A preferred AMPK phosphorylation site adjacent to the inhibitory loop of cardiac and skeletal troponin I

Raquel Sancho Solis,¹ Ying Ge,^{1,2*} and Jeffery W. Walker³

¹Department of Physiology, School of Medicine and Public Health, University of Wisconsin-Madison, WI 53706

²Human Proteomics Program, School of Medicine and Public Health, University of Wisconsin-Madison, WI 53706

³Department of Physiology, University of Arizona, Tucson, Arizona 85724

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Abstract: 5'-AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that is activated when cellular AMP to ATP ratios rise, potentially serving as a key regulator of cellular energetics. Among the known targets of AMPK are catabolic and anabolic enzymes, but little is known about the ability of this kinase to phosphorylate myofilament proteins and thereby regulating the contractile apparatus of striated muscles. Here, we demonstrate that troponin I isoforms of cardiac (cTnI) and fast skeletal (fsTnI) muscles are readily phosphorylated by AMPK. For cTnI, two highly conserved serine residues were identified as AMPK sites using a combination of high-resolution top-down electron capture dissociation mass spectrometry, ³²P-incorporation, synthetic peptides, phospho-specific antibodies, and site-directed mutagenesis. These AMPK sites in cTnI were Ser149 adjacent to the inhibitory loop and Ser22 in the cardiac-specific N-terminal extension, at the level of cTnI peptides, the intact cTnI subunit, whole cardiac troponin complexes and skinned cardiomyocytes. Phosphorylation time-course experiments revealed that Ser149 was the preferred site, because it was phosphorylated 12–16-fold faster than Ser22 in cTnI. Ser117 in fsTnI, analogous to Ser149 in cTnI, was phosphorylated with similar kinetics as cTnI Ser149. Hence, the master energy-sensing protein AMPK emerges as a possibly important regulator of cardiac and skeletal contractility *via* phosphorylation of a preferred site adjacent to the inhibitory loop of the thin filament protein TnI.

Keywords: phosphorylation; troponin I; AMPK; top-down high-resolution mass spectrometry; electron capture dissociation

Introduction

5'-AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that is thought to act as a

cellular energy sensor. AMPK is activated in response to energetic stressors that inhibit ATP production or consume ATP, such as ischemia, hypoxia,

Abbreviations: AMPK, 5'-AMP-activated protein kinase; cTn, cardiac troponin; cTnI, cardiac troponin I; cTnC, cardiac troponin C; cTnT, cardiac troponin T; ECD, electron capture dissociation; fsTn, fast skeletal troponin; fsTnI, fast skeletal troponin I; MO25, mouse protein 25; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PAK, p21-activated kinase; PKA, protein kinase A; PKC, protein kinase C; PKD, protein kinase D; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of the mean; STRAD, STE20 related adapter protein; Tn, troponin.

Additional Supporting Information may be found in the online version of this article.

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*Correspondence to: Ying Ge, 1300 University Ave., SMI 130, Madison, WI 53706. E-mail: yge@physiology.wisc.edu

and exercise.¹ Once activated, AMPK stimulates ATP-producing pathways and inhibits ATP-consuming pathways, resulting in an effort to maintain high cellular ATP levels. In skeletal and cardiac muscle, AMPK has been shown to stimulate glucose uptake, glycolysis, fatty acid oxidation, and mitochondrial biogenesis and to inhibit biosynthesis of lipids, glycogen, cholesterol, and proteins^{2–6} by phosphorylation of key metabolic enzymes and activation of transcription factors.⁷ Newer roles for AMPK now include regulation of cell growth and proliferation, development of cell polarity, and global regulation of food intake.^{7,8} Despite extensive research on the physiological role of AMPK in the metabolism of heart and skeletal muscle,^{3–6,9,10} the ability of AMPK to phosphorylate myofilament proteins and, thereby, regulate the contractile apparatus in striated muscle remains unclear.

