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Review

Role of the Alpha-B-Crystallin Protein in Cardiomyopathic Disease

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Abstract: Alpha-B-Crystallin, a member of the small heat shock family of proteins, has been implicated in a variety of cardiomyopathies and in normal cardiac homeostasis. It is known to function as a molecular chaperone, particularly for desmin, but also interacts with a wide variety of additional proteins. The molecular chaperone function is also enhanced by signal-dependent phosphorylation at specific residues under stress conditions. Naturally occurring mutations in *CRYAB*, the gene that encodes alpha-B-crystallin, have been suggested to alter ionic intermolecular interactions that affect dimerization and affect chaperone function. These mutations have been associated with myofibrillar myopathy, restrictive cardiomyopathy, and hypertrophic cardiomyopathy, and promote pathological hypertrophy through different mechanisms such as desmin aggregation, increased reductive stress or activation of calcineurin-NFAT signaling. This review will discuss the known mechanisms by which alpha-B-crystallin functions in cardiac homeostasis and the pathogenesis of cardiomyopathies and provide insight into potential future areas of exploration.

Keywords: alpha-B-crystallin; cryab; molecular chaperone; desminopathy; hypertrophic cardiomyopathy; dilated cardiomyopathy; restrictive cardiomyopathy; calcineurin; NFAT

1. Introduction

Proteins are the molecular effectors of cell function, by providing structure and function in support of the essential biomolecular processes necessary for organism survival and proliferation. Alterations in protein folding have profound effects on protein structure, function, and stability. Chaperone proteins are present in a wide variety of organisms across the evolutionary spectrum and function to promote and maintain proper folding of proteins, especially under stress conditions. A universal environmental stressor that promotes protein unfolding is exposure to heat. Elevations in temperature can promote protein unfolding that can be detrimental to cell function and survival [1]. In response to increased temperatures, organisms initiate a heat shock response based on activation of, and increased expression of, heat shock proteins (Hsp) [1]. The family of heat shock proteins consists of numerous classes divided based on their function within cells, from induction of proteolysis to changes in cellular metabolism and regulation of transcription [1]. The first class of heat shock proteins to be discovered and the most abundantly expressed within cells are molecular chaperones [1]. Molecular chaperones help stabilize, fold, and refold proteins even at physiologic temperatures but become even more critical for survival during times of cellular stress [1]. Molecular chaperones are divided into groups based on their function. One such group includes the small heat shock protein (sHsp) family whose members prevent aberrant protein interactions [1]. However, the sHsp also overlap with another family of proteins, the crystallins. The crystallin family is comprised of two unrelated superfamilies, the alpha crystallins which make up the small heat shock proteins and then the beta/gamma crystallins [2]. The former is made up of two genes: *CRYAA* and *CRYAB* which encode alpha A and alpha B crystallins, respectively. Due to the ubiquitous nature of molecular chaperones, both these proteins are involved in a myriad of cellular functions and processes [1]. However, this also means that mutations in *CRYAA* and *CRYAB* have a wide array of deleterious effects from cancer to eye disorders and cardiac diseases. Decades ago, a novel CRYAB mutation was

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found to cause hypertrophic cardiomyopathy [3], sparking multiple studies into the effect of CRYAB mutations in cardiovascular disease. In this review we focus on the cellular functions of CRYAB and the broad set of consequences associated with its dysfunction with a particular focus on its role in heart disease highlighting decades of research and exciting new developments.

