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Experimental research

Polyglutamined expanded androgen receptor interacts with chaperonin CCT

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ABSTRACT

CCT chaperonin is a highly conserved molecular chaperone, which plays an important role in the folding of complex proteins in mammalian cells. CCT chaperonin interacts with huntingtin and results in decrease of aggregate formation followed by increase of cell survival. Using yeast-two-hybrid system, we screen for specific CCT chaperonin subunit, which can recognize and bind to androgen receptor. We show that subunit 6 of CCT chaperonin interacts with androgen receptor. Interestingly, CCT chaperonin shows higher binding affinity to polyglutamine expanded androgen receptor than that of the wild-type. We prove this interaction in mammalian cell models, which show co-localization of androgen receptor and subunit 6 of CCT in cellular cytosol. Therefore, not only huntingtin but also androgen receptor is a polyglutamine expanded protein, which is a substrate of CCT chaperonin. Our results suggest that CCT might play an essential role in modulation of folding of polyglutamine expanded proteins and could be another target for further therapeutic studies.

1. Introduction

Spinobulbar muscular atrophy (SBMA) or Kennedy's disease is one of neurodegenerative disorders caused by polyglutamine expansion [1]. This mutation causes nine diseases which, altogether, constitute the most common inherited neurodegenerative disorder including Huntington's disease (HD), spinocerebellar ataxia type 1, 2, 3, 6, 7, and 17 (SCA 1, 2, 3, 6, 7, and 17), dentatorubral-pallidoluysian atrophy (DRPLA), and SBMA [2]. SBMA is caused by CAG expansion mutation in *androgen receptor* (*AR*) gene, which results in progressive apoptosis of motor neurons in brain stem and anterior horn cells in spinal cord [3,4]. Expansion of polyglutamine tract affects folding of androgen receptor (AR) into toxic oligomers, which are highly hydrophobic [5–9]. The toxic proteins are subjected to cleavage into short, N-terminal, polyglutamine containing fragments, which are resistant to cellular degradation [10–12]. These insoluble fragments finally aggregate as intracellular amyloid plaque and apoptotic cell death takes place [13,14].

CCT chaperonin or chaperonin containing T-complex polypeptide 1 (TCP-1) or the so called TRiC (TCP-1 ring complex) is a large molecular weight chaperone containing 8 subunits those form a chamber within its cylindrical shaped structure. It is structurally conserved and is localized in cytoplasm [15–17]. The main function of CCT chaperonin is to promote correct folding of newly synthesized proteins. Each CCT subunit contains a different substrate binding site, which specifically binds to different proteins prior to regulate their folding. Unlike most molecular chaperones, CCT is not induced by stress, but is instead transcriptionally and functionally linked to protein synthesis [9]. Recent studies have demonstrated the ability of specific subunits of CCT chaperonin, to specifically bind and reduce aggregation of polyglutamine expanded huntingtin protein [18–21]. CCT chaperonin may prevent aggregate formation by specifically binding to mutant huntingtin and further refolding it into more soluble conformation.

In the present study, we reported the genotype–phenotype correlation of a SBMA patient, who had a polyglutamine expanded AR and characteristics of spinal and bulbar motor neuron degeneration, and showed for the first time for the contribution of CCT chaperonin in modulation of polyglutamine expanded AR by its interaction with polyglutamine expanded AR.

2. Materials and methods

2.1. DNA samples and polymerase chain reaction (PCR)

With approval by the Human Research Ethics Committee, Siriraj Institutional Review Board, Mahidol University (#SI054/2012),

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human DNA containing *AR* gene with 21 and 64 CAG repeats representing wild-type and expansion mutations were amplified using a primer pair encompassing AR exon 1. Primers were 5′-TTG ACT GTA TCG CCG ATG GAA GTG CAG TTA-3′ and 5′-CCG GAA TTA GCT TGG CTG CAG TCA ACG CAT GTC CC-3′, which incorporated PCR products with 5′ and 3′ flanking recombination tags (RTs). The PCR in total volume of 50 μl contained 400 ng of genomic DNA, 10 μl of 10× platinum® *Pfx* (Invitrogen) reaction buffer, 1 μL of 50 mM $MgSO_4$, 15 pmol of each primer, 15 mM of deoxynucleotide triphosphate (dNTPs), 15 μL of 10× platinum® *Pfx* enhancer and 1 units of platinum® *Pfx* DNA polymerase. The reaction was carried out in a GeneAmp PCR System 2400 (Applied Biosystems), starting with an initial denaturation step of 94 °C for 10 min and followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 52 °C for 30 s, and extension at 68 °C for 2 min, and one cycle of final extension at 68 °C for 7 min.

The entire coding sequences of 8 subunits of CCT chaperonin were amplified from human cDNA using each subunit specific primer pairs. All primer pairs also incorporated the 5′ and 3′ flanking recombination tags (RT) to the amplified products. Sequences of the primer used were shown in Table 1. The PCR in total volume of 50 μl contained 2 μL of cDNA, 10 μl of 10× platinum® *Pfx* (Invitrogen) reaction buffer, 1 μL of 50 mM $MgSO_4$, 7.5 pmol of each primer, 15 mM of deoxynucleotide triphosphate (dNTPs), 15 μL of 10× platinum® *Pfx* enhancer and 1 units of platinum® *Pfx* DNA polymerase. The reaction was carried out in a GeneAmp PCR System 2400 (Applied Biosystems), starting with an initial denaturation step of 94 °C for 10 min and followed by 35 cycles of denaturation at 94 °C for 15 s. Then, the reaction was kept up with 30 s of each specific annealing temperature, which is 54 °C for CCT 1, 2, 3, 5, and 7, 52 °C for CCT 4 and 6, or 48 °C for CCT 8. Lastly, extension at 68 °C for 2 min, and one cycle of final extension at 68 °C for 7 min were performed. An aliquot (5 μl) of the PCR reaction was loaded onto 2% agarose gel to verify the size and quantity of the PCR product.

2.2. Yeast two-hybrid system

Two-hybrid screening was performed as described by Finley and Brent [22]. The galactose-inducible pJZ4-NRT [23] was used as the bait plasmid to express AR fused to B42 activation domain, while the constitutively expressed pEG-NRT [23] was used as the prey plasmid to express CCT subunit fused to LexA DNA binding domain. *Saccharomyces cerevisiae* RFY231 strain (MATα *his3 leu2::3Lexop-LEU2 ura3 trp1 LYS2*) containing *LEU2* gene, which is a leucine reporter system, was transformed with the bait vector, pJZ4-NRT, to express AR fusion protein. *S. cerevisiae* RFY206 strain (MATa *his3Δ200 leu2-3 lys2Δ201 ura3-52 trp1Δ::hisG*) was transformed with the prey vector, pEG-NRT, to express CCT subunit fusion protein together with pSH18-34, which contains a *LacZ* reporter system.

In vivo homologous recombination was performed using 300 ng of linearized vectors and 100 ng of the PCR product using the LiOAc method [24]. RFY231 containing bait vector was selected on glucose drop-out medium lacking tryptophan (glu/-w), while RFY206 containing prey vector and pSH18-34 was selected on glucose drop-out medium lacking histidine and leucine (glu/-h/-l). Plasmids were harvested, purified and DNA sequence analysis was then performed to ensure correct DNA insert. Primers were 5′-CTT GCT GAG TGG AGA TGC CTC-3′ and 5′-CGG TTA GAG CGG ATG TGG G-3′ for pJZ4-NRT and 5′-TCG TTT TAA AAC CTA AGA GTC-3′ and 5′-AGC TTC ACC ATT GAA GGG CTG-3′ for pEG-NRT. The PCR reaction was carried out in a Gene-Amp PCR System 9700 (Applied Biosystems) consisting of 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for pJZ4-NRT or 48 °C for pEG-NRT for 30 s, and extension at 72 °C for 2 min, and one cycle of final extension at 72 °C for 7 min.

Each of the bait and prey strains were mated and plated on the indicator plates, including glucose or galactose drop-out medium containing X-Gal but lacking histidine, tryptophan and uracil (glu/X-Gal/-u/-h/-w or gal/raf/X-Gal/-u/-h/-w/). The interaction of bait and prey fusion proteins was expected to activate 3Lexop-*LacZ* reporters, which gave blue colony on gal/raf/X-Gal/-u/-h/-w/ but remained white on glu/X-Gal/-u/-h/-w. Transforming the positive interaction control plasmids (pCDI2 and pRFHM12), and the negative interaction control plasmids (pCDI2 and pRFHM1) into the yeast cells were also performed. In addition, mating with empty vectors (pEG-NRT and pJZ4-NRT) was performed to exclude auto-activation [25].

2.3. Cell culture and transfection assays

Directional TOPO® cloning system (Invitrogen) was used as manufacturer's instruction. Briefly, human DNA containing *AR* gene with 21 and 64 CAG repeats and human DNA containing *huntingtin* gene with 22 and 50 CAG repeats were each amplified and cloned into pENTR™/SD/D-TOPO® vector (Invitrogen). Sequences of primers used in mammalian expression study were shown in Table 2. Positive clones were selected in LB agar plates containing kanamycin. The purified plasmids were then recombined into pcDNA-DEST-53 vectors (Invitrogen) using LR recombination reaction to generate pDEST53-AR-21Qs, pDEST53-AR-64Qs, pDEST53-HD-22Qs, and pDEST53-HD-50Qs as expression vectors for AR-21Qs, AR-64Qs, HD-22Qs, and HD-50Qs, respectively. Plasmid pcDNA-DEST53 contains N-terminal GFP-tag to incorporate green fluorescent protein into N-terminus of the expressed protein. The constructed plasmids were propagated in *E. coli* and positive clones were selected in LB agar plates containing

Table 1
Primers used in amplification of AR and CCT chaperonin for yeast-two-hybrid study.

Names	Sequences
AR-ex1F	5′-TTG ACT GTA TCG CCG ATG GAA GTG CAG TTA-3′
AR-ex1R	5′-CCG GAA TTA GCT TGG CTG CAG TCA ACG CAT GTC CC-3′
CCT1-F	5′-TTG ACT GTA TCG CCG ATG GAG GGG CCT T-3′
CCT1-R	5′-CCG GAA TTA GCT TGG CTG CAG TCA ATC ATT AAG GGC-3′
CCT2-F	5′-TTG ACT GTA TCG CCG ATG GCA TCC CTT TCC CTT GC-3′
CCT2-R	5′-CCG GAA TTA GCT TGG CTG CAG TTA ACA GGG GTG GTG ATC-3′
CCT3-F	5′-TTG ACT GTA TCG CCG ATG ATG GGA CAC CGT CCA GTG CTC GTG-3′
CCT3-R	5′-CCG GAA TTA GCT TGG CTG CAG TCA CTC TTG ACC AGC ATC AGG AGC CCC GCC TTG C-3′
CCT4-F	5′-TTG ACT GTA TCG CCG ATG CCC GAG AAT GTG-3′
CCT4-R	5′-CCG GAA TTA GCT TGG CTG CAG TTA TCG AGT GTT TAC-3′
CCT5-F	5′-TTG ACT GTA TCG CCG ATG GCA TCC ATG GGG ACC CTC GC-3′
CCT5-R	5′-CCG GAA TTA GCT TGG CTG CAG TCA TTC TTC AGA TTC TCC AG-3′
CCT6-F	5′-TTG ACT GTA TCG CCG ATG GCA GCA GTG AAG ACC CTG AAC-3′
CCT6-R	5′-CCG GAA TTA GCT TGG CTG CAG TCA ACC TTT CAG AGA AG-3′
CCT7-F	5′-TTG ACT GTA TCG CCG ATG ATG CCC ACA CCA GTT ATC-3′
CCT7-R	5′-CCG GAA TTA GCT TGG CTG CAG TCA GTG GGG GCG GCC-3′
CCT8-F	5′-TTG ACT GTA TCG CCG ATG GCA CTT CAC GTT C-3′
CCT8-R	5′-CCG GAA TTA GCT TGG CTG CAG TCA ATC ATT TTG GTC-3′

Table 2
Primers used in mammalian expression study.

Names	Sequences
ARtopo-ex1F	5′-CAC CCA GAT GGA AGT GCA GTT AGG GCT G-3′
ARtopo-ex1R	5′-CGC ATG TCC CCG TAA GGT CCG-3′
HDtopo-ex1F	5′-CAC CCA GAT GGC GAC CCT GGA AAA GCT G-3′
HDtopo-ex1R	5′-GGT CGG TGC AGC GGC TCC TCA-3′