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The A31P missense mutation in cardiac myosin binding protein C alters protein structure but does not cause haploinsufficiency

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ABSTRACT

Mutations in *MYBPC3*, the gene encoding cardiac myosin binding protein C (cMyBP-C), are a major cause of hypertrophic cardiomyopathy (HCM). While most mutations encode premature stop codons, missense mutations causing single amino acid substitutions are also common. Here we investigated effects of a single proline for alanine substitution at amino acid 31 (A31P) in the C0 domain of cMyBP-C, which was identified as a natural cause of HCM in cats. Results using recombinant proteins showed that the mutation disrupted C0 structure, altered sensitivity to trypsin digestion, and reduced recognition by an antibody that preferentially recognizes N-terminal domains of cMyBP-C. Western blots detecting A31P cMyBP-C in myocardium of cats heterozygous for the mutation showed a reduced amount of A31P mutant protein relative to wild-type cMyBP-C, but the total amount of cMyBP-C was not different in myocardium from cats with or without the A31P mutation indicating altered rates of synthesis/degradation of A31P cMyBP-C. Also, the mutant A31P cMyBP-C was properly localized in cardiac sarcomeres. These results indicate that reduced protein expression (haploinsufficiency) cannot account for effects of the A31P cMyBP-C mutation and instead suggest that the A31P mutation causes HCM through a poison polypeptide mechanism that disrupts cMyBP-C or myocyte function.

1. Introduction

Hypertrophic cardiomyopathy (HCM) is the most common genetic cause of cardiomyopathy and is estimated to affect 1 in 500 people [1–3]. Hundreds of mutations in genes encoding sarcomeric proteins are thought to cause HCM [3]. However, the majority of mutations occur in two genes, *MYH7* and *MYBPC3*. *MYH7* encodes the β-myosin heavy chain, the primary force generating protein of cardiac muscle, while *MYBPC3* encodes cardiac myosin binding protein C (cMyBP-C), an essential regulatory protein that modulates the force and speed of cardiac contraction. Although the majority of mutations in *MYH7* cause single amino acid substitutions, most *MYBPC3* mutations are non-sense or frameshift mutations that encode premature termination codons and are thus predicted to cause early termination of cMyBP-C. However, truncated cMyBP-C proteins have not been detected in myocardium from HCM patients [4–6], most likely because cell quality control mechanisms either efficiently degrade mRNAs that contain premature termination codons or because truncated or misfolded proteins are rapidly degraded thus preventing their accumulation [4,7,8]. The apparent lack of expression of mutant truncated proteins combined with observations that the total amount of cMyBP-C protein is reduced in some patients with HCM has led to the hypothesis that haploinsufficiency causes sub-stoichiometric amounts of cMyBP-C within the sarcomere which impair contractile function and cause disease. Because reduced cMyBP-C protein expression was also found in myocardium of some patients with cMyBP-C missense mutations that cause single amino acid substitutions [5], both truncation and missense mutations could cause disease through a

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common mechanism of reduced cMyBP-C expression. In support of this idea, partial extraction of MyBP-C from cardiac or skeletal myocytes increased calcium sensitivity of force [9] and increased shortening velocity in a dose dependent matter [10], suggesting that insufficient amounts of cMyBP-C could disrupt muscle function and thereby trigger disease. However, the idea that haploinsufficiency can account for effects of cMyBP-C mutations has been challenged [6] and other mechanisms are possible that can account for deleterious effects of cMyBP-C missense mutations. For instance, missense mutations in cMyBP-C could directly disrupt the function of cMyBP-C by acting as poison polypeptides or by causing protein misfolding that impairs protein function. Protein misfolding could also affect cMyBP-C protein stability and abundance and/or impact overall cell homeostasis by increasing the burden on protein quality control clearance mechanisms [7,8]. Given the complex basis of HCM where hundreds of different cMyBP-C sequence variants are believed causative for the disease, it is likely that both haploinsufficiency and poison polypeptide mechanisms will be relevant depending on the individual mutation.

