The Chaperonin Containing TCP-1: Interactions with the Mammalian Cytoskeleton

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Abstract

The eukaryotic Chaperonin Containing TCP-1 (CCT) is a heterooligomeric chaperonin essential for enabling the cytoskeletal proteins actin and tubulin to fold to their native state. The eight CCT subunits are encoded by individual genes and are present in cells as the ~960kDa oligomer, as components of micro-complexes, and as monomeric subunits. In addition to the well-characterised substrate folding mechanism of the CCT oligomer, roles for CCT subunits as monomeric proteins are emerging. The work in this thesis illustrates the dependence upon functional CCT and its roles in cytoskeletal organisation.
Levels of functional CCT have far-reaching implications for cellular functions dependent upon an intact cytoskeleton and siRNA targeting of CCT subunits results in growth arrest and reduced levels of native actin and tubulin. Targeting each CCT subunit individually by siRNA revealed different effects upon cytoskeletal organisation, suggestive of distinct roles for specific CCT monomers. Cell shape and microfilament polymerisation are influenced by CCT monomers with CCTε levels appearing particularly important for these processes. Furthermore, CCTε and to a lesser extent ζ and θ were found to co-localise to microfilaments and CCT subunits associated with non-soluble protein assemblies following detergent extraction are predominantly monomeric. Gelsolin, an actin filament severing and capping protein was identified as a CCT-binding protein, providing another link between CCT activity and cytoskeletal organisation. Although shown to bind the CCT oligomer with some degree of specificity, it is most likely that gelsolin does not represent a CCT folding substrate. siRNA of CCT subunits influences gelsolin levels differently depending upon the target subunit, suggestive of some regulation between CCT monomers, F-actin, and gelsolin levels.
The work presented in this thesis indicates that CCT influences the mammalian cytoskeleton far beyond its involvement in folding newly synthesised actin and tubulin polypeptides and implicates CCT subunits in their monomeric assembly state as likely perpetrators of such activity.

Keywords: CCT, TRiC, Chaperonin, Molecular chaperone, Cytoskeleton, Actin, Tubulin, Gelsolin

Publications

This thesis is based upon the following papers, which will be referred to in the text by their roman numerals

Paper I

Grantham J, **Brackley KI**, Willison KR.
Substantial CCT activity is required for cell cycle progression and cytoskeletal organization in mammalian cells.
Exp Cell Res. 2006 Jul; 312(12):2309-24

Paper II

Brackley KI, Grantham J.
Subunits of the chaperonin CCT interact with F-actin and influence cell shape and cytoskeletal assembly.
Exp Cell Res. 2010 Feb; 316(4):543-553

Paper III

Brackley KI, Grantham J.
Interactions between the actin filament capping and severing protein gelsolin and the molecular chaperone CCT: evidence for non-classical substrate interactions.
Manuscript

Other publications, not included in this thesis

Paper IV

Martín-Benito J, Grantham J, Boskovic J, **Brackley KI**, Carrascosa JL, Willison KR, Valpuesta JM.
The inter-ring arrangement of the cytosolic chaperonin CCT.
EMBO Rep. 2007 Mar; 8(3):252-7.

Paper V

Brackley KI, Grantham J.
Activities of the chaperonin containing TCP-1 (CCT): implications for cell cycle progression and cytoskeletal organisation.
Cell Stress Chaperones. 2009 Jan;14(1):23-31. Review.

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The cytoskeleton is composed of filamentous protein polymers called microfilaments, microtubules and intermediate filaments. These networks along with families of accessory proteins enable many important cellular processes.

The cytoskeleton is often regarded as scaffolding running throughout the cell. This is suggestive of a rigid supportive structure and although filaments of these networks are strong enough to withstand forces both from outside and within cells, this is a huge injustice to the complex and dynamic nature of the cytoskeleton.

Without an intact functional cytoskeleton, we would be unable to contract our muscles (important not only for movement, but also for a heartbeat), our neurons would be unable to branch out and make new connections, cells would be unable to move and unable to divide. Obviously, based upon these very few examples alone, we have a lot to thank these proteins for.

Therefore, it is crucial that cells are able to maintain their supplies of the proteins which form these cytoskeletal structures, and also, and maybe more importantly that these building blocks have the correct structure to allow them to be incorporated correctly into these filaments. For actin (the protein microfilaments are made from) and tubulin (the building blocks of microtubules), this is the responsibility of the molecular chaperone Chaperonin Containing TCP-1 (CCT). CCT actively assists during the folding of these proteins allowing them to achieve their correct 3-dimensional structure, a process vital for a functional cytoskeleton and all downstream processes dependent upon it.

The work in this thesis looks more closely at the relationship between the cytoskeleton and this molecular chaperone, establishing that interactions extend beyond those enabling folding of newly synthesised proteins to include interactions between CCT subunits and native proteins in established cytoskeletal networks.

Mammalian actin and tubulin cytoskeletal networks

Within mammalian cells there are three cytoskeletal networks, microfilaments (composed of actin monomers) microtubules (built from tubulin heterodimers) and intermediate filaments (assembled from different intermediate filament proteins), together these constitute the mammalian cytoskeleton. These cytoskeletal structures are important for a whole host of cellular functions and many of their properties are dependent upon their dynamic nature and a host of accessory proteins. The assembly and dynamics of microtubules and, more relevantly for the work presented in this thesis, microfilaments, will be briefly discussed here.

Microtubules are hollow tubes composed of tubulin α/β-heterodimers. Tubulin binding co-factors enable α/β-tubulin heterodimer formation from α- and β-tubulin monomers. Heterodimers then align to form protofilaments, which subsequently associate laterally forming cylindrical microtubules. Microtubules are polarised relative to their nucleation from the microtubule organising centre (MTOC) and most dynamic at their plus end, located towards the cell periphery. Rapid assembly and disassembly of microtubules is pivotal to their functions. Microtubules experience "dynamic instability" whereby they can rapidly fluctuate between elongation and shrinkage. Microtubules are often considered as transportation networks throughout the cell as the motor proteins kinesins and dyneins travel along microtubules transporting cargo proteins and vesicles to intracellular locations. In addition to motor proteins, a whole host of other proteins, collectively referred to as Microtubule Associated Proteins (MAPs) bind to microtubules, regulating their activity and assembly (reviewed by e.g. Desai and Mitchison, 1997; Valiron et al., 2001).

Microfilaments are composed of actin monomers, the requirements of actin and tubulin differ prior to filament incorporation in that no equivalents of tubulin co-factors are necessary in the actin system. Like microtubules, microfilament formation begins with a nucleation step dependent upon actin monomer concentrations. The plus and minus ends of actin filaments are also referred to as the barbed and pointed ends respectively; following nucleation, monomers associate most rapidly with the barbed end of filaments. Again, as for microtubules, it is essential that microfilaments are dynamic, thereby enabling cell motility and morphological changes. Actin filaments are regulated by a host

of proteins termed actin binding proteins. Actin binding proteins can be sub-divided into a few key categories, including: i) Actin monomer-binding proteins, these control the pool of unpolymerised actin within cells, e.g. β-thymosins and profilin. ii) Actin filament-capping proteins, that can bind barded or pointed ends of actin filaments and prevent subunit association/disassociation. iii) Actin filament-severing proteins create short fragmented filaments and iv) Actin filament cross-linking proteins, which enable microfilaments to assemble into higher order structures such as stress fibres. Stress fibres contain many actin filaments bundled together with alternating polarities and myosin II filaments that give stress fibres their contractile properties. As with individual actin filaments, stress fibres are also regulated by an array of actin binding proteins. Stress fibre assemblies can be classified into three main types with differing assembly mechanisms and intracellular locations; traverse arcs lay parallel to the lamellipodia structures at cell edges, dorsal stress fibres elongate from the cell edge towards the dorsal surface, and ventral stress fibres, which are anchored by focal adhesions at both ends (reviewed by e.g. dos Remedios et al., 2003; Hotulainen and Lappalainen, 2006; Naumanen et al., 2008; Pellegrin and Mellor, 2007; Pollard, 1990).

The structural and dynamic nature of the cytoskeleton is paramount to cell function. During protein folding it is the molecular chaperone CCT which ensures that actin and tubulin attain their native conformations ready for incorporation into cytoskeletal networks. Incorporation of misfolded proteins into filaments would have potentally detrimental affects upon cytoskeletal functions, therefore demonstrating the importance of CCT.

Chaperonin Containing TCP-1

Chaperonin Containing TCP-1 (CCT, also known as TCP-1 Ring Complex or TRiC) is a eukaryotic chaperonin essential for the folding of newly synthesised actin and tubulin monomers. Chaperonins are a specialised sub-group of a class of proteins, known as molecular chaperones, which assist in the folding of newly synthesised proteins. Before describing the chaperonins in more detail, some basic principals need to be considered, namely, why do proteins require assistance to fold and how do molecular chaperones achieve this?

