Published in final edited form as:

J Mol Cell Cardiol. 2010 December ; 49(6): 918–930. doi:10.1016/j.yjmcc.2010.09.004.

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

Huali Zhang^a, Namakkal S. Rajasekaran^a, Andras Orosz^a, Xianzhong Xiao^b, Martin Rechsteiner^c, and Ivor J. Benjamin^{a,c,*}

^a Center for Cardiovascular Translational Biomedicine, Division of Cardiology, Department of Internal Medicine, University of Utah School of Medicine, Salt Lake City, UT 84132, USA

^b Department of Pathophysiology, Central South University, Changsha City, Hunan 41008, China

^c Department of Biochemistry, University of Utah, School of Medicine, Salt Lake City, UT 84132, USA

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 overexpression 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.

Keywords

CryAB; HSPB1; Aggregate; Degradation; Solubility; Stability; Proteasome; Macroautophagy

1. Introduction

CryAB (α B-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

*Corresponding author. Department of Internal Medicine and Biochemistry, 30N 1900 E, Room 4A100, Salt Lake City, UT 84132, USA. Tel.: +1 801 581 6785; fax: +1 801 585 1082. Ivor.benjamin@hsc.utah.edu (I.J. Benjamin).

Disclosures

None declared.

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-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% CO₂ 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 CO₂ 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 cm²), 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. H9c2 cells 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

H9c2 cells 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 12,000g 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.

3. Results

3.1. Aggregate-prone CryAB mutants display differential solubility in H9c2 cells

In striated tissues, the CryAB chaperone interacts with client proteins such as desmin and titin to maintain muscle integrity and to prevent protein aggregation and denaturation. In contrast, loss of chaperone activity and increased aggregation of the R120G CryAB mutant are associated with crystallinopathies [14]. We and others have shown that CryAB R120G mutant is present in insoluble fractions of myopathic hearts [8,9]. To determine whether CryAB mutants also aggregate in H9c2 cells, we probed immunoblots of whole cell lysates from cells expressing Myc-tagged wild-type (WT), R120G, 450delA and 464delCT CryAB two days after transfection with the corresponding plasmids (Fig. 1A) using either anti-c-Myc and/or anti-CryAB antibodies. We observed similar levels of protein expression for WT, R120G, 450delA and 464delCT CryAB (Fig. 1B and C). By design, anti-CryAB antibody recognizes the C-terminal residues contained in either WT or R120G but neither 450delA nor 464delCT lacking similar residues. In H9c2 cells, anti-CryAB antibody recognizes basal levels of endogenous CryAB and 11-fold increases in exogenous WT or R120G 48 h after transfections (Fig. 1B). To assess the consequences of different C-terminal CryAB mutants on intracellular solubility, we examined detergent-soluble and detergent-insoluble fractions by SDS-PAGE and immunoblotting using the anti-c-Myc antibody that exclusively recognizes all exogenous gene products (Fig. 1D–G). As expected, WT CryAB was found almost entirely in the soluble fraction (Fig. 1D). R120G partitioned almost equally between soluble and insoluble fraction (Fig. 1D–G), whereas 450delA and 464delCT were almost entirely in the insoluble fractions (Fig. 1D–G). Our results recapitulate the biochemical hallmarks of decreased solubility of CryAB mutants *in vivo* and *in vitro*.

3.2. Cytoplasmic aggregate formation by CryAB mutants in H9c2 cells

To determine whether decreased solubility of CryAB mutants was due to aggregate formation, we examined the intracellular distribution of CryAB mutant and WT proteins by immunofluorescence microscopy. As expected, WT CryAB was distributed homogeneously in the cytoplasm of H9c2 cells, whereas some of H9c2 cells expressing disease-causing CryAB mutants contained cytoplasmic and/or peri-nuclear aggregates (Fig. 2). We counted the number of c-myc positive cells containing aggregates relative to the total number of c-myc positive cells in 10 randomly selected microscope fields. None of the cells expressing WT CryAB contained aggregates, whereas the R120G -expressing cells contained $15\pm4\%$ aggregates as compared with 450delA- and 464delCT-expressing cells whose aggregate formation reached $50\pm7\%$ and $30\pm3\%$, respectively (Fig. 2A–D). The abundance of 450delA and 464delCT aggregates is consistent with their being detected almost entirely in the insoluble fractions.

