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Phosphorylation of myosin regulatory light chain controls myosin head conformation in cardiac muscle

Thomas Kampourakis *, Malcolm Irving

Randall Division of Cell and Molecular Biophysics and British Heart Foundation Centre of Research Excellence, King's College London, London SE1 1UL, United Kingdom

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ABSTRACT

The effect of phosphorylation on the conformation of the regulatory light chain (cRLC) region of myosin in ventricular trabeculae from rat heart was determined by polarized fluorescence from thiophosphorylated cRLCs labelled with bifunctional sulforhodamine (BSR). Less than 5% of cRLCs were endogenously phosphorylated in this preparation, and similarly low values of basal cRLC phosphorylation were measured in fresh intact ventricle from both rat and mouse hearts. BSR-labelled cRLCs were thiophosphorylated by a recombinant fragment of human cardiac myosin light chain kinase, which was shown to phosphorylate cRLCs specifically at serine 15 in a calcium- and calmodulin-dependent manner, both *in vitro* and *in situ*. The BSR-cRLCs were exchanged into demembranated trabeculae, and polarized fluorescence intensities measured for each BSR-cRLC in relaxation, active isometric contraction and rigor were combined with RLC crystal structures to calculate the orientation distribution of the C-lobe of the cRLC in each state. Only two of the four C-lobe orientation populations seen during relaxation and active isometric contraction in the unphosphorylated state were present after cRLC phosphorylation. Thus cRLC phosphorylation alters the equilibrium between defined conformations of the cRLC regions of the myosin heads, rather than simply disordering the heads as assumed previously. cRLC phosphorylation also changes the orientation of the cRLC C-lobe in rigor conditions, showing that the orientation of this part of the myosin head is determined by its interaction with the thick filament even when the head is strongly bound to actin. These results suggest that cRLC phosphorylation controls the contractility of the heart by modulating the interaction of the cRLC region of the myosin heads with the thick filament backbone.

1. Introduction

Contraction of both heart and skeletal muscle is driven by cyclic interactions between myosin and actin [1,2]. In each cycle, the light-chain domain (LCD) of the myosin head amplifies small conformational changes induced by the release of ATP hydrolysis products from its catalytic domain to produce large rotational motions of the LCD. One function of the LCD is thus to act as a 'lever arm' to generate nm-scale sliding between the myosin-containing thick filaments and the actin-containing thin filaments [3,4]. The part of the LCD next to the myosin head-rod junction is formed by the cardiac regulatory light chain (cRLC) winding around a short α-helix of the myosin heavy chain [5] (Fig. 1). The cRLC is thought to be partly phosphorylated *in vivo* under resting conditions, and some studies suggest that its phosphorylation level depends on the physiological state of the myocardium, implying a role for cRLC phosphorylation in the regulation of cardiac contractility [6–9]. Reported values of basal cRLC phosphorylation differ substantially however [10–12], and their interpretation is complicated by heterogeneity between different parts of the myocardium [7,13,14]. At a functional level, a significant role for cRLC phosphorylation is indicated by the effects of hypertrophic cardiomyopathy (HCM) mutations that abolish cRLC phosphorylation *in vitro* [15], and the severe cardiac dysfunction in transgenic mouse models expressing non-phosphorylatable cRLCs [12,16]. cRLC is phosphorylated by a cardiac-specific isoform of myosin light chain kinase (cMLCK) [17,18], and ablation of cMLCK expression is associated with a decrease in cRLC phosphorylation level in transgenic mice under basal conditions, leading to an impaired beta-adrenergic response and ventricular hypertrophy [19]. cMLCK activity has also been associated with control of sarcomere organisation in isolated mammalian cardiomyocytes [20] and with cardiac development in a zebrafish model [17]. However, the molecular mechanisms of these effects remain elusive.

The effects of cRLC phosphorylation on the mechanical properties of the myocardium have been extensively studied. Increased cRLC phosphorylation has been associated with an increase in calcium sensitivity [21], and in the rate of stretch activation [22] and cross-bridge cycling [23] in demembranated cardiac muscle preparations. The changes in

Abbreviations: cRLC, cardiac regulatory light chain; BSR, bifunctional sulforhodamine; cMyBP-C, cardiac myosin binding protein C; cMLCK, cardiac myosin light chain kinase; HMM, heavy meromyosin; ELC, essential light chain; LCD, light chain domain of myosin; ME, maximum entropy.

