Elucidation of the subunit orientation in CCT (chaperonin containing TCP1) from the subunit composition of CCT micro-complexes

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A collection of chaperonin containing TCP1 (CCT) micro-complexes that are comprised of subsets of the constitutively expressed CCT subunits have been identified. These CCT micro-complexes have mol. wts ranging from 120 to 250 kDa and are present in cells at lower abundance (<5%) as compared with intact CCT. Biochemical characterization of these micro-complexes has shown that several are comprised of two different types of CCT subunit. Furthermore, it was observed that each subunit associates with only one or two other different types of subunit, suggesting that each subunit has fixed partners. This observation, together with CCT gene counting being concordant with the 8-fold structural symmetry, is consistent with predictions derived from analysis of the primary structures of these subunits concerning inter-subunit interactions, and implies a unique topology of the subunits constituting the torodial ring in CCT. The series of subunit–subunit association patterns determined from CCT micro-complexes has provided information to infer, from the 5040 (7!factorial) combinatorial possibilities, one probable subunit orientation within the torodial ring.

Keywords: CCT/folding/micro-complexes/molecular chaperones/subunit

Introduction

The proper folding of cytoskeletal proteins (tubulins and actins) requires the participation of the eukaryotic chaperonin CCT [chaperonin containing T-complex polypeptide 1 (TCP1)], also called TRiC (TCP1 ring complex), under both *in vitro* and *in vivo* conditions (Frydman *et al.*, 1992; Gao *et al.*, 1992, 1993; Melki *et al.*, 1993; Sternlicht *et al.*, 1993; Melki and Cowan, 1994; Lewis *et al.*, 1996; Tian *et al.*, 1996). Similarly to other chaperonins, CCT is a high molecular weight protein complex (950 kDa), whose subunits are arranged into two stacked multimeric rings, with a central cavity on each side of the molecule (Gao *et al.*, 1992; Lewis *et al.*, 1992; Marco *et al.*, 1994). However, unlike other chaperonins which are assembled from one or two different types of subunit, the eukaryotic chaperonin CCT is comprised of eight different types of subunits and it is probable that each of them is present in the same CCT complex. If this is the case, there are many

combinations of possible subunit arrangements within the torodial ring of CCT.

Immunoprecipitation of intact CCT with a monoclonal antibody recognizing a single subunit, CCT α /TCP1, has shown that all the other seven TCP1-related subunits can be co-precipitated and are of approximately equal molar amounts (Lewis *et al.*, 1992; Kubota *et al.*, 1994). This subunit composition, together with the 8-fold symmetry in each ring of CCT, observed using electron microscopy (Lewis *et al.*, 1992; Marco *et al.*, 1994), favours a model whereby each different subunit type is recruited to form a component of the ring. This unique subunit arrangement within the ring has probably been fixed by evolution and has become critical for the chaperoning activity of CCT. Thus, elucidation of the subunit arrangement may provide insights into the mechanistic functions of this chaperonin.

In this study, complexes (termed CCT micro-complexes) comprising more than one type of CCT subunit were identified and isolated from mouse testis and human cells. A series of subunit–subunit association patterns were observed and, by consolidating the observed association patterns, a probable subunit arrangement is proposed.

Results

Identification of CCT micro-complexes

The enrichment of intact CCT from mouse testis post-nuclear supernatant (PNS) by a 10.2–40% continuous sucrose gradient has shown that this protein complex consistently sediments in fractions corresponding to 19–23% sucrose. The localization of CCT in the sucrose gradient can be determined through probing, with the monoclonal antibody 91A that recognizes mouse CCT α , a Western blot of the gradient fractions (Lewis *et al.*, 1992). However, on prolonged exposure of the blot, via enhanced chemiluminescence, the distribution of CCT α extends to fractions corresponding to 10.2–18% sucrose (data not shown). These light sucrose fractions, when resolved by non-denaturing polyacrylamide gel electrophoresis followed by probing with monoclonal antibody 91A, showed several bands in addition to that representing intact CCT (Figure 1A and B). This distribution pattern of CCT α suggests the possible existence of smaller complexes comprising CCT subunits in these sucrose fractions. From here on, these smaller complexes are termed CCT micro-complexes to distinguish them from intact CCT.

Using the alternative technique of semi-native diagonal electrophoresis (SNaDE; non-denaturing gel electrophoresis in the first dimension followed by SDS–PAGE in the second dimension) and Western blotting, the presence of CCT micro-complexes in sucrose fraction 14 corresponding to a sucrose density of 1.039 g/cm³ was examined in detail. Figure 2 shows a single Western blot probed

Fig. 1. Multiple smaller complexes containing CCT α in sucrose fractions 13–16 (corresponding to sucrose densities 1.054–1.030 g/cm³). Mouse testis sucrose gradient fractions 7–16 (corresponding to sucrose densities 1.132–1.030 g/cm³) were resolved in a 6% non-denaturing polyacrylamide gel followed by Western blotting. The blot was then probed with monoclonal antibody 91A, which recognizes mouse CCT α . The distribution pattern of CCT α is revealed by chemiluminescence, and two different exposure times are shown; (A) 10 s; (B) 2 min. The presence of smaller complexes containing CCT α is clearly shown in the longer exposure (B).

sequentially with eight specific antibodies, recognizing different CCT subunits (CCT α , CCT β , CCT γ , CCT δ , CCT ϵ , CCT ζ , CCT η and CCT θ), and with each antibody two signals are observed consistently on the blot. The signal on the left represents intact CCT since a similar and co-incident signal is obtained with all eight specific antibody probings. On the other hand, the signals on the right are generated from CCT subunits present as components of smaller complexes. The non-superimpossibility of these signals when probed with different specific antibodies suggests the co-existence of many species of smaller complexes, each comprising a subset of the eight constitutively expressed CCT subunits, CCT α –CCT θ . The abundance of these various CCT micro-complexes is much less than of intact CCT, probably <5% in total.

In Figure 2, the complex patterns exhibited on the Western blots (particularly with the anti-CCT θ and the anti-CCT ζ antibodies) were the result of non-specific cross-reactivity of the polyclonal antibodies used in the analysis. Nevertheless, the signals representing bona fide CCT subunits can be located by comparing their mobilities with the left-most signal representing the corresponding CCT subunit that migrates as a component of intact CCT. In this manner, the signals on the blots representing non-CCT subunits were identified and were excluded during subsequent analysis.

Size distribution of CCT micro-complexes

In order to determine the size distribution of these CCT micro-complexes and ensure that all of them are included in this analysis, sucrose fractions 14–16 (which correspond to sucrose densities of 1.068–1.030 g/cm³) were pooled and concentrated before being subjected to gel filtration chromatography using a Superose 6 column. Within the 40 resultant fractions obtained, all the CCT subunits were located within the molecular weight range of 2700–5 kDa (Figure 3). Similar to the results obtained from the SNaDE analysis, all the CCT subunits were located broadly in

Fig. 2. The presence of CCT micro-complexes in sucrose gradient fraction 14 (corresponding to a sucrose density of 1.039 g/cm³) from analysis by SNaDE and Western blotting. SNaDE analysis of the mouse testis sucrose fraction 14 shows the presence of intact CCT (left most spot on the blot) and CCT micro-complexes (indicated by inset arrows). The 'Top' and 'Bottom' in the figure represent the orientation of the 6% non-denaturing gel slice that was resolved further in an 8% SDS-PAGE gel. A Western blot of the SDS-PAGE gel was probed sequentially with specific antibodies indicating the distribution patterns of CCT α , CCT β , CCT γ and CCT ϵ . Another identical Western blot was probed sequentially with the other four specific antibodies indicating the distribution patterns of CCT δ , CCT θ , CCT η and CCT ζ .

two regions. In the first region, centering around fraction 19, a protein complex that has a mol. wt of ~920 kDa and is reactive with all the eight specific antibodies is clearly intact CCT. On the other hand, we attribute the distinct clusters of bands in the second region to CCT micro-complexes. During each probing with the different subunit-specific antibodies, the distribution of these bands reflects the size distribution of the subset of CCT micro-complexes containing the respective CCT subunit type. Each CCT subunit type shows a different distribution pattern (Figure 3), demonstrating a distinct size distribution for the micro-complexes containing each of these subunit types.

From the size distribution of each type of CCT subunit, it is probable that they exist not only as free subunits, but also as components of many larger complexes. To convey this point, we have classified CCT micro-complexes into three categories, (60–100 kDa, 100–150 kDa, 150–250 kDa) and we infer that they represent monomeric, dimeric and trimeric molecular states (Table I). The multiplicity of the molecular states for the subset of CCT micro-complexes containing each CCT subunit is obvious. For example, it seems that CCT θ exists only as monomer (i.e. 60 kDa) whereas CCT γ and CCT ϵ are found to be present solely as components of larger complexes (i.e. >120 kDa). The remaining CCT subunits seem to exist

as monomers and as components of larger complexes. It is probable that a subset of these complexes may comprise more than one type of CCT subunit.

CCT subunit association patterns within the CCT micro-complexes

Although the results from SNaDE analysis and gel filtration (Figures 2 and 3) show partial overlap of some of the

signals in regions not occupied by intact CCT, the resolution of both these techniques is insufficient to ascertain definitively the association patterns between different CCT subunits. Therefore, non-denaturing gel electrophoresis and Western blotting analysis have been used to determine the association patterns within these micro-complexes. Using sucrose gradient fractions 13 and 14 (corresponding to sucrose densities of 1.054 and 1.039 g/cm³ respectively) as a source of micro-complexes and by probing the blot sequentially with specific antibodies, the protein bands representing micro-complexes containing more than one type of CCT subunit were characterized (Figure 4). The multiple bands in each panel indicate the multiple species of CCT micro-complexes containing distinct CCT subunit types.

Again, due to the non-specific cross-reactivity for some of the polyclonal antibodies used, it is essential to identify the signals representing CCT subunits in order to ensure the accuracy of subsequent analysis. This was done by taking an identical gel lane containing all these bands and resolving them by SDS-PAGE in the perpendicular direction (data not shown). All bands that contain CCT subunits produce signals at their corresponding molecular weights. For example, any micro-complexes containing CCTα will yield a signal in the 57 kDa region after they are resolved by SDS-PAGE. In this manner, all bands observed on the non-denaturing gel lanes that were not comprised of CCT subunits were determined and were not considered during further analysis. However, all the signals that seem to represent bona fide CCT micro-complexes and which were superimposable provided the subunit-subunit association patterns.

For clarity, all the overlapping signals are indicated by inset arrows, and each association between different types of subunit is indicated. In Table II, all the observed subunit-subunit association patterns are tabulated.

Determination of the subunit orientation for the toroidal ring in CCT

Analysis, based on the protein sequence of the CCT subunits and the structure of GroEL (Kim *et al.*, 1994), predicts that each type of CCT subunit will only associate with two other different types of subunit within each ring of CCT. As predicted, each type of CCT subunit associates only with one or two other different types of CCT subunit (Table II), with the exception of CCTθ which remains mainly as free subunit. This feature of association specificity for each CCT subunit type suggests a unique orientation for the subunits forming the ring.

From Table II, there are sufficient sets of association

Fig. 3. Molecular size distribution of each CCT subunit type when present as components of CCT micro-complexes. CCT micro-complexes present in sucrose fractions 14–16 (corresponding to sucrose densities 1.039–1.030 g/cm³), were separated by gel filtration chromatography and the chromatographic fractions 14–37 (corresponding to a mol. wt range of 2700–5 kDa) were resolved in an 8% SDS-polyacrylamide gel followed by Western blotting. The blot was probed sequentially with specific antibodies recognizing different CCT subunits to indicate their distribution patterns. The molecular size distributions of CCT micro-complexes containing CCTα; CCTβ and CCTε; CCTγ; CCTδ; CCTθ; CCTη and CCTζ are shown. The band observed in fraction 19 which cross-reacted with all the specific antibodies used to identify CCT subunits and which has an estimated mol. wt of 920 kDa is intact CCT.

Table I. Multiplicity of molecular states of CCT micro-complexes containing each CCT subunit type

Sizes (kDa)	Types of CCT subunit in micro-complexes							
	CCT α	CCT β	CCT γ	CCT ϵ	CCT δ	CCT θ	CCT η	CCT ζ
60–100	+	+	–	–	+	+	+	+
100–150	+	+	+	+	+	–	+	+
150–250	+	+	+	+	–	–	+	+

Subunit present (+) or absent (–) in complexes of the respective size class.

patterns to enable the construction of a probable subunit orientation within each CCT ring (Figure 5). However, in mouse testis, the absence of an observable association pattern between CCT γ and CCT β gives rise to an additional possible arrangement for the subunits in the ring. Nevertheless, the association of these two subunit types observed in 293-T cells (Figure 4J and K) allows discrimination between the two alternative arrangements from the mouse testis data.

Discussion

Unique subunit orientation in the torodial ring of CCT

Publication of the complete protein sequences for the eight constitutively expressed murine CCT subunits (Willison *et al.*, 1986; Kubota *et al.*, 1994, 1995) and the crystal structure of GroEL (Braig *et al.*, 1994) enables topographical modelling of CCT to be performed. Comparison of the protein sequences of the subunits for these two chaperonins allows the mapping of regions of the protein sequence to the corresponding spatial location on the GroEL crystal structure. From this analysis, it is found that among the CCT subunits, the regions of sequence corresponding to subunit–subunit interaction domains within the same torodial ring in GroEL are highly divergent (Kim *et al.*, 1994). Consequently, the subunit–subunit interaction domains of each CCT subunit are predicted to have specific recognition motifs, and hence every CCT subunit is expected to associate with two and only two other different types of subunit within the same ring in CCT. The possibility of identical subunit types associating with themselves within the torodial ring is discounted, as this would give rise to homo-oligomeric or mixed-symmetry rings in the protein complex. This mixed ring composition model is also unlikely since equi-molar ratios for every different subunit type have been observed for immunoprecipitated CCT (Lewis *et al.*, 1992) and biochemically purified CCT (Frydman *et al.*, 1992; Lewis *et al.*, 1992; Rommelaere *et al.*, 1993; Kubota *et al.*, 1994).

In addition, the crystal structure of GroEL (Braig *et al.*, 1994) revealed that the subunit–subunit contact area within the same ring is 982 Å, which is more than double the magnitude of the contact area between associating subunits across different rings, 418 Å. These subunit contact areas in the radial and axial directions are assumed to be similar in CCT, given the clear structural similarities between these two chaperonins. Hence, the strength of association between two subunits of the same ring is expected to be stronger than the subunit association between the two

Fig. 4. Mouse testis sucrose gradient fractions 13 and 14 (which correspond to sucrose densities of 1.054 and 1.039 g/cm³ respectively) analysed by non-denaturing polyacrylamide gel electrophoresis reveals the subunit–subunit association patterns in CCT micro-complexes. Sucrose gradient fractions 13 and 14 were resolved in a 6% non-denaturing polyacrylamide gel followed by Western blotting. The CCT subunit–subunit association patterns were determined by probing the blot sequentially with specific antibodies recognizing the eight CCT subunits to locate superimposable bands. (A–E) The distribution patterns of CCT α , CCT η , CCT δ , CCT θ and CCT γ respectively, when existing either as components of intact CCT or CCT micro-complexes or present as free subunits, on one Western blot. (F–I) The distribution patterns of CCT α , CCT ϵ , CCT ζ and CCT β respectively when existing either as components of intact CCT or CCT micro-complexes or present as free subunits on another equivalent Western blot. (J) and (K) The distribution patterns of CCT γ and CCT β respectively (as components of intact CCT or CCT micro-complexes as well as free subunits) in sucrose gradient fractions 13 and 14 of 293 cells. Inset arrows indicate the corresponding superimposable protein bands, and the symbols indicate the two CCT subunit types whose association is inferred by this band, e.g. the band α – η refers to the superimposable band on the blot when probed sequentially with specific antibodies recognizing CCT α and CCT η respectively.

rings. Therefore, any assembly intermediates comprised of different types of subunits would probably be assembly fragments of the same ring.

Origins of CCT micro-complexes

It is possible that CCT micro-complexes originate from fragmentation of intact CCT during purification. Neverthe-

Table II. Subunit-subunit association patterns observed in CCT micro-complexes

	CCT α	CCT β	CCT γ	CCT ϵ	CCT δ	CCT θ	CCT η	CCT ζ
CCT α	n.d.							
CCT β	–	n.d.						
CCT γ	–	+ ^a	n.d.					
CCT ϵ	+	–	–	n.d.				
CCT δ	–	–	–		n.d.			
CCT η	+	–	–	–	+	n.d.		
CCT θ	–	–	–	–	–		n.d.	
CCT ζ	–	+	–	+	–	–	–	n.d.