Protein folding in the crowded cell environment

In order to be functional, proteins must have adopted their correct three-dimensional structure. There are different occasions when proteins will be unfolded and therefore require assistance from the cellular protein folding machinery in order to prevent their aggregation. If proteins are localised to particular cellular compartments, during translocation they may adopt linear formations to enable passage through membranes and therefore need refolding upon reaching their destination. Additionally, proteins can experience off-pathway folding reactions during conditions of cellular stress and require assistance to try to regain native structure and prevent aggregation. However, the bulk of cellular protein folding activity occurs when proteins are newly translated as polypeptide chains and need to fold to their correct conformation.

As newly synthesised polypeptides emerge from the ribosome into the cell they are confronted with the daunting task of reaching their native conformation whilst in a crowded environment, i.e. surrounded by high macromolecule concentrations (200-400mg/ml protein and RNA (Ellis, 1997)). Proteins of approximately 300 amino acid residues can take up to 1 minute to be translated and since the newly synthesised polypeptide becomes available in a vectoral manner during translation, this means that regions which are translated first may be susceptible to aggregation before the rest of the protein has been translated (Hartl and Hayer-Hartl, 2002). The exit site of the ribosome itself is too narrow to allow formation of protein structure (Nissen et al., 2000) and therefore newly synthesised polypeptide chains must emerge during translation, vulnerable to the crowded, protein-rich conditions of the cell environment. This increases the

possibility that proteins may fail to reach their native conformations, either by failing to fold correctly upon exit from the ribosome or by later "off-pathway" events resulting in loss of native structure. Either way, non-native protein conformations can result in protein aggregation, a potentially dangerous threat to the cells' survival. Protein aggregation is often associated with disease; particularly neurological disorders such as Alzheimer's and Parkinson's diseases that arise when proteins misfold and resulting aggregates are deposited in cells or tissues (e.g. Dobson, 2003; Stefani and Dobson, 2003). Although individual diseases are usually associated with misfolding of one particular protein, Bucciantini et al., have shown that proteins other than those classically associated with amyloid diseases are capable of aggregate formation, producing levels of cytotoxicity comparable to those of Aβ (1-42) (common in Alzheimer's disease) (Bucciantini et al., 2002).

Anfinsen (e.g. 1973) proposed a model whereby the amino acid sequence of a nascent polypeptide will dictate the protein's native state, and whilst this remains true, we now know that as well as the polypeptide sequences' intrinsic properties, the cell contains proteins intent on ensuring other proteins can fold to their correct conformation. Molecular chaperones are a group of proteins, often working together, assisting nascent polypeptide chains to reach their native conformation, smoothing folding energy landscapes and offering protection from the crowded cytoplasm, allowing safe release of newly synthesised proteins (e.g. Ellis, 1997). Most molecular chaperones require ATP hydrolysis for their mechanism of action. Although this investment of ATP in the folding of newly synthesised polypeptides and those that have gone off-pathway may seem expensive to the cell, the alternative may be protein aggregation, with potentially catastrophic consequences. Furthermore, the cost of re-folding a protein that has gone off-pathway is only ~10% of the energy required for initial translation (Horwich et al., 2007). Therefore, cells may save energy "repairing" misfolded proteins using molecular chaperones as opposed to re-synthesising new ones to replace them.

Mammalian molecular chaperone systems

More than 20 years ago the term 'molecular chaperones' was designated to describe "a class of cellular proteins whose function is to ensure that the folding of certain other polypeptide chains and their assembly into oligomeric structures

occur correctly" (Ellis, 1987). Chaperones are therefore often responsible for the final step in producing biologically active proteins following transcription and translation. Many molecular chaperones are designated (and were first identified) as heat shock proteins (Hsps) based upon their up-regulation during cellular stress. However, they are also necessary for many of the 'housekeeping' processes in cells growing under normal conditions. Mammalian chaperones include the Hsp70, Hsp90 and chaperonin systems. Some chaperones may cooperate with each other and many newly synthesised proteins will interact with more than one type of chaperone and their host of accessory proteins / co-chaperones before reaching their native conformation. Figure 1 demonstrates the different pathways newly synthesised proteins may take and the chaperones they can interact with between exiting the ribosome and reaching their correct conformation in the cell cytosol. The sooner proteins can interact with chaperones the less likely they are to go off pathway. The first line of defence against protein aggregation are chaperones which are ribosome bound, interacting with nascent polypeptide chains co-translationally (Craig et al., 2003). The eukaryotic NAC (nascent chain-associated complex) binds and protects stretches of hydrophobic residues in emerging nascent chains. This activity is ATP independent and is not known to actively prevent aggregation, instead it appears as more of a stabilising, protective measure whilst peptide chains wait for completion of their translation and release from the ribosome, following which NAC complex dissociates from polypeptide chains (reviewed by e.g. Hartl and Hayer-Hartl, 2002; Young et al., 2004). The constitutively expressed Hsp70 homologue Hsc70 can also interact with nascent chains and has a very general clientele as it recognises exposed hydrophobic sequences, common to most nascent polypeptide chains. Interactions with Hsp70 vary depending upon a protein's size. The larger a protein is, the more likely an interaction is needed for efficient protein folding. Up to 20% of bacterial proteins interact with the Hsp70 homologue DnaK whereas eukaryotic proteins are generally larger, and therefore a higher percentage will utilise the Hsp70 chaperone family during their folding pathways (Mayer and Bukau, 2005). Unlike the ribosome-tethered NAC complex, the activity of Hsp70 chaperones is ATP dependent and their ATP hydrolysis cycle controls the rate of protein folding. The rate-limiting ATP hydrolysis step of Hsp70 protein folding can be stimulated by Hsp40, a co-chaperone that mediates interactions with newly synthesised proteins, subsequently transferring them to Hsp70 (Wegele et al 2006).

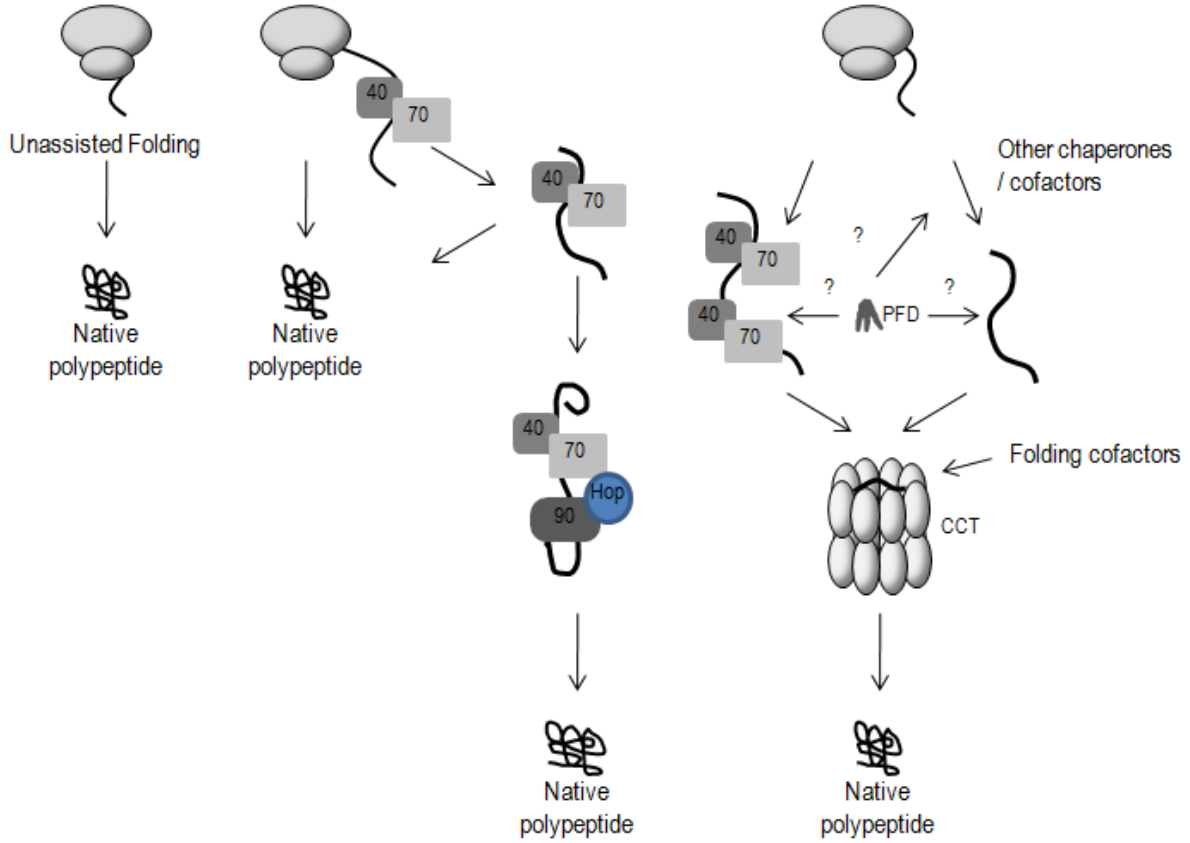

Figure 1: Mammalian pathways of chaperone assisted protein folding. Whilst some simple proteins may be able to reach their native conformation without assistance from molecular chaperones, many cannot. The cell contains multiple chaperone pathways. Proteins may utilise cross talk between pathways and a host of co-chaperones and folding cofactors to become folded to their correct three-dimensional structure. Based upon Young et al., (2004).

