Intrasarcoplasmic Amyloidosis Impairs Proteolytic Function of Proteasomes in Cardiomyocytes by Compromising Substrate Uptake

Quanhai Chen,* Jin-Bao Liu,* Kathleen M. Horak, Hanqiao Zheng, Asangi R.K. Kumarapeli, Jie Li, Faqian Li, A. Martin Gerdes, Eric F. Wawrousek, Xuejun Wang

Abstract—The presence of increased ubiquitinated proteins and amyloid oligomers in failing human hearts strikingly resembles the characteristic pathology in the brain of many neurodegenerative diseases. The ubiquitin–proteasome system (UPS) is responsible for degradation of most cellular proteins and plays essential roles in virtually all cellular processes. UPS impairment by aberrant protein aggregation was previously shown in cell culture but remains to be demonstrated in intact animals. Mechanisms underlying the impairment are poorly understood. We report here that UPS proteolytic function is severely impaired in the heart of a mouse model of intrasarcoplasmic amyloidosis caused by cardiac-restricted expression of a human desmin–related myopathy-linked missense mutation of αB-crystallin (CryABR120G). The UPS impairment was detected before cardiac hypertrophy, and failure became discernible, suggesting that defective protein turnover likely contributes to cardiac remodeling and failure in this model. Further analyses reveal that the impairment is likely attributable to insufficient delivery of substrate proteins into the 20S proteasomes, and depletion of key components of the 19S subcomplex may be responsible. The derangement is likely caused by aberrant protein aggregation rather than loss of function of the CryAB gene because UPS malfunction was not evident in CryAB-null hearts and inhibition of aberrant protein aggregation by Congo red or a heat shock protein significantly attenuated CryABR120G-induced UPS malfunction in cultured cardiomyocytes. Because of the central role of the UPS in cell regulation and the high intrasarcoplasmic amyloidosis prevalence in failing human hearts, our data suggest a novel pathogenic process in cardiac disorders with abnormal protein aggregation. (Circ Res. 2005;97:1018-1026.)

Key Words: proteasome ■ ubiquitin ■ protein aggregation ■ αB-crystallin ■ desmin-related cardiomyopathy ■ amyloidosis ■ transgenic mice

Most cellular proteins are degraded through the ubiquitin–proteasome system (UPS). UPS-mediated proteolysis includes 2 major steps: attachment of a chain of ubiquitin to the target protein molecule through a process known as ubiquitination and degradation of the ubiquitinated proteins by the 26S proteasome. The latter consists of a barrel-shaped 20S core and the 19S cap on 1 or both ends of the 20S. The actual proteolytic activity resides in the interior of the 20S, whereas the 19S plays a critical role in channeling ubiquitinated protein molecules into the 20S. Ubiquitinated proteins accumulate in the cell when the proteasome is inhibited. Aberrant protein aggregation is a common process in many neural degenerative diseases such as Alzheimer’s, Parkinson’s, and Huntington’s diseases. Remaining to be demonstrated in intact animals, this process was shown in cell culture to impair UPS-mediated proteolysis. Therefore, UPS malfunction is considered an important pathogenic mechanism in neurodegeneration.