To investigate the cellular mechanisms by which a missense mutation in cMyBP-C leads to HCM, we used a naturally occurring feline model of HCM caused by a single amino acid substitution in cMyBP-C. The missense mutation, a proline for alanine substitution at amino acid 31 in the C0 domain of cMyBP-C (A31P, Fig. 1A, B), was first identified as a cause of HCM in a research colony of domestic cats (Maine Coon/mixed breed) with inherited HCM [11]. HCM is the most common cause of heart failure in cats and, similar to HCM in people, is characterized by abnormal regional or global thickening of the left ventricle, left ventricle outflow tract obstruction including systolic anterior motion of the mitral valve (SAM), and sudden cardiac death [12–14]. Since its original description, the A31P allele has been identified with high frequency in outbred Maine Coon cat populations throughout the world where homozygous inheritance of the A31P allele is associated with an increased odds ratio for HCM in cats [15–17]. Because inheritance of the A31P allele was associated with reduced cMyBP-C protein in the original report of this mutation [11], we sought to determine whether cMyBP-C protein structure was altered by the A31P mutation, whether the A31P mutation affects localization of cMyBP-C within the sarcomere, and to quantify cMyBP-C expression in a larger population of cats heterozygous and homozygous for the A31P allele. Circular dichroism analysis demonstrated that the mutation disrupted the structure of C0. Consistent with altered structure, recombinant proteins with the A31P mutation showed altered susceptibility to trypsin digestion. Western blots using an antibody that preferentially recognizes N-terminal domains of cMyBP-C including C0 demonstrated diminished epitope recognition for proteins containing the A31P mutation *in* vitro, as well as in cardiomyocytes from cats with the A31P mutation. The total amount of cMyBP-C was found similar in cats with or without the A31P mutation and immunohistochemistry revealed proper incorporation of the mutant A31P protein into sarcomeres. However, the amount of A31P mutant protein was relatively low compared to normal cMyBP-C in the myocardium of cats heterozygous for the A31P mutation, suggesting altered protein synthesis or stability of A31P cMyBP-C. Therefore, our results suggest that the A31P mutation alters cMyBP-C structure, potentially leading to altered protein stability and function, rather than causing disease through haploinsufficiency.

2. Materials and methods

2.1. Recombinant protein expression and purification

Recombinant proteins encoding the C0 domain of feline cMyBP-C or domains C0–C2 (inclusive of C0, the proline–alanine rich region, C1, the M-domain and C2 (Fig. 1A)) were expressed with or without the A31P mutation (C0-WT, C0-A31P, C0C2-WT and C0C2-

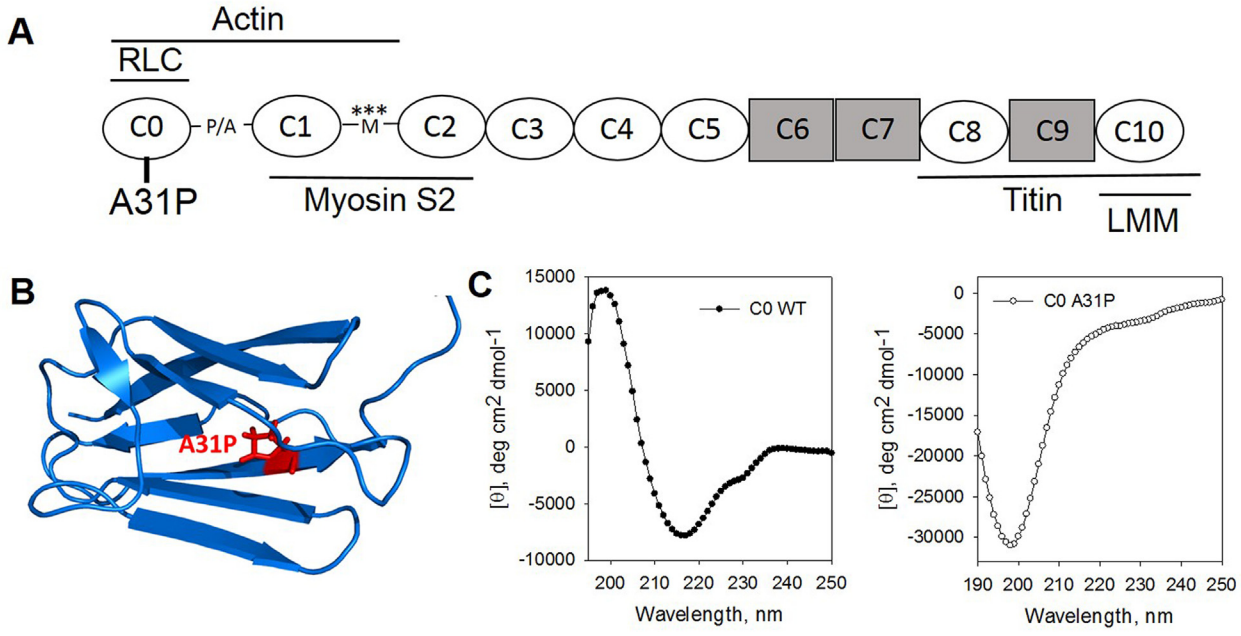

Fig. 1. A. Cartoon depicting the domain structure of cMyBP-C. cMyBP-C consists of 8 immunoglobulin-like domains (ovals) and 3 fibronectin type III-like domains (rectangles) along with an unstructured proline–alanine rich linker sequence (P/A) between C0 and C1 and the regulatory M-domain (M) between C1 and C2 with protein kinase phosphorylation sites indicated by asterisks. The A31P mutation is located in C0, the first immunoglobulin domain unique to the cardiac isoform of MyBP-C. The positions of binding sites for other sarcomeric proteins are indicated by lines above or below the domains. B. Ribbon diagram of C0 domain structure (PDB 2K1M) showing the location of the A31P mutation. C. Circular dichroism spectra (mean residue ellipticity versus wavelength) of C0-WT (*left*) and C0-A31P (*right*) showing that C0-WT is high in β-sheet content while C0-A31P showed a spectrum consistent with disordered proteins.

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