Antibody characterisation of two distinct conformations of the chaperonin-containing TCP-1 from mouse testis

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Abstract We describe a panel of antibodies specific to individual subunits of the chaperonin-containing TCP-1 (CCT) and one antibody that reacts with all the subunits of CCT. Immunoblot analysis of CCT purified from mouse testis suggests that the testis-specific subunit, S6, may be related to CCTζ and that a co-purifying 63 kDa protein may be a novel subunit of CCT. Using these antibodies in the analysis of CCT subjected to non-denaturing IEF we observed the resolution of two distinct conformations of CCT, which differ in their susceptibility to proteolysis and in the number of associated polypeptides.

Key words: Molecular chaperone; CCT; Mouse testis; Anti-peptide antibody; Non-denaturing IEF

1. Introduction

Chaperonins are double torus protein complexes essential in mediating ATP-dependent polypeptide chain folding in the cell. In eukaryotes, two families of chaperonins have been identified in evolutionarily unrelated cellular compartments. Group I chaperonins are found in eubacteria (GroEL) and in the endosymbiotically derived organelles, chloroplasts (RUBISCO binding protein) and mitochondria (hsp 60). Group II includes chaperonin-containing TCP-1 (CCT) in eukaryotic cytosol and archaebacterial chaperonins (TF55 and thermosome) [1].

One striking difference between CCT and other chaperonins is its hetero-oligomeric composition [2–6]. In contrast to all the other chaperonins, which are composed of one or two subunit species, CCT consists of at least eight polypeptide species encoded by independent genes. Evolutionary analysis of CCT primary structure suggests that each CCT subunit has evolved an independent function related to specific substrate binding in addition to maintaining common properties such as ATPase activity [5]. In vitro studies have shown that CCT can bind to [7] and mediate the folding of [3,8] a variety of proteins, however, to date only actin [4,9] and tubulin [9,10] have been established as physiological substrates of CCT.

We have made polyclonal anti-peptide antibodies to all subunits of mouse testis CCT and have used these antibodies in the analysis of distinct populations of hetero-oligomeric CCT separated by non-denaturing isoelectric focusing. Two conformations of CCT are resolved, which differ in their susceptibility to proteolysis and in the number of associated polypeptides.

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2. Materials and methods

2.1. 2D PAGE

Mouse testis CCT was purified by sucrose gradient fractionation followed by ATP-affinity column chromatography as previously described [2]. Isoelectric focusing (IEF) was carried out according to [11], and was followed by SDS-PAGE on 8% gels and either silver staining or electrotransfer of proteins to nitrocellulose, immunoblotting and detection by the ECL system (Amersham), as previously described [2].

2.2. Non-denaturing isoelectric focusing

Mouse testis CCT was partially purified by sucrose gradient fractionation according to [2]. The fraction corresponding to 20% sucrose was mixed with an equal volume of sample buffer (40% sucrose w/v, 2% ampholytes (Resolyte 4–8; BDH)) and subjected to non-denaturing isoelectric focusing at 500 V for 4 h. The non-denaturing IEF was performed in the same way as denaturing IEF described in [11] with the substitution of 40% sucrose for 50% urea and the omission of CHAPS in the acrylamide gel mixture. Non-denaturing IEF was followed by SDS-PAGE on 8% gels and either silver staining or electrotransfer of proteins to nitro-cellulose, immunoblotting and detection by the ECL system (Amersham).

2.3. Production of anti-peptide antibodies

Peptides were synthesized by Fmoc chemistry (Severn Biotech, Kidderminster, UK). Each peptide (9 mg) was coupled through an amino-terminal cysteine residue to the PPD (purified protein derivative) carrier protein (10 mg). Polyclonal antibodies were raised in rabbits by performing four immunisations over a 96 day schedule. Whole antisera were used at a dilution of 1/500 to 1/2000 for immunoblot analysis.

3. Results

3.1. Polyclonal antibodies to CCT subunits

Mouse testis CCT contains nine subunit species (S1–S9) by 2D PAGE analysis. (Fig. 1A and Table 1). *Tcp-1* and seven *Tcp-1*-related genes encoding the α, β, γ, δ, ε, ζ, η and θ subunits of mouse CCT have been cloned [5,12,13]; the testis-specific subunit, S6, and a co-purifying 63 kDa protein (arrowed in Fig. 1A), which may be a further subunit of mouse CCT, remain to be cloned.

Polyclonal rabbit antibodies were made to carboxy-terminal amino acid sequences deduced from the primary structures of seven subunits of mouse CCT (Table 1), because this region is highly divergent between all CCT primary structures identified so far. A previously characterised monoclonal antibody, 91a [2,5], was used to detect CCTα (Fig. 1D). Six antibodies recognising single subunit species, BC-1, GC-1, DC-1, EC-1, TC-1 and THC-2 were produced, which reacted specifically with the β, γ, δ, ε, η and θ subunits of CCT, respectively, as shown in Fig. 1. In addition to CCTζ, the antibody ZC-1 also reacted with the testis-specific subunit, S6 (Fig. 1G), which indicates that these two subunits have related carboxy-terminal sequences and may be encoded by closely related genes. A polyclonal rabbit antibody, UM-1, was made to an amino-terminal consensus motif which is highly conserved between all

chaperonin sequences and is proposed to be involved in ATP binding and hydrolysis [5,14] (Table 1). UM-1 reated with all nine subunits of mouse testis CCT, including the testis-specific subunit, S6. CCTβ, CCTε, CCTγ, CCTζ and S6 were recognised strongly (Fig. 1J), whilst CCTα, CCTδ, CCTη and CCTθ were recognised weakly (not shown in Fig. 1J but seen on a longer exposure). In addition to the nine subunits described above, UM-1 strongly reacted with the co-purifying 63 kDa protein, which suggests that this protein could be an additional subunit species of mouse testis CCT or alternatively a modified isoform of a previously identified CCT subunit.

3.2. Resolution of native populations of CCT

Partially purified CCT from mouse testis germ cells resolves into two distinct populations, I and II, when subjected to non-denaturing isoelectric focusing (Fig. 2). These populations were further analysed in a second dimension by SDS-PAGE and visualised by silver staining (Fig. 2A) or immunoblotting with specific antibodies (Fig. 2B and C). A striking difference between the two populations is that a large number of polypeptides, in addition to subunits of the CCT complex, are associated with II, whilst I has only a few co-migrating species (Fig. 2A). It is likely that many of these associated polypeptides are substrates for folding by CCT and that II has a high affinity for binding polypeptide chain substrates and I has a low affinity. To date, only tubulin and actin have been established as substrates for folding by CCT in vivo [4,9,10], and both β-tubulin (Fig. 2C) and actin (data not shown) are found associated with II but not with I. Arrowheads show hsp 70 proteins (Fig. 2A). Small amounts of hsp 70 proteins co-immunoprecipitate with CCT under native conditions [2,5], suggesting that hsp 70 is found associated due to its interaction with polypeptide chains undergoing folding on CCT. Hsp 70 proteins resolve away from CCT under these conditions, possibly due to the high pH conditions during sample loading, which indicates that this technique is a stringent analysis of protein interactions and a reflection of the high affinity of CCT form II for substrates. Fig. 2B shows CCT populations I and II immunoblotted with a polyclonal rabbit antibody to CCTε, however, both populations contain all nine subunits of mouse testis CCT as demonstrated by immunoblotting with the antibodies described in section 3.1 (data not shown). Since resolution of native IEF markers in this system demonstrates that separation is on the basis of charge and not size (data not shown), we propose that I and II represent two distinct conformations of hetero-oligomeric CCT which are resolved under these conditions due to different complements of surface charges being exposed in each conformation. During the biochemical extraction of CCT from mouse testis and from other sources, we often observe limited proteolysis of all the subunits in preparations of intact 900 kDa CCT complex. A 30 kDa fragment of CCTε (arrowed in Fig. 2B) is detected only in form II. This differential susceptibility to proteolysis indicates that the cleavage site is more accessible in II, which is supporting evidence that I and II adopt different conformations. Similarly sized proteolytic fragments of all the CCT subunits are detected by immunoblotting only in form II.
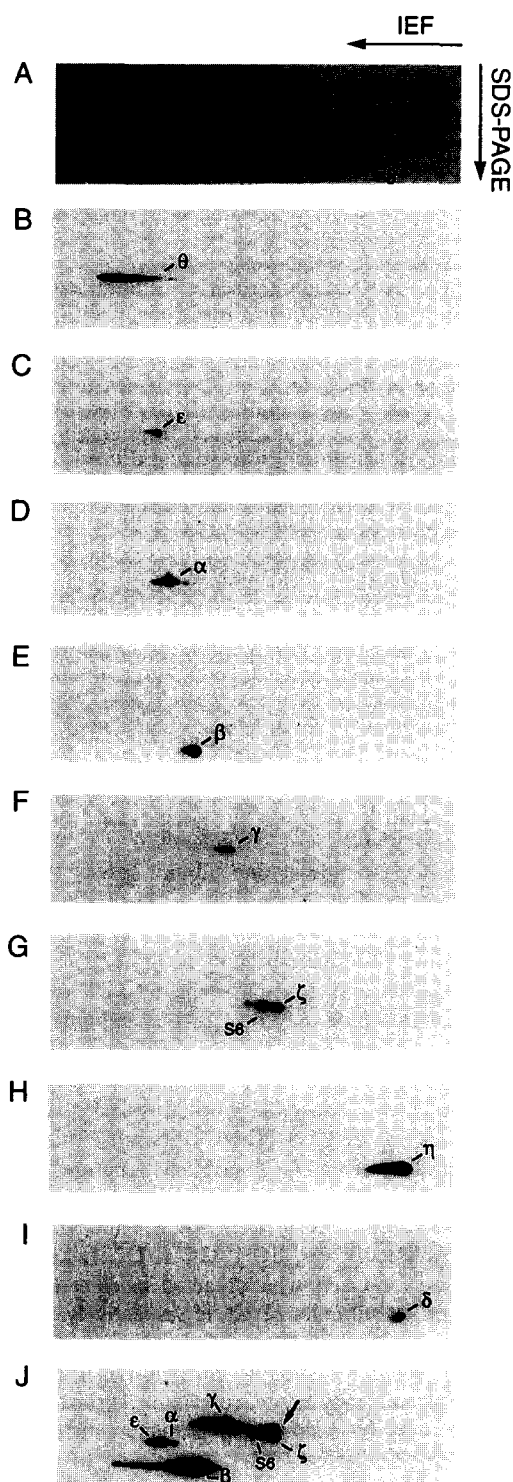

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Fig. 1. 2D PAGE analysis of CCT. Subunits of ATP-affinity purified CCT from mouse testis were separated by 2D PAGE and proteins were visualised by silver staining (A). Greek letters indicate the eight subunit species the genes of which have been cloned, and S6 shows the testis-specific subunit. A novel co-purifying 63 kDa protein of pI 6.93 is indicated by an arrow, and hsp 70 proteins are shown by arrowheads. CCT subunits were immunoblotted with rabbit antibodies against carboxy-terminal amino acid sequences of CCTθ (B), CCTε (C), CCTβ (E), CCTγ (F), CCTζ (G), CCTη (H), CCTδ (I), and a monoclonal antibody, 91a, against CCTα (D). Panel J shows CCT subunits immunoblotted with a rabbit antibody to a chaperonin consensus sequence thought to be involved in ATP-binding. Sequences of the peptide immunogens are shown in Table 1. In all panels the acidic side is to the left.
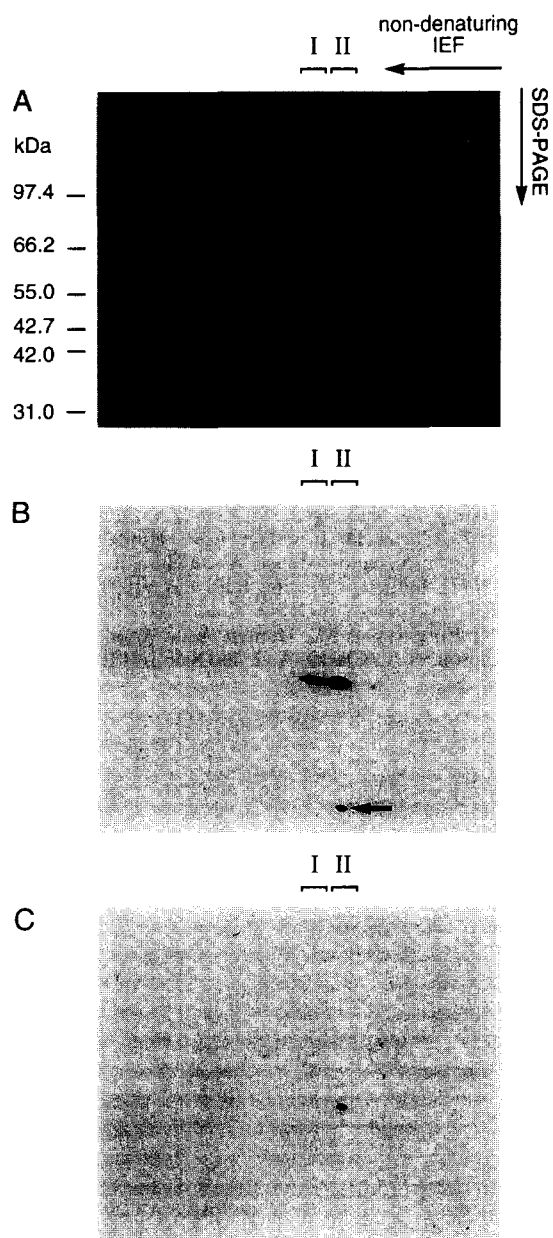
Fig. 2. Analysis of native populations of CCT. Partially purified CCT from mouse testis was subjected to non-denaturing isoelectric focusing followed by SDS-PAGE. Proteins were visualised by silver staining (A). Native populations of CCT resolved by non-denaturing IEF are indicated by I and II and arrowheads show hsp 70 proteins. Native populations of CCT were immunoblotted with a rabbit antibody against CCTε (B), a 30 kDa proteolytic fragment of CCTε is indicated by an arrow, or a monoclonal antibody against β-tubulin (C). In all panels the acidic side is to the left.