Recent studies revealed that abnormal protein aggregation, intrasarcoplasmic amyloidosis (IA), and accumulation of ubiquitinated proteins are also very common phenomena in human hearts with idiopathic or ischemic cardiomyopathies. These observations, especially the discovery that very abundant amyloid-positive substance is strikingly associated with myofibrils in most human hearts with dilated or hypertrophic cardiomyopathies, suggest IA as a potential major pathogenic process in congestive heart failure (CHF). Hence, elucidation of the effect of IA on UPS-mediated proteolysis may provide significant insight into the molecular pathogenesis of CHF.
A missense mutation (R120G) in the αB-crystallin (CryAB) gene encoding a molecular chaperone highly expressed in the heart has been shown to cause aberrant protein aggregation in the heart and result in desmin-related cardiomyopathies (DRC) in humans and transgenic (Tg) mice. Mice carrying 3 copies of a Tg consisting of the mouse α-myosin heavy chain promoter and the CryABR120G cDNA show no apparent cardiac abnormality at 1 month but develop concentric cardiac hypertrophy with diastolic dysfunction at 3 months and die of CHF between 5 and 7 months. It was recently demonstrated that CryABR120G mice were a model of cardiac IA, resembling a prominent feature in failing human hearts. Therefore, CryABR120G mice are used as an animal model to study the pathogenesis of aberrant protein aggregation and IA in the present study. We hypothesized that aberrant protein aggregation induced by CryABR120G impairs UPS proteolytic function in the heart, representing a nodal pathogenic process in cardiac remodeling and failure in DRC. We present here several lines of evidence that clearly support this hypothesis. We have demonstrated in intact animals that expression of a misfolded and aggregation-prone cytosolic protein impairs the proteolytic function of the UPS in the heart before other cardiac pathology becomes discernible. The defect appears to reside in the delivery of ubiquitinated protein molecules into the proteolytic cavity of the 20S proteasomes, and depletion of key components of 19S proteasomes may be responsible. Using cultured cardiomyocytes, we have further proven that aberrant protein aggregation is an essential process for CryABR120G to induce proteasomal malfunction. Because aberrant protein aggregation and accumulation of ubiquitinated proteins, both indicative of malfunction in protein quality control, have been frequently observed in failing human hearts, protein aggregation-induced UPS malfunction likely represents a potentially important pathogenic process in not only DRC but also other cardiac disorders with protein misfolding.

**Materials and Methods**

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

**Tg Mice**

FVB/N Tg mice with cardiac-specific overexpression of CryABR120G or wild-type (WT)-CryAB have been described. Generation of CryAB-null mice has been reported previously. FVB/N Tg mice ubiquitously expressing a reporter substrate (GFPdgn) of the UPS are described elsewhere. GFPdgn is created by fusion of degron CL-1 to the carboxyl terminus of the conventional green fluorescent protein (GFP). Previous studies have proven that this modification renders GFP a specific substrate for the UPS and that resultant GFPdgn can serve as a dynamic indicator for the proteolytic function of the UPS in cardiomyocytes. All animals used in this study were produced by the Laboratory Animal Facility of the University of South Dakota. Institutional guidelines for the care and use of the animals were followed.

**In Vitro GFPdgn Degradation Assay**

To assess the proteolytic function of the entire UPS, GFPdgn was purified from the skeletal muscle of GFPdgn mice by immunoaffinity chromatography (Pierce). Equal amounts of GFPdgn protein were incubated with 10 μg of soluble protein from ventricular myocardiun. The reactions were performed in 25 mmol/L Tris-HCl buffer containing ATP (2 mmol/L) and MgCl₂ (2 mmol/L) for 30 minutes in the presence or absence of MG-132 (10 μmol/L). The reactions were stopped by adding 3 × SDS-PAGE sampling buffer and immediately followed by boiling for 5 minutes. The end product was then fractionated by 12% SDS-PAGE and immunoprobed for GFPdgn.

**Results**

**Ubiquitinated Proteins Were Progressively Increased in CryABR120G Mouse Hearts**

Because cardiac expression of CryABR120G not only recapitulates manifestations of human DRC but also displays IA that is frequently observed in non-DRC failing human hearts, CryABR120G Tg mice serve as a valuable model to dissect mechanisms underlying CHF. To explore the pathogenesis of CryABR120G, we found that ubiquitinated proteins in both the soluble fraction and total protein extracts from the heart progressively increased in CryABR120G Tg but not WT-CryAB Tg mice at 1 month and 3 months, whereas free ubiquitin remained unchanged compared with Ntg littermates (Figure 1A through 1D). Ubiquitinated proteins are normally degraded efficiently by the 26S proteasomes. An increase in ubiquitinated proteins indicates either proteasomal malfunction or enhanced ubiquitination. Because the overexpression of CryABR120G at the transcript level is much less than overexpression of WT-CryAB in these Tg mice, increased ubiquitination resulting from CryAB protein overexpression could not account for the significant difference in ubiquitinated protein levels between CryABR120G and WT-CryAB.
intensities in the cardiomyocytes of CryABR120G/GFPdgn myocardium showed clearly increased green fluorescence tentatively, direct fluorescence confocal microscopy of GFPdgn double-Tg hearts (Figure 2A through 2C). Consistent with these tests prove that cardiac UPS proteolytic function is significantly impaired by expression of CryABR120G.