2. Alpha-crystallin B chain (CRYAB)

2.1. Wild Type CRYAB

Crystallins were initially discovered in the eye lens, where they are the predominant structural protein [4]. From there more and more crystallins have been found in organs across the body where they serve to prevent improper protein folding and aggregation. Of the two alpha crystallin members, CRYAA is mainly found in the eye, while CRYAB is widely expressed across all organs and is highly expressed in skeletal and cardiac muscle. Wild type CRYAB functions as a molecular chaperone, where its main functions are to prevent improper protein folding and aggregation and thus prevent proteotoxicity in cells [5–7]. Proteotoxicity is the state in which unfolded and aggregated proteins negatively impact cellular function [13]. Additional functions include regulation of calcium signaling [8], autophagy [9,10] and cellular survival [11]. CRYAB binds to denatured proteins and enhances their solubility which plays an important role in preventing protein precipitation in cells [12]. Through multiple independent studies, the effects of CRYAB are not mediated by a single pathogenic mechanism but instead are based on specific protein defects [13]. Proteotoxicity can be divided into 4 classes based on functional effects: 1) improper protein folding or structural preservation resulting in altered degradation, 2) poor protein function due to dominant negative mutations, 3) toxic functions due to gain of function mutations, and 4) toxic aggregation of multiple misfolded proteins [13]. Wild type CRYAB functions to prevent the first and the fourth of the proteotoxic classes from occurring in cells. Improper protein folding is a universal problem that can occur in all cells. Protein folding is in part based on the amino acids sequences that make up the protein and their respective side chains. The side chains of each amino acid lend its chemical properties to the folding process, for native protein folding the hydrophobicity of the side chains play a major role [15]. However, given that the free energy, and therefore the stability, of native proteins is only a few kcal/mol higher than their unfolded counterparts, other intramolecular forces, such as backbone hydrogen bonding, cannot be excluded [15]. The relatively small amount of free energy separating folded and unfolded proteins also highlights the fact that even single amino acid mutations can result in consequential changes to protein structure and function. Despite the remarkable success of native protein folding, a persistent degree of misfolding and unfolding occurs in cells. Due to this pressure, cells have developed mechanisms to counter this process, one of which involves the molecular chaperones. As a molecular chaperone, wild type CRYAB plays a major role in preventing aberrant misfolding and the ensuing associated proteotoxicity. Therefore, it is not surprising that wild type CRYAB is upregulated in a number of cardiovascular disorders [11]. It should be noted that a wide variety of proteins and cellular functions attempt to maintain normal protein folding and mutations that affect these mechanisms lower cellular capacity to maintain order but in general do not fully abolish proper protein folding [13]. Therefore, it might take years or decades for the emergence of clinically apparent pathology and additional stress on the system may be necessary to provoke pathological changes [13]. CRYAB as a molecular chaperone is able to recognize and respond to a variety of stressors, as there are numerous stressors that cause alterations in protein folding. CRYAB is activated in response to stress through post-translational modification. In response to a number of stresses, including heat, okadaic acid and high concentrations of NaCl, CRYAB is phosphorylated at three different serine residues Ser-19, Ser-45 and Ser-59. Interestingly, no phosphorylation has been seen in response to agents that increase intracellular cAMP [16]. When phosphorylated, CRYAB translocates from the cytosol to the cytoskeleton presumably to prevent protein destabilization [16]. CRYAB phosphorylation is likely driven by MAP kinase-activated protein 2 which is itself activated by p38 MAP kinase, suggesting its role in the regulation of CRYAB activity [16]. Studies have shown that wild type CRYAB overexpression is benign in itself and protective against ischemia and reperfusion

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injury in vitro and in vivo in transgenic mouse models [14]. Furthermore, cardiovascular diseases are often associated with increased oxidative stresses. In that vein, overexpression of wild type CRYAB in H9C2 cells has been shown to protect against oxidative stress and the apoptosis that accompanies it [17]. The reduction in apoptosis occurs in association with decreased release of cytochrome c and downregulation of apoptosis regulator BCL2, which might be mediated thought the PI3K/AKT pathway [17]. Therefore, wild type CRYAB is upregulated as an apoptosis inhibitor in certain cancers, and although this article will focus on the cardiovascular system it is interesting to see the wide range of biological processes influenced by CRYAB [12]. The roles of wild type CRYAB as a molecular chaperone can most well be understood through its mutations and the pathology that follows which we will discuss in the next sections.

2.2. CRYAB 109 Mutations

Multiple papers documenting mutations of the 109th amino acid of CRYAB, have deciphered its role in a range of pathologies from cataracts to myopathies [18,19]. In terms of cardiac dysfunction, one of the more common mutations noted is CRYAB^{D109G}, a missense mutation, that has been implicated in the development of restrictive cardiomyopathy [19]. Two additional mutations have been noted at the 109th amino acid, CRYAB^{D109A} described by Fichna et al. in 2017 [20] in which patients develop isolated myofibrillar myopathy without cardiac involvement, and CRYAB^{D109H} described by Sacconi et al. in 2012 [21] in which a single patient presented with late stage dilated cardiomyopathy [19]. The CRYAB protein spontaneously forms dimers which then form oligomers at physiologic conditions minimizing activity [22,23]; these structures are disrupted in response to stress resulting in its activation and chaperone function [24,25]. The amino acid D109 is highly conserved across species as it forms an integral ionic bridge stabilizing the CRYAB dimer [19], loss of which appears to lead to aberrant chaperone function. The pathology of CRYAB^{D109G} often revolves around abnormal desmin aggregation. A large portion of noted mutations causing cardiac dysfunction are in sarcomeric and cytoskeletal genes due to their importance in maintain cardiac structure; interestingly CRYAB^{D109G} affects cardiac cell structure indirectly through improper desmin function [19]. Desmin is a muscle specific intermediate filament that helps stabilize the contractile mechanism and nucleus in sarcomeres and plays a role in sarcomere architecture. Additionally, desmin plays a role in maintaining tissue structure by tightly associating with cell-cell adhesion complexes [26]. Desmin is highly expressed in muscle tissue and proper organization of the desmin filaments is key to maintaining cellular function. Pathologies arising from desmin-related dysfunction and aggregation are termed desminopathies, and when they involve muscle tissue are named desmin-related myopathies. Desminopathies can arise from mutations within desmin itself and a number of pathogenic desmin mutations have been described, however they can also arise from dysfunctions of proteins involved in protein folding and stability [26]. In this vein when CRYAB^{D109G} mutations are present, the protein is no longer able to efficiently prevent the desmin filaments from aggregating together. Desmin aggregation in cells is broadly characterized by two criteria defined by Goebel et al. in 1997, 1) multifocal cytoplasmic inclusions or spheroid bodies, and 2) disseminated accumulation of granulofilamentous material [26]. Wild type CRYAB forms stable dimers through ionic bridges at D109 and R120, which are disrupted by mutations in the region and is a particularly common site of missense mutations in patients with myopathies [19]. Therefore, instead of binding desmin to stabilize the Z-bands and intercalated disks in muscles, they form cytoplasmic aggregates in conjunction with the mutated CRYAB protein, falling into the first classification of desmin aggregation [19]. When the desmin filaments then aggregate, they cause cellular dysfunction which in the heart manifests mostly as forms of cardiomyopathies both hypertrophic and restrictive, although hypertrophy is more commonly noted [19].

2.3. CRYAB 120 Mutations

Mutations at the 120th amino acid of CRYAB, similar to mutations at the 109th amino acid, are also involved in various pathologies across the body. The most common mutation associated with cardiovascular disease is the germline CRYAB^{R120G} missense mutation, which is inherited in an

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autosomal dominant manner [27]. As was noted in the previous section CRYAB forms dimers, that are stabilized by ionic bonds at the D109 and R120 amino acids [19]. Interestingly, cryoelectron microscopy has shown that CRYAB^{R120G} has an abnormal quaternary structure with a molecular weight at least twice that of wild type CRYAB, suggesting it disrupts normal oligomerization [28]. Interestingly, in vitro studies indicate that CRYAB^{R120G} acts in a dominant negative manner with the mutant protein compromising the function of wild type proteins in the dimerized form [14]. CRYAB mutant aggregation then suggests that even in the cases of heterozygous mutations in CRYAB that mutant protein might cause wild type proteins to form aggregates resulting in the development of cardiac pathology. As was seen in the mutations at D109, mutations at R120 also lead to desmin aggregation and subsequent cellular dysfunction with loss of normal muscular striations [27]. Desmin-related myopathies are defined by electron-dense granular aggregates in the cytoplasm seen on electron microscopy [27]. These structures are divided into two classes; Type I structures had a relevantly low electron density, were large and regularly shaped, and tend to occupy a large portion of the central part of the cardiomyocyte while Type II structures were composed of finer and smaller granules that are more numerous than Type I granules, irregularly shaped and surrounded by many fine filaments [27]. It appears that Type I granules were mainly composed of mutant CRYAB aggregates, while Type II aggregates were composed of CRYAB mutants and desmin filaments [27]. Although some aggregates contained both desmin and CRYAB mutant protein, interestingly it was most common for CRYAB and desmin to aggregate independent of the other protein [27]. Mice overexpressing the CRYAB^{R120G} variant additionally are under reductive stress, with myopathic hearts showing increased recycling of oxidized glutathione to reduced glutathione due to augmented expression and enzymatic activity of glucose-6-phosphate dehydrogenase(G6PD), glutathione reductase and glutathione peroxidase. Crossing of these mice with mice expressing reduced levels of G6PD rescued the cardiomyopathic and proteotoxic phenotype [29]. In cells with the CRYAB^{R120G} mutation, autophagy, a process by which dysfunctional cellular organelles are removed, is inhibited, suggesting another mechanism by which mutant CRYAB negatively impacts the function of cells [30]. Furthermore, inducing autophagy in CRYAB^{R120G} cultured cardiomyocyte reduces the aggregation burden and cytotoxic aggregation intermediates, referred to as pre-amyloid oligomers [30]. An earlier study observed that autophagy in hearts of mice overexpressing CRYAB^{R120G} demonstrate increased autophagy that is an adaptive response to proteotoxic aggregates. Crossing these mice with mice deficient in autophagy due to Beclin deficiency developed worsened proteotoxicity and cardiomyopathy [9]. Although the measured levels of autophagy may differ in cultured cardiomyocytes and mouse hearts overexpressing CRYAB^{R120G}, the effects of autophagy augmentation and inhibition on improving and worsening CRYAB^{R120G}-associated proteotoxicity and cardiomyopathy is consistent. It is important to note that although protein aggregates are the hallmark of desmin-related cardiomyopathy, their accumulation is only weakly correlated with disease severity while the amount of pre-amyloid oligomers more strongly correlate with human cardiovascular disease [30]. As was noted in the D109 mutants, the cardiac pathology that is most often associated is the development of desmin-related cardiomyopathy.

2.4. CRYAB 123 Mutations

A recently identified mutation in CRYAB by Maron et al., the CRYAB^{R123W} mutation, was discovered through genetic analysis in twins that developed hypertrophic cardiomyopathy with astonishing concordance [31,32]. Follow up mouse studies revealed that unlike the previous two mutations, CRYAB^{R123W} does not cause desmin aggregation but rather leads to cardiac dysfunction through sarcomere independent mechanisms. Knock-in mice with the CRYAB^{R123W} mutation do not develop hypertrophic cardiomyopathy spontaneously but undergo a distinct remodeling process upon pressure overload via transaortic constriction. Wild type CRYAB has been previously reported to play a protective role against the development of pathological hypertrophy in pressure overloaded hearts [8]. As for the mechanism behind the protective effects of wild type CRYAB in this setting, it has been proposed that CRYAB prevents the interaction between Calcineurin and NFAT and inhibits the subsequent downstream activation. Whereas the CRYAB^{R123W} mutant is unlikely to block that

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interaction as efficiently therefore leading to aberrant activation. Crossing of Cryab^{R123W} mice with NFAT-luciferase reporter mice resulted in an increase of NFAT-luciferase reporter activity, while overexpression in H9c2 cells also led to increased NFAT-luciferase reporter activity [32]. Five NFAT transcription factors have been discovered; NFATc1-c4 are regulated by calcineurin, whereas NFAT5 resides in the nucleus and is not under calcineurin regulation. Calcineurin is activated by intracellular calcium and serves many important cellular functions. Calcineurin is a serine/threonine phosphatase activated by sustained high levels of calcium. Calcium bound calmodulin binds to calcineurin leading to a conformational change in which the calcineurin C-terminal autoinhibitory domain is disengaged. Once active, calcineurin binds NFAT and de-phosphorylates several serine motifs in its regulatory domain of NFAT, exposing its nuclear localization signal. This enables NFAT translocation to the nucleus where it acts as a transcription factor [33]. In order for proper signaling, the calcineurin catalytic domain must be able to bind to the conserved PxIxIT motif on NFAT which is located N terminal to its phosphorylation sites [33]. As this interaction is integral to NFAT activation by calcineurin, blocking this site represses NFAT activity. It is possible that wild type CRYAB blocks this interaction, as it has been shown that wild type CRYAB inhibits the activation of NFAT and its nuclear translocation [34]. CRYAB^{R123W} would thus be expected to bind less efficiently and facilitate calcineurin/NFAT activation through a de-repression mechanism. Interestingly, however, overexpression of CRYAB^{R123W} in H9c2 leads to activation of NFAT activity, despite the presence of WT CRYAB, suggesting that an activation mechanism is present rather than a simple de-repression mechanism [32].

3. CRYAB Mouse Models

Mutations in CRYAB have been studied in various cellular and animal models. Here we will discuss the mouse models that have been generated and used to study CRYAB related cardiovascular diseases.