* Corresponding author.
E-mail address: thomas.kampourakis@kcl.ac.uk (T. Kampourakis).

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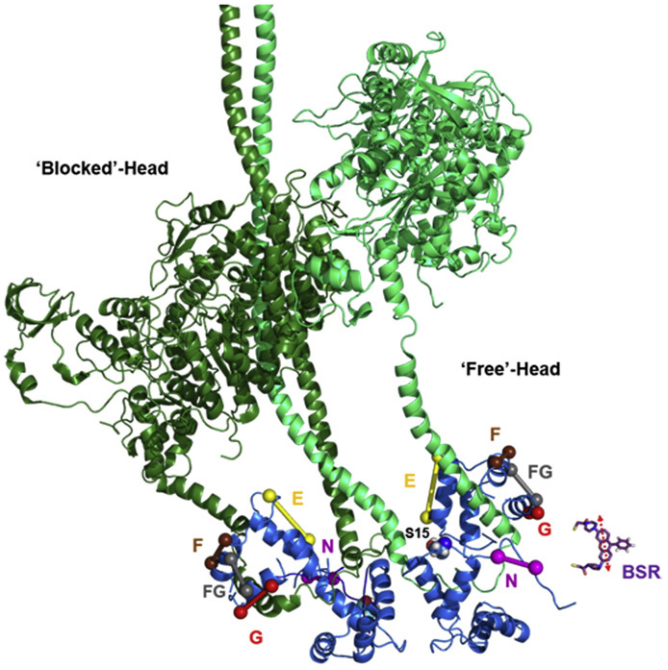

Fig. 1. Bifunctional rhodamine probes on the cRLC. The regulatory light chains (blue) are shown bound to the myosin heavy chain (green) in the atomic model for those regions of myosin in isolated thick filaments from invertebrate muscle (PDB 3DTP). The so-called 'blocked' and 'free' heads are shown in dark and light green, respectively. The essential light chains were removed for clarity. The model was built by superimposing the RLC structure of the nucleotide-free scallop myosin S1 (PDB 1SR6) onto the RLC of the free head, and by superimposing the RLC N- and C-lobes separately for the blocked head. The N-terminal extension of the RLC was built in Pymol using the sequence of human ventricular RLC. The phosphorylatable serine 15 is shown in Van der Waals representation. BSR probes were introduced on the RLC N-terminus (magenta), and helices E (yellow), F (brown), and G (red) and crosslinking helices F and G (grey) in the C-terminal lobe. The Cβ-atoms (or Cα-atoms in case of glycine residues) of mutated residues are shown as coloured spheres and the expected probe dipole orientations are indicated by sticks. The atomic structure of BSR (purple) is shown to scale in the right lower corner with the orientation of the fluorescence dipole indicated by a red double arrow.

the equatorial X-ray reflections from cardiac trabeculae produced by *in situ* cRLC phosphorylation indicate a transfer of myosin heads towards the thin filaments [23], suggesting that these mechanical effects may be mediated by an increased probability of actin attachment (for review see [11]). Electron microscopy studies of isolated thick filaments from mammalian and invertebrate skeletal muscles [24,25] showed that the helically ordered organisation of myosin heads on the thick filament surface in the unphosphorylated state is lost on incubation with active MLCK. However the molecular mechanism by which cRLC phosphorylation controls the conformation of the myosin heads is unknown.

