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Highlighted Article

Selective degradation of aggregate-prone CryAB mutants by HSPB1 is mediated by ubiquitin–proteasome pathways

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ABSTRACT

Disease-causing mutations of genes encoding small MW heat shock proteins (sHSPs) constitute a growing family of inherited myofibrillar disorders. In the present work, we found that three structurally-distinct CryAB mutants R120G, 450delA and 464delCT are mostly present in the detergent-insoluble fractions when overexpressed in H9c2 rat heart cells. We found that either over-expression or knockdown of HSPB1, a related sHSP, affects the solubility, stability, and degradation of aggregation-prone CryAB mutants. HSPB1 overexpression has negligible effects on the solubility and protein aggregates of either R120G and/or 450delA but increased the solubility and prevented formation of 464delCT aggregates. HSPB1 knockdown decreased solubility and increased protein aggregates of all CryAB mutants, indicating a key role for HSPB1 in clearance of CryAB mutants under basal conditions. We provide four lines of evidence that such selective clearance of R120G, 450delA and 464delCT mutants by HSPB1 is mediated by the ubiquitin–proteasome system (UPS). First, we found that treatment with the proteasome inhibitors increased the levels of all CryAB mutants. Second, R120G and 450delA overexpression corresponded to the accumulation of their specific ubiquitin conjugates in H9c2 cells. Third, HSPB1 knockdown directly increased the levels of all polyubiquitin conjugates. And fourth, the selective attenuation of 464delCT expression by HSPB1 over-expression was abrogated by the proteasome inhibition. We conclude that such selective interactions between CryAB mutants and HSPB1 overexpression might have important implications for the clinical manifestations and potential treatment.

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1. Introduction

CryAB (αB-crystallin; HSPB5), a small heat shock protein (sHSP) with chaperone-like properties, is abundantly expressed in the ocular lens, heart and skeletal muscle [1]. sHSPs function as molecular chaperones to prevent protein aggregation and to accelerate the clearance of unfolded proteins under normal and especially during stressed conditions [2]. CryAB has also been shown to bind and increase the unfolding force of the filamentous protein, titin, through direct interactions of the NN2B-U and Ig domains [3].

Several mutations in CryAB have been identified that lead to the degeneration of distinct tissues, including the lens of the eye and/or cardiac and skeletal muscles. At the biochemical level, mutant CryAB proteins lose their chaperone-like properties when assessed *in vitro* with client proteins [4–6]. The first discovered mutation in CryAB is the missense mutation R120G; it results in dominant gain-of-function properties, and causes myofibrillar myopathy as well as cataract formation [7]. Overexpression of R120G in cardiomyocytes of transgenic mice results in a phenotype strikingly similar to that observed in patients with R120G CryAB-associated cardiomyopathies [8,9]. This phenotype is characterized by protein misfolding and the presence of large cytoplasmic aggregates of mutant CryAB.

Three truncated versions of CryAB (450delA, Q151X and 464delCT) have also been identified [10,11]. Berry and coworkers first reported that isolated congenital cataracts arising from 450delA CryAB produced an aberrant protein of 184 residues from a frameshift mutation in exon 3 at codon 150 [10]. Selcen and Engel [11] first reported that 464delCT CryAB in a patient with myofibrillar myopathy from peripheral weakness of the limb girdle, paralysis of the diaphragm, and who died from respiratory failure. This mutation, from a 2 base pair deletion at position 464, produced reduced amounts of the truncated protein of 162aa instead of 175 residues. Q151X and 464delCT were reported to have an increased tendency to form cytoplasmic aggregates in transfected COS-7 cells or neonatal cardiomyocytes [12,13]. The truncated CryAB mutations tend to self-

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aggregate, suggesting that the C-terminal extension is important for oligomerization [12]. Although aggregation-prone CryAB mutations are restricted in their pathology to either the lens (450delA) or muscle (Q151X, 464delCT) or both (R120G), protein aggregation is a key histopathological feature of all four mutations.

Such pathological manifestations are often age-dependent in onset, distributed in a tissue restricted manner, and have variable penetrance in severity. However, the underlying mechanism(s) and etiologic factors that might increase the resistance and/or susceptibility in selective cells and tissues remain partially understood. Among resistance factors, the genes encoding the family of heat shock proteins have been implicated in biological processes that prevent protein misfolding and improve cellular function among protein conformation diseases. In the present study, we hypothesized that HSPB1 (Hsp27), a major 27 kDa protein in eukaryotic cells, might confer such beneficial properties through its molecular interactions with client proteins related to protein degradation, chaperone-like activities in mitigating protein folding, apoptosis, mitochondrial interactions and disease progression.

Given the lack of information on the degradation pathways responsible for the catabolism of mutant CryAB proteins, we have asked whether the ubiquitin–proteasome system (UPS) or autophagy–lysosome pathways are involved into their degradation. Indeed, our findings indicate for the first time that HSPB1 plays a central role in the UPS-dependent degradation of mutant CryAB client proteins—with strikingly different efficiencies.

2. Materials and methods

2.1. Vector constructs

Wild type human CryAB and HSPB1 constructs were made using the vector pCMV. The CryAB mutant plasmids R120G, 450delA, 464delCT and Q151X were produced by in vitro site-directed mutagenesis system (Promega) using pCMV-myc-WT CryAB plasmid and complementary primers. Constructs were sequenced and compared for fidelity to the Gen-Bank TM database (accession number NM_001885).