The free ubiquitin levels were not altered. Moreover, ubiquitinated proteins were significantly increased in CryABR120G hearts (Figure 1), suggesting that the primary cause of UPS malfunction in these hearts is not at ubiquitin conjugation but rather in the 26S proteasome. The latter is composed of the 19S and the 20S subcomplexes. The 19S is believed to recognize ubiquitinated protein molecules, deubiquitinate and unfold them, and channel the unfolded protein to the 20S, where actual proteases reside. The 20S proteasome is a hollow cylindrical protein complex composed of 2 central antiparallel β rings flanked by 2 identical α rings. Each α or β ring consists of 7 protein molecules (α7 to α7 and β1 to β7). The unfolded protein is believed to be degraded in the cavity of the 20S by 3 major peptidase activities: chymotrypsin-like, trypsin-like, and caspase-like (also known as peptidylglutamyl-peptide hydrolase) activities. These peptidase activities were not reduced, whereas proteasomal peptidase activity in CryABR120G/GFPdgn double-Tg hearts was significantly compromised compared with Ntg littermates or WT-CryAB Tg controls (Figure 2F). These results prove that cardiac UPS proteolytic function is significantly impaired by expression of CryABR120G.

GFPdgn transcript level was not increased in CryABR120G/GFPdgn double-Tg hearts compared with GFPdgn single-Tg hearts (Figure 2D and 2E). Furthermore, the ability of crude protein extracts from CryABR120G hearts to degrade in vitro GFPdgn protein immunoprecipitated from the skeletal muscle of GFPdgn mice was significantly compromised compared with Ntg littermates or WT-CryAB Tg controls (Figure 2F). These results prove that cardiac UPS proteolytic function is significantly impaired by expression of CryABR120G.

The free ubiquitin levels were not altered. Moreover, ubiquitinated proteins were significantly increased in CryABR120G hearts (Figure 1), suggesting that the primary cause of UPS malfunction in these hearts is not at ubiquitin conjugation but rather in the 26S proteasome. The latter is composed of the 19S and the 20S subcomplexes. The 19S is believed to recognize ubiquitinated protein molecules, deubiquitinate and unfold them, and channel the unfolded protein to the 20S, where actual proteases reside. The 20S proteasome is a hollow cylindrical protein complex composed of 2 central antiparallel β rings flanked by 2 identical α rings. Each α or β ring consists of 7 protein molecules (α7 to α7 and β1 to β7). The unfolded protein is believed to be degraded in the cavity of the 20S by 3 major peptidase activities: chymotrypsin-like, trypsin-like, and caspase-like (also known as peptidylglutamyl-peptide hydrolase) activities, which reside in the β5, β6, and β7 subunits, respectively. 

We measured these peptidase activities using synthetic fluorogenic substrates and crude protein extracts from the heart. WT-CryAB Tg mice do not show any abnormal phenotype,
and the UPS proteolytic function in their hearts does not differ from Ntg littermates (Figures 1 through 3). Therefore, for brevity, WT-CryAB Tg mice were not included in studies described in Figures 4 and 5. Compared with Ntg littermates, all 3 forms of peptidase activities were, surprisingly, not decreased in CryABR120G hearts at 1, 3, or 6 months. On the contrary, trypsin-like activities were significantly increased at all of the 3 time points, especially at 3 and 6 months, and the chymotrypsin-like and the caspase-like activities were also significantly increased at 6 months when CHF becomes overt (Figure 4). These findings suggest that the impairment of proteosomal proteolytic function in CryABR120G hearts is attributable to a defect in the uptake of ubiquitinated proteins into 20S proteasomes rather than altered peptide cleavage activities of the 20S. It is generally accepted that the 19S
proteasome and its interaction with the α rings of the 20S regulate and mediate the entry of targeted protein molecules into the 20S.15 Consistently, the protein abundance of the 20S components (α6, β2, β5, and the precursor of β5) was significantly increased, whereas key components (Rpt3 and Rpt5) of the 19S evidently decreased in CryABR120G hearts at 3 and 6 months (Figure 5).

Aberrant Protein Aggregation Plays an Essential Role in UPS Impairment by CryABR120G

CryABR120G is misfolded and aggregation prone. Its presence dominantly inhibits the chaperone function of normal CryAB.16,17 To ascertain whether UPS impairment by CryABR120G is caused by loss of function of CryAB, we examined UPS functional status in CryAB-null mouse hearts at ~2 to 3 months, when no discernible phenotype is evident. Compared with age-matched wild-type mice of the same genetic background, neither the abundance of ubiquitinated proteins nor proteasomal peptidase activities were significantly altered in the CryAB-null hearts (data shown in the online data supplement). This suggests that UPS malfunction observed in CryABR120G expressing hearts is not caused by loss of function of the CryAB gene. Because CryAB can oligomerize with other small heat shock proteins (Hsp) and may function in heterooligomers,17 the results from CryAB-null mice do not rule out a possible contribution of the dominant negative activity of CryABR120G to the UPS malfunction.

In cultured HEK cells, aberrant protein aggregation of mutant huntingtin and cystic fibrosis membrane conductor protein was shown to be sufficient to impair the UPS.4 Adenovirus-mediated overexpression of truncated cardiac myosin-binding protein C mutants were recently shown to impair the UPS in cultured NRVMs and form abnormal aggregates, but a causal relationship between aberrant protein aggregation and UPS impairment was not tested.18 We have further addressed these critical issues in a well-established NRVM culture system. CryABR120G, WT-CryAB, and a UPS function reporter (GFPu or GFPu) similar to GFPdgn were introduced into cultured NRVMs via adenovirus infection. We reported that changes in GFPu protein levels in cultured NRVMs inversely reflect the proteolytic function of the UPS.14 As observed in Tg mouse hearts, expression of CryABR120G but not WT-CryAB caused significant increases of GFPu protein in cultured NRVMs in a dose-dependent manner (Figure 6A). The increase in GFPu protein levels marks a decrease in UPS proteolytic function because the synthesis of GFPu, as demonstrated by steady-state GFPu transcript levels, was not increased by coexpression of CryABR120G compared with coexpression of either WT-CryAB or β-galactosidase (Figure 6B). These findings demonstrate that modest overexpression of CryABR120G, but not

Figure 5. Changes in the abundance of key components of 19S and 20S proteasomes. Total protein extracts from the ventricles of CryABR120G Tg (R120G-TG) and Ntg littermates were fractionated by SDS-PAGE and analyzed quantitatively using Western blots for the indicated subunits. Western blot images and densitometry data are shown in A and B, respectively. At 1 month (1m), the Rpt3 and Rpt5 subunits of the 19S and the α6 (20S-α6), β2 (20S-β2), and matured β5 (the lower band of 20S-β5) subunits of the 20S was significantly increased, whereas the β5 precursor (boxed in A; pr-β5 in B) of the 20S remained unchanged, whereas all of the examined subunits of the 20S was significantly increased in R120G Tg hearts. *P<0.01, **P<0.05, compared with Ntg; Student’s t test; n=3. AU indicates arbitrary units.
WT-CryAB, is sufficient to impair the UPS in cultured cardiomyocytes. Similar to what was observed in the heart, the abundance of free ubiquitin was not affected; however, the ubiquitinated proteins were significantly increased by CryABR120G but not WT-CryAB overexpression in cultured NRVMs (Figure 6C and 6D). These findings indicate that changes in ubiquitin conjugation do not likely contribute to the UPS proteolytic malfunction in CryABR120G-expressing cells. This is consistent with in vivo findings and implicates that the primary defect is in the proteasome.

The major pathological change in CryABR120G-expressing human and mouse hearts and in CryABR120G-expressing NRVMs is aberrant protein aggregation. CryAB-positive abnormal protein aggregates were observed in CryABR120G but not WT-CryAB Tg hearts.11 In addition to relatively large and microscopically visible aggregates, SDS-soluble high molecular–weight CryAB oligomers, which are unable to pass through the nitrocellulose membrane with a pore size of 0.25 μm, were significantly increased in the CryABR120G hearts (Figure 7A, a) and CryABR120G-expressing NRVMs (Figure 7B). It has been well documented that molecular chaperones, such as Hsp and certain pharmacological agents (eg, Congo red), can bind misfolded proteins and effectively prevent them from aggregating, thereby assisting protein quality control in the cell.19–22 To test the necessity of abnormal protein aggregation in UPS impairment by CryABR120G, we determined the effect of reduction of protein aggregation by Congo red treatment or by overexpressing inducible Hsp70 in cultured NRVMs. Interestingly, as shown by the nitrocellulose filter-trapping assay, the prevalence of CryAB oligomers in the myocardial homogenate from CryABR120G hearts was dramatically reduced by in vitro treatment of Congo red (Sigma) in a dose-dependent manner (Figure 7A, b). Consistent with a previous report,8 the treatment of Congo red on NRVMs in primary culture significantly reduced the formation of both the microscopically visible protein aggregates (Figure 7D) and the soluble CryAB oligomers induced by expression of CryABR120G (Figure 7B and 7C). Importantly, this inhibition of aberrant protein aggregation by Congo red led to a significant attenuation of UPS malfunction, as evidenced by a significantly decreased GFPu accumulation (Figure 7B through 7D). Similar reduction in both CryAB oligomers and GFPu accumulation was also observed with coexpression of inducible Hsp70 (data not shown). These findings prove that aberrant protein aggregation plays an essential role in CryABR120G induced proteasomal malfunction.

**Discussion**

UPS-mediated proteolysis degrades abnormal proteins such as misfolded, oxidized, and mutant proteins, thereby serving as a critical quality control in the cell. The UPS also degrades normal proteins that are no longer needed. This regulatory degradation ensures timely removal of normal proteins, including signaling proteins and transcription regulators such as mitotic cyclins, CDK inhibitors, IκB, β-catenin, and calcineurin when their jobs are done.1,23 Therefore, the UPS plays critical roles in virtually all important cellular pro-
cesses, such as cell-cycle control, transcription regulation, signal transduction, and cell survival and homeostasis. Until very recently, the elucidation of the involvement of UPS dysfunction in pathogenesis of disease has been hindered by a lack of reliable methods to measure dynamic changes of UPS function in vivo. Recently established UPS functional reporter Tg mouse models make it possible to monitor in vivo dynamic changes in the proteolytic function of the entire UPS. Taking advantage of 1 such mouse model that we have recently created and validated, we have collected in the present study definitive in vivo evidence that modest cardiac overexpression of a human DRC-linked mutant CryAB impairs the proteolytic function of the UPS in the heart. This impairment occurred before cardiac hypertrophy, and malfunction became discernible. Cardiac remodeling and malfunction progressed as UPS function in the heart deteriorated progressively, suggesting that UPS dysfunction likely participates in the pathogenesis of the mutant CryAB. Further analyses reveal that the UPS malfunction is mainly attributable to a defect in the delivery of ubiquitinated proteins into the proteolytic chamber of the 20S proteasome, and the depletion of key components of the 19S proteasome may be responsible. Because of accumulation of ubiquitinated proteins and alteration of proteasomal peptidase activities were not evident in CryAB-null mice, proteasomal malfunction in CryABR120G Tg hearts is unlikely caused by loss of function of the CryAB gene. Our findings from experiments with cultured NRVMs further suggest that aberrant protein aggregation is a major underlying mechanism by which misfolded CryAB causes proteasomal malfunction.

**Tg Expression of CryABR120G Impairs the Delivery of Ubiquitinated Proteins Into 20S Proteasomes**

By crossbreeding UPS functional reporter (GFPdgn) mice with CryABR120G Tg mice, we are able to prove that proteolytic function of the UPS is severely impaired in CryABR120G Tg hearts (Figures 1 through 3). However, conventional proteasomal peptidase activity assays failed to detect the impairment. On the contrary, all of the 3 peptidase activities showed significant increases in the CryABR120G heart at the CHF stage (Figure 4). These peptidase assays use synthetic fluorogenic substrates, which are small peptides and can easily diffuse into the proteolytic chamber of the 20S proteasome; therefore, they only measure the function of the 20S proteasomes. In the CryABR120G heart, ubiquitin conjugation does not appear to be a problem because ubiquitinated...
proteins are progressively increased (Figure 1A through 1D). The function of 20S proteasomes is unlikely to be the primary cause of observed proteasomal malfunction because conventional in vitro assays showed that their peptidase activities are not reduced at all but rather significantly increased at the CHF stage (Figure 4). These analyses suggest that the primary defect likely resides at the transport of ubiquitinated proteins to the 26S proteasome and the entry of ubiquitinated proteins into the 20S proteasome. The process by which target proteins are delivered into the proteolytic core of the 20S proteasome is incompletely understood. Although other proteins, such as the CDC48 protein complex, may be involved, it is generally believed that 19S proteasomes play a primary role in this process. Therefore, we assessed the abundance of key components of the 19S and the 20S proteasomes and discovered that Rpt3 and Rpt5 of the 19S were significantly decreased, whereas all of the examined subunits of the 20S were markedly increased in CryABR120G hearts at 3 and 6 months, when overt cardiac hypertrophy and malfunction were evident (Figure 5). It was reported that inhibition of proteasome activity in cultured mammalian cells induces concerted expression of proteasome genes including components of the 19S and the 20S and de novo formation of the 26S proteasome. This is considered a compensatory response to acquired proteasome malfunction. Hence, in CryABR120G hearts, which clearly show proteasome malfunction, the upregulation of 20S proteasome components is likely a compensatory response, whereas the depletion of 19S proteasome components is probably a cause for the deficiency in the delivery of ubiquitinated proteins into the 20S.

It is also worthwhile to note that discoveries presented here also illustrate that it can be very misleading to use proteasomal peptidase activity assays alone to evaluate the proteolytic function of the UPS or the proteasome.

**UPS Impairment by Aberrant Protein Aggregation Is Potentially a Novel Pathogenic Process for Cardiac Remodeling and Failure**

This postulate remains to be further tested, but it is consistent with multiple lines of direct and indirect evidence. First, aberrant protein aggregation is not just a feature for DRC but is rather a common phenomenon in human CHF. It was recently discovered that IA (a form of aberrant protein aggregation) from unknown proteins was frequently present in the cardiomyocytes of failing human hearts with either hypertrophic or dilated cardiomyopathies. Aberrant protein aggregation was also observed in ischemic cardiomyopathy and CHF resulting from dilated cardiomyopathy. Second, proteasomal malfunction has been implicated in failing human hearts. Immunohistologic studies of failing human hearts revealed that increased ubiquitin conjugates were colocalized with autophagic cell death, an important form of cell death in the failing heart. It was also reported that ubiquitinated protein levels were significantly increased in the heart with either dilated or ischemic cardiomyopathies, suggesting that proteasomal function is likely impaired in failing human hearts. Third, studies with cell culture have previously shown impairment of the UPS by aberrant protein aggregation. The present study proves that UPS proteolytic function is severely impaired by expression of an aggregation-prone protein in the heart of intact mice. Our additional tests with cultured NRVMs demonstrate that aberrant protein aggregation is not only sufficient but also essential for a mutant CryAB to impair UPS function. Although it has not been tested whether UPS malfunction causes or potentiates cardiac hypertrophy and/or failure, it has been reported that several signaling proteins, such as β-catenin and calcineurin, which mediate cardiac growth, including pathological hypertrophy, are degraded by the UPS. Indeed, we found that cytosolic β-catenin was significantly increased in CryABR120G Tg hearts at the CHF stage (supplemental Figure I). Activated calcineurin was previously found to be markedly increased in failing human hearts. It will be very important to determine whether the increase in these signaling proteins in CHF is attributable to a decrease in their degradation. Finally, proteasome inhibition leads to apoptosis, a major form of cell death that is considered an important mechanism underlying CHF. Cells, at least proliferating cells, defective in the UPS most often arrest near the G1/M boundary of the cell cycle and undergo apoptosis. We have previously observed that proteasomal inhibition by MG-132 increased cell death in cultured NRVMs. It has not been reported but should be extremely important to ascertain whether proteasome malfunction affects cardiomyocyte survival in vivo.

Sarcomeres are the most fundamental function units of cardiac contractility. Normal turnover of most sarcomeric proteins depends on the UPS. Failure to replace aged or damaged sarcomeric proteins would conceivably afflict the behavior and performance of myofibrils. Furthermore, the endoplasmic reticulum (ER) plays critical roles in the quality control of membrane proteins and proteins for secretion. The ER accomplishes this important task in partnership with the UPS. In fact, the proteasome is believed to be responsible for the degradation of all abnormal cellular proteins, thereby executing quality control in the cell. Prolonged ER stress has been reported as a mechanism underlying heart failure. It would not be surprising if UPS malfunction played an even greater role than prolonged ER stress in cardiac remodeling and failure.

It should be pointed out that the UPS is the major, but by no means the only, proteolytic pathway responsible for protein turnover in cardiomyocytes. Other pathways, such as the calpain proteolytic pathway and the lysosomal, autophagic pathway, also exist and likely play a role in cardiomyocytes. It is unknown but should be very interesting to investigate whether these other pathways compensate for UPS proteolytic deficiency.

