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CUEDC2 interacts with heat shock protein 70 and negatively regulates its chaperone activity

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ABSTRACT

Recently studies have revealed that CUEDC2, a CUE domain-containing protein, plays critical roles in many biological processes, such as cell cycle, inflammation and tumorigenesis. In this study, to further explore the function of CUEDC2, we performed affinity purification combined with mass spectrometry analysis to identify its interaction proteins, which led to the identification of heat shock protein 70 (HSP70). We confirmed the interaction between CUEDC2 and HSP70 *in vivo* by co-immunoprecipitation assays. Mapping experiments revealed that CUE domain was required for their binding, while the PBD and CT domains of HSP70, mediated the interaction with CUEDC2. The intracellular Luciferase refolding assay indicated that CUEDC2 could inhibit the chaperone activity of HSP70. Together, our results identify HSP70 as a novel CUEDC2 interaction protein and suggest that CUEDC2 might play important roles in regulating HSP70 mediated stress responses.

1. Introduction

CUEDC2 is a CUE domain containing protein. CUE domain is an approximately 40 amino acids containing motif, which is able to bind mono-ub or poly-ub, and protein containing CUE domain is proposed to function as scaffolds for complexes that involve in trafficking and ubiquitination pathways [1]. Until recently, the function of CUEDC2 has not been uncovered. Zhang et al. first reported that CUEDC2 interacts with the progesterone receptor (PR) and promote progesterone-dependent PR degradation by ubiquitination [2]. After that, accumulating evidences revealed that CUEDC2 is a multi-functional protein playing important roles in many biological events, such as cell cycle regulation, inflammation, and tumorigenesis [3–5]. Gao et al. reported that CUEDC2 plays critical roles in metaphase–anaphase transition, during which CUEDC2 is phosphorylated by Cdk1 and controls anaphase-promoting complex or cyclosome (APC/C) activation [5]. Another study demonstrated that CUEDC2 interacts with IkB kinase (IKK) and has an inhibitory role in the activation of transcription factor nuclear factor-κB signaling, which play pivotal roles in inflammatory responses and tumorigenesis [6–8]. More recently, Pan et al. reported that CUEDC2 plays an important role in downregulating the expression of hormone receptors estrogen receptor-α, thereby impairing the responsiveness of breast cancer to endocrine therapies [3]. Although recent studies have uncovered some features of CUEDC2, and established its important roles in inflammation and tumorigenesis, the more detailed functions of CUEDC2 and the underlying mechanisms remain to be clarified.

Heat shock proteins (HSPs) are initially described as a group of proteins which could be induced by heat shock [9]. Later, it is acknowledged that they play important physiological roles in normal conditions and pathological situations upon various cellular stresses [10]. HSPs are categorized into four major families on the basis of their molecular weights: HSP90, HSP70, HSP60, and small HSPs including HSP27 and HSP10 [11]. Among them, HSP70 is a major member of molecular chaperones which assists various processes involving folding, unfolding and homeostasis of cellular proteins [12]. Through interaction with different partners and cofactors, it carries out diverse functions, such as refolding denatured proteins, transporting proteins into organelles, targeting substrates for degradation and regulating the heat-shock response [13]. Upon heat shock and other environmental stresses, HSP70 is induced and exerts protective effects to enhance cell survival. Studies indicate that HSP70 protect cells and organs against vari-

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ous deleterious effects and is involved in many human diseases, including infection, ischemia, and tumorigenesis [10,14,15]. For example, HSP70 is reported to play important roles in gastric inflammation and ulcer healing [10]. Other studies showed that HSP70 is overexpressed in majority of cancers, and its expression level is correlated with tumor grade and poor prognosis [14]. Targeting this protein for cancer therapy is promising, and several inhibitors have been tested as anticancer agents in pre-clinical or clinical trials [14,15].

HSP70 contains three domains, namely the ATPase domain (AD), peptide-binding domain (PBD) and C-terminal domain (CT) [16]. ATPase domain is involved in ATP hydrolysis, and PBD domain is required for substrates connection. C-terminal of HSP70 contains four conservative amino acids, Glu–Glu–Val–Asp, and could bind co-chaperones. Several co-chaperones, such as FAF1 [17], HIP [18] and BAG-1 [18,19] have been reported to modulate the chaperone activity of HSP70, and consequently affect its function in various stress responses.

In this study, using affinity purification combined with mass spectrometry analysis, we identified HSP70 as a new CUEDC2-binding protein. We confirmed the interaction between CUEDC2 and HSP70 with co-immunoprecipitation assays in mammalian cells. Through mapping experiments, we determined that CUE domain of CUEDC2 mediated their binding, while both the PBD and CT domain of HSP70 was required for the interaction with CUEDC2. The intracellular Luciferase refolding assay demonstrated that overexpression of CUEDC2 inhibited the chaperone activity of HSP70. Therefore, CUEDC2 is a novel negative regulator of chaperone activity of HSP70, and it might play important roles in regulating HSP70 function in response to stresses.

2. Materials and methods

2.1. Plasmid constructions

GST-tagged different truncate mutants of HSP70, FLAG–HSP70 and Myc–HSP70 were kindly provided by Professor Alexandra C. Newton (University of California, San Diego). pRSVLL/V encoding cytoplasmic luciferase was gift from by S. Subramani (University of California, San Diego). For mapping assay, the corresponding CUEDC2 cDNAs (1–287, 1–226, 1–180 and 1–133aa) were amplified by PCR and cloned in-frame into pCDNA3.0-FLAG vectors.

2.2. Cells and reagents

HEK293T cells and HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, GIBCO BRL/Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) in the cell incubator (37 °C, 5% CO_2). Cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instruction. Monoclonal anti-FLAG antibody M2, anti-FLAG-coupled agarose (M2) beads was purchased from Sigma. Anti-c-Myc (sc-40) was purchased from Santa Cruz Biotech. Anti-HSP70 (ADI-SPA-810), anti-HSP27 (ADI-SPA-800), anti-HSP40 (ADI-SPA-450), anti-HSP90 (ADI-SPA-831) were obtained from Enzo Life Sciences. Monoclonal anti-CUEDC2 antibody was prepared in our laboratory.

2.3. Affinity purification and mass spectrometric analysis

For identification of CUEDC interaction proteins, FLAG–CUEDC2 was transiently transfected into HEK293T cells. 48 h after transfection, the cells were harvested for affinity purification with anti-FLAG agarose beads. Immunoprecipitations were subjected to SDS–PAGE and the gels were Coomassie Blue G-250 (CBB G250)-stained for visualization. The interested bands were cut out and subjected to in-gel digestion with trypsin and mass spectrometry analysis as previously described [20]. Briefly, protein bands were analyzed by NanoLC-HDMS MS/MS on an Acquity™ Nano UPLC system (Waters Corp., Milford, USA), and then Synapt high-definition mass spectrometry (HDMS) was performed with a nanospray ion source (Waters). Peak lists were generated using PLGS 2.2 software and automatically combined into a single pkl file for every LC-MS/MS run. The MS/MS data were acquired and processed using MassLynx V4.1software (Micromass). Mascot from Matrix Science in March 2013 was used to search the database. Protein identification was repeated at least twice using bands from different gels.

2.4. Co-immunoprecipitation and immunoblotting

HEK293T cells were transfected as indicated and harvested 48 h after transfection. For immunoprecipitation experiments, cells were lysed in E1A lysis buffer (50 mM Hepes pH 7.6, 250 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 mM DTT, 1 mM PMSF and cocktail). The whole cell lysates were collected and centrifuged at 4 °C, 10,000 rpm for 10 min. Supernatants were incubated with anit-FLAG agarose beads for 6–8 h at 4 °C after preincubating with protein A/G-Sepharose (Santa Cruz Biotechnology). The immunoprecipitations were washed three times with E1A wash buffer (50 mM Hepes pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.1% NP40 and 1 mM PMSF), boiled in sample buffer, and immunoblotted with anti-Myc and anti-FLAG antibodies.

2.5. Luciferase reactivation assay

HEK293T cells were transfected with pRSVLL/V and indicated plasmids. After 24 h, the cells were treated with 20 μg/ml cycloheximide for 30 min. Luciferase was inactivated by heating the cells at 45 °C for 30 min and recovered at 37 °C for various times. Luciferase activities of the harvested cells were measured using Dual-Luciferase Reporter Assay System (Promega) as described by the manufacturer's instructions. All experiments were repeated at least three times.

3. Results

3.1. Identification of HSP70 as CUEDC2-interacting protein

To identify CUEDC2 interacting proteins, affinity purification and mass spectrometry analysis were applied in this study. FLAG-tagged CUEDC2 was transiently overexpressed in HEK293T cells. 48 h after transfection, immunoprecipitaion were performed using anti-FLAG M2 agarose beads. Following affinity purification, immunoprecipitates were separated by SDS–PAGE and CBB G250-stained for visualization (Fig. 1A). Antibody chains, FLAG–CUEDC2 and its phosphorylated form are indicated [5] (Fig. 1A). Protein bands detected in FLAG–CUEDC2 transfected cells, rather than in FLAG vector transfected cells, were cut out from gels, subjected to in-gel digestion with trypsin and followed by mass spectrometry analysis. The protein band with molecular weight of about 70 kD was identified as HSP70 (Fig. 1B).

3.2. Confirmation of interaction between HSP70 and CUEDC2

To confirm the interaction between HSP70 and CUEDC2, pCDNA3.0-FLAG-CUEDC2 was transfected into HEK293T cells (Fig. 2A). FLAG–CUEDC2 was immunoprecipitated from cell lysates by anti-FLAG and analyzed for heat shock proteins binding by immunoblotting. As indicated in Fig. 2A, HSP70 could be co-immunoprecipitated in the presence of FLAG–CUEDC2, while other heat

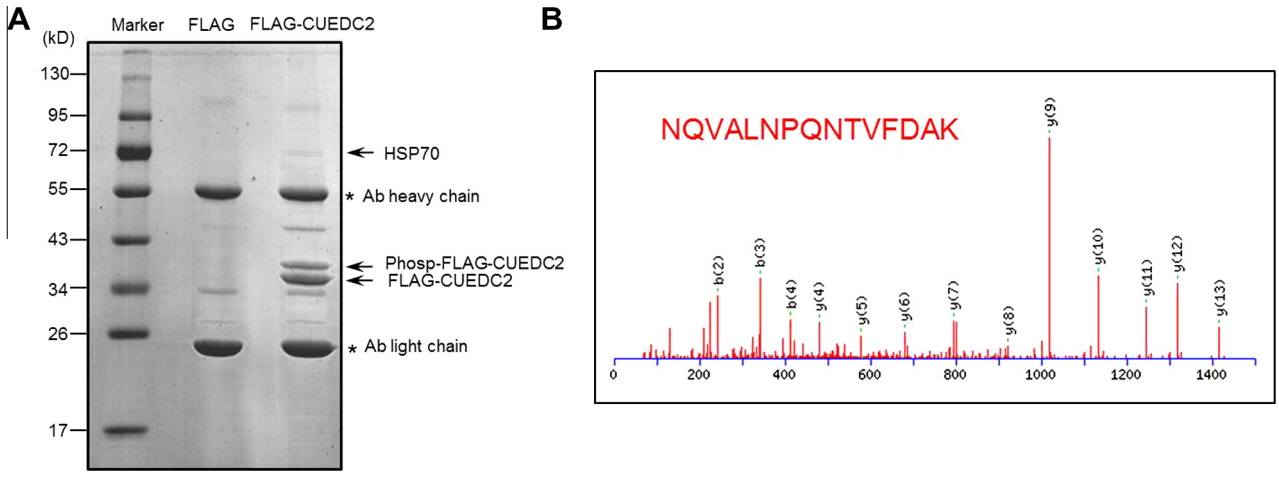

Fig. 1. Identification of HSP70 as CUEDC2-interacting Protein. (A) Affinity purification and Mass spectrometry analysis were used to identify proteins that interact with CUEDC2. HEK293T cells were transfected with pcDNA 3.0-FLAG vector (lane 2) or FLAG-tagged CUEDC2 (lane 3). Cell were lysed and immunoprecipitated with anti-FLAG M2-agarose beads. Precipitates were separated with SDS–PAGE and visualized by CBB G250 staining. Protein bands only detected in FLAG–CUEDC2 transfected cells, rather than in FLAG vector transfected cells were indicated by numbers with arrows. Candidate protein bands were cut out from the gel and subjected to in-gel digestion with trypsin, and Mass spectrometry analyses were conducted. The peptide of 1657.83 for band 1 was sequenced by nano-ESI-MS/MS and the deduced sequence is indicated (B).

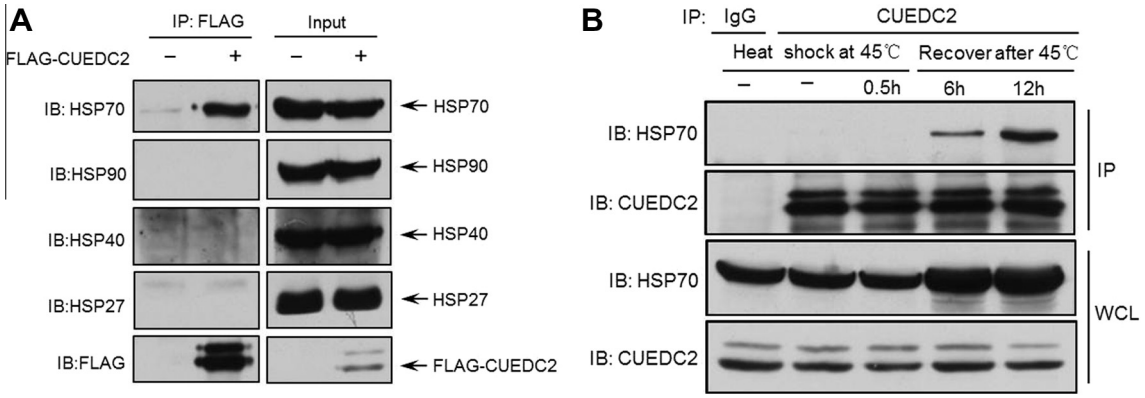

Fig. 2. Associated of HSP70 with CUEDC2 *in vitro* and *in vivo*. (A) FLAG–CUEDC2 and Myc–HSP70 plasmids were co-transfected into HEK293T cells and Immunoprecipitation was performed using anti-Flag agarose beads, followed by the western blot analyses of immunoprecipitates and whole cell lysate using anti-Flag or anti-Myc antibodies. (B) Hela cells were heated at 45 °C for 30 min, and recovered for various times at 37 °C. Lysates were subjected to immunoprecipitation with anti-CUEDC2 antibody or a control mouse IgG.

shock protein family members, including HSP90, HSP40, and HSP27 could not be detected in the immunocomplex. The co-immunoprecipitation experiment confirmed that HSP70 could specifically bind CUEDC2 in mammalian cells. We next investigated whether endogenous CUEDC2 interacts with endogenous HSP70, and whether this interaction is regulated by heat shock. Hela cells were exposed to heat shock (45 °C) for 30 min, recovered for various times at 37 °C, and then immunoprecipitated with anti-CUEDC2 antibody. The immunocomplexes were subjected to SDS–PAGE, followed by Western blot analysis. HSP70 could hardly be detected in immunocomplex when the cells were untreated or heat treated for 30 min. In contrast, during recovery after heat shock, significant interaction between CUEDC2 and HSP70 were detected, and the interaction enhanced gradually over time (Fig. 2B). These data confirmed the interaction between the two proteins, and the binding between CUEDC2 and HSP70 is dynamic.

3.3. CUEDC2 interacts with HSP70 through its CUE domain

To define the domain in CUEDC2 that is critical for interaction with HSP70, we generated a series of truncation mutants of CUEDC2 (1–133, 1–180, 1–226aa) (Fig. 3A). The full length CUEDC2 and three CUEDC2 fragments were transiently expressed in HEK293 cells, and co-immunoprecipitation was performed. As shown in Fig. 3B, HSP70 associates with all the CUEDC2 deletion mutants except CUEDC2 (1–133), which lacks of CUE domain. These data suggest that the CUE domain of CUEDC2 is indispensible for interaction with HSP70.

3.4. HSP70 interacts with CUEDC2 through its PBD and CT domains

To delineate which region of HSP70 domains is necessary for association with CUEDC2, plasmids expressing different truncated forms of HSP70 as well as CUEDC2 were cotransfected into the HEK 293T cells and assess their interaction by co-immunoprecipitation experiments (Fig. 3C). The results showed that both PBD and CT, but not AD of HSP70, were required for its interaction with CUEDC2, suggesting that the interaction is mediated by the PBD and CT domains together (Fig. 3D).

3.5. CUEDC2 inhibit chaperone activity of HSP70

Since CUEDC2 interacts with C-terminal of HSP70, we next investigated the possibility of CUEDC2 regulating the chaperone activity of HSP70. The intracellular luciferase refolding assay, a widely applied system to evaluate the chaperone activity of

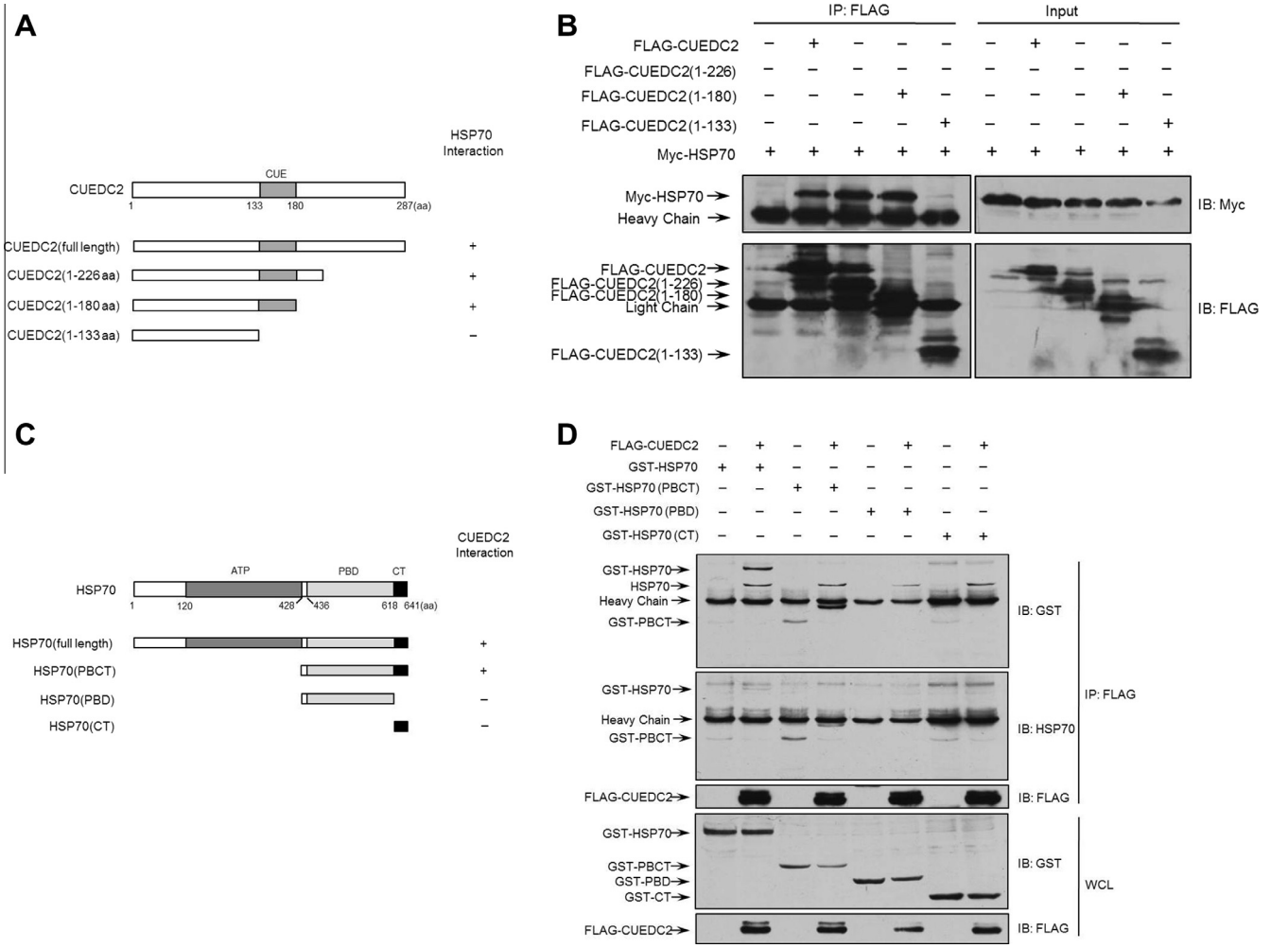

Fig. 3. Mapping of the interaction domains of CUEDC2 and HSP70. (A) Schematic diagram of the CUEDC2 protein, illustrating the regions of various mutants. (B) 293T cells were cotransfected with vectors expressing Myc-tagged HSP70 and Flag-tagged CUEDC2 or its Truncation mutants (1–133, 1–180, 1–226aa). Lysates immunoprecipitated with anti-Flag agarose beads and whole cell lysates were analyzed by immunoblot with anti-Myc and anti-Flag. (C) Schematic diagram of the deletion mutants of HSP70 used in domain-mapping experiments. (D) Immunoassay of 293T cells transfected Flag–CUEDC2 together with GST-HSP70 or its deletion mutants. Cell lysates immunoprecipitated with anti-Flag and whole cell lysates were analyzed by immunoblot with indicated antibodies.

HSP70 [18,19], was performed. The accordingly plasmids were transfected into HEK293T cells, and the overexpression of each protein was confirmed by Western blot analysis (Fig. 4A,C). The cells were heated at 45 °C for 30 min and recovered for the indicated times at 37 °C, harvested, and lysed, and luciferase assay was performed. The results showed that overexpression of CUEDC2 can inhibit the chaperone activity of HSP70 (Fig. 4B), while the deletion mutant of CUEDC2 (1–133aa), which failed to bind HSP70, could not inhibit the chaperone activity of HSP70 (Fig. 4D). These data suggest that CUEDC2 can modulate the chaperone activity of HSP70, and this capability is dependent on their interaction.

4. Discussion

Accumulating evidences have demonstrated that CUEDC2 is a multifunctional protein and play critical roles in many biological events, such as cell proliferation, inflammation and tumorigenesis [2–6]. Through inactivating IκB kinase (IKK), CUEDC2 negatively regulate NF-κB signaling pathway, which plays crucial roles in inflammatory responses and tumorigenesis [6]. Recent studies show that CUEDC2 is highly elevated in several cancers and play important roles in breast cancer progression [3,5]. In an attempt to further explore the function of CUEDC2 and the underlying mechanisms, we performed affinity purification followed by mass spectrometry analysis to identify its interaction proteins. Our studies demonstrate that CUEDC2 interacts with HSP70 and negatively regulates its chaperone activity.

Substantial evidences demonstrated that HSP70, a central component of molecular chaperons, play important physiological roles in normal conditions and pathological situations involving in various stresses [10,12,13,21]. Molecular chaperones are a ubiquitous feature of cells in which they can recognize and selectively bind nonnative proteins under physiological and stress conditions, which provide a first line of defense against misfolded proteins [12,22]. HSP70 could be induced upon infection and plays protective roles in inflammation [23]. Accumulating studies demonstrated that HSP70 plays complex roles in cancer development and progression [10,14,24,25]. It is acknowledged that HSP70 exert its biological functions mainly through its chaperon activity [26]. Researchers also indicate that cancer cells rely on HSP70's chaperon activity for survival, and depletion of Hsp70 could activate a tumor-specific apoptosis [24,27]. HSP70 is believed to be a promising target for cancer therapy, and HSP70 inhibitors have been tested as anticancer agents in pre-clinical or clinical trials