^aThis association is not very clear in mouse testis but is substantiated in human 293-T cells.
n.d., not determined.

less, the subunit-subunit association patterns exhibited in these CCT micro-complexes would be identical to that within the torodial ring in CCT.

The attempts to assemble intact CCT by simultaneously expressing the eight CCT subunits in rabbit reticulocyte lysate only met with limited success. In the reaction, a small percentage (<10%) of the expressed subunits is assembled into the protein complex (unpublished data). This observation suggests that intact CCT may not be assembled via a spontaneous single step process by the recruitment of the eight different subunits. One interpretation is that there may be regulated exchange between subsets of CCT subunits forming the rings and those in the cytosol, and this exchange may be part of the life cycle of this chaperonin. In other words, the assembly of intact CCT may involve intermediates. It is probable that the CCT micro-complexes observed in fractions of low sucrose percentages may be the intermediates in the assembly pathway of intact CCT.

In addition, there are significant differences in the mRNA levels for different CCT subunits in mouse testis. The level of mRNA of CCT α is >5-fold that of CCT θ , which has the lowest mRNA level among all the subunits detected during Northern analysis (H.Kubota and K.Willison, unpublished results), suggesting that CCT micro-complexes may be present naturally in cells and at different abundances.

Specific binding domain for different substrates

The presence of a unique arrangement for the subunits suggests that the binding of unfolded/folding substrate within the cavity of CCT may be ordered. As a consequence, for the substrate to be bound successfully, each CCT subunit should bind to a specific region of the unfolded polypeptide. Unlike GroEL, which is comprised of a single subunit type, each CCT subunit type may have evolved with a different binding motif that functions cooperatively to facilitate the optimal capture and/or folding of the substrate. This model also infers that all the different subunit types are unlikely to be complementary to each other, which agrees with the non-complementarity observed between CCT α and CCT β in yeast (Miklos *et al.*, 1994). Also, based on this model, it is probable that one of the eight different subunits would behave as a position marker, which associates with a substrate protein early in its folding pathway, in order to orient the binding of the folding substrate on the torodial ring.

Fig. 5. The proposed CCT subunit orientation in each of the two stacked rings in CCT.

The protein sequence diversity between actins and tubulins (known substrates of CCT) together with a unique subunit orientation in CCT suggests that these two families of substrate may bind to different domains within the cavity of CCT. In yeast, mutants with a defective CCT α or CCT β gene disrupt microtubule-mediated processes but not actin-mediated processes (Ursic and Culbertson, 1991; Miklos *et al.*, 1994). On the other hand, genetic mutations in CCT δ affect actin-associated phenotypes, like bud formation, with no observable effects on microtubule organization (Vinh and Drubin, 1994). Looking at the spatial arrangement of these subunits based on the proposed subunit orientation (Figure 5), CCT α and CCT β , together with CCT ϵ and CCT ζ , form a continuous domain which may therefore be the substrate-binding domain for tubulin within the cavity. However, CCT δ and its immediate neighbours may form the domain where unfolded actin interacts with CCT within the cavity. Despite the prediction that actin and tubulin may have different binding domains on CCT, it is unlikely that a single CCT ring can be occupied by two substrates at the same time. This notion is supported by the competitive nature in the binding of unfolded actin and unfolded tubulin to CCT reported by Melki *et al.* (1993).

The presence of a large number of related CCT subunits together with their pattern of evolution implicates not only functional independence among themselves but also suggests that their arrangement in the final structure is of importance to the functional activity of CCT. In the absence of a crystal structure of CCT, which would provide the complete structural solution to the topology of this protein complex, the knowledge of the subunit orientation may allow further understanding both of the relationships between different subunits and the relationships between the subunits and their target substrates, all of which are essential for the future study of the structure and function of this chaperonin.

Materials and methods

Preparation of post-nuclear supernatant

In this study, CCT micro-complexes were isolated from mouse testis and human 293-T cells. Mouse testes were excised directly from young adult male MF1 mice of age 56–70 days, and human 293-T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS).