3.3. HSPB1 overexpression selectively increases the solubility of aggregation-prone CryAB mutants

Although the precise pathogenic roles of protein aggregates remain controversial in disease progression [15,16], such pathogenomic hallmarks are associated with increased morbidity and decreased lifespan in both experimental models and in humans [17]. As a result, there is intense focus on strategies to ameliorate protein aggregation using chemical, genetic and other maneuvers. HSPB1, the major 27 kDa member of the sHSPs family, forms hetero-oligomers with native CryAB. HSPB1 has been reported to accumulate in aggresomes, which are composed of mutant misfolded protein, ubiquitin, β -tubulin, SEC61 α and chaperones, presumably to aid in protein resolubilization and/or clearance [18]. In R120G myopathic hearts, we found that HSPB1 was modestly increased in the soluble fraction but was more than 25-fold higher in the insoluble fraction. Such co-aggregation with R120G CryAB might increase disease susceptibility of R120G cardiomyopathic hearts [9]. In H9c2 cells, we found that all of these mutant CryAB proteins can interact with HSPB1 (Fig. 3A, lower panel). HSPB1 was clearly increased in the detergent-insoluble fractions from cells expressing R120G and 450delA mutants but not in fractions from cells expressing 464delCT mutant (Fig. 3C, lane 3, lane 4 and lane 5, respectively).

To investigate the role of HSPB1 in aggregate formation of CryAB mutants, we co-transfected HA-tagged human HSPB1 with myc-tagged CryAB WT and mutants. In H9c2 cells, HSPB1 overexpression had negligible effects on either WT, R120G or 450delA expression (Fig. 4A, lanes 3–8, upper panel and 4B) but significantly reduced the levels of 464delCT in whole cell lysates (Fig. 4A, lanes 9–10, upper panel and Fig. 4B). In addition, there was a marked redistribution of 464delCT upon HSPB1 over-expression. In H9c2 cells co-transfected with the vector control, 464delCT was almost entirely present in the insoluble fraction. By contrast, 464delCT was exclusively present—albeit at reduced amounts—in the soluble fractions upon HSPB1 over-expression (Fig. 4C, lanes 9 and 10, upper panel and Fig. 4D; Fig. 4E, lanes 9 and 10, upper panel and Fig. 4F). Intriguingly, R120G and 450delA, but not 464delCT, sequestered exogenous HSPB1 in the insoluble fractions (Fig. 4C and E, lanes 6, 8, middle panel). The apparent dominance of the mutants R120G and 450delA over HSPB1 perhaps explains why overexpression of HSPB1 did not increase the solubility of either R120G and/or 450delA.

Consistent with the fractionation results, confocal images revealed the homogenous diffuse fluorescence in the cytoplasm of 464delCT co-transfected with HSPB1, indicating the decrease of aggregate formation in 464delCT (Fig. 4G). By contrast, HSPB1 overexpression had negligible effects on the aggregate formation of either R120G or 450delA in H9c2 cells (Fig. 4G). Taken together, our findings indicate distinct but hitherto undefined interactions of CryAB

mutants and HSPB1 appear to affect the solubility of between CryAB mutants and HSPB1 in H9c2 cells.

3.4. Down-regulation of HSPB1 increases accumulation of CryAB mutant proteins

To address the effects of HSPB1 on mutant protein degradation, we used siRNA to reduce the levels of HSPB1. We observed that knockdown of HSPB1 by 75% increased the concentration of all three CryAB mutant proteins (Fig. 5A and B). Biochemical fractionation studies, however, showed that the reduced levels of soluble 450delA and 464delCT CryAB expression paralleled the lower levels of soluble HSPB1; knockdown of HSPB1 modestly increased all three CryAB mutant proteins in the insoluble fractions (Fig. 5C–F). Using immunocytochemistry, we confirmed that HSPB1 siRNA consistently increased the number of cells expressing CryAB aggregates (Fig. 5G). We conclude that HSPB1 expression increases the solubility of CryAB mutant proteins under basal conditions.