A number of myofilament proteins associated with the contractile apparatus are subject to regulation by direct phosphorylation including myosin regulatory light chain,^{11,12} myosin binding protein C¹³ and the troponin-tropomyosin complex.¹⁴ Among these, the inhibitory subunit of the cardiac troponin (Tn) heterotrimer (cTnI) has emerged as a key regulatory phosphoprotein in the heart.¹⁵ Under the influence of β -adrenergic stimulation, cTnI becomes phosphorylated at Ser22 and Ser23 (also known as Ser23 and Ser24 including initial methionine) by cyclic AMP-activated protein kinase (PKA).^{14,16} Phosphorylation of these “PKA sites” in cTnI regulates the Ca²⁺ sensitivity of force development and MgATPase activity and may modulate the rate of cross-bridge cycling.¹⁵ cTnI and other myofilament proteins are also targeted by additional protein kinases involved in signaling such as protein kinase C (PKC),^{17–22} protein kinase D (PKD),^{23,24} and p21-activated kinase (PAK).²⁵ For example, PKC is able to phosphorylate cTnI at Ser41, Ser43, and Thr142 (human cTnI sequence excluding the initial methionine), although it can also cross-phosphorylate the PKA sites, albeit slowly.^{18,19} Phosphorylation of each of these sites on cTnI has been associated with functional changes,¹⁵ although the precise assignment of a specific altered function to the phosphorylation of a specific site has proven difficult. cTnI, through its ability to modulate myofilament activation properties, may play an important role in regulating energy consumption by the contractile apparatus.²⁶ Multiple studies suggested that AMPK is the most important regulator of energy consumption in the heart.^{3,5,6} Hence, we reasoned that AMPK, a critical regulator of cardiac energetics, may be able to regulate energy consumption by the contractile apparatus through altering the phosphorylation status of cTnI.

In this study, we have used a comprehensive approach with a combination of high-resolution top-down mass spectrometry (MS), ³²P-incorporation,

synthetic peptides, phospho-specific antibodies, and site-directed mutagenesis to investigate the phosphorylation of TnI by AMPK. The top-down MS strategy allows analysis of intact proteins to obtain a comprehensive assessment and localization of post-translational modifications (PTMs) and point mutations with full sequence coverage and without a priori knowledge.^{27–37} It starts with measuring molecular weights of intact proteins followed by fragmentation of the protein in the mass spectrometer with tandem mass spectrometry (MS/MS) such as electron capture dissociation (ECD)³⁸ to obtain sequence information. ECD has been demonstrated to be especially valuable for mapping phosphorylation sites, because it preserves the labile PTMs during MS/MS process.^{32–34,39,40}

To obtain a complete view of the phosphorylation state of whole cardiac troponin (cTn) complexes that have been treated with AMPK, purified whole Tn complexes from a single rat heart were analyzed by high-resolution top-down MS.³² This approach, combined with the use of synthetic peptides, site-directed mutagenesis, and phosphospecific antibodies, and identified Ser149 and Ser22 as the only sites targeted by AMPK, with Ser149 as the preferred site. AMPK was found to phosphorylate cTnI at the level of cTnI peptides, the intact cTnI subunit, whole cTn complexes and skinned cardiomyocytes, indicating that cTnI is a substrate for AMPK even when assembled with other contractile proteins in highly ordered myofilaments. Similar analyses of fast skeletal TnI (fsTnI) revealed that it is also a good substrate for AMPK at a site homologous to Ser149 of cTnI. Hence, a conserved serine residue adjacent to the inhibitory loop of TnI emerges as a preferred target of AMPK in both cardiac and fast skeletal muscle myofilaments. Through the phosphorylation of TnI, AMPK may function as a novel regulator of myofilament contractile properties.

Results

Incorporation of ³²P into purified human cTn subunits by AMPK

The first indication that AMPK might recognize and phosphorylate cTn subunits was inferred from upon ³²P-incorporation into subunits obtained commercially. Purified human cTnI and cTnT were treated with active AMPK^Δ in the presence of γ -³²P-radiolabeled ATP. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), stained with Coomassie blue, and subjected to autoradiography. Stoichiometry and quantification were determined by scintillation counting of excised bands, normalization of cpm to ³²P- γ -ATP specific activity, densitometry of cTn bands and amino acid analysis to determine cTnI content in

Figure 1. Phosphorylation of purified human cTn subunits. (A) Effects of active AMPK α on 32 P-incorporation into purified human cTnI and cTnT. (B) Quantification of 32 P-incorporation from gel and autoradiogram shown in (A).

moles. cTnI was readily phosphorylated by AMPK, with an incorporation of 1.0 μ mol phosphate per μ mol of cTnI. In comparison, cTnT was a poor substrate showing incorporation of 0.08 μ mol phosphate per μ mol cTnT under the same conditions (Fig. 1). Incubation of cTn subunits with only the AMPK upstream kinase LKB1/STRAD/MO25 and no AMPK α resulted in no 32 P-incorporation (data not shown).

Identification of cTnI sites phosphorylated by AMPK in synthetic peptides.