3.1. CRYAB R120G Mouse Models

Robbins et al. reported the construction of transgenic mice expressing three different expression levels of the CRYAB^{R120G} mutant [27]. Germline transmission was confirmed with normal mendelian offspring ratios indicating no embryonic lethality across the expression levels. Protein analysis of the transgenic mutant hearts showed elevated levels of proteins, especially of insoluble proteins likely representing protein aggregates seen on stained myocardial sections. As the mutant mice aged, the number and size of aggregates increased. Higher expression of mutant CRYAB^{R120G} increased mortality indicating a possible dose dependent phenotype. Mice with the highest expression level died around 5-7 months while mice with intermediate expression levels showed a similar phenotype in 12-16 months. Extracted hearts were grossly enlarged and dilated. Autopsy also revealed pulmonary and hepatic congestion, pleural effusion, and subcutaneous edema consistent with congestive heart failure. The mutant line 708, whose expression was intermediate, and mutant line 134, whose expression was the highest, were chosen for further study and compared to mice expressing transgenic wild type CRYAB with expression and protein level comparable to the mutants. On a molecular basis early in the development of hypertrophic cardiomyopathy in humans, there is an upregulation of atrial natriuretic peptide and B-myosin and a downregulation of a-myosin, phospholamban and sarcoplasm reticulum calcium. This molecular pattern was seen in young mice harboring the CRYAB^{R120G} mutant. By 3 months, hypertrophy was grossly apparent based on increased ventricular weight/tibial length ratios and continued to worsen as the mutant mice aged. Cardiomyocyte size progressively enlarged and at 3 months the increase heart size was attributed to concentric hypertrophy. However, as the mice aged the increased size was due to heart dilation suggestive of failure. Both the early molecular changes and physiologic progression were consistent with the clinical progression seen in human cardiovascular diseases. Comparable to the human pathophysiology of desmin-related cardiomyopathy, 3-month-old CRYAB^{R120G} mice maintained contractile function but relaxation impairments were noted. With age, however, there was progression to severe disease comparable to humans with loss of contractile function and relaxation

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becoming load dependent. A knock-in mouse model expressing normal levels of CRYAB^{R120G} also demonstrates lens and myopathy phenotypes [35].

3.2. CRYAB R123W Mouse Models

Mice harboring the CRYAB^{R123W} mutation were generated by Chou et al. using C57BL/6 mice with CRISPR/Cas9-mediated homology directed repair to knock-in the mutant allele [32]. In this model mice did not develop hypertrophic cardiomyopathy at a steady state which is not unexpected given that many models require additional stress for pathology to emerge. At steady state, young mice homozygous for the CRYAB^{R123W} mutation were found to have increased E_{max}, a load-independent measure of contractility, compared to wild type and heterozygous mice; interestingly this seems to decrease with age. Steady state mice were also found to have an elevated E/E' indicative of diastolic dysfunction commonly seen in hypertrophic cardiomyopathy patients that developed with age. However, using this model in combination with transaortic constriction generated pressure overload resulting in the development of marked pathological hypertrophy in homozygous and heterozygous CRYAB^{R123W} mutants not seen in wild type mice. Similar to other mouse models of hypertrophic cardiomyopathy (HCM), these mice generated circumferential hypertrophy as opposed to the asymmetric septal hypertrophy seen in humans. But otherwise CRYAB^{R123W} mutant mice hearts showed a greater extent of cellular hypertrophy compared to wild type and large areas of parenchymal fibrosis were consistent with key features of human HCM. It should also be noted that mice carrying the CRYAB^{R123W} mutation developed progressive systolic dysfunction post transaortic constriction, which did not worsen in mice with dual CRYAB^{R123W} mutation and heterozygous MYBPC3 truncation suggesting that CRYAB^{R123W} acts in a sarcomere independent manner. Overall, the CRYAB^{R123W} mutant mice displayed key elements of human HCM pathology and were stable during steady state conditions indicating that these mice are easy to maintain and readily induced to develop pathological hypertrophy with the addition of pressure overload. Of note, however, these mice did not develop proteotoxic desmin or CRYAB aggregates and demonstrated increased calcineurin/NFAT activation, indicating a distinct mechanism of promoting pathological hypertrophy compared to the CRYAB^{R120G} variant.

5. Conclusions and Future Directions

A large body of evidence has accumulated in support of the essential role of α-B-crystallin in normal cardiac homeostasis through its function as a molecular chaperone to reduce proteotoxic aggregation and to attenuate pathological calcineurin/NFAT signaling. Naturally occurring mutations that lead to desmin-related cardiomyopathy, restrictive cardiomyopathy and hypertrophic cardiomyopathy underscore its relevance to human disease. Analysis of the pathological mechanisms in these various conditions underscore the broad effects of CRYAB on cellular function and how different mutations can have distinct effects on either protein aggregation or calcineurin/NFAT signaling to promote divergent phenotypes. Future work to determine the specific effects of pathological mutations on CRYAB structure, function and interacting proteins will likely provide further insight into downstream pathological mechanisms and identify future targets for therapeutic intervention.

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