3.5. Metabolism of aggregate-prone CryAB mutants

Mutations that promote aggregate formation may affect protein turnover, which can contribute to the pathogenesis of disease. To determine whether mutations in CryAB may affect the protein's turnover, we treated transiently transfected H9c2 cells with cycloheximide. As shown in Fig. 6, pulse chase experiments showed that the level of R120G was stable for at least 24 h after cycloheximide treatment (Fig. 6A and B), but 450delA level decreased to $77 \pm 3.6\%$, $53.4 \pm 4\%$, $48.6 \pm 2.2\%$ of control after 6 h, 12 h and 24 h of treatment, respectively (Fig. 6C and D) and 464delCT mutant was degraded to $83.3 \pm 3.7\%$ compared to control after 24 h of treatment (Fig. 6E and F). As might be expected, the different CryAB mutant proteins have different half-lives in H9c2 cells.

Since HSPB1 affects the solubility of all three CryAB mutants, we next determined whether HSPB1 is involved in the turnover of CryAB mutant proteins. As shown in Fig. 7, the level of R120G CryAB mutant remained stable in the presence of HSPB1 overexpression (Fig. 7A and B). Interestingly, over-expression of HSPB1 decreased the degradation of 450delA (Fig. 7C and D), but enhanced the degradation of 464delCT (Fig. 7E and F). A possible explanation is that HSPB1 over-expression increased the solubility of 464delCT, making it a better substrate for the degradative pathway.

3.6. Degradation of aggregate-prone CryAB mutants is mediated by UPS but not macroautophagy pathway

Ubiquitin–proteasome and autophagy–lysosome pathways are the two main routes of protein degradation in eukaryotes. In order to determine the role of proteasome pathway in the clearance of aggregate-prone CryAB mutants, the proteasome inhibitors MG132 and epoxomicin were applied to H9c2 cells transfected with the appropriate plasmids. We observed that the inhibition of proteasome pathway resulted in the accumulation of all three CryAB mutants (Fig. 8A–D), suggesting that they are degraded at least, in part, by the UPS.

There are three types of autophagy: macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy. Rapamycin is an antifungal antibiotic that is widely used to stimulate macroautophagy [19], whereas 3-methyladenine (3-MA) and Bafilomycin A1 are well-characterized inhibitors of the process [20,21]. Since macroautophagy is considered the major type of autophagy, we used these compounds to ask whether aggregate-prone CryAB mutants were degraded through the macroautophagy–lysosome pathway in H9c2 cells. After transient transfection with plasmids encoding aggregate-prone CryAB mutants, H9c2 cells were exposed to rapamycin for various time points. Western blot analysis showed that the levels of CryAB mutant proteins were not decreased after 24 h or 48 h of treatment with Rapamycin, but beclin and LC3-II, two markers of autophagic activity, were elevated following Rapamycin

treatment and p62, a selective substrate of autophagy, was decreased after Rapamycin treatment (Fig. 8E). Inhibiting macroautophagy with 3-MA and Bafilomycin A1 for 24 h also did not lead to an obvious increase in the levels of mutant CryAB proteins (Fig. 8F). The absence of any effect of macroautophagy activator or inhibitor compounds on CryAB mutant protein levels argues against their degradation by macroautophagy pathways in H9c2 cells.

3.7. Accumulation of ubiquitin conjugates in H9c2 cells expressing aggregate prone CryAB mutants

A number of studies have implicated UPS dysfunction in a range of aggregation-prone polyglutamine neurodegenerative diseases. We have previously demonstrated that ubiquitinated proteins progressively increase in hearts from mice expressing CryAB R120G mice but not WT-CryAB mice [22]. To explore any effects of aggregation-prone CryAB mutants might have on UPS proteolytic function, we measured the levels of polyubiquitylated proteins in whole cell lysate from transiently transfected H9c2 cells. Several distinct ubiquitin conjugates accumulated in H9c2 cells expressing R120G and 450delA mutants (Fig. 9A, lane 3 and lane 4), which are different from the striking increase in the levels of various types of polyubiquitin conjugates observed upon proteasome inhibition (Fig. 9A). Their molecular weights suggested these unique species were CryAB-ubiquitin conjugates. To test this supposition, myc-tagged CryAB mutants were immunoprecipitated using anti-c-Myc agarose and analysis of immunoprecipitates confirmed the increased ubiquitination of R120G or 450delA or their interacting proteins (Fig. 9B, lane 3 and lane 4). Our data demonstrated that the accumulation of ubiquitin conjugates in R120G and 450delA mutants in H9c2 cells is not a consequence of global proteasome inhibition.