To identify possible phosphorylation sites, candidate residues in cTnI were selected based on similarities in sequence with the described recognition motif of AMPK ($\Phi(X,\beta)XXXS/TXXX\Phi$, with Φ and β representing hydrophobic and basic residues, respectively).⁴¹ Peptides containing the selected residues were synthesized and treated with active AMPK α . Phosphorylated peptides were then purified by an ion exchange HPLC column and identified by MALDI-TOF MS. Peptides containing residues Ser22Ser23 and Thr142/Ser149 were monophosphorylated, while peptides containing Ser41/Ser43, Thr30 and Ser76Thr77 were not phosphorylated even after 8 h incubations with AMPK (data not shown). High resolution top-down ECD MS of the monophosphorylated peptides revealed residues Ser22 and Ser149 as the sites targeted by AMPK (Fig. 2). For the monophosphorylated peptides containing Ser22Ser23 [Fig. 2(A)], the extensive fragmentation pattern including the key cleavage between Ser22 and Ser23 (pc_7 , and z_8^+) revealed that Ser22 was the phosphorylated residue since the fragment ion containing Ser22, but not Ser23, (*i.e.*, pc_7 , which includes the first seven amino acid resi-

dues from the N-terminal) was detected phosphorylated, whereas the fragment ion containing Ser23, but not Ser22, was not phosphorylated (*i.e.*, z_8^+ , which includes the first eight amino acid residues from the C-terminal). Likewise, the fragmentation pattern observed for the monophosphorylated peptides containing Thr142/Ser149 clearly located the phosphorylation site at residue Ser149, as all the fragment ions that contained Thr142 but not Ser149 were detected unphosphorylated (*i.e.*, c_8-c_{12}), whereas fragment ions containing Ser149 but not Thr142 were detected only in their phosphorylated forms (*i.e.*, z_{10}^+ , z_{11}^+ , z_{13}^+ , z_{14}^+). Therefore, phosphorylation was quite selective for Ser22 and Ser149, as no phosphorylation of other potential sites in the same peptides, that is, Ser23 or Thr142, was observed. In addition, residues surrounding both Ser22 and Ser149 matched fairly well the described AMPK recognition motif.

Incorporation of 32 P into recombinant cTnI by AMPK

To confirm potential phosphorylation sites in intact cTnI, candidate residues Ser22Ser23 and Ser149

Figure 2. Localization of phosphorylation sites in synthetic phosphopeptides by high-resolution MS/MS. Peptides were phosphorylated by active AMPK α . Location of the phosphorylation site is indicated in the peptide sequence as a "p" before the phosphorylated residue. ECD spectra of monophosphorylated peptides of cTnI (rodent sequence) containing residues Ser22Ser23 (A) and Thr142/Ser149 (B), respectively. Insets, expanded isotopically resolved precursor ions.

Figure 3. Phosphorylation of mouse recombinant WT and mutant cTnI (Ala₂ and Ala₂ S149A) by AMPK. (A) Diagram depicting mutations in GST-cTnI (right) and a representative gel of purified GST-cTnI constructs (left). (B) A representative gel and autoradiogram showing ³²P-incorporation into WT and mutant GST-cTnI treated with active AMPK^Δ.

were mutated to nonphosphorylatable alanines in recombinant mouse cTnI to evaluate their contribution to phosphorylation by AMPK. Recombinant mouse cTnI wild-type (WT) and mutants were expressed and purified from *E. coli* as GST-fusion proteins [Fig. 3(A)]. An Ala₂ mutant was created by mutating residues Ser22Ser23 (*i.e.*, the PKA sites) to alanine. An Ala₂ S149A mutant had an additional mutation of Ser149 (*i.e.*, the PAK site) to alanine. Single amino acid mutants (S22A, S23A) were not created because compensatory phosphorylation can occur at the unmutated adjacent residue due to similarities in the amino acid sequence when the preferred site of a kinase is mutated and unavailable for phosphorylation.⁴²

Recombinant mouse cTnI was treated with active AMPK^Δ in the presence of γ-³²P-radiolabeled ATP and then subjected to SDS-PAGE, autoradiography [Fig. 3(B)], and quantification of ³²P-incorporation. Compared to cTnI WT, ³²P-incorporation into cTnI Ala₂ decreased by 42 ± 3% ($P < 0.05$, $n = 8$), confirming the involvement of the PKA sites in the phosphorylation of cTnI by AMPK. Further mutation of Ser149 to alanine resulted in a further 16 ± 5% decrease ($P < 0.05$ compared to WT and Ala₂, $n = 8$) in ³²P-incorporation, indicating that this site was also targeted by AMPK. However, ~42% of the phosphorylation seen in WT was still present in the Ala₂ S149A mutant, suggesting the possible existence of unidentified sites, compensatory phosphorylation or that the phosphorylation of the PKA sites is permissive to AMPK phosphorylation at other sites. There was no ³²P-incorporation into the GST protein tag itself (data not shown). Top-down MS analysis of recombinant mouse cTnI WT and mutants treated with AMPK was attempted in an effort to characterize the phosphorylation pattern and to identify possible additional sites. However, top-down MS analysis of recombinant cTnI was unsuccessful since

recombinant GST-cTnI protein precipitated during the desalting procedures.

Top-down MS analysis of rat cTn complexes treated with AMPK

Purified rