 Interruption of CryAB-Amyloid Oligomer Formation by HSP22*  

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An R120G missense mutation in α-B-crystallin (CryAB), a small heat-shock protein (HSP), causes a desmin-related cardiomyopathy (DRM) that is characterized by the formation of aggregates containing CryAB and desmin. The mutant CryAB protein leads to the formation of inclusion bodies, which contain amyloid oligomer intermediates (amyloid oligomer) in the cardiomyocytes. To further address the underlying mechanism(s) of amyloid oligomer formation in DRM linked to the CryAB R120G, a recombinant CryAB R120G protein was generated. The purified CryAB R120G protein can form a toxic amyloid oligomer, whereas little immunoreactivity was observed in the wild-type CryAB protein. A native PAGE showed that the amyloid oligomer may be more important in pathogenesis than the insoluble fibrillar amyloid deposits (8–11). An antibody that specifically recognizes the structure of the amyloid oligomer reacts with oligomers generated from all types of amyloidogenic proteins and peptides, such as Aβ-(1–42), α-synuclein, polyglutamine, and prions (6). This result implies that the amyloid oligomer has a shared structure with diverse proteins (6). Furthermore, amyloid oligomers from different diseases share the ability to permeabilize cell membranes and lipid bilayers, indicating that this may represent the primary toxic mechanism of amyloid pathogenesis (12). This suggests that the amyloid oligomer may share a common mechanism of pathogenic action as a primary toxic species (12).

The R120G missense mutation in α-B-crystallin (CryAB) or HSP B5, which is a small heat-shock protein (HSP), can cause a desmin-related cardiomyopathy (DRM) (13). This disease, which is characterized by the formation of aggregates containing CryAB and desmin, can be recapitulated in transgenic mice by expression of the mutant protein specifically in the heart (14). We showed that the R120G missense mutation in the CryAB protein led to perinuclear aggresome formation and that these aggresomes contained the amyloid oligomer (15). These results suggest that CryAB-DRM is a subclass of the aggresomal and amyloid-related diseases (15). Furthermore, using a cardiac-specific inducible transgenic system (16), we found that continuous expression of the mutant protein is needed to sustain the high concentrations of the amyloid oligomer that correlate with depressed cardiac function as well as unfolded proteins in the cells can contribute to or be causal for at least some of these neurodegenerative and systemic diseases (2). However, the direct relationship between the protein deposition and the disease pathology is still controversial. Recent reports suggest that amyloid β (Aβ) 2 and other amyloidogenic proteins exert their cellular toxicity as soluble amyloid oligomeric intermediates (amyloid oligomer) but not as insoluble aggregates or fibrils (3–6). These soluble amyloid oligomer proteins generally appear to have β-sheet structures, whose formation is correlated with the appearance of a hydrophobic environment (6, 7). Several reports indicate that the soluble amyloid oligomer may be more important in pathogenesis than the insoluble fibrillar amyloid deposits (8–11). An antibody that specifically recognizes the structure of the amyloid oligomer reacts with oligomers generated from all types of amyloidogenic proteins and peptides, such as Aβ-(1–42), α-synuclein, polyglutamine, and prions (6). This result implies that the amyloid oligomer has a shared structure with diverse proteins (6). Furthermore, amyloid oligomers from different diseases share the ability to permeabilize cell membranes and lipid bilayers, indicating that this may represent the primary toxic mechanism of amyloid pathogenesis (12). This suggests that the amyloid oligomer may share a common mechanism of pathogenic action as a primary toxic species (12).