The sizes of these proteolytic fragments are between 28 and 30 kDa, suggesting that the protease-sensitive sites reside in the putative apical polypeptide chain binding region of each CCT subunit [14].

4. Discussion

Antibodies specific for each subunit species will be useful to investigate the combination and arrangement of subunits in the CCT complex, and the antibody, UM-1, which recognises all the subunits, can be used as a general reagent to identify CCT from other eukaryotes. Characterisation of mouse testis CCT revealed that the testis-specific subunit, S6 [5], may be related to CCTζ, and that a co-purifying 63 kDa protein may be a novel subunit of mouse testis CCT. These two species are probably encoded by novel Tcp-1-related genes (Hynes and Kubota, unpublished results).

A problem with the biochemical analysis of large molecular weight complexes such as CCT is in resolving different states even when these states differ by the addition of accessory polypeptides, such as substrates or co-factors. We have described a non-denaturing isoelectric focusing technique that facilitates the resolution of two forms of CCT. One form is bound to many other polypeptides and is susceptible to proteolysis, whilst the other form is bound to only a few other polypeptides and is resistant to proteolysis. These results suggest that CCT in the cell may adopt two distinct conformations with different affinities for polypeptide chain substrates. The conformation with high binding affinity for substrates may expose a domain which is susceptible to proteolysis in each CCT subunit, and this site may be located within the substrate binding domain since it is in a similar position to the substrate binding domain of GroEL [15,16]. In GroEL, dramatic structural changes have been associated with ATP binding [17,18]. One interpretation of our data is that binding or hydrolysis of ATP acts as a switch between conformational forms of CCT that interact strongly or weakly with polypeptide chain substrates. The wide range of polypeptides found associated with CCT suggests that the physiological substrates for CCT may not be limited to actin and tubulin, and implies that CCT has a general role in protein folding in the eukaryotic cytosol. The resolution of different conformations of protein complexes should prove useful in analysing aspects of the functions and properties of all types of chaperonins.

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Table 1
Immunochemical analysis of CCT subunits

Antibody used for detection	Sequence of peptide immunogen	Corresponding subunit of mouse CCT	Species recognised on 2D gel
THC-2	SGKKDWDDDQND	CCTθ	S1
EC-1	IDDIRKPGESEE	CCTε	S2
91a*	–	CCTα	S3
BC-1	APRKRVPDHHPC	CCTβ	S4
GC-1	NRQTGAPDAGQE	CCTγ	S5
ZC-1	EIMRAGMSSLKG	CCTζ	S6, S7
TC-1	SAGRGRGQARFH	CCTη	S8
DC-1	SILKIDDVVNTR	CCTδ	S9
UM-1	QDDEVGDGTTSVV	chaperonin consensus motif	S1, S2, S3, S4, S5, S6, S7, S8, S9, p63**

*Denotes a rat monoclonal antibody which recognises the C-terminus of CCTα [2].
**Denotes a co-purifying 63 kDa protein of pI 6.93.

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