2.2. Cell culture and transfection

H9c2 embryonic rat heart cells were grown in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS; Invitrogen) and 100 u penicillin/100 μg streptomycin/ml medium in a 5% CO_2 humidified atmosphere. $CHIP^{+/+}$ or $CHIP^{-/-}$ MEF were kindly provided by Cam Patterson (University of North Carolina).

One day prior to transfection, cells were trypsinized and plated in growth medium without antibiotics to achieve 80% confluence. Transient transfections were carried out using Lipofectamine 2000 (Invitrogen) with 3 μg for a single plasmid or 6 μg for two plasmids in 6-cm dishes. Cells were incubated in a CO_2 incubator for 48 h prior to testing for transgene expression.

ON-TARGET plus SMART pool siRNA Rat HSPB1 (Catalog #L-080155-00) and Non-targeting siRNA (Catalog #D-001810-01-05) were from Thermo Scientific Dharmacon RNAi Technologies. Co-transfections were carried out using Lipofectamine 2000 (Invitrogen) with 2 μg constructs and 200 pmol HSPB1 siRNA or Non-targeting siRNA in a 6-well plate. Cells were incubated for 72 h before testing.

2.3. Cell fractionation, SDS-PAGE, and immunoblotting

Cells grown on 6-cm dishes were washed twice with ice-cold phosphate-buffered saline before being lysed with 0.25 ml of 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA , 1% Triton X-100. Following incubation on ice for 30 min, the lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4 °C. The supernates were used as detergent-soluble fractions. The resulting pellet fractions were washed twice and sonicated in 100 μl 1× SDS buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 100 mM DTT, 10% Glycerol) for 30 s as detergent-insoluble fractions. The whole cell lysate was extracted from transfected cells using 1× SDS buffer. The detergent-soluble fraction, detergent-insoluble fractions and whole cell lysate were separated on 12% glycine SDS-PAGE gel and transferred to PVDF membrane. Membranes were blocked in 5% dry milk in TBS with 0.1% TWEEN-20 (TBST) for 1 h at room temperature followed by incubation with indicated primary antibodies in TBST with 0.2% BSA. Anti-c-Myc (sc-40, Santa Cruz biotechnology), HRP-conjugate anti-ubiquitin (PW0150, Biomol international), anti-CHIP (#2080, Cell Signaling Technology) and anti-Beclin (#3738, Cell Signaling Technology) were used at 1:1000 dilution. Anti-β-actin (A5441, Sigma Aldrich), anti-GAPDH (#2118, Cell Signaling Technology), anti-HSP25 (SPA-801, Assay Designs, for rat species HSPB1) and anti- HSP27 (sc-1049, Santa Cruz biotechnology, for human species HSPB1) were used at 1:2000 dilution. Immunoblot signals were visualized with Super Signal West Pico chemiluminescent substrate (Pierce Biotechnology).

2.4. Immunofluorescence microscopy

H9c2 cells were grown in a 2 well chamber (3.8 cm^2), transfected with all CryAB mutants, and 48 h later were washed twice with PBS, fixed with 4% (v/v) paraformaldehyde, and permeabilized with 0.2% Triton X-100. Subsequently, cells were incubated for 2 h at room temperature with primary mouse monoclonal c-myc antibody (diluted 1:200 in PBS, 1% BSA) and with secondary goat anti-mouse antibody (diluted 1:1000 in PBS) coupled to Alexa Fluor (Invitrogen). Images were collected using a FV1000-xy inverted confocal microscope.

2.5. Cycloheximide chase assay

Protein stability was assessed using cycloheximide-chase. H9c2cells were transiently transfected with plasmids encoding myc-tagged CryAB mutants, and 24 h later, cells were treated with 20 μg/ml cycloheximide (Sigma) for the indicated time prior to harvesting in 1× SDS buffer. Lysates (20 μg protein/lane) were subjected to Western blot analysis with anti-myc antibodies.

2.6. Immunoprecipitation/immunoblotting

H9c2cells were transiently transfected with empty vector or CryAB mutant-encoding plasmids, and 48 h later, cells were washed with cold PBS and harvested in RIPA buffer (50 mM Tris–HCl, pH = 7.4, 150 mM NaCl, 1%NP-40, 1% sodium deoxycholate, and 0.1% SDS). The lysate was gently shaken at 4 °C for 30 min, followed by centrifugation at 12,000*g* for 10 min. An aliquot of supernatants (400 μg protein) were incubated with 10 μl of anti-c-Myc agarose from Pierce mammalian c-Myc Tag IP/Co-IP kit (Thermo Scientific) in spin columns, followed by rotation at 4 °C overnight. The agarose was washed 3 times using TBS plus 0.05% Tween-20 and then eluted using non-reducing sample buffer to minimize interference from co-eluting antibody fragments. The eluted proteins were resolved on SDS/PAGE gel and subjected to Western blot analysis.

2.7. Data analysis

All the experiments were repeated 3 times. For western blots, one representative image is shown in figures. Densitometry for the Western blot signal was performed using Image J software. Values are represented as means ± SE. Student's *t* test was used for statistical analysis.