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2 The abbreviations used are: Aβ, amyloid β; amyloid oligomer, positive immunoreactive material against the anti-oligomer antibody; R120G, missense mutation from arginine to glycine at the 120th residue of α-β-crystallin; polyglutamate, expanded polyglutamate tract of n residues; native PAGE, PAGE under a native condition; MTI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; m.o.i., multiplicity of infection; DTT, dithiothreitol; PVDF, polyvinylidene difluoride; HA, hemagglutinin; UPS, ubiquitin-proteosome system; GFP, green fluorescent protein; HSP, heat-shock protein; DRM, desmin-related cardiomyopathy; WT, wild type; TBS, Tris-buffered saline.
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premature lethality (17). These results indicate that the CryAB-DRM disease is reversible and that the amyloid oligomer is critical for the DRM disease (17). The oligomer amyloid was also present in cardiomyocytes derived from multiple human dilated and hypertrophic cardiomyopathies (15). Thus, potential commonalities due to the presence of the amyloid oligomer may exist in cardiovascular diseases (15). This suggests that the reduction and/or prevention of toxic amyloid oligomer formation may represent a new therapeutic approach for the DRM disease as well as other amyloid-related diseases. To address this therapeutic approach for the DRM disease, it will be important to analyze the molecular mechanisms of amyloid oligomer formation by the CryAB R120G protein.

Small HSP families, such as HSP22 (HSP B8 or H11kinase) and HSP25 (HSP B1), share their sequence similarity within the α-crystallin domain but exhibit different patterns of gene expression, transcriptional regulation, and subcellular localization (18). The chaperone-like activity of small HSPs, which are classified according to their ability to prevent protein aggregation and/or restore the biological activity of the substrates, is widely believed to be a protective mechanism for protein misfolding and denaturation triggered by noxious environmental stimuli, such as hyperthermia stress, heavy metals, ischemic injury, and some genetic diseases (1, 18, 19). At least 10 mammalian small HSPs have been identified in recent genome surveys of mice, rats, and humans, but their functions have remained poorly understood (18). In this study, we show the results of amyloid oligomer formation by self-polymerization of the CryAB R120G protein. Recombinant HSP25 or HSP22 proteins can interrupt oligomer formation by the CryAB R120G protein in both the recombinant protein and cardiomyocyte systems. Blockade of amyloid oligomer formation by either HSP22 or HSP25 recovered the ubiquitin proteosomal activity as well as the cellular toxicity. Blockade of oligomer formation by small HSPs, such as HSP22 and HSP25, may be a new therapeutic strategy for treating the DRM disease as well as other types of amyloid-based degenerative diseases.

EXPERIMENTAL PROCEDURES

Cardiomyocyte Cultures and Adenovirus Infection—After isolation of rat neonatal cardiomyocytes, cells were grown on glass slides coated with a gelatin as described previously (15, 17). Replication-deficient recombinant adenoviruses were made using an AdEasy system (15, 17). cDNAs were isolated using reverse transcription-PCR and used to generate adenoviral constructs as described previously (15). The cells were transfected by adenovirus containing the modified GFP at an m.o.i. of 1, and the GFP signal was traced as a parameter of UPS in the cardiomyocytes.

Co-immunoprecipitation Assay Under Native Conditions—Co-immunoprecipitation was performed under a native condition as described previously (21). Cells were scraped into 0.5 ml of a TEPT buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM NaF, 0.5% Triton X-100, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride). The extracts were vortexed and centrifuged at 18,000 × g for 15 min at 4 °C. The supernatants were mixed with 1.5 volumes of a TEBS buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM NaF, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100) and an anti-FLAG antibody conjugated to agarose beads (Sigma) followed by incubation at 4 °C overnight. The beads were washed three times with the TEBS buffer. The complexes were analyzed by Western blot after SDS-PAGE as described above (16).

Immunohistochemistry—Immunohistochemical analyses were performed as described (15, 17). All cell culture media were from Alexa 488-conjugated anti-rabbit and Alexa 568-conjugated anti-mouse antibodies (Invitrogen), and TO-PRO-3 nuclear staining was from Molecular Probes. The anti-CryAB antibody (SPA-223) and anti-HSP25 (SPA-801) were from StressGen; anti-FLAG was from Stratagene, and anti-HA (Y11) was from Santa Cruz Biotechnology. The anti-cTnI antibody (MAB1691) was from Chemicon International, and the anti-HSP22 antibody was from Upstate Biotechnology, Inc., and the anti-oligomer antibody was from BIOSOURCE. The cellular viability was measured 4 days after transfection using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Image J 1.36b was used to quantify the immunofluorescent intensity. The results from 30 to 50 cells were averaged for cohort comparison. The area stained with the oligomer antibody was defined, and the average pixel intensity of the cardiomyocyte was determined for comparison.

Recombinant Protein—To produce a recombinant protein, His epitope-tagged wild-type CryAB-FLAG, CryAB R120G-FLAG, and HSP22HA were overexpressed in BL21 cells or Rosetta™ cells using the pET system (Novagen, Madison, WI) and purified with a nickel-nitrilotriacetic acid column (Qiagen, Santa Clarat, CA). After dialysis or dilution with a Tris-Cl buffer, pH 7.5, amyloid oligomer dot-blot analysis was performed. To quantify the amyloid oligomer level, each recombinant protein or protein mixture was incubated at 37 °C for 3 h and blotted on a nitrocellulose membrane. After fixation with 4% paraformaldehyde in TBS for 3 min, blots were incubated with the anti-oligomer antibody (1:1000) (BIOSOURCE). Image J 1.36b was used to quantify the intensity of the amyloid oligomer. For Western blot analysis, protein samples were loaded onto a 12% SDS-polyacrylamide gel and electrophoresed at 100 V for ~3 h. The separated proteins were then transferred onto PVDF or nitrocellulose membranes (Amersham Biosciences) at 4 °C overnight. Native gel analysis was performed using the PAGEL® native gel system (Atto Corp., Tokyo, Japan). In some experiments, the recombinant protein was separated by the native gel and extracted from the gel into
The extract from the native gel was used for dot-blot analysis as described above. Western blot was performed using a standard method described previously (16). The cellular toxicity of the recombinant CryAB R120G protein in HEK293T cells was determined using an MTT assay. Recombinant proteins were added to give a final concentration of 0.2 or 0.4 mg/ml in a serum-free Dulbecco’s modified Eagle’s medium and incubated for 4 h. After incubation, MTT assays were performed.

Statistics—Data are expressed as the means ± S.E. Statistical analysis was performed using the unpaired Student’s t test, and the analysis of variance was followed by a post hoc comparison with Fisher’s PLSD using Statview version 5.0 software (Concepts, Inc., Berkeley, CA). Differences between groups were considered statistically significant at \( p < 0.05 \).

**RESULTS**

The Recombinant CryAB R120G Protein Is Immunoreactive against the Anti-oligomer Antibody—Recent studies support the toxic amyloid oligomer hypothesis for amyloid-based disease (12). The amyloid oligomer structure, which is observed in several distinct amyloidogenic proteins, including CryAB R120G, Aβ, α-synuclein, and polyglutamine proteins, shares a common epitope that is recognized by an oligomer-specific antibody (6, 15, 17). To address the molecular mechanisms of amyloid oligomer formation by the CryAB R120G, a recombinant CryAB R120G protein was generated and purified using a standard Ni\(^{2+}\) column. The CryAB R120G protein was quite difficult to isolate in a soluble form because it easily forms an inclusion body in bacteria. Thus, we tried to isolate the CryAB R120G protein with Rossetta and Rossetta 2 (Novagen, Madison, WI) with incubation at 30 °C to increase the soluble protein level of CryAB R120G. SDS-PAGE showed a single band in both the wild-type CryAB-FLAG protein (>95% of total protein) and the CryAB R120G-FLAG protein (>95% of total protein) (Fig. 1A). The recombinant proteins were incubated at 37 °C for 2 h and blotted on a nitrocellulose membrane. The CryAB R120G-FLAG proteins showed strong immunoreactivity against the anti-oligomer antibody, and their reactivity was relatively stable in TBS solutions from pH 6.5 to 8.5 (Fig. 1B and C). The CryAB R120G-FLAG proteins (0.4 ng/ml) showed cellular toxicity in HEK 293 cells, whereas the wild-type CryAB was neither cytotoxic nor immunoreactive against the anti-oligomer antibody (1.2 and 2.5 μg) (Fig. 1B and D). This result suggests that the missense mutation in the CryAB protein can alter the entire protein conformation and/or the polymerized form to be reactive against the anti-oligomer antibody.

Characterization of the CryAB-Amyloid Oligomer—The missense mutation of the CryAB protein led to toxic amyloid oligomer formation. To address the biochemical characteristics of the mutant CryAB-amyloid oligomer, we tried a native PAGE system. The CryAB R120G protein showed multiple bands with various molecular weights in the native gel (Fig. 2A, right panel indicated as arrows), whereas most of the wild-type CryAB protein formed a high molecular mass protein (Fig. 2A, left panel). Immunoreactivity against the oligomer antibody (Fig. 2B) as well as multiple bands with various molecular weights (Fig. 2A) was lost by treatment with either 5% β-mercaptoethanol or 0.1% SDS for 10 min, suggesting that immunoreactivity against the anti-oligomer antibody is dependent on the polymerized protein structure. This oligomer conformation was also partially blocked by metal ion chelating (data not shown). It is unlikely that β-mercaptoethanol affects a disulfide bond in the CryAB R120G proteins, because no cysteine residue is present in the CryAB protein (22, 23). To confirm the effect of β-mercaptoethanol on the amyloid oligomer, we examined the effect of 5 mM DTT and ethanol on CryAB R120G amyloid formation. Immunoreactivity against the anti-oligomer antibody was slightly increased by treatment with 10 or 20% ethanol but reduced by treatment with 5 mM DTT (Fig. 2C). These results suggest that a chemical compound containing a thiol residue can interfere with the conformation of the amyloid oligomer. To analyze the possibility of interaction between a sulfur-containing amino acid, i.e. methionine, at position 68 of the CryAB protein and chemical compound-containing thiol, such as β-mercaptoethanol and dithiothreitol, we generated a recombinant protein in which the methionine residue at position 68 was changed to alanine (Fig. 2D). The immunoreactivity of the CryAB R120G protein containing an additional missense mutation (M68A) against the anti-oligomer antibody was the same as that of the CryAB R120G protein (Fig. 2D), whereas the CryAB M68A protein without an R120G mutation was scarcely immunoreactive against the anti-oligomer antibody. Thus, these results suggest that the effect of a thiol residue on the CryAB-amyloid oligomer is independent of the sulfur-containing amino acid.

To further address the biochemical characteristics of the CryAB-amyloid oligomer, in particular the molecular size of the immunoreactive oligomer mass protein of the mutant CryAB R120G Oligomer and HSP22

![Figure 1](image)

**FIGURE 1.** Formation of an amyloid oligomer by the recombinant CryAB R120G protein. **A**, a typical picture of the SDS-PAGE of the recombinant wild-type CryAB-FLAG (WT) and CryAB R120G-FLAG (R120G). **B**, dot blotting shows the presence of the amyloid oligomer in the recombinant CryAB R120G protein, whereas no detectable amyloid oligomer is seen in the wild-type CryAB (CryAB WT). The amyloid oligomer is detected by the anti-oligomer antibody. **C**, formation of an amyloid oligomer by the CryAB R120G protein (2.5 μg) was neither cytotoxic nor immunoreactive against the anti-oligomer antibody. **D**, cellular toxicity of recombinant proteins. The cellular toxicity of the CryAB R120G proteins in HEK293 cells is determined using the MTT method. Values are the fold increase relative to untreated cells (UN), whose value was arbitrarily set to 1. **, \( p < 0.001 \) versus untreated cells.
CryAB R120G protein (R120G) disappeared as a result of the treatment with EtOH, we analyzed the interaction between the CryAB-amyloid molecular mass complex by self-polymerization and/or binding. Because the CryAB is present as a high amyloid is a potent therapeutic target for amyloid-related diseases, including DRM. This suggests that the soluble oligomer may represent the primary toxic mechanism of amyloid pathogenesis (12). This implies that the soluble amyloid oligomer structure formed by the 12–24-merized CryAB R120G protein as well as the molecular weight of the complex.

**Effects of Small HSPs on CryAB R120G Aggregates and Amyloid Oligomer in Cardiomyocytes**—The recombinant HSP22 and HSP25 proteins can interfere with oligomer formation by the CryAB R120G protein. To extend this finding to the cardiomyocyte level, we used isolated neonatal rat cardiomyocytes (17). The level of the amyloid oligomer was sustained by co-transfection of the wild-type CryAB with CryAB R120G in cardiomyocytes (17). The previous report, we presented the effects of co-transfection of the wild-type CryAB with CryAB R120G in cardiomyocytes (17). The level of the amyloid oligomer was sustained by co-transfection of the wild-type CryAB with CryAB R120G in cardiomyocytes, whereas the aggresome disappeared (17). Fig. 3A shows that the mixture of the purified CryAB R120G protein with the wild-type CryAB protein can be immunoreactive against the anti-oligomer antibody and that its intensity was elevated in the mixed sample rather than the CryAB R120G protein (Fig. 3A). Thus, the wild-type CryAB can directly enhance amyloid oligomer formation. To analyze the effect of HSP25 and HSP22 on the CryAB-amyloid oligomer, recombinant HSP25 and HSP22 proteins were overexpressed and purified from the bacteria (Fig. 3B). SDS-PAGE showed a single band of each of the HSP25 and the HSP22 proteins (>95% of the total protein) (Fig. 3B). Either the HSP25 or the HSP22 protein can reduce the immunoreactivity against the anti-oligomer antibody in the mutant CryAB protein (Fig. 3, C and D). These results suggest that small HSPs, such as HSP25 and HSP22, can interfere with amyloid oligomer formation by the mutant CryAB at the purified protein level, although the wild-type CryAB, also a small HSP, can enhance oligomer formation. To further address the inhibitory effect of small HSPs on CryAB-amyloid formation, we performed the native gel analysis. An obvious inhibitory effect of HSP25 on the CryAB-amyloid oligomer formation was seen at around 240–480 kDa, whereas no reduction was observed over a 720-kDa fraction. The CryAB R120G protein was present from 143 kDa to over 720 kDa, whereas the HSP25 treatment altered the CryAB R120G protein distributions (Fig. 3G). Most CryAB protein was present over 480 kDa in the mixture of CryAB R120G and HSP25 (Fig. 3G). The distribution of HSP25 was similar to that of CryAB R120G (Fig. 3H). This result implies that HSP25 can directly interfere with the amyloid oligomer structure formed by the 12–24-merized CryAB R120G protein as well as the molecular weight of the complex.

**HSP25 and HSP22 Can Interrupt CryAB R120G Oligomer Formation**—The polymerized mutant CryAB protein can form an amyloid oligomer. As described above, the soluble amyloid oligomer may represent the primary toxic mechanism of amyloid pathogenesis (12). This suggests that the soluble oligomer amyloid is a potent therapeutic target for amyloid-related diseases, including DRM. Because the CryAB is present as a high molecular mass complex by self-polymerization and/or binding with other small HSP families, such as HSP25 (24) and HSP22 (18), we analyzed the interaction between the CryAB-amyloid oligomers HSP25 and HSP22. In our previous report, we presented the effects of co-transfection of the wild-type CryAB with CryAB R120G in cardiomyocytes (17). The level of the amyloid oligomer was sustained by co-transfection of the wild-type CryAB with CryAB R120G in cardiomyocytes, whereas the aggresome disappeared (17).

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**FIGURE 2.** Characterization of an amyloid oligomer formed by the recombinant CryAB R120G protein. A, native gel. Although the wild-type CryAB (CryAB WT) showed self-polymerized protein over 720 kDa, CryAB R120G showed several bands (indicated by arrows) at 143–480 kDa, which disappeared as a result of treatment with 5% β-mercaptoethanol (BME) or 1% SDS. B, the amyloid oligomer formed by the recombinant CryAB R120G protein (R120G) disappeared as a result of the treatment with β-mercaptoethanol or SDS. C, effect of ethanol (EtOH) and 5 mM DTT on the amyloid oligomer formed by the CryAB R120G. D, amyloid oligomer formation by CryAB R120G containing the substitution from methionine to alanine at 68 residues (CryAB R120G M68A). CryAB R120G M68A as well as CryAB R120G can form the amyloid oligomer. E–G, separation of the CryAB R120G protein by the native gel. E, the CryAB R120G protein is separated by the native gel and extracted into TBS. F and G, dot-blot in extracts from the native gel. The amyloid oligomer is present from fraction 1 (>720 kDa) to fraction 4 (>143 kDa and <242 kDa). The most potent signal as the amyloid oligomer is detected at fraction 3 (>242 kDa and <480 kDa). G, quantitative analysis of the amyloid oligomer (n = 5).

CryAB, we performed Western blot analysis using a native gel without any reducant or detergent treatment. However, no detectable amyloid oligomer was observed as a result of Western blot analysis, although numerous CryAB proteins were present in the blot (data not shown). The reason that it was difficult to detect the amyloid oligomer by Western blot is unclear. We hypothesized that the protein conformation of the CryAB-amyloid is unstable during transfer to PVDF or nitrocellulose membranes. Thus, we tried to extract the protein from the native gel after the native PAGE. We divided the gel sample into 11 pieces by molecular weight and performed protein extraction from the gel (Fig. 2E). Of these extracts, a protein mixture with a molecular mass from 240 to 480 kDa showed the most potent immunoreactivity against the anti-oligomer antibody (Fig. 2, F and G). These results suggest that the polymerized CryAB R120G protein at 240–480 kDa, an ~12–24-merized CryAB R120G protein, could potentially be a major source of the amyloid oligomer.

**Effects of Small HSPs on CryAB R120G Aggregates and Amyloid Oligomer**—The recombinant HSP22 and HSP25 proteins can interfere with oligomer formation by the CryAB R120G protein. To extend this finding to the cardiomyocyte level, we used isolated neonatal rat cardiomyocytes and the adenoviral transfection system (15, 17). Fig. 4 shows the effects of either HSP25 or HSP22 on CryAB R120G aggregate formation (Fig. 4). Adenoviral transfection increased the protein levels of both HSP25 (Fig. 4, A and B) and HSP22 (Fig. 4, D).
and that these interactions can inhibit the aggregate formation of the mutant CryAB proteins. We then analyzed the amyloid oligomer levels in the cardiomyocytes transfected with the CryAB R120G (Fig. 6). As described above, the amyloid oligomer level was sustained, whereas the aggregate formation was inhibited by co-transfection of the wild-type CryAB with the CryAB R120G in cardiomyocytes (17). In contrast to the wild-type CryAB, both HSP25 and HSP22 transfection significantly reduced amyloid oligomer formation in cardiomyocytes transfected with the mutant CryAB R120G (Fig. 6). This result suggests that, although the amino acid sequence reveals homology among small HSPs, small HSPs have some divergent effects of amyloid oligomer formation.

**Inhibitory Effects of HSP22 and HSP25 on Amyloid Oligomer Formation Are Protective for Cardiomyocytes**—The UPS controls many cellular processes, and the disturbance of this system is thought to be one of the causal mechanisms of the amyloid degenerative disease (20). Recent findings indicate that in situ UPS activity using modified GFP signals correlates much more with cellular conditions than does enzyme activity in the lysate (25). In this study, we used an adenovirus containing modified GFP to analyze the UPS activity in cardiomyocytes transfected with CryAB R120G. The adenovirus containing modified GFP at an m.o.i. of 1 induced none or very little GFP protein in the untreated cardiomyocytes and cardiomyocytes transfected with the wild-type CryAB, whereas a UPS inhibitor (MG132 100 nM) induced marked up-regulation of the GFP signals (Fig. 7, A and B). The GFP signal was also undetectable in cardiomyocytes treated with three times the amount of adenovirus containing the wild-type CryAB (m.o.i. of 30) (data not shown). This implies that adenovirus transfection did not disturb the UPS activity in cardiomyocytes under the present experimental condition. In contrast to the case of the wild-type CryAB, a significant increase in the GFP signals was observed in cardiomyocytes transfected with the CryAB R120G (Fig. 7, A and B), suggesting that CryAB R120G impairs the UPS activity. After co-transfection of either HSP25 or HSP22 with the CryAB R120G in cardiomyocytes in

![FIGURE 3. Interference of the CryAB-amyloid oligomer by treatment with either HSP25 or HSP22. A, effect of the wild-type CryAB (CryAB WT) on the CryAB R120G (CryAB R120G)-amyloid oligomer. Wild-type CryAB increases the amyloid oligomer level. B, typical picture of the recombinant HSP25 and HSP22HA proteins. C, effect of either HSP25 or HSP22 proteins on the CryAB R120G amyloid oligomer. Incubation of the CryAB R120G amyloid oligomer with either HSP25 or HSP22 decreases the amyloid oligomer level. D, quantitative analysis of the amyloid oligomer formed by the CryAB R120G protein by either HSP25 or HSP22 treatment (n = 3–4). E and F, effect of the HSP25 on the CryAB amyloid oligomer. HSP25 interferes with the CryAB-amyloid oligomer at 240-480 kDa. G, quantitative analysis of the amyloid oligomer (n = 3–4). H, distribution of the HSP25 protein in the mixture of the CryAB R120G with the HSP25. ***p < 0.001 versus CryAB WT. ###p < 0.001 versus CryAB R120G.](https://example.com/fig3.png)
which both the aggregates and the amyloid oligomer were attenuated, the up-regulation of the GFP signals was completely attenuated in the cardiomyocytes (Fig. 7, C and D). The cell viability estimated by the MTT assay was well correlated with the UPS activity. The CryAB R120G transfection significantly reduced the cardiomyocyte viability, and co-transfection of the HSP25 and HSP22 rescued the reduction in cell viability (Fig. 8). Thus, these results indicate that HSP25 and HSP22 have powerful protective effects against cellular toxicity induced by the mutant CryAB.

**DISCUSSION**

In our previous study, we showed that the R120G missense mutation in the CryAB protein that is a cause of DRM led to perinuclear aggresome formation and that these aggresomes contained the amyloid oligomer (15). Furthermore, we demonstrated that the continuous expression of the mutant protein is needed to sustain the high concentrations of the amyloid oligomer that correlate with depressed cardiac function as well as premature lethality (17). These previous studies indicate that the amyloid oligomer is critical for the DRM disease as well as other amyloid-related neurodegenerative diseases (17). In this study, we show that the CryAB R120G protein can form a toxic amyloid oligomer that is generated by self-oligomerization of the mutant protein. In addition, the polymerized CryAB R120G proteins can be a potential major source of the amyloid oligomer. HSP22 and HSP25, members of the small HSP family that can directly bind to the mutant CryAB, block the amyloid oligomer formation of the mutant CryAB at both the purified protein and the cardiomyocyte levels, whereas the wild-type CryAB can deteriorate the cell viability, which correlates with the sustained level of the amyloid oligomer. These findings suggest that both the polymerized CryAB R120G proteins and the protein complex made up of the mutant protein and the wild-type CryAB can form an amyloid oligomer that is a toxic species for cardiomyocytes. HSP22 and HSP25 can directly interact with the mutant CryAB protein and block CryAB-amyloid oligomer formation. We also analyzed the inhibitory effects of small HSPs on the other amyloid oligomer protein. HSP22 and HSP25 can block a reduction of the cellular viability and UPS activity in cardiomyocytes expressing a peptide containing 81 repeats of polyglutamine (PQ81) without reduction at the
aggregate level (data not shown). These data imply that the protective effect of small HSPs is not specific to the CryAB mutation. Thus, the attenuation in the amyloid oligomer by small HSPs may be a powerful therapeutic strategy for the treatment of DRM as well as other amyloid-related neurodegenerative diseases.

Disturbance of the UPS activity by the CryAB R120G was observed in a previous study (26). As in previous results, we observe the impairment of UPS activity estimated by the modified GFP signals in cardiomyocytes transfected with CryAB R120G, and we demonstrate that small HSPs, such as HSP25 and HSP22, can rescue this impairment of UPS activity. UPS protects cells against the toxicity of protein aggregation (27). The significance of UPS impairment in neurodegenerative diseases was confirmed by the discovery of genes encoding UPS components, in which the loss of function was found to be the cause of dominantly inherited neurodegenerative diseases (28, 29). Because the direct effects of the amyloid oligomer on UPS activity are unclear (25), further studies will be needed to address the protective effect of small HSPs on the impairment of UPS activity by the amyloid oligomer.