Cytoplasmic Hsp90 is essential for eukaryotic cell viability and is one of the most abundant proteins in cells under non-stressed conditions, accounting for 1-2% of soluble cell protein (e.g. Lai et al., 1984), however it is not required for the de novo protein folding of most proteins (Nathan et al., 1997). Instead, the ATP dependent Hsp90 chaperone machinery appears to regulate the activity of proteins, especially those involved in signalling pathways. Hsp90's functional mechanisms, selectivity for client proteins and their binding mechanisms are still not well understood (reviewed by e.g. Pearl and Prodromou, 2006; Wegele et al., 2004; Young et al., 2004). The Hsp organising protein (Hop) links the Hsp70 and Hsp90 chaperones in mammalian systems, facilitating substrate transfer without influencing the ATPase activity of either (Johnson et al., 1998; Wegele et al., 2006). Hsp90 client proteins can be divided into three main groups; protein kinases, transcription factors and structurally unrelated proteins

(reviewed by Pearl et al., 2008). Hsp90 selectively interacts with kinases which act as informational hubs in signalling networks (Citri et al., 2006), including many involved in oncogenesis such as c-Src, PKB/Akt1 and Cdk4. Hsp90 plays important roles in disease states and especially cancers. Its function has been implicated in many of the hallmark steps of cancer and elevated Hsp90 levels are linked to poor prognosis. Hsp90 inhibitors are being developed for cancer treatment and should have wide reaching impacts due to the large number of client proteins involved in oncogenesis (reviewed by Neckers, 2007; Pearl et al., 2008). Additionally, Hsp90's interaction with the mutant form of cystic fibrosis transmembrane conductance regulator has been suggested to contribute to the disease pathology of cystic fibrosis (Wang et al., 2006) and Hsp90 is also involved in viral replication complex assembly.

The chaperonins

Chaperonins are a sub-class of molecular chaperones. Chaperonins are sub-divided into Group I chaperonins (found in eubacteria and endosymbiotic organelles), of which the GroEL/GroES system is the most well characterised example, and Group II chaperonins (from archeabacteria and the eukaryotic cytosol) which include the thermosome and CCT. The chaperonins all have a cylindrical shape composed of two back-to-back rings surrounding a central folding cavity. This cavity offers a protective environment, reducing the effects of molecular crowding during a substrate's folding cycle. Demonstrating how important they are for protein folding, chaperonins are present in all three kingdoms of life and moreover are essential proteins, *E.coli* and *S. cerevisiae* deficient for GroEL or CCT subunits respectively are non-viable (Fayet et al., 1989; Stoldt et al., 1996).

Each chaperonin ring contains between seven and nine individual subunits, of approximately 60kDa resulting in the total molecular weight of chaperonins reaching up to 1MDa. Features, such as dependence upon a functional ATP cycle and the domain architecture of chaperonin subunits are conserved amongst all chaperonins. Within each subunit there are three distinct domains (figure 2c), the apical domain is responsible for substrate capture whilst the equatorial domain contains the highly conserved ATP nucleotide binding site and mediates inter- and intra-ring communication. Communication between the apical and equatorial domains within each subunit occurs via the flexible hinge-like

intermediate domain. The eubacterial GroEL system works with a lid-forming co-chaperone GroES. Flexible loops of sequence bind to substrate binding regions in the GroEL apical domains causing dissociation of substrates and their enclosure in the cavity (e.g. Bukau and Horwich, 1998; Horwich et al., 2009; Sigler et al., 1998; Xu et al., 1997). For the eukaryotic chaperonin CCT no such co-chaperone exists and cavity enclosure is achieved by conformational rearrangements of the helical protrusion regions of CCT subunits' apical domains (e.g. Ditzel et al., 1998; Klumpp et al., 1997; Llorca et al., 1999b; Llorca et al., 1998).

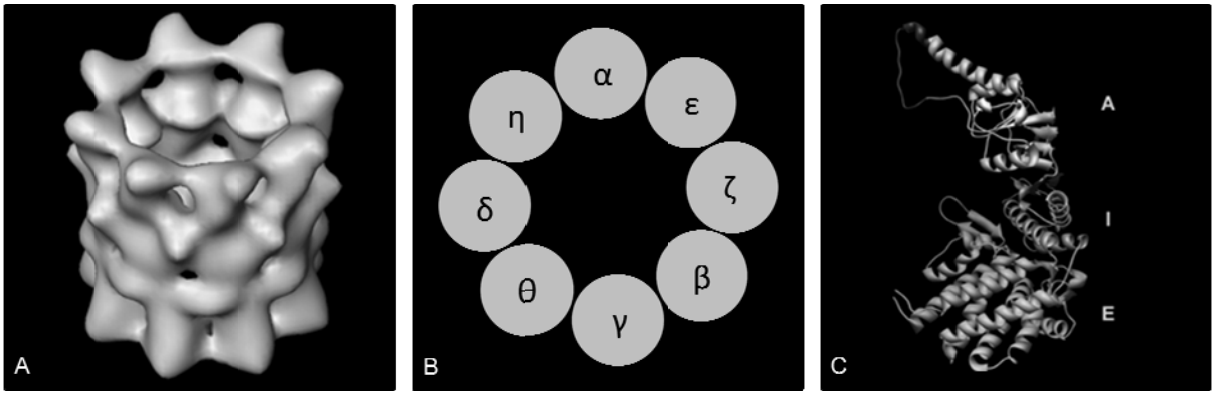

Figure 2: 3D reconstruction of the CCT oligomer from cryo-electron microscopy reprinted by permission from Macmillan Publishers Ltd. The EMBO Journal (Llorca et al., 2001b) copyright 2001 (A). The fixed arrangement of the CCT subunits within the chaperonin ring as determined by Liou and Willison (1997) (B), the arrangement of CCT domains within individual subunits of the chaperonin (Martin-Benito et al., 2007b), based upon the crystal structure of the thermosome (Ditzel et al., 1998) (C).

The Chaperonin Containing TCP-1

The number of different genes encoding the subunits of a chaperonin can vary from one in the case of GroEL with its homoheptameric rings, one, two or three in the archeal thermosome, and up to eight or nine in the eukaryotic chaperonin CCT. CCT is therefore unique in that each of its back-to-back rings contains eight subunits derived from individual genes (Kubota et al., 1994). In yeast, subunits are known as Cct1-8p whilst mammalian nomenclature for the subunits is CCTα, β, γ, δ, ε, ζ, η and θ (in testis a ninth, tissue-specific, subunit is present, sharing greater than 80% homology with CCTζ and is therefore termed CCTζ-2

(Kubota et al., 1997)). Liou and Willison deduced that these subunits have a fixed position within the rings, as shown in Figure 2b (Liou and Willison, 1997). CCT subunits are most divergent in their apical domain sequences and CCT orthologues have areas of related sequence in polypeptide binding regions across species (Kim et al., 1994). GroEL and CCT share sequence similarities in both the equatorial and intermediate domains but display no significant sequence homology in the polypeptide binding regions of the apical domains. Differences between the two chaperonins in this region suggest that substrate-binding mechanisms may differ. Substrates binding GroEL have only one type of subunit protein to bind, whereas CCT offers eight different proteins with diverse sequences. Therefore, CCT has a much more complex binding interface to offer its client proteins compared to its bacterial counterpart. Furthermore, CCT substrate binding sites have been aligned to specific resides underneath the apical domain helical protrusions (Pappenberger et al., 2002). The fact that these are polar amino acid residues suggests that the binding of actin and tubulin could be sequence-specific and not just via general hydrophobic binding mechanisms that would generally detect non-native proteins. The different mechanisms by which GroEL and CCT interact with their client proteins indicates much more general substrate-capture mechanisms for GroEL compared to the CCT chaperonin system. Furthermore, GroEL, mitochondrial Hsp60 and the thermosome are all heat inducible whereas CCT is not up-regulated during heat shock (Horwich et al., 2007). Therefore, whilst other chaperonins are able to assist with general protein folding, under stress conditions, CCT does not mediate general refolding of heat induced off-pathway conformations suggesting that under normal cell conditions CCT satisfies a very specific folding requirement within the eukaryotic cytosol.

From cryoelectron microscopy studies of actin and tubulin bound CCT, it seems that CCT substrates remain bound to the chaperonin (Llorca et al., 2001b). In the GroEL/GroES system substrates are released into the cavity for folding. Therefore, if substrates remain bound, CCT potentially offers greater levels of active assistance in protein folding compared to the GroEL/GroES system, which can be considered as a more passive, yet protected, folding environment. In agreement with this is the way in which different chaperonins execute their ATP hydrolysis cycles. GroEL subunits hydrolyse their ATP nucleotides in a concerted manner (Yifrach and Horovitz, 1995) whilst CCT subunits hydrolyse their ATP in a defined order as a wave around the CCT ring (Lin and Sherman,

1997; Rivenzon-Segal et al., 2005), a process which allows conformational rearrangements of substrates bound across the cavity (Llorca et al., 2001b).

CCT substrates

CCT was originally identified as a chaperone mediating actin and tubulin folding (Gao et al., 1992; Sternlicht et al., 1993; Ursic and Culbertson, 1991; Yaffe et al., 1992) and these are still considered the archetypal obligate substrates of this chaperonin. Tubulin-CCT interactions involve all eight of CCT's subunits, suggesting that CCT may have evolved for tubulin folding requirements and later adapted during actin evolution to accommodate its folding requirements. Actin has ancestral genes dating back ~three billion years (Pollard and Cooper, 2009) and prokaryotic actin predecessors such as MreB can fold to their native conformation and even polymerise into functional filamentous structures (Carballido-Lopez and Errington, 2003; Michie and Lowe, 2006; van den Ent et al., 2001) without CCT. However, eukaryotic actin is unable to reach a native conformation unassisted and GroEL cannot replace the need for CCT (Pappenberger et al., 2006; Stemp et al., 2005). Altschuler and Willison (2008) propose that CCT overcomes particular folding difficulties faced by eukaryotic actin. CCT's adaptation to accommodate actin folding function is supported by the fact that during the evolution of individual CCT subunits by gene duplications, CCTδ/ε and α/β/η clades are those that evolved most recently (Archibald et al., 2000) and CCTβ, δ and ε are those responsible for actin-specific substrate interactions.

Apart from actin and tubulin, until recently only a limited number of proteins had been identified as CCT substrates (reviewed by Dunn et al., 2001; Valpuesta et al., 2002; Willison and Grantham, 2001)

A number of cell cycle related proteins have been identified as CCT substrates, including Polo-like kinase 1 (Plk1) involved in G_2/M cell-cycle progression (Liu et al., 2005) and in CCT siRNA targeted cells, Plk1 levels are reduced, consistent with this (paper I). Cyclin E has also been identified as a CCT binding protein (Won et al., 1998), (although data in paper I suggest that it does not behave as a CCT substrate). Cdc20 and Cdh1 are cell cycle proteins responsible for APC/C activation at mitosis and G_1 respectively, these proteins rely upon CCT for two aspects of their function (Camasses et al., 2003).

Firstly, Cdc20 and Cdh1 rely upon CCT as substrates to achieve their native protein structures. Both of these proteins are WD repeat proteins, a class of proteins that have been shown to commonly use CCT for their protein folding requirements. Ho et al., (2002) performed a screen for protein-protein interactions using immunoprecipitation and mass-spectrometry and from the 21 proteins interacting with 3 or more CCT subunit proteins, 16 were WD repeat proteins (Ho et al., 2002), representing 17% of the total yeast WD repeat proteins (Valpuesta et al., 2002). This protein fold may require assistance from CCT; moreover, these propeller-shaped β-sheet structures would fit within the dimensions of the CCT cavity (Craig, 2003; Valpuesta et al., 2002). Despite having 7-blade β-sheet propeller structures, the interaction between CCT and Cdc20 / Cdh1 was mapped to blades III through V, suggesting that specific recognition mechanisms exist allowing CCT to distinguish not only between different WD repeat proteins, but also between their different blades (Camasses et al., 2003). Secondly, following folding to their native structure, Cdc20 and Cdh1 then use CCT as a platform for assembly into functional complexes with APC/C. This mechanism may allow a high degree of regulation by "just-in-time" assembly, in other words if Cdc20-APC/C complex assembly is the rate limiting step for cell cycle progression, this is regulated by CCT activity levels (Dekker, 2010).

In a similar manner to Cdc20 and Cdh1, the von Hippel-Lindau (VHL) tumour suppressor protein also uses CCT for folding and assembly. VHL represents a genuine folding substrate of CCT but, after folding, utilises CCT as a platform for its assembly into a functional complex with elongin B and elongin C (Feldman et al., 1999; Melville et al., 2003).

Recently, two studies in yeast greatly contributed to our current knowledge of CCT interacting proteins (Dekker et al., 2008; Yam et al., 2008). 136 CCT interacting genes/proteins were identified using both proteomic and genomic approaches (Dekker et al., 2008) and via a genome-wide small pool expression cloning approach a higher estimation was made that 6-7% of cytosolic proteins interact with CCT (Yam et al., 2008). Proteins that interact with CCT were shown to have a propensity toward being components of oligomeric assemblies and/or enriched for regions of β-sheet (Yam et al., 2008). CCT interacting proteins identified using these methods will not all represent proteins which have an obligate requirement for CCT and cannot reach their native state in systems lacking the chaperonin (such as actin and tubulin). Additional

categories of interacting partners include: i) proteins that bind to CCT as regulatory proteins, assisting with protein folding, either by delivering substrates to the ring or by regulating the folding activity/rate of CCT; ii) proteins using CCT as a platform for oligomerisation and iii) proteins which may have developed off-pathway conformations and bound to CCT opportunistically to try and re-fold. Proteins belonging to any of these three categories do not represent obligate folding substrates of CCT.

Dekker and colleagues (2008) identified components of the septin ring complex as a novel group of CCT-interacting cytoskeletal proteins. They also identified a number of proteins with nuclear functions, including proteins that make up the nuclear pore complex and those involved in nuclear transport. Additionally histone deacetylases were identified as CCT binding partners, consistent with a previous report that HDAC3 deacetylase requires CCT for its activation, via assembly into a complex with SMRT (Guenther et al., 2002).

It can be seen that CCT interacts with a diverse range of interacting proteins which utilise the chaperonin to satisfy different requirements during their maturation into functional proteins. Techniques to detect protein-protein interactions are increasing in sensitivity, allowing interactions that occur with lower abundance or more transiently to be detected; consequently, the pool of known CCT interacting proteins is expanding. As this number increases, it will become easier to establish if any shared characteristics / folding requirements exist amongst them as currently very little is known. It will also be important to determine which proteins represent those with an absolute requirement for CCT in order to achieve their native structure.

Actin and tubulin – obligate CCT folding substrates

The main substrates of CCT are the essential cytoskeletal proteins actin and tubulin (Gao et al., 1992), both of which depend upon CCT to reach their native conformations (Tian et al., 1995b). Following translation, actin and tubulin polypeptides are presented to CCT in a quasi-native state (discussed below) and it has been proposed that the ATP independent chaperone prefoldin (GimC in yeast), binds to nascent chains and delivers them to chaperonins for further folding (e.g. Hansen et al., 1999; Siegers et al., 2003; Vainberg et al., 1998). Eukaryotic prefoldin has more complex subunit compositions and substrate binding mechanisms (Leroux et al., 1999; Martin-Benito et al., 2007a) than its

archeal counterpart and three-dimensional reconstructions have shown interactions between prefoldin and specific CCT apical domains, presenting a mechanism for delivery of partially folded actin to the chaperonin (Martin-Benito et al., 2002).

Both actin and tubulin are able to adopt two different orientations across the chaperonin cavity, binding subunits on opposing sides of the ring. Actin interacts with two CCT subunits upon binding with the small (N-terminal) domain of actin binding constitutively to CCTδ whilst the larger (C-terminal) domain of actin binds to either CCTε or β (Llorca et al., 1999a). The interaction of CCT with tubulin also occurs via two different binding orientations, however, in the case of tubulin each binding orientation utilises five CCT subunits. The N-terminal binds CCTθ and δ and the C-terminal binds to CCTε, ζ and β or the N-terminal binds CCTη and α and the C-terminal binds CCTβ, γ and θ (Llorca et al., 2000), as shown in Figure 3A.