3.8. Over-expression or knockdown of HSPB1 leads to the differential change of polyubiquitin conjugates

Our previous data demonstrated that either over-expression or knockdown of HSPB1 affects the solubility, stability, and degradation of aggregation-prone CryAB mutants. Colocalization of HSP27 and the 26S proteasome in aggresome suggested a role for HSP27 in ubiquitin-dependent degradation. Recently, Garrido et al. [23,24] reported that HSPB1 overexpression in various cell types enhanced the degradation of ubiquitinated proteins by 26S proteasome. Our present data showed that HSPB1 over-expression decreased the abundance of ubiquitinated proteins in basal condition and 464delCT-transfected cells, respectively, but not in R120G and 450delA mutants (Fig. 10A), which is consistent with that HSPB1 overexpression decrease the level of 464delCT. The degradation of 464delCT by HSPB1 over-expression was abolished in the presence of a proteasome inhibitor MG132 (Fig. 10B and C). Furthermore, HSPB1 knockdown increased the polyubiquitinated conjugates in cells expressing all three CryAB mutants. Taken together, the selective degradation of R120G, 450delA and 464delCT mutants by HSPB1 is mediated by the ubiquitin-proteasome system.

4. Discussion

Following the discovery that the missense R120G hCryAB causes a multisystem disease in humans, termed “desmin-related myopathy,” the quest to understand the molecular pathogenesis of mutant chaperones has attracted much attention. HSPs are known to play protective roles in protein conformation diseases and to provide a first line of defense against aggregation-prone proteins because of their ability to enhance appropriate folding of misfolded proteins [25,26]. Our present study sought to examine whether the cellular mechanism(s) involving two other disease-causing CryAB mutants, 450delA and 464delCT, associated protein aggregates might be related to their biophysical and structural properties in the context of HSPB1 expression when transiently transfected into H9c2 cells.

R120G represents the prototypical missense CryAB mutation, whereas both 450delA and 464delCT result in an extension and truncation compared with full-length CryAB, respectively. Previously, both mutants were shown to be purified from inclusion bodies and could only be refolded in the presence of wild type CryAB *in vitro* [12]. Our present work has demonstrated that 450delA and 464delCT are found mainly in the insoluble fraction from transiently transfected H9c2 cells (Figs. 1 and 2). Because the C-terminal region contributes to the solubility of 450delA and 464delCT, it is conceivable that the replacement of the normal C-terminus with missense residues in the truncated protein alters either the solubility or chaperoning interactions with HSPB1.

HSPB1, which is under the transcriptional control of HSF1[27], accumulates into inclusion bodies in many protein conformation diseases [28]. For example, Chavez Zodel and colleagues demonstrated that HSPB1 prevented R120G CryAB misfolding, oligomerization and aggresome formation in PTK2 cells [18]. Likewise, Ito and coworkers [29] reported that HSPB1 expression in HeLa cells reduced the number of inclusion bodies and increased the solubility of R120G CryAB. In the present study, our data show that HSPB1 overexpression had negligible effects on the solubility of either R120G or 450delA but significantly increased the solubility of 464delCT CryAB (Fig. 4). Such selective effects of HSPB1 overexpression do not exclude its well-established biological role in protein degradation under basal conditions. Specifically, we found that HSPB1 knockdown significantly decreased the solubility and clearance of all three CryAB mutants (Fig. 4), suggesting that these divergent responses between individual CryAB mutants and HSPB1 might provide novel insights in the context of key biological properties in H9c2 cells.

Small HSPs characteristically display three structural domains: a centrally conserved α -crystallin domain flanked by N- and C-terminal variable domains. N-terminal regions have been assigned properties of oligomerization, subunit assembly, and substrate binding; in parallel, C-terminal domains play key roles for solubility and chaperoning functions [30]. As illustrated in Fig. 1A, recent structure-functional analyses have subdivided the C-terminal region into “tail” and “extension” subregions, which are functionally linked to homodimerization and substrate recognition, respectively [31]. The tail subregion promotes the binding of sHSP dimers, the building blocks for characteristic oligomerization, whereas the extension subregion disaggregates dimers and increases solubility.

Such opposing effects of C-terminal subregions suggest several potential outcomes. One possibility is that length of the C-terminal subregion influences its protein solubility. Among CryAB mutants, 464delCT truncation is solubilized by HSPB1 overexpression. Alternatively, the shorter extension of 464delCT increases its client protein recognition. Of interest, the C-terminal extension for the aggregation-prone R120G and 450delA are both longer than 464delCT, suggesting a potential mechanism for solubilization by HSPB1 overexpression. When combined with their disease-causing functions *in vivo*, we believe that these sequence changes among CryAB mutants contribute to our novel findings related to HSPB1-dependent interactions on protein solubility and aggregation.

What might be consequences of increased solubilization for the selective degradation by HSPB1 of 464delCT but neither R120G nor 450delA in H9c2 cells? Proteins with short half-lives are mostly degraded by the proteasome and cytosolic proteins with long half-lives are degraded by autophagic pathways as well [32,33]. The ubiquitin–proteasome system (UPS) degrades short-lived nuclear or cytosolic proteins and misfolded proteins extruded from the endoplasmic reticulum [34]. By recognizing and selectively degrading misfolded proteins, the UPS protects cells against the potentially toxic effects of protein aggregation [34]. Substrates of UPS need to be unfolded to pass through the narrow barrel-shaped proteasome, which makes aggregate-prone proteins poor substrates for UPS [35,36]. Our data indicate that *soluble* but

not insoluble CryAB mutants are degraded through ubiquitin–proteasome pathway in H9c2 cells, and that either inhibition of proteasome markedly increased mutant CryAB levels. We found that combined 464delCT/HSPB1 expression in the presence of cycloheximide treatment significantly decreases protein content compared with 464delCT over 24 h (Fig. 7E and F), strongly supporting the notion that HSPB1 overexpression exerts direct effects on either protein clearance or stability, or both. Such evidence suggests the intriguing possibility that therapeutic interventions aimed at 464delCT might reverse the dominant negative effects *in vivo* [11].

Autophagy can relieve proteasome inhibition by removing aggregates too large for efficient proteasome-mediated clearance by mediating bulk degradation of cytoplasmic proteins in lysosomes [37]. Surprisingly, we found that CryAB mutants R120G, 450delA and 464delCT in H9c2 cells were not dependent on the macroautophagy pathway for their degradation. However, lack of evidence for macroautophagy might be limitation of the experimental system—CryAB mutant expression for 24 h or 48 h in H9c2 cells. This would be consistent with recent report of Wong *et al.* [38], suggesting that not all protein inclusions or aggresomes are degraded by autophagy. It is conceivable that mutant CryABs overexpression do not faithfully recapitulate the (patho)physiologic factors metabolism, chronicity, and other environment factors found in intact organs or organisms.

Notwithstanding defining the factors that influence the degradation of aggregated proteins might uncover new pathways as drug targets for therapeutic interventions [39]. It is tempting to speculate that HSPB1 overexpression might have direct beneficial effects on disease manifestation and/or progression such as 464delCT, whereas similar maneuvers might have insignificant or deleterious consequences for R120G and/or 450delA in the intact organism. Our foregoing studies would provide proof of concept for examining these hypothesis in the (patho)physiologically relevant animal models of human diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Heart, Lung and Blood Institute (ARRA Award 2 R01 HL063834-06 to IJB), 2009 NIH Director's Pioneer Award 1DP1OD006438-01, Christi T. Smith Foundation (IJB), American Heart Association, Grant-in-Aid 0755022Y(IJB) and an American Heart Association Postdoctoral Fellowship (09POST2251058 to Huali Zhang). We thank Justin Benesch (University of Oxford) for his helpful suggestions. Jennifer Schroff provided excellent editorial assistance during preparation of this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.yjmcc.2010.09.004.

Fig. 1.