We showed that the R120G missense mutation in the CryAB protein led to perinuclear aggresome formation and that these aggresomes contained the amyloid oligomer (15). However, no molecular information is available at present about the CryAB-amyloid oligomer, particularly the molecular size of the immunoreactive oligomer-mass-protein of the mutant CryAB. In this study, we showed that the mutant CryAB protein, whose molecular mass is from 240 to 480 kDa, can have the most potent immunoreactivity against the anti-oligomer antibody (Fig. 2, F–H). These results suggest that the polymerized CryAB R120G protein at 240–480 kDa, an ~12–24-merized CryAB R120G protein, can be a potential major source of the amyloid oligomer. In addition, HSP25 dramatically reduced the amyloid oligomer formed by the CryAB R120G protein at 240–480 kDa, although the amyloid oligomer at over 720 kDa was unchanged. Both HSP25 and HSP22 dramatically reduced the CryAB-amyloid oligomer level in both the recombinant protein and the cardiomyocytes as well as improved the cell viability in the cardiomyocytes expressing CryAB R120G. These results strongly suggest that the amyloid oligomer formed by the polymerized CryAB R120G protein at 240–480 kDa may play a critical role in cytotoxicity and that the interference of the CryAB-amyloid oligomer at 240–480 kDa by small HSP may be a powerful therapeutic strategy for the treatment of DRM.

The recombinant CryAB R120G protein can contribute to amyloid oligomer conformation. However, oligomer formation by the recombinant protein was undetectable by Western blot using a native gel system, although the oligomeric CryAB R120G protein (~143–480 kDa) was observed (data not shown). Because we can detect the amyloid oligomer in the extract from the native gel, the self-oligomeric form of CryAB R120G dramatically lost its reactivity against the anti-oligomer antibody in the course of transfer to the PVDF membrane. This suggests that the CryAB R120G amyloid oligomer is relatively unstable. Consistent with the result using recombinant proteins, no positive immunoreactivity against the oligomer antibody was detected in dot-blot analysis using the lysate from the CryAB R120G transgenic mouse, although the oligomer was detected in a study using immunohistochemistry (Fig. 6). Because Western blot analysis works for some types of amyloid oligomers that seem to be stable in SDS, the stability of the oligomer probably depends on the characteristics of each protein (30). Thus, the CryAB-amyloid oligomer conformation may be more fragile than that of other amyloidogenic proteins, such as the Aβ peptide and polyglutamine.
oligomer by the thiol residue is independent of the sulfur-containing amino acid. Further studies will be needed to identify the cytotoxic protein states of CryAB R120G as an amyloid oligomer.

The CryAB is a small HSP and probably works as a molecular chaperone, which is usually present as a complex with partners, such as HSP25 and target proteins (31, 32). It is known that the CryAB R120G protein, which loses its chaperon-like function (33), can also bind to a molecular partner, such as a wild-type CryAB, HSP25, and form a high molecular mass complex (24, 31, 32). It is known that the molecular weight of the high molecular mass complex by CryAB R120G was larger than that of the complex by the wild-type CryAB (21, 31, 32). In this study, we observed that some of the oligomerized CryAB R120G protein (~66–480 kDa) was present as a self-oligomerized form in the native gel, whereas the amount of the self-oligomerized form was much smaller in the wild-type CryAB. Because we used the purified CryAB protein in this study, the high molecular mass was only formed by the polymerized wild-type CryAB and CryAB R120G proteins. As described above, the polymerized CryAB R120G may be unstable in the lysate, and these experimental conditions may explain the difference between previous results and our findings.

In summary, we show the results of CryAB R120G amyloid oligomer formation by mutant protein self-interaction. Recombinant HSP22 and HSP25 interrupt the oligomer formation by the CryAB R120G protein in both the recombinant protein and cardiomyocyte systems. The blockade of amyloid oligomer formation by either HSP22 or HSP25 reduced the ubiquitin proteosomal activity with concomitant recovery of the cellular viability. The blockade of oligomer formation by small HSPs, such as HSP22 and HSP25, may be a new therapeutic strategy for rescuing DRM as well as other types of amyloid-based degenerative diseases.

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