CCT subunits involved in actin interactions have been identified, shown in Figure 3B (Hynes and Willison, 2000; Llorca et al., 2001a; Llorca et al., 1999a), and the actin sequences responsible for CCT binding and folding have been mapped (Hynes and Willison, 2000; Neirynck et al., 2006; Rommelaere et al., 1999). These sites are interesting because normally chaperone-substrate interactions occur via hydrophobic interactions in areas of the protein that will be buried inside the native protein structure. However, in the case of actin, CCT interacting sequences have been mapped to the surface of native actin, suggesting that actin interacts with the chaperonin as a quasi-native folding-intermediate with a high-degree of folded structure (Llorca et al., 2000; Tian et al., 1995a). Different sub-domains of the actin molecule bind CCT subunits on opposing sides of the chaperonin ring, stretching it across the chaperonin ring (Llorca et al., 1999a), separating sub-domains 2 and 4 and expanding the ATP binding cleft (Villebeck et al., 2007). GroEL is unable to fold native actin (Stemp et al., 2005) and this may result from the absence of such expansion during β-actin–GroEL interactions (Villebeck et al., 2007). ATP binding promotes the closure of an in-built lid from the apical domains of CCT, thereby closing the cavity (Llorca et al., 2001b), which has been shown to be essential for productive folding of actin to its native state (Reissmann et al., 2007). However, unlike the GroEL system cavity closure does not induce release of substrate into the cavity. ATP hydrolysis induces conformational changes in

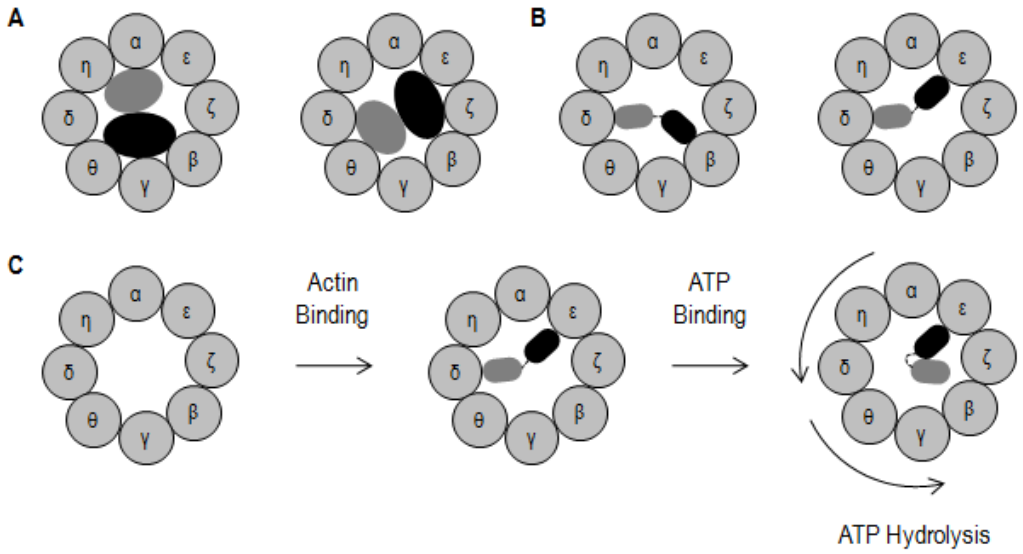

Figure 3: Possible binding orientations of tubulin (A) and actin (B) to CCT. Conformational rearrangements of actin are determined by the ATP hydrolysis cycle of CCT (C). The N-termini of CCT substrates (grey) are more easily released from the ring than the C-termini (black) which are bound with higher affinity (Hynes and Willison, 2000; Llorca et al., 2001b). Upon ATP hydrolysis, which occurs sequentially around the ring (Lin and Sherman, 1997), actin moves across the cavity creating a more compact structure, but one which remains attached to the chaperonin (Llorca et al., 2001b).

CCT sequentially around the ring in a defined order (Lin and Sherman, 1997), resulting in movement of substrates from being extended across the cavity to a more compact structure as shown in figure 3C. This hinge-like movement of actin around its nucleotide cleft is important and mutants in this region prevent actin maturing to its native state and result in lack of substrate release from the chaperonin (McCormack et al., 2001a; Neirynck et al., 2006). Nucleotide hydrolysis by CCT subunits therefore directly influences actins' structural conformation, demonstrating that the chaperonin plays an active role in protein folding as opposed to merely providing an environment to allow passive folding to occur (as reviewed by, e.g. Brackley and Grantham, 2009; Gutsche et al., 1999; Horwich et al., 2007). Actin is released from CCT as a productive folding intermediate which is stabilised by nucleotide and cation binding (Altschuler and Willison, 2008). It is uncertain whether actin needs to bind cyclase associated protein (CAP) to become loaded with nucleotide, or whether this final

step towards the maturation of actin occurs whilst bound to CCT, but once achieved, actin monomers are native and able to be incorporated into microfilaments.

CCT regulation by phosducin-like proteins

Recently a role for the family of phosducin-like proteins as regulators of CCT activity has emerged. Originally linked to G-protein signalling, these proteins have been shown to bind to CCT in their native state and regulate CCT-mediated folding (reviewed by Willardson and Howlett, 2007). Three phosducin like proteins have been shown to interact with CCT, but so far their roles are not clearly defined. For example, using an *in vitro* yeast CCT-actin folding system, McCormack et al., (2009) have shown that, via direct interactions between the C-terminus of Plp2p and actin subdomain 4, Plp2p stimulates actin folding, increasing yields by up to 30 fold (McCormack et al., 2009). This is contradictory to the inhibitory effect of human PDCL3 (PhLP2) on CCT folding activity (McCormack et al., 2009; Stirling et al., 2007), suggesting that higher eukaryotes may have developed additional regulatory mechanisms governing phosducin-like protein mediated CCT-substrate folding (McCormack et al., 2009). Our understanding of CCT function *in vivo* will be greatly enhanced as we begin to understand more about the complex regulation of CCT substrate folding by co-chaperones and regulatory proteins.

Roles for CCT monomeric subunits:

CCT's assistance during the folding of newly synthesised proteins fulfils the classic job description of a molecular chaperone. However, it has been shown that CCT has other functional roles, and the focus of the work presented in this thesis extends our knowledge of such non-classical CCT interactions. Although the majority of cellular CCT is in the oligomeric assembly state, the cell also contains CCT subunits as monomeric entities or within micro-complexes composed of a few CCT subunits (Liou and Willison, 1997), additionally, the CCT oligomer is dynamic and can assemble and disassemble under physiological conditions (Roobol et al., 1999a). Interestingly, some CCT interactions occur independently of the chaperonin oligomer and roles for monomeric CCT subunits are emerging. For example, CCT α, γ, ζ and θ have

been identified as microtubule associated proteins (Roobol et al., 1999b) and CCTα (independently or possibly within the oligomer) has been shown to localise to the centrosome (Brown et al., 1996) and to microtubule derived structures important for spermiogenesis (Soues et al., 2003). CCT subunits have recently been implicated in the suppression of polyglutamine aggregates (Behrends et al., 2006; Kitamura et al., 2006; Tam et al., 2006; Tam et al., 2009). Tam et al., (2006) found that in yeast, aggregation can be suppressed by over-expression of CCT1 or CCT4 and interestingly, the apical domain of CCT1 alone is sufficient to suppress aggregation, whilst other subunits do not mediate similar effects (Tam et al., 2006). This once again demonstrates the specific binding characteristics of individual CCT subunits and effectively rules out a requirement for the oligomer in polyglutamine aggregation suppression. Similarly, overexpression of CCT6 can, in an ATP binding dependant manner, rescue abnormal cell phenotypes in *S. cerevisiae* resulting from either *tor2-21*, *lst8-2* and *rsp5-9* conditional mutants or over-expression of Sit4p and Sap155p (Kabir et al., 2005).

These examples, along with the data from paper II, demonstrate that CCT monomers appear to perform a diverse range of functions independently of the oligomer.

Results

Paper I

The extent to which cells rely upon CCT function was investigated in paper I using 2 different approaches to reduce functional levels of the chaperonin.

Firstly, an anti-CCTε antibody (recognising an epitope located in the helical protrusion of the apical domain) was microinjected into synchronised cell populations, leading to an observed delay in cell cycle progression at G_1 to S phase transition. To investigate the effect of the antibody on CCT's substrate folding cycle, α-tubulin, β-actin and a fragment of actin containing subdomains 3 and 4 were translated *in vitro* in rabbit reticulocyte lysate (a system rich in endogenous CCT). In the presence of this antibody, rates of substrate processing were delayed whilst substrate capture rates were unaffected.

The second way in which levels of functional CCT were reduced was by siRNA targeting of CCTζ-1 mRNA resulting in a reduction of total levels of CCT. Targeting cells with siRNA probes against the CCTβ, δ or ζ-1 subunits led to growth arrest without cell-cycle checkpoint activation. CCT protein knockdown did not have general affects upon total levels of newly synthesized cytoplasmic proteins but dramatically affected levels of native cytoskeletal proteins. Whilst total levels of tubulin were significantly reduced just 1 day post-transfection (by up to as much as 70% compared to control cells), total actin levels were relatively unaffected even after 3 days. However, although total actin levels were unaffected, inability of actin to bind DNase I showed that CCT depleted cells have a high proportion of non-native actin. Observed cell phenotypes showing altered cell shape, irregular cytoplasmic actin organisation (as shown by immunofluorescence and phalloidin staining) and altered leading and retracting cell edges resulting in abnormal cell motility were all indicative that the usually meticulously organised actin cytoskeleton had been disrupted by reduced levels of functional CCT.

Therefore, these data demonstrate the dependence upon CCT for cell cycle progression and cytoskeletal organisation. Cell cycle delay can be induced by even a fairly subtle disruption to CCT-substrate folding activity (by antibody microinjection) and when levels of the CCT complex are greatly reduced by siRNA, cytoskeletal organisation is disrupted and cells undergo growth arrest.

An important observation from paper I led to the study in paper II. Biochemical analysis of the assembly state of CCT following siRNA targeting of the CCTζ-1 subunit revealed that levels of the targeted subunit are, as expected, reduced. However, as oligomer assembly cannot occur as one monomeric component is missing, cells will have increased levels of the other seven non-targeted subunits in their monomeric assembly states.

Paper II

As observed in paper I, siRNA targeting of CCTζ-1 influences cell shape resulting in flatter cells with more F-actin staining at the leading edges compared to control cells, so in paper II we investigated the effect on cell shape of targeting each CCT subunit individually. Upon the targeting of CCTα, β, γ, δ, ζ-1, η or θ subunits, cells appeared larger and flatter relative to control cells, however CCTε knockdown cells adopted a spindle-like formation, becoming longer and thinner. This suggests that the presence of monomeric CCTε (found in excess when other CCT subunits are the siRNA target) is responsible for flattened cell morphology and that spindle shaped cells are most likely a result of a lack of CCTε monomer. Supporting this reasoning, double knockdown experiments with CCTε and either CCTβ or δ resulted in spindle shaped cells. Cell shape determination is dependent upon cytoskeletal networks and therefore we investigated the influence of disrupted CCT levels upon microfilament and microtubule re-polymerisation following drug treatment. The drugs used were nocodazole and latrunculin-A which depolymerise microtubules or microfilaments by binding β-tubulin or monomeric actin respectively. Upon removal of nocodazole, microtubule re-growth occurs starting with aster formation and then elongation of the microtubules towards the cell periphery. Compared to control cells, the rate of re-polymerisation is enhanced and the amount of unpolymerised tubulin after 1 hour of re-growth is reduced in the presence of excess CCT monomers resulting from CCTε and ζ-1 siRNA treatment. Re-growth of actin microfilaments is also affected following reduction in CCT levels, but differently than microtubule re-growth. The effect upon F-actin re-growth depends upon the siRNA targeted subunit and whilst CCTζ-1 knockdown cells were able to recover, those depleted of CCTε were unable to re-polymerise their actin microfilaments.

Localisation of both endogenous and epitope-tagged transfected CCT subunits was analysed by immunofluorescence. Cell staining using antibodies recognising endogenous CCT subunits was performed to see if any CCT subunits could possibly influence polymerisation by co-localising to cytoskeletal filaments. Interestingly CCTε staining was fibrous in appearance, reminiscent of F-actin staining. Co-staining with phalloidin and vinculin confirmed that CCTε co-localises to actin microfilaments whilst CCTδ does not. Staining using different endogenous CCTε antibodies confirmed its co-localisation to F-actin and additionally revealed a differently localised sub-population of CCTε at the cell periphery. Different antibodies may preferentially stain CCTε in different intra-cellular locations due to varying ease of accessibility to different antibody epitopes. The availability of epitopes may depend upon, for example, CCT assembly state, protein conformation or protein-protein interactions. Immunofluorescence staining for transiently transfected CCT subunits allowed them to be compared using an antibody to an incorporated epitope tag, thereby eliminating differences in antibody sensitivities for individual subunits. Whilst no subunits were found to localise to microtubules, co-staining with phalloidin revealed that CCTε and to a lesser extent CCTθ were localised to F-actin. Performing the same experiment with N-terminally truncated CCT subunits that are unable to enter the oligomer and therefore remain entirely monomeric, revealed three monomeric subunits, CCTε, ζ and θ, localising to F-actin structures. Biochemical analysis of cell extracts following detergent based removal of soluble cytosolic proteins, revealed that the CCT remaining associated with the non-extracted fraction (containing intact cytoskeletal networks) was found predominantly as monomeric subunits. This is consistent with monomeric CCT subunits being involved in cytoskeletal organisation downstream of the folding of newly synthesised actin and tubulin polypeptide chains.

Paper III

In paper III the actin severing / capping protein gelsolin is identified as a novel CCT-binding protein via a proteomics approach using immunoprecipitation and liquid chromatography – mass spectrometry / mass spectrometry. Potential gelsolin-CCT interactions *in vivo* were also shown by using proximity ligation assays with anti-CCTε and anti-gelsolin primary antibodies.

Interactions between the CCT oligomer and its folding substrates actin and tubulin are well characterised in a rabbit reticulocyte lysate *in vitro* translation system. Translation of gelsolin in the same system demonstrated CCT binding kinetics distinct from those of actin – an obligate CCT substrate. As opposed to binding to, being folded by and subsequently released from CCT, a small percentage of total gelsolin slowly binds and accumulates on the chaperonin oligomer. Structurally, gelsolin is composed of six individual domains, each of which were individually translated in rabbit reticulocyte lysate. Comparing the binding of these six domains to CCT, domain 4 shows higher affinity binding than the other 5 domains, suggesting that gelsolin may bind CCT with some degree of specificity.

Due to the role of gelsolin in microfilament re-modelling, identifying it as a CCT binding protein raises the question as to whether disruption of CCT subunit levels affects gelsolin levels as the findings of papers I and II implicate CCT subunit activity in cytoskeletal organisation and cell shape determination. If gelsolin were an obligate folding substrate of CCT, reducing levels of CCT oligomer by siRNA would have a knock-on effect and functional gelsolin levels should be reduced similarly regardless of the targeted CCT subunit. However, the fact that gelsolin levels are affected differently depending upon the CCT subunit targeted by siRNA, demonstrates that gelsolin does not behave as an obligate substrate. When CCTγ, δ or θ are targeted, levels of gelsolin are elevated relative to the control, whereas targeting CCTβ with siRNA reduces gelsolin levels. Cell staining shows that in both control and siRNA treated cells higher gelsolin levels appear to correlate to cells that are narrower as opposed to those flatter in appearance.

The fact that gelsolin is a CCT-binding protein identifies another link between CCT and cytoskeletal organisation, further broadening the role of this essential chaperonin beyond its protein folding activity essential for newly synthesised proteins.

Discussion

Levels of functional CCT are important for cell cycle progression and cytoskeletal integrity

In paper I microinjection of an anti-CCT antibody (which delays the rate at which the CCT chaperonin is able fold its substrates) induces a delay in cell cycle, whilst siRNA targeting of CCT has a more severe effect, resulting in complete cell cycle arrest. These data demonstrate the importance of CCT for cell cycle progression. Consistent with the timing of the observed cell cycle delay, under normal conditions levels of CCT subunits (at both the mRNA and protein levels) are most up-regulated during G_1/S phase transition of the cell cycle (Yokota et al., 1999). The fact that CCT levels fluctuate throughout cell growth suggests that the requirement for native CCT substrates varies similarly and that chaperonin levels are up-regulated to satisfy such demands. Furthermore, tubulin synthesis levels peak around the G_1/S transition whereas actin levels remain fairly consistent throughout the cell cycle (Yokota et al., 1999). This suggests that, in the presence of the CCTε antibody, levels of newly folded tubulin monomers may be reduced below a functional threshold, causing the observed delay in cell cycle progression. Cdh1, a protein involved in G_1/S transition relies upon CCT for folding to its functional conformation (Camasses et al., 2003). However, native levels of this protein were unaffected by the presence of CCTε antibody (paper I), indicating that loss of its function was not the cause of the observed cell cycle delay.

Whilst cells injected with the antibody were able to progress through S phase, albeit at a delayed rate, when cells were treated with siRNA the effect is more severe and cell growth was arrested. This arrest occurred non-synchronously and without checkpoint activation. This suggests that growth arrest occurs whenever levels of CCT substrates required for subsequent cell cycle progression are insufficient and cannot be replenished as CCT is depleted.

