cct4G345D cannot rescue growth. Interestingly, cells expressing GFP-Cct4D91E also grow at the non-permissive temperature and fluorescence microscopy of GFP-Cct4D91E expressed in the cct4-1 strain demonstrates some enrichment at the cell periphery, similar to that seen for GFP-Cct4 in wt yeast (Fig. 4e). When the D91E mutation is present in the endogenous Cct4 and thus also in the CCT oligomer, it is lethal [15,21], presumably because the Cct4 ATPase is essential for CCT oligomer folding activity [21]. Although we cannot exclude that the GFP-Cct4 and GFP-Cct4D91E incorporated into the CCT oligomer could account for the rescue of the growth of cct4-1 at the non-permissive temperature, the fact that GFP-Cct4D91E is able to rescue supports our hypothesis that a monomer function is being compensated for. This is consistent with GFP-Cct4D91E being able to accumulate at the cell periphery in the cct4-1 cells, where it is the only source of a wt Cct4 apical domain sequence.

The mammalian protrusion phenotype is dependent upon ATPase activity, while in yeast Cct4, monomer function appears to be independent of the ATPase activity. This could reflect that the roles of CCTδ and Cct4 are not independent in mammalian cells and yeast. However, the localization of both GFP-CCTδ and GFP-Cct4 to the periphery is suggestive of a similar function being conserved between yeast and mammals. Interestingly, in yeast Cct4 is the most abundant of the CCT subunits [22], an observation that is consistent with functions additional to placement in the CCT oligomer.

In summary, the data presented here reveal a subunit-specific activity of CCTδ that induces a protrusion phenotype when there are increased levels of CCTδ monomer in mammalian cells and that a conserved glycine residue in the apical domain is important for monomer activity. This work establishes the concept of CCT subunits possessing monomer activities in addition to their role as components of the CCT oligomer and illustrates the complexity of the CCT chaperone function.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2015.06.007.

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Fig. 4. GFP-Cct4 and GFP-Cct4\textsuperscript{D91E} rescue growth of yeast strain cct4-1 at 37 °C. (a) Fluorescence microscopy of yeast transformed with GFP-Cct4, GFP-Cct4\textsuperscript{D91E} and GFP-Cct4\textsuperscript{G345D}. GFP-Cct4 shows enrichment at the periphery, while the two mutants are located throughout the cell. (b) Yeast cells were lysed in ice-cold 10 mM Tris (pH 7.8), 150 mM NaCl, 5 mM MgCl\textsubscript{2}, 10% glycerol, 0.5 mM DTT and protease inhibitors (Sigma) using a bead mill, and the resulting lysates clarified by centrifugation (firstly by centrifugation at 5000 rpm in a benchtop micro-centrifuge for 20 s, then at top speed in a benchtop micro-centrifuge for 5 min) and loaded onto 40–10% sucrose gradients containing 10% glycerol and centrifuged using a Beckman ultracentrifuge with a SW55Ti rotor at 85,000g for 22 h at 4 °C. Gradients were then fractionated into 12 equal volume fractions for analysis by SDS-PAGE and Western blotting using either anti-GFP (Roche 11814460001) or an antibody to Cct5 (monoclonal \(\varepsilon\)AD2). While the endogenous Cct5 is present as a component of the oligomer and as monomers, the GFP-Cct4 and GFP-Cct4\textsuperscript{D91E} proteins elute predominantly as components of the oligomer. GFP-Cct4\textsuperscript{G345D} does not show a distinct oligomeric peak. The fractions numbered 1–6 were then analyzed in a co-immunoprecipitation assay. (c) Fractions were mixed with GFP-nanobody beads (Chromotek) and incubated for 1 h on a rotating wheel at 4 °C, then washed three times in lysis buffer. Proteins were eluted with SDS-PAGE sample buffer, resolved by SDS-PAGE and compared to a sample of CCT oligomer prepared by acetone precipitation of the 20S sucrose fraction of a gradient containing non-GFP-Cct4. This indicated that, unlike GFP-Cct4 and GFP-Cct4\textsuperscript{D91E}, GFP-Cct4\textsuperscript{G345D} was not incorporated into endogenous oligomers. (d) Yeast expressing GFP-Cct4 were analyzed as total cell extract (Total) by lysing cells in sodium hydroxide and compared to the pellets from clarification of the lysate (P1 and P2) and the lysate (L) obtained when lysing cells with the bead mill method used for sucrose gradient fractionation. Samples were resolved by SDS-PAGE and Western blotting was performed using an antibody to yeast Cct4. The upper band corresponds to GFP-Cct4 and the lower band corresponds to Cct4. (e) The temperature-sensitive yeast strain cct4-1, harboring the Cct4\textsuperscript{G345D} mutation, was transformed with plasmids expressing GFP, GFP-Cct4, GFP-Cct4\textsuperscript{G345D} and GFP-Cct4\textsuperscript{D91E}. A serial dilution was dropped on selective SC-URA Agar plates and incubated at 25 °C or 37 °C for 3 days. All constructs are able to grow at the permissive temperature (25 °C); however, only GFP-Cct4 and GFP-Cct4\textsuperscript{D91E} are able to rescue growth at 37 °C. Cells expressing GFP-Cct4\textsuperscript{D91E} were imaged using a Zeiss Observer Z1 microscope.
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Abbreviations used:
CCT, Chaperonin containing tailless complex poly
peptide 1; SIM, structured illumination microscopy; wt, wild type.

References


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