Overexpression of aggregate-prone CryAB mutants in H9c2 Cells. (A) Schematic representation of single amino acid substitution (R120G) and deletions (450delA and 464delCT). (B) H9c2 cells were transiently transfected with Myc-tagged WT, R120G, 450delA, and 464delCT plasmids. Steady-state CryAB levels in whole cell lysates were determined on western blots probed with anti-Myc and anti-CryAB. Equal loading was confirmed by reprobing the blots with an antibody against β -actin (lower panel); (C) quantitation of CryAB WT and mutants, normalized against β -actin, was carried out with NIH ImageJ software. The levels were averaged over three experiments and plotted. (D–E) Detergent-soluble and (F–G) detergent-insoluble fractions prepared from transfected cells were separated by electrophoresis and analyzed by western blot using the anti-Myc antibody to determine the solubility of WT and CryAB mutants. Equal loading was confirmed by reprobing the blots with an antibody against β -actin.

Fig. 2.

Aggregate formation of CryAB mutants in H9c2 Cells. Representative confocal microscopic immunofluorescence images for WT (A), R120G (B), 450delA (C) and 464delCT (D). H9c2 cells were transfected with the indicated expression constructs. After 48 h, cells were fixed and immunostained with anti-Myc antibody followed by goat anti-mouse IgG conjugated to Alexa Fluor 488 (green). Nuclei are stained blue with DAPI. Arrows indicate protein aggregates.

Fig. 3.

Distribution of HSPB1 into subcellular fraction from transfected H9c2 cells with CryAB mutants. (A) Lysates from H9c2 cells transfected with vector, CryAB WT, and CryAB mutant plasmids were subjected to IP with anti-Myc, followed by anti-Myc antibody or anti-HSPB1 antibody (SPA-801). (B, C) Detergent-soluble (B) and detergent-insoluble (C) fractions prepared from transfected cells were separated by electrophoresis and analyzed by Western blot using the anti-HSPB1 antibody to determine the distribution of HSPB1. Equal loading was confirmed by reprobing the blots with an antibody against β -actin.

Fig. 4.

Effect of HSPB1 overexpression on the expression and solubility of CryAB mutants. (A, C, E) H9c2 cells were co-transfected with Myc-tagged R120G, 450delA or 464delCT plus pCMV-HA vector or HA-tagged human HSPB1. The whole lysates (A), detergent-soluble (C) and detergent-insoluble (E) fractions were analyzed by immunoblotting using the anti-Myc and anti-HSPB1 antibody (sc-1049) after 48 h of transfection. Arrow indicates the endogenous HSPB1 in H9c2 cells. Reprobed the blot for β -actin to normalize protein loading. (B, D, F) Densitometry values are represented as relative intensities to β -actin in mean arbitrary units. * $p < 0.05$, compared with pCMV-HA vector group. (G) Percentage of c-Myc-positive cells with aggregates in H9c2 cells co-transfected with Myc-tagged CryAB mutants and either pCMV-HA vector or HA-tagged human HSPB1 was determined from 10 different confocal microscopy fields. * $p < 0.01$, compared with pCMV-HA vector.

Fig. 5.

Effect of HSPB1 knockdown on the expression and solubility of CryAB mutants. (A, C, E) H9c2 cells were co-transfected with Myc-tagged R120G, 450delA or 464delCT and with either a non-target siRNA or HSPB1 siRNA. The whole lysates (A), detergent-soluble (C) and detergent-insoluble (E) fractions were analyzed by immunoblotting using the anti-Myc and anti-HSPB1 antibody at 72 h after transfection. Reprobing the blot for GAPDH or β -actin confirmed equal loading. (B, D, F) Densitometry values are represented as relative intensities to GAPDH or β -actin in mean arbitrary units. * $p < 0.05$, compared with non-target siRNA group. (G) Percentage of c-Myc-positive cells with aggregates in H9c2 cells co-transfected with Myc-tagged CryAB mutants and either a non-target siRNA or HSPB1 siRNA was determined from 10 different confocal microscopy fields. * $p < 0.02$, compared with non-target siRNA group.