PNS from 15 confluent P90 plates of 293-T cells (2×10^8 cells) was applied to each sucrose gradient and, in the case of mouse testis cells, the cells from six testes (1×10^9 cells) were required. Spermatogenic cells from mouse testis were obtained using the method outlined in West and Willison (1996). Mouse testis and 293-T cells were washed with Harms buffer (250 mM sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, pH 7.5) at 0°C, pelleted by centrifugation (1500

r.p.m., 10 min, 4°C) and resuspended in 2 ml of Harms buffer at 0°C. The cells were then homogenized using a steel ball-bearing homogenizer as outlined in Lewis *et al.* (1992). Finally, the PNS was recovered by centrifugation (2000 g, 10 min, 4°C). Protease inhibitors [0.3 U/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mg/ml chymostatin, 5 mg/ml pepstatin A, 5 mg/ml antipain, 10 mg/ml leupeptin] were added to the PNS to minimize protein degradation by proteases.

Sucrose gradient centrifugation and fractionation

PNS was layered carefully on a continuous sucrose gradient at 0°C [10.2–40% (w/w) in 90 mM KCl, 50 mM HEPES pH 7.2] (Lewis *et al.*, 1992). The loaded gradient was centrifuged in a Beckman SW-28 rotor (25 000 r.p.m., 16 h, 4°C). Upon completion of the run, the gradient was fractionated into 16 fractions of ~1 ml each, with fractions 1 and 16 corresponding to the highest and lowest sucrose percentages respectively. Sucrose densities were determined by refractometry.

Non-denaturing polyacrylamide gel electrophoresis

To determine the CCT subunit association patterns, sucrose gradient fractions 13 and 14 of both mouse testis and 293-T cells were analysed by non-denaturing polyacrylamide gel electrophoresis. The electrophoresis was carried out in a 6% polyacrylamide gel of constant pH in Tris-glycine buffer (25 mM Tris-HCl, 192 mM glycine, pH 8.8) at 4°C and at a constant voltage of 80 V until the dye front was 1 cm from the end of the gel. The markers used in the electrophoresis were thyroglobulin (669 kDa), apoferritin (443 kDa), bovine serum albumin (66 kDa) and chicken ovalbumin (45 kDa). All the markers used in non-denaturing polyacrylamide gel electrophoresis were obtained from Sigma, UK.

Semi-native diagonal electrophoresis

SNaDE is an adaptation of two-dimensional polyacrylamide gel electrophoresis. For the first dimension, protein samples were resolved in a 6% non-denaturing gel as described above. For the second dimension, lanes of interest were excised and immersed in gel loading buffer [25 mM dithiothreitol (DTT), 1% SDS, 40mM Tris pH 6.8, 5% glycerol, bromophenol blue as required] at room temperature for 15 min to equilibrate and denature the proteins. Each gel slice was layered on a denaturing 8% polyacrylamide gel and electrophoresed at a constant current of 20 mA until the dye front was 1 cm above the end of the gel. Each second dimension gel was Western blotted and probed sequentially with specific antibodies which recognize the eight different CCT subunit types (Willison *et al.*, 1989; Hynes *et al.*, 1995).

Gel filtration chromatography

To prepare the sample for gel filtration chromatography, 0.4 ml samples from each of the sucrose fractions 14–16 (which correspond to sucrose densities of 1.039–1.030g/cm³) were pooled and concentrated to 50 µl by using the micro-concentrator centricon-30 (Amicon, Canada). Traces of cell debris in the sample were removed by a further pulse centrifugation (13 000 r.p.m., 10 min, 4°C). Then, the sample was subjected to gel filtration chromatography in the SMART system (Pharmacia, Milton Keynes, UK) eluting with Breaking buffer (50 mM HEPES, 90 mM KCl, pH 7.10) using a Superose 6 column (Pharmacia, Milton Keynes, UK) pre-equilibrated with Breaking buffer, and 50 fractions of 40 µl each were obtained. Fractions 14–37, which correspond to a mol. wt range of 2700–5 kDa, were analysed by SDS-PAGE and Western blotting. The molecular size distribution of the CCT micro-complexes after chromatography was determined by probing blots sequentially with specific antibodies recognizing the eight CCT subunits (Willison *et al.*, 1989; Hynes *et al.*, 1995).

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