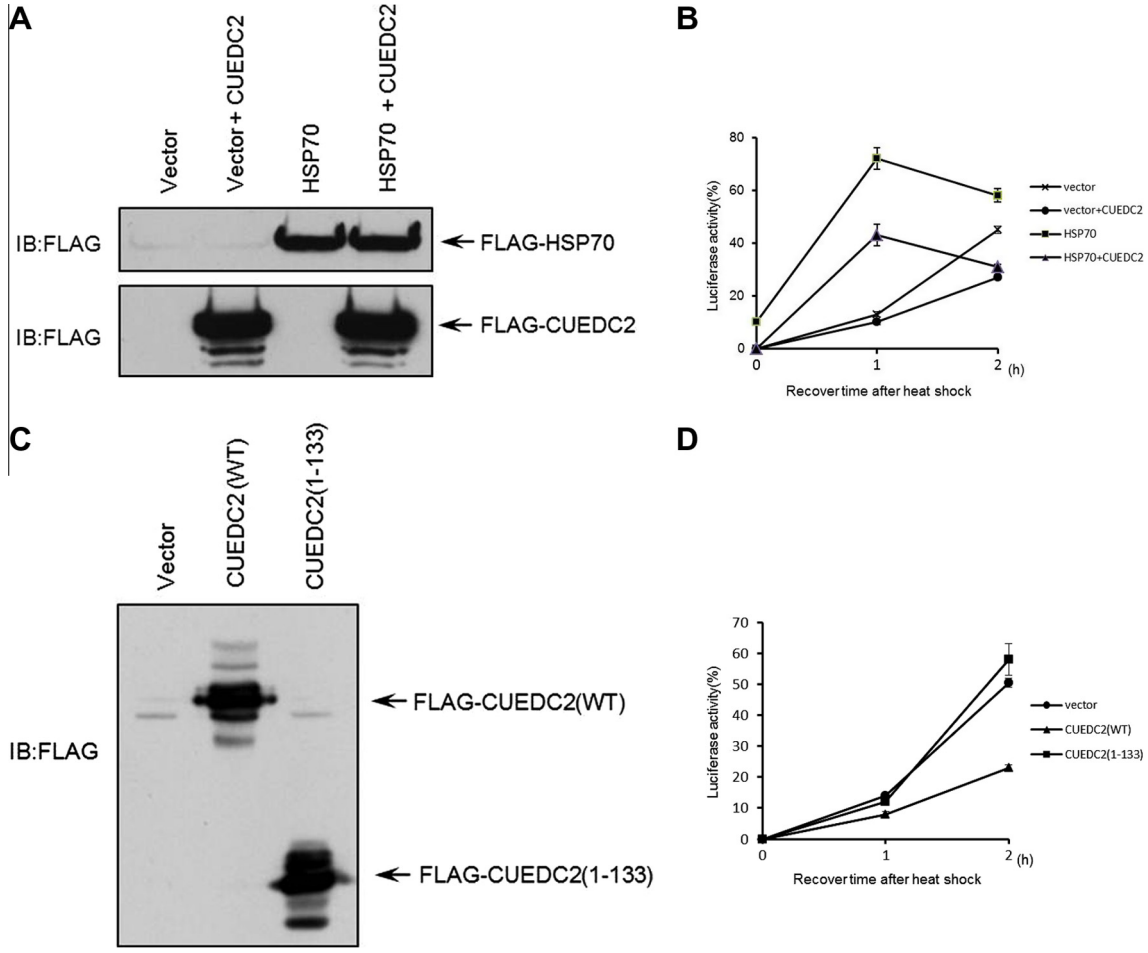

Fig. 4. Inhibition effect of CUEDC2 on HSP70-mediated reactivation of heat-denatured firefly luciferase. HEK293 cells were transiently transfected with pRSVLL/V together with pcDNA 3.0-FLAG-vctor, pcDNA 3.0-FLAG-CUEDC2, pcDNA 3.0-FLAG-CUEDC2 (1–133) or pCMV-2-HSP70 as indicated. At 24 h after transfection, the cells were treated with cycloheximide to inhibit new luciferase synthesis, heated for 30 min at 45 °C, and reincubated at 37 °C to allow luciferase refolding. Cell lysates were collected at the indicated time points, and the luciferase activity was measured and plotted relative to the activity prior to treatment. (A) Expression levels of Flag–CUEDC2 and Flag–HSP70 after transfection. (B) Luciferase refolding in cells expressing CUEDC2 alone (solid squares), HSP70 alone (solid triangles), or HSP70 and CUEDC2 (solid circles) compared to cells transfected with an empty vector (control, open circles). (C) Expression levels of Flag–CUEDC2 (full-length) and Flag–CUEDC2 (1–133) after transfection. (D) Luciferase refolding in cells expressing Flag–CUEDC2 (full-length) or its truncation mutant Flag–CUEDC2 (1–133).

[14,24,28,29]. Our finding that CUEDC2 can attenuate the chaperon activity of HSP70 provides new insights into the complexity for CUEDC2-regulatory events in tumorigenesis. Further studies need to determine the biological effect and significance of CUEDC2 and HSP70 interaction. For examples, whether CUEDC2 affect protective roles of HSP70 upon various stresses, and whether HSP70 could regulate the function of CUEDC2 in inflammation and tumorigenesis.

With mapping experiment, we demonstrated that CUE domain of CUEDC2 interacts with the C terminus of HSP70. It is believed that PBD domain is responsible for substrate-binding sites of HSP70, whereas ATPase domain and CT domain could bind co-chaperones, which either regulate chaperon activity or determine the fate of substrate [16,26]. Our studies revealed that CUEDC2 did not bind to ATPase, but rather bound to C terminus (428–641), which composed of PBD domain and CT domain. However, neither PBD domain alone nor CT domain could bind CUEDC2, and whether CUEDC2 is the substrate of HSP70 still needs further investigation. We next examined whether CUEDC2 regulate the chaperon