Fig. 6. Metabolism of aggregate-prone CryAB mutants. (A, C, E) H9c2 cells transfected with R120G (A), 450delA (C) or 464delCT (E) plasmid for 24 h were treated with 20 μ g/ml cycloheximide, and cells were harvested using 1 $\times$ SDS buffer at indicated time points. C-myc antibody was used to detect CryAB mutants by Western blot analysis. GAPDH was used as loading control. (B, D, F) Quantification of CryAB mutant protein levels at different times. Values represent means $\pm$ SE, $n=3$.

Fig. 7.

Effect of HSPB1 overexpression on the metabolism of aggregate-prone CryAB mutants. H9c2 cells co-transfected with Myc-tagged R120G (A), 450delA (C) or 464delCT (E) plus pCMV-HA vector or HA-tagged human HSPB1 for 24 h were treated with 20 μ g/ml cycloheximide, and cells were harvested using 1 $\times$ SDS buffer at indicated time points. C-myc antibody was used to detect CryAB mutants by Western blot analysis. GAPDH was used as loading control. (B, D, F) Quantification of CryAB mutant protein levels was performed at different time points. Values represent means $\pm$ SE, $n=3$.

Fig. 8.

Aggregate-prone CryAB mutants are degraded via a ubiquitin–proteasome pathway but not macroautophagy pathway. (A, C) H9c2 cell transfected with CryAB mutant plasmids for 24 h were exposed to 5 μ M MG-132 (A), 1 μ M Epoxomicin (C) or DMSO as controls in cell culture medium for another 15 h. Whole-cell lysates were separated on 12% SDS-PAGE gel. CryAB mutants were detected using C-myc antibody and loading was checked by probing for β -actin. (B,D) Quantification of CryAB mutant protein levels after MG-132 (B) or Epoxomicin (D) treatment. Values represent means $\pm$ SE, $n=3$. * $p<0.05$, compared with DMSO group. (E, F) H9c2 cells transfected with R120G, 450delA or 464delCT were exposed to 0.4 μ g/ml Rapamycin (E), 10 mM 3-MA or 80 nM Bafilomycin A1 (F) for the indicated times. Whole-cell lysates were separated on 12% SDS-PAGE gel and detected by anti-myc, anti-LC3, anti-beclin and anti-p62 antibody. Equal loading was confirmed by reprobating the blots with an antibody against β -actin. All the experiments were repeated 3 times. One representative blot is shown.

Fig. 9.

Ubiquitinylated conjugates accumulating in R120G and 450delA transfected H9c2 cells. (A) H9c2 cells were transiently transfected with vector, CryAB WT, and CryAB mutant plasmids for 48 h and ubiquitin antibody was used to detect mono- or polyubiquitinylated proteins. Arrows indicated the abnormal ubiquitinylated conjugates. 5 or 10 μ M MG132 was used to treat H9c2 cells for showing the global polyubiquitinylated proteins under proteasome inhibition. (B) Lysates from H9c2 cells transfected with vector, CryAB WT, and CryAB mutant plasmids were subjected to IP with anti-Myc, followed by anti-ubiquitin immunoblotting (upper) or anti-Myc antibody (middle).

Fig. 10.

Selective degradation of Aggregate-prone CryAB mutants by HSPB1 is through ubiquitin–proteasome pathway. (A) whole-cell lysates from Myc-tagged R120G, 450delA or 464delCT co-transfected with pCMV-HA vector or HA-tagged human HSPB1 were detected using anti-ubiquitin antibody. Reprobing the blot for β -actin confirmed equal loading. (B) H9c2 cell transfected with CryAB mutant plasmids plus pCMV-HA vector or HA-tagged human HSPB1 for 24 h were exposed to 5 μ M MG-132 in cell culture medium for another 15 h. β -actin was used as loading control. (C) Quantification of CryAB mutant protein levels after MG-132 treatment. Values represent means $\pm$ SE, $n=3$. * $p<0.05$, compared with vector group. # $p<0.05$, compared with HA-HSPB1 group. (D) whole-cell lysates from Myc-tagged R120G, 450delA or 464delCT co-transfected with non-target siRNA or specific HSPB1 siRNA were detected using anti-ubiquitin antibody. Reprobing the blot for β -actin confirmed equal loading.