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Stephanne Levin
Lake Forest College

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Stephanne Levin*
Department of Biology
Lake Forest College
Lake Forest, Illinois 60045

Introduction

Hypertrophic cardiomyopathy (HCM) is a disease of the myocardium that is associated with cardiac dysfunction due to asymmetrical thickening of the left ventricular wall of the heart (Richard et al., 2003; Richard et al., 2006). HCM is believed to be the most common genetic form of cardiovascular disease in humans and has been identified as the most common form of cardiac disease in the domestic cat (Marian & Roberts, 2001; Maron, 2002; Kittleson et al., 1999). Mutations in the cardiac myosin binding protein C (MYBPC3) gene are the most frequent genetic cause of HCM in humans (Richard et al., 2003), and MYBPC3 is currently the only gene with identified mutations causing HCM in cats (Meurs et al., 2005; Meurs et al., 2007).

MYBPC3 encodes the cardiac isoform of myosin binding protein-C (cMyBP-C), which is found almost exclusively in heart muscle (Harris et al., 2002). cMyBP-C is believed to play a role in cardiac contraction and structure, but its specific functions are not well understood (Harris et al., 2002). The N-terminus of the protein contains a binding site for myosin, a separate binding site for actin, as well as three potential phosphorylation sites (Gautel et al., 1995; Kulikovskaya et al., 2003). A study by Kulikovskaya et al. (2003) demonstrated that the degree of phosphorylation of cMyBP-C determines whether the N-terminal region binds to myosin or actin, and may thus regulate thick filament structure and cardiac contractility.

In 2005, Sadayappan et al. utilized transgenic mice in which the phosphorylation sites of cMyBP-C were changed to non-phosphorylatable alanines to demonstrate that phosphorylation of cMyBP-C is essential for normal cardiac function. These mice showed severe cardiac hypertrophy consistent with hypertrophic cardiomyopathy (Sadayappan et al., 2005). It is possible that the inability to phosphorylate cMyBP-C contributed to the observed phenotype by altering the protein's ability to interact with other proteins, such as actin and myosin. This, however, was not investigated by Sadayappan et al. and is thus the focus of the proposed study. Determining whether the altered cMyBP-C protein is capable of binding to actin and myosin may provide important insight into the role of cMyBP-C phosphorylation in normal cardiac function and may also lead to a better understanding of the molecular basis for hypertrophic cardiomyopathy.

Specific Aims

1. The first goal of this proposal is to use the techniques developed by Sadayappan et al. (2005) to create transgenic mice in which the three known phosphorylation sites of cMyBP-C, Ser-273, Ser-282, and Ser-302, are changed to alanine. It is hypothesized that this change in the amino acid sequence will prevent phosphorylation of cMyBP-C by Ca^{2+}-regulated kinases, thus resulting in depressed cardiac function due to reduced interactions with actin.
2. The second goal is to determine whether the non-phosphorylatable cMyBP-C is able to modify thick filament structure and cardiac contractility by decreasing binding to actin thin filaments. Reduced or abolished interactions with actin in mice expressing non-phosphorylatable cMyBP-C would provide direct evidence for the importance of phosphorylating cMyBP-C to regulate bonds with actin necessary for maintaining sarcomeric integrity and regulating cardiac contraction.

Experimental Proposal

Previous research has shown that phosphorylation of cMyBP-C increases the likelihood that it will bind to actin thin filaments (Kulikovskaya et al., 2003). However, it is not clear whether phosphorylation and subsequent binding to actin contribute to sarcomeric integrity and cardiac contractility. To test the hypothesis that preventing cMyBP-C phosphorylation will reduce thick filament stability and significantly diminish binding with actin, a line of transgenic mice will be generated which express a version of cMyBP-C that cannot be phosphorylated. The procedure for generating the transgenic mice will be performed as previously described (Sadayappan et al., 2005). RT-PCR using RNA isolated from mouse cardiac cells will be used to obtain cDNA for mouse cMyBP-C, which will then be used to convert Ser-273, Ser-282, and Ser-302 to alanine. Additionally, a myc epitope will be incorporated in each cDNA for later identification of the transgenically encoded proteins using anti-myc monoclonal antibodies. The PCR product will be linked to the promoter for murine α-myosin heavy chain, which will be used to generate multiple lines of transgenic mice. To ensure that no endogenous phosphorylatable cMyBP-C is present, the mice will be bred to MYBPC3 transgenic mice lacking normal cMyBP-C. These knockouts will be produced as described by Yang et al. (1998). Wild-type mice will also be bred to the cMyBP-C null mice, and the offspring of this cross will serve as a control.

SDS-PAGE followed by Western blot analysis will be performed as described by Sadayappan et al. (2005) to verify that the system has indeed worked and the cMyBP-C produced in the transgenic mice is incapable of being phosphorylated. Anti-myc monoclonal antibodies will detect the presence of myc-tagged transgenic cMyBP-C in both the transgenic controls and the mice encoding non-phosphorylatable cMyBP-C. Additionally, anti-cMyBP-C monoclonal antibodies will be used to identify the endogenous protein in wild-type mice, which will serve as a second control. The null mice should show an absence of both the transgenically encoded and the endogenous forms of the protein. Treatment of protein extracts from the hearts of the mice with PKA or phosphatase should show that only cMyBP-C from the transgenic mice expressing the non-phosphorylatable protein remain dephosphorylated in both conditions.

If the results are consistent with those obtained by Sadayappan et al. (2005), depressed cardiac function should be evident in the transgenic mice lacking phosphorylatable cMyBP-C. To ascertain whether this may be a consequence of altered interactions with actin, immunoprecipitation will be utilized to demonstrate if the chronically dephosphorylated

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cMyBP-C binds to actin *in vivo*. This will be accomplished using techniques described by Kulikovskaya et al. (2003). First, cMyBP-C will be immunoprecipitated from cardiac cells from the wild-type and transgenic mice using rabbit polyclonal antibodies raised against the C0-C1 domains of the protein. The protein-antibody complexes will then be run on polyacrylamide gels and probed with antibodies to actin obtained from Santa Cruz Biotechnology, Inc. (Kulikovskaya et al., 2003). As a positive control, filamentous actin will be blotted with the actin antibodies. Additionally, the cMyBP-C from wild-type mouse hearts will serve as another positive control since at least some of the endogenous protein should be bound to actin. Samples from the cMyBP-C null mice will act as negative controls due to the fact that the cells from these mice are deficient of normal cMyBP-C and therefore no protein-actin bonds should be possible.

If the hypothesis is supported, then the lane on the blot with the non-phosphorylatable cMyBP-C should not show any band for actin, suggesting that the protein does not bind to actin thin filaments. The hypothesis may also be supported if a faint band for actin appears, suggesting that there has been a reduction in the number of interactions with actin. Both of these scenarios would support the proposition that actin can only bind to the phosphorylated form of cMyBP-C, and that this interaction is essential for the protein's function. They would also provide evidence that cMyBP-C's inability to bind to actin may have contributed to the cardiac hypertrophy and overall cardiac dysfunction observed in these mice. However, if cMyBP-C from the transgenic mice results in a band comparable to that from the wild-type mice, it would imply that the perpetually dephosphorylated cMyBP-C binds to actin as often as the normal form of the protein. Therefore, if this is the case, the absence of these interactions could not be implicated as a cause of the structural and functional abnormalities seen within the hearts of the transgenic mice.

It is important to note that, since a negative result would support the given hypothesis, it may be necessary to conduct further investigations to confirm that the results are not a consequence of other factors besides the inability to phosphorylate cMyBP-C.

Conclusion

The proposed experiment will provide valuable information about the function of cardiac myosin binding protein C and how its function is altered due to phosphorylation and subsequent binding to actin. Furthermore, it may provide insight into the molecular basis for hypertrophic cardiomyopathy, which may allow for the identification of prospective treatments of the disease.

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