activity of HSP70. Transient overexpression of CUEDC2 inhibited the chaperon activity of HSP70, whereas transient expression of the truncated mutant that fail to bind HSP70 has little effect, indicating that CUEDC2 serves as a co-chaperone and attenuates the chaperon activity of HSP70. So far, several co-chaperons of HSP70 have been identified, such as FAF1 [17], Hip [18], Bag1 [18], CHIP [30], and so on. FAF1 binds to the N terminus of HSP70, inhibits the HSP70 chaperone activity and increases heat-shock induced cell death [17]. Bag1 forms a stoichiometric complex with HSP70 and functions as another negative regulator of HSP70 chaperone activity [18]. CHIP acts on HSP70 in the ATP-bound state, and inhibits the ATPase activity of HSP70 [26]. In this study, we identify CUEDC2 as a novel negative regulator of HSP70, however, compared to other co-chaperons of HSP70, whether it exerts similar or unique function in HSP70 mediated stress response needs further investigation.

It is reported that some co-chaperones of HSP70 contained ubiquitin-related domains and are involved in the proteasome degradation of HSP70 substrates [19,31]. For examples, Bag1 contains a ubiquitin-like domain which bind to proteasome and CHIP has U box domain that has ubiquitin ligase activity [18,19]. Similarly, CUEDC2 also contained an ubiquitin-related domain, CUE domain and the present study showed that it was required for interaction with HSP70. CUE domain is a ubiquitin-binding motif consisting of 40 amino acids and could interact with both mono- and poly-ubiquitin [1,4]. It has been reported that some CUE domain containing proteins involved in degradation of misfolded proteins in the endo-

plasmic reticulum. We previously reported that CUEDC2 promoted the degradation of PR and ER-α [2,3], and the present data further suggest its role in ubiquitin system for protein degradation.

In conclusion, our results demonstrated that CUEDC2 interacts with HSP70 and inhibits its chaperon activity. Our results identify a novel negative regulator of HSP70 and for the first time provide new insights into the function of CUEDC2 in stress response. Further studies need to investigate the biological significance of their interaction.

Acknowledgments

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