We recently described a new method to determine the orientation of the cRLC region of the myosin heads with respect to the filament axis in demembranated ventricular trabeculae, based on measuring polarized fluorescence intensities from bifunctional sulforhodamine (BSR) probes on the cRLC [26]. We showed that, in the unphosphorylated state, cRLC orientation did not change significantly during calcium activation, and that its N-lobe did not change orientation even during strong attachment of myosin heads to actin in rigor. These results suggested that the cRLC interacts with the thick filament backbone *via* its N-lobe. Since the N-lobe contains the phosphorylation site, modulation of this interaction by cRLC phosphorylation might provide a mechanism for the control of myosin head conformation. Here we tested that hypothesis using a recombinant C-terminal fragment of the human cardiac isoform of MLCK to thiophosphorylate BSR-labelled human cRLCs *in vitro*. The BSR-cRLCs were exchanged into demembranated right ventricular trabeculae with a very low endogenous cRLC phosphorylation background, and the orientation of the phosphorylated cRLC C-lobe was determined during relaxation, active isometric contraction and rigor. An important advantage of this *in vitro* labelling/cRLC exchange approach is that the orientation of the phosphorylated cRLCs can be determined directly in the presence of a mixed phosphorylated/unphosphorylated population, since only the phosphorylated cRLCs carry the probe. Comparison with our previous results for unphosphorylated BSR-cRLCs using the same cRLC exchange protocol [26] then allowed us to determine the change in the *in situ* orientation of the C-lobe produced by cRLC phosphorylation in each state.

2. Materials and methods

Details of protein production, preparation of cardiac trabeculae, protein exchange protocols, fluorescence polarization experiments and tissue sampling procedures are provided in the supplemental materials.

3. Results

3.1. Preparative thiophosphorylation of BSR-cRLCs by cMLCK

A fragment of the human cardiac isoform of myosin light chain kinase (cMLCK, UniProtKB entry: Q32MK0) spanning the catalytic and regulatory domains was expressed in and purified from Sf9 cells to over 95% homogeneity as described in the Materials and methods section. The purified cMLCK fragment was partially phosphorylated (34% unphosphorylated, 55% mono- and 11% bis-phosphorylated as determined by Phostag™-SDS-PAGE; data not shown) and the phosphate groups were removed by Lambda Protein phosphatase treatment. There was no significant difference between the catalytic activities of the untreated and dephosphorylated cMLCK fragments.

Previous studies have led to contradictory conclusions about the calcium- and calmodulin-dependence of cMLCK activity [17,18]. In the present work, *in vitro* kinase assays at different enzyme-to-substrate ratios in the presence and absence of Ca^{2+}, calmodulin and EGTA revealed a strong dependence of cMLCK activity on Ca^{2+} and calmodulin (Fig. 2A), as expected from the canonical C-terminal calmodulin binding site of cMLCK. However, some calcium-independent catalytic activity was observed at high enzyme-to-substrate ratios.

Multiple phospho-species of cRLC have been identified in rodent cardiac muscle [27], and human cRLC contains several residues that could serve as potential substrates for protein kinases. Two serines in the N-terminal extension (S15 and S19) of cRLC and a tyrosine (Y118) in its C-lobe have been identified by mass spectrometry and site-specific methods as potentially phosphorylated *in vivo* (PhosphoSitePlus®, www.phosphosite.org) [28]. To unambiguously identify which residue is phosphorylated by the purified cMLCK (and to exclude the possible contribution of any co-purified kinases) we mutated the two serine residues (S15 and S19) in the N-terminal extension separately or together to alanines. The human wildtype and mutant cRLCs were tested in *in vitro* kinase assays (Fig. 2). Only the wildtype and S19A mutant could be phosphorylated, indicating that the purified cMLCK specifically phosphorylates serine 15 in the human cRLC N-terminal extension (Fig. 2A). Specific mono-phosphorylation of the recombinant wildtype cRLC was confirmed by ESI mass spectrometry. The measured (calculated) masses (in Da) for the cRLC before and after phosphorylation by cMLCK were 19020.6 (19020.5) and 19101.6 (19100.5), respectively. Additionally, although rat cRLC has an additional phosphorylatable serine at position 14 that is replaced by asparagine in the human cRLC sequence, we found that the recombinant cMLCK also mono-phosphorylates rat cRLC *in vitro* (Fig. S1), indicating that cMLCK has a high specificity for serine 15.

ATPγS was used to preparatively thiophosphorylate BSR-labelled cRLCs, on the basis that thiophosphorylated proteins are expected to be relatively resistant to dephosphorylation by any protein phosphatases that might be present in freshly skinned trabeculae. In this study,