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References


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Running title: Proteasome Malfunction in Crystallinopathy

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Supplemental Materials and Methods

Transgenic (Tg) Mice
FVB/N Tg mice with cardiac-specific overexpression of CryAB<sup>R120G</sup> or wild type (WT-) CryAB have been described.<sup>1,2</sup> Generation of CryAB null mice was reported previously.<sup>3</sup> Wild type mice with the same background (129X1/SvJ) from the Jackson Laboratory (Bar Harbor, Maine), were used as controls for the CryAB null mice. FVB/N Tg mice ubiquitously expressing a reporter substrate (GFPdgn) of the UPS are described elsewhere.<sup>4</sup> GFPdgn is created by fusion of degron CL-1 to the carboxyl terminus of the conventional green fluorescence protein (GFP). Previous studies have proven that this modification renders GFP a specific substrate for the UPS and resultant GFPdgn can serve as a dynamic indicator for the proteolytic function of the UPS in cardiomyocytes.<sup>5,6</sup>

Antibodies and Protein Detection
Rabbit polyclonal anti-ubiquitin and anti-β-catenin antibodies (Ab’s) were purchased from Sigma. Horseradish peroxidase-conjugated goat anti-mouse or rabbit secondary Ab’s, mouse monoclonal Ab’s for GFP and for Thr41/Ser45 phosphorylated β-catenin were from Santa Cruz. Rabbit polyclonal Ab’s for CryAB were from Stressgen. Rabbit polyclonal Ab’s for the 19S proteasome components Rpt5 and Rpt3, and for the 20S proteasome subunits α<sub>6</sub> and β<sub>2</sub> were from BIOMOL. Polyclonal Ab’s against the β<sub>5</sub> subunit of the 20S proteasome were raised in rabbits (Genemed) using synthetic peptide corresponding to residues 249-262 (DNVADLHDKYSSVS) of the mouse proteasome subunit β<sub>5</sub> precursor (pre-β<sub>5</sub>) sequence (NCBI accession number AAH12246). Pilot studies showed that this Ab detects both pre-β<sub>5</sub> and mature β<sub>5</sub> subunit in western blot analysis. Protein concentration determination with BCA reagents (Pierce), SDS-PAGE, western blot analysis, and densitometry were performed as described.<sup>6</sup>

Proteasomal Peptidase Activity Assays
The synthetic fluorogenic substrate Suc-LLVY-aminomethycoumarin (AMC, 25 µM) (CALBIOCHEM), Z-LLE-β naphthylamide (NA, 25µM), and Bz-VGR-AMC (40µM)
(BIOMOL) were respectively used for measuring chymotrypsin-like, caspase-like, and trypsin-like activities. Assays were performed essentially as described.\(^6\) Peptide cleavage reactions were carried out in the absence or presence of the proteasome-specific inhibitor MG-132 (N-Cbz-LLL, 20 \(\mu\)M) for chymotrypsin-like and caspase-like activities or epoxomicin (5\(\mu\)M, CALBIOCHEM) for trypsin-like activity. The portion of activities that was inhibited by respective inhibitors is attributed to the proteasome.

**Fluorescence Microscopy**

Cryosections from paraformaldehyde perfusion-fixed mouse hearts were visualized using an Olympus I-70 confocal microscope (Olympus). Green fluorescence images from different myocardial samples in the same figure were obtained using the same optical and imaging settings. To observe the distribution relationship between CryAB aggregation and GFPu accumulation, cultured NRVMs in fibronectin-coated chamber slides were fixed with 4% paraformaldehyde, immunolabeled for CryAB (Alex 565, red), and visualized and imaged for CryAB (indirect red fluorescence) and GFPu (direct green fluorescence) with an epi-fluorescence microscope equipped with an IX-71 digital camera.