No global effects upon the levels of newly synthesised proteins were observed in cells with reduced levels of functional CCT, this could be expected due to the limited range of CCT obligate substrates, but contrasts the effects observed upon GroEL deletion in *E. coli* (Chapman et al., 2006), whereby 30-40% of cytoplasmic proteins become insoluble. However, CCT siRNA severely affected levels of native actin and tubulin. The observed decreases in total

tubulin levels and native actin levels demonstrate that both tubulin and actin rely upon CCT activity for folding to their native state. Within a time frame where at least 30% of CCT remains relative to the control, it appears that total tubulin levels have already halved, consistent with reports that tubulin levels are tightly regulated. For example, tubulin mRNA stability and synthesis rates are controlled by cytosolic tubulin concentrations and α- and β-tubulin levels are regulated relative to one other (Cleveland and Theodorakis, 1994).

CCT subunit deletion mutants are non-viable (Stoldt et al., 1996), as would be expected if functional CCT is essential for cell homeostasis. Cytoskeletal abnormalities in papers I and II support the hypothesis that CCT function is necessary for cytoskeletal organisation; actin and tubulins' dependence upon functional CCT levels should result in a disrupted cytoskeleton upon reduced CCT function. This also holds true when considering the effect of CCT mutants. A point mutation *anc2-1* in the CCT4 subunit (Vinh and Drubin, 1994) results in a disorganised actin cytoskeleton (Vinh and Drubin, 1994). Although mapped to the outside surface of the apical domain (Llorca et al., 1999a), a region not involved in CCT-substrate interactions, yeast cells carrying this mutation have reduced actin processing rates, due to altered allostery of CCT's ATPase activity (Shimon et al., 2008). Temperature sensitive *tcp1-1-3* mutants of CCT1 also result in cytoskeletal disorganisation and growth defects, which, can be partially rescued by CCT1p expression (Ursic and Culbertson, 1991; Ursic et al., 1994). Furthermore, *plp2* yeast mutants have cytoskeletal and cell cycle defects consistent with CCT folding substrates essential for these processes (Stirling et al., 2007).

In paper I although levels of total actin remained the same, the ratio between G/F-actin was affected upon siRNA targeting of CCT subunits. The pool of G-actin normally localised at the cells' leading edge is absent when CCT levels are reduced, whilst F-actin levels in these regions increase (paper I). This results in altered cytoskeletal organisation at the leading and retracting edges of cells, and consequently disrupted cell motility.

These examples demonstrate both the importance and fragility of the CCT system, which if even mildly disrupted has such far-reaching implications for cytoskeletal processes and ultimately cell viability.

CCT's influence upon the cytoskeleton extends beyond the folding of newly synthesised substrate proteins; monomeric subunits influence cell shape and cytoskeletal dynamics

In paper II the influence of CCT on cytoskeletal organisation was investigated and these effects were considered from the perspective of CCT monomeric subunits. Whilst different cell lines and tissues have varying CCT expression levels, levels of the 8 individual subunits appear to be tightly regulated (Kubota et al., 1999; Yokota et al., 1999). This, combined with the fact that null mutants of CCT subunits are lethal, is consistent with one copy of each subunit occupying a fixed position within each ring (Liou and Willison, 1997). When siRNA targets one CCT subunit, levels of the targeted subunit will be reduced whilst levels of the other subunits as monomers will increase (as oligomers cannot be formed) (paper I) and in paper II this was used to study the effects of individual CCT monomers. Previous reports have suggested roles for CCT monomers in cytoskeletal organisation, CCTα, γ, ζ and θ subunits have been shown to act as microtubule associated proteins (MAPs) (Roobol et al., 1999b) and CCTα has been shown to localise to centrosomes (Brown et al., 1996).

In paper II, CCTε was revealed as a key player in cytoskeletal organisation, influencing cell shape and actin polymerisation. *In vitro* studies have shown previously that CCT can interact with actin during polymerisation (Grantham et al., 2002) influencing the rate of polymerisation but not the final yield. Following polymerisation, not all subunits were found associated to the same degree and CCTδ, a keystone in CCT-actin interactions during folding, was not found associated with microfilaments (Grantham et al., 2002) consistent with paper II where no co-localisation of CCTδ to microfilaments was seen. This suggests that different CCT subunits are involved in interactions with actin monomers during folding and with polymerised F-actin. Rates of actin re-polymerisation following drug induced depolymerisation are dependent upon which monomeric CCT subunits are present in excess (paper II) and cells lacking CCTε cannot re-polymerise their microfilaments, indicative of this subunit, when monomeric, influencing F-actin. CCTε and to a lesser extent ζ and θ co-localise to actin stress fibres, only a subset of which are decorated with CCT subunits, possibly representing the dynamic and heterogeneous nature of F-actin filaments and stress fibres. The actin turnover dynamics of individual stress fibres vary, and the less dynamic they are the more prominent they become (Bertling et al., 2004; Hotulainen et al., 2005). Therefore, as the stress

fibres decorated with CCT subunits tend to be those with weaker phalloidin staining; this may represent those that are more dynamic with higher rates of actin monomer exchange. As well as varying dynamics, F-actin filaments can also have different structural conformations (Egelman and Orlova, 1995a; 1995b). The structure they adopt may depend upon proteins bound to the filament and likewise F-actin binding proteins may only be able to bind to one or other form (Reisler and Egelman, 2007), therefore perhaps the sub-population of CCT bound filaments represents F-actin in a certain conformation.

Together papers I and II demonstrate the importance for CCT for the integrity of the cytoskeleton. Furthermore, CCT subunits in their monomeric form have been implicated as potential regulators of cell shape and mammalian cytoskeletal dynamics. These emerging roles for CCT allow a model to develop describing its interactions with actin and tubulin from nascent polypeptide chains to fully assembled cytoskeletal structures (Figure 4).

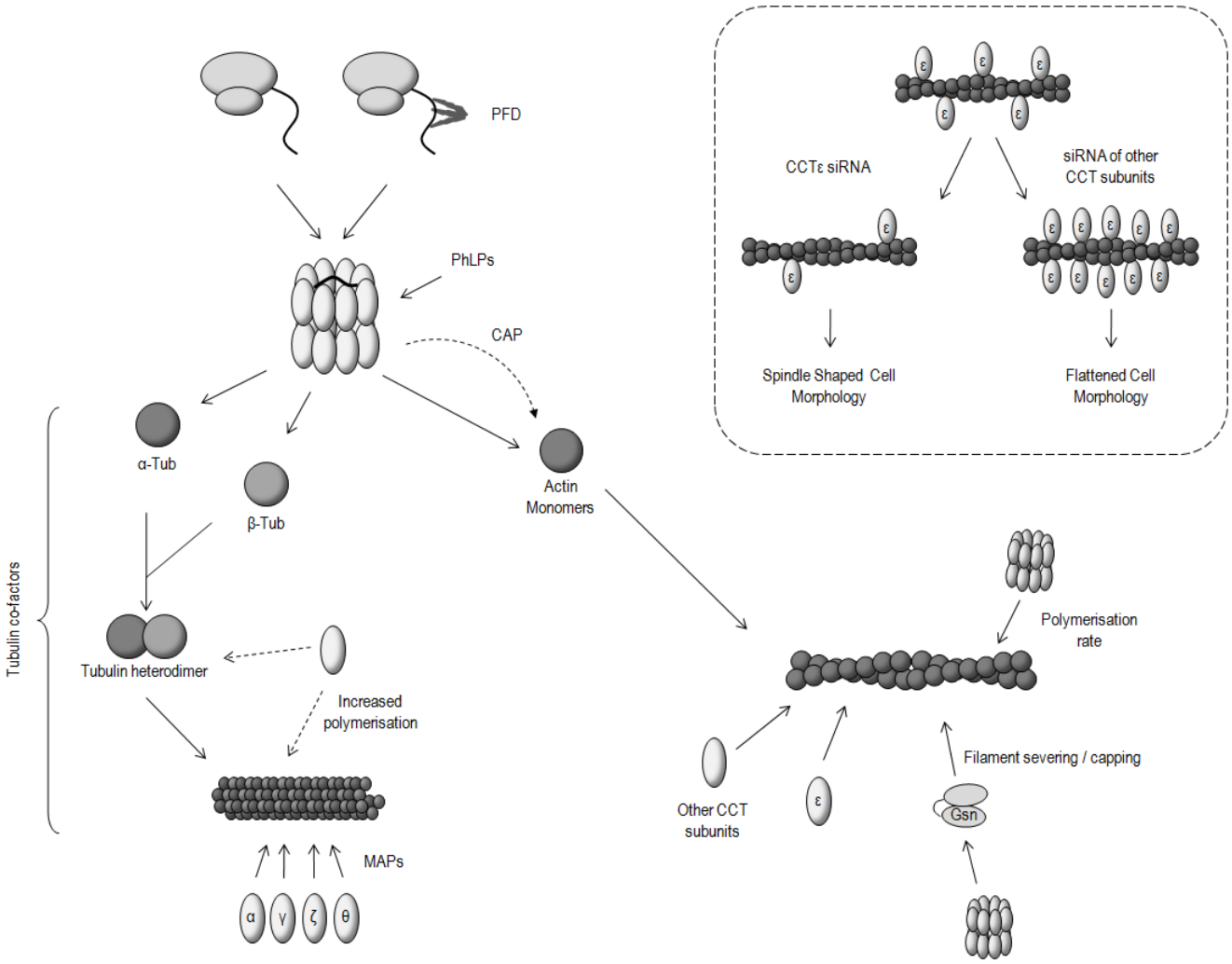

Figure 4: The interaction between CCT and the cytoskeleton extends beyond the folding of newly synthesised polypeptides, and involves interactions with monomeric CCT subunits. CCT monomers have been shown to influence microtubule and microfilament polymerisation and cell shape. Levels of CCTε have been implicated as a factor for cell shape determination and this subunit has been shown to co-localise to microfilaments. As shown in the insert, when CCTε is targeted by siRNA, levels of monomer decrease and cells adopt a spindle-shaped morphology, conversely when other CCT subunits are targeted, levels of monomeric CCTε will increase and result in flattened cell shapes.

Gelsolin binds CCT, but not as an obligate folding substrate

Gelsolin is a protein involved in regulating and remodelling the actin cytoskeleton via its role as an actin severing and capping protein. Gelsolin promotes actin dynamics more effectively than filament-capping actin binding proteins, because as a result of its severing action it increases the number of actin filaments. Although filament capping prevents elongation, this is reversible and in presence of PIP_2, ADF or cofilin, uncapped filament ends will experience rapid turnover (e.g. Sun et al., 1999), therefore gelsolin's influence upon cytoskeletal dynamics extends beyond its severing mechanism.

Gelsolin is the founding member of a family of actin binding proteins containing gelsolin-like structural repeats. Gelsolin has six such domains whose structure and interactions with actin filaments have been characterised. Calcium activation is required to induce conformational changes in gelsolin, exposing actin binding sites and thereby facilitating gelsolin-actin interactions. A schematic representation of gelsolin structure and the way in which conformational changes facilitate F-actin severing and capping are shown in figure 5.

Following the identification of gelsolin as a CCT-interacting protein, the binding of gelsolin to CCT was investigated. Gelsolin appears to bind CCT most strongly via domain 4, which may indicate that gelsolin binds to the CCT chaperonin with some degree of sequence specificity. Gelsolin's six domains share a high degree of structural similarly and conserved sequence (Burtnick et al., 1997). However functional interactions such as gelsolin-actin binding are mediated via residues which vary between the different domains (Kwiatkowski, 1999). This may also hold true for gelsolin-CCT binding, consistent with domain 4 containing sequence specific CCT-binding determinants, which may not be present in the other domains.

Despite this specificity of its interaction, gelsolin does not appear to be "processed" by CCT in the same way as the CCT substrate actin (paper III). Gelsolin slowly accumulates on CCT over time, whereas actin binds to, and is subsequently released from the chaperonin (paper III and e.g. Grantham et al., 2000; McCormack et al., 2001a; McCormack et al., 2001b). This difference suggests that it is unlikely that gelsolin is dependent upon CCT to reach its native conformation. Furthermore, functional gelsolin and individual domains / groups of domains are commonly made in the absence of CCT, for example

when expressed recombinantly in *E. coli* (e.g. Azuma et al., 2000; Nag et al., 2009; Robinson et al., 1999). Gelsolin is a calcium regulated protein and in the presence of calcium undergoes major conformational rearrangements, shifting from a closed, inactive form to an extended, active conformation (Figure 5). Structural studies on the effects of gelsolin domains' calcium binding properties were performed with recombinantly expressed gelsolin (e.g. Choe et al., 2002; Robinson et al., 1999), therefore suggesting that CCT interactions are not required for gelsolin's ability to respond to calcium activation.

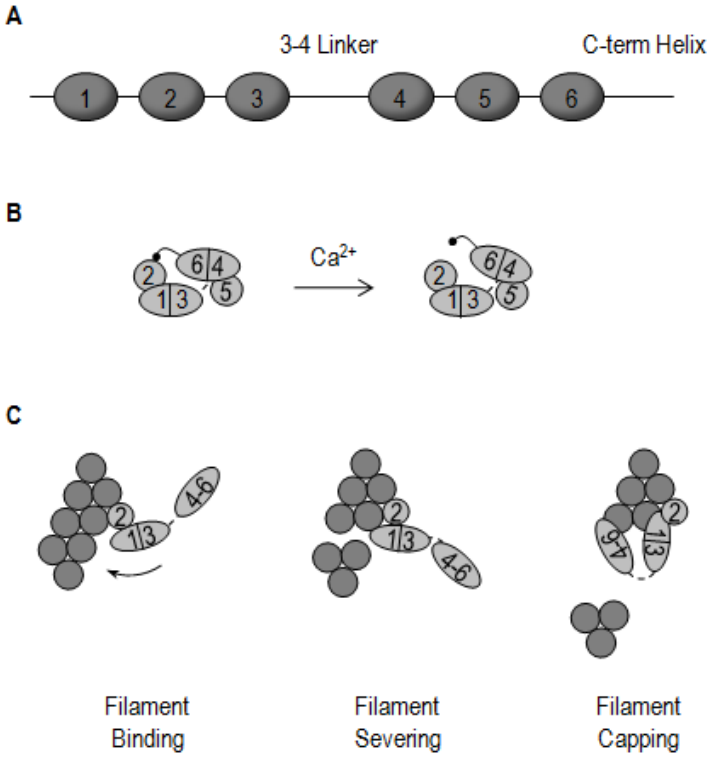

Figure 5: A cartoon representation of gelsolin's structure (A). In the inactive form of gelsolin, a "latch" helix from the C-terminus of domain 6 blocks actin filament binding sites in domain 2, upon calcium activation this latch is released from domain 2 (B). Conformational changes occur around the 53 amino acid linker between domains 3 and 4 and domain 2 binds F-actin thereby positioning domain 1 correctly for actin interactions. Domain 1 disrupts actin-actin interactions causing filament severing and when the C-terminal domains 4-6 swing across, binding a different actin monomer of the opposite side of the filament, capping is complete (C) (Figure based upon Burtnick et al., 1997).

In paper III we show that gelsolin levels are affected by CCT subunit levels. When CCTβ is depleted by siRNA, total gelsolin levels are reduced, whereas CCTγ, δ or θ targeted cells display elevated gelsolin levels. This is once again supportive of the fact that gelsolin is probably not a CCT substrate; if it were, gelsolin levels would be expected to decrease when the oligomer levels are depleted by siRNA. Furthermore, the effect of each individual CCT subunit would be the same. The observation that gelsolin levels are affected differently depednign upon the CCT subunit targeted by siRNA suggests some level of inter-dependence between CCT and gelsolin.

Levels of gelsolin have been linked with cancer cell motility and tumour invasiveness both of which depend upon the actin cytoskeleton. Gelsolin expression levels are reduced in 60-90% of tumours from a wide variety of tissues (reviewed by e.g. Kwiatkowski, 1999) and although this role of gelsolin as a tumour suppressor is consistent with reduced gelsolin mediated apoptosis, gelsolin null mutants do not have increased tumour incidence rates. Furthermore, other reports suggest that increased levels of gelsolin increase cell motility and invasiveness (Van den Abbeele et al., 2007) and can be linked to poor disease prognosis (Shieh et al., 1999), therefore suggestive of gelsolin as a tumour activator. Gelsolin's role in cancer progression is obviously complicated and so far largely undefined. However, as a mediator of actin cytoskeleton remodelling and apoptosis, both of which can influence cancer development, gelsolin may hold important clues for cancer research.

In paper III we have shown that there is a close association between gelsolin, an F-actin severing and capping protein and CCT, suggestive of another possible way in which CCT contributes to the regulation of cytoskeletal organisation.

Concluding Remarks

The work described in this thesis has established new links between the mammalian cytoskeleton and the molecular chaperone CCT.

Functional CCT has been shown to be essential for cell cycle progression and cytoskeletal organisation. CCT monomers have been implicated in influencing cell shape and the polymerisation of microfilaments and microtubules. Furthermore, the actin regulating protein gelsolin has been identified as a CCT-binding protein.

Together these findings suggest that mammalian cytoskeletal organisation is governed by mechanisms dependent upon CCT. Identifying gelsolin as a CCT-interacting protein may broaden the ways in which CCT can regulate the actin cytoskeleton.

Interactions between CCT and cytoskeletal proteins therefore extend beyond CCT's role in the folding of newly synthesised polypeptides to influencing established cytoskeletal networks. Furthermore, this involves CCT subunits in their monomeric assembly state, suggesting a role independent of the CCT oligomer.

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