**In vitro GFPdgn Degradation Assay**

To assess the proteolytic function of the entire UPS, GFPdgn was purified from the skeletal muscle of GFPdgn mice by immuno-affinity chromatography (Pierce). The GFPdgn eluates were then neutralized with neutralizing buffer and dialyzed in 25mM Tris-HCl buffer (pH 7.5) at 4\(^\circ\)C overnight. Equal amounts of GFPdgn protein were incubated with 10 \(\mu\)g of soluble protein from ventricular myocardium. The reactions were carried out in 25mM Tris-HCl buffer containing ATP (2 mM) and MgCl\(_2\) (2 mM) for 30 min in presence or absence of MG-132 (10\(\mu\)M). The reactions were stopped by adding 3X SDS-PAGE sampling buffer and immediately followed by boiling for 5 minutes. The end product was then fractionated by 12\% SDS-PAGE and immuno-probed for GFPdgn. The residual GFPdgn protein was quantified using the Quantity-One software (BioRad). Equal amounts of GFPdgn, without addition of myocardial extracts, were included as control.

**Cardiomyocyte Culture and Adenovirus Infection**
Isolation and culture of neonatal rat ventricular myocytes (NRVMs) were performed as previously described.\textsuperscript{6} Replication deficient recombinant adenoviruses harboring the GFP\textsuperscript{u} (Ad-GFP\textsuperscript{u}) or β-galactosidase (Ad-β-Gal) expression cassette have been described.\textsuperscript{6, 7} Adenoviruses harboring WT-CryAB expression cassette (Ad-CryAB) and Ad-CryAB\textsuperscript{R120G} were made using the AdEasy System.\textsuperscript{6} To distinguish the Tg products from endogenous CryAB protein, a HA-epitope was added to the carboxyl termini of both the WT-CryAB and CryAB\textsuperscript{R120G}. Infection of cultured NRVMs with any of these adenoviruses at an indicated multiplicity of infection (MOI) was generally started at 48 to 72 hours after the cells were plated.

**Filter-Trap Assay for CryAB Aggregates**

This was performed essentially as described.\textsuperscript{2, 8} Ventricular myocardium homogenates or Cultured NRVMs were ultrasonicated in 1X SDS-PAGE sampling buffer on ice, and the homogenates were boiled for 5 min and then centrifuged at 5,000 xg for 5 min. The supernatant was collected and its protein concentration determined as described earlier. The protein extract (5 µg) in a volume of 200 µl was filtered through a 0.25-µm nitrocellulose membrane, using a dot blot apparatus. The high molecular weight CryAB on the membrane was detected via immunoblotting.

**Northern Blot**

This was done as described.\textsuperscript{1} A P\textsuperscript{32}-labeled GFP\textsubscript{dgn} cDNA probe was created using the nick-translation method. The probe-bound membrane was exposed to a phosphor screen (BioRad) and detected with a Personal Molecular Imager (BioRad). Densitometry of northern blot signals was performed with the Quantity One image analysis software (BioRad).

**Statistical Analysis**

Results are presented as mean ± s.d.. For cell culture studies, quantitative results were collected from 4 independent repeats. Unless indicated otherwise, significance levels were calculated using one-way ANOVA, followed by the Scheffe test. $P < 0.05$ was considered significant.
Supplemental Figure Legends

**Figure 1.** Western blot analyses of cytosolic β-catenin levels in the heart. (A) Representative western blots for β-catenin and Thr41/Ser45 phosphorylated β-catenin (p-β-catenin) in the cytosolic fraction of ventricular myocardium from littermate Ntg, WT-CryAB Tg (WT-Tg), and CryAB<sup>R120G</sup> Tg (R120G) mice at 6 months. (B) Densitometric data from 3 different hearts of each group are presented. *: p<0.01, **: p<0.05, compared to Ntg or WT-TG. AU: arbitrary unit (the same for other figures).

**Figure 2.** UPS function remains unaltered in the heart of young CryAB-null mice. (A) Total protein extracts from the heart of 2~3 months old mice were fractionated by SDS-PAGE and immunoblotted for free ubiquitin (Ub) and ubiquitinated proteins (upper panel). In a separate western blot, CryAB protein levels in the heart were probed (lower panel). Compared to age-matched wild type controls, the levels of both free Ub and ubiquitinated proteins remained unchanged while CryAB protein was completely missing in the CryAB/HspB2 double knockout mouse hearts (CryAB<sup>-/-</sup>). MM: molecular weight markers. (B) Chymotrypsin-like, caspase-like, and trypsin-like activities in the soluble protein extracts from CryAB-null (CryAB<sup>-/-</sup>) and wild type (CryAB<sup>+/+</sup>) mouse hearts were compared. No statistically significant difference was detected between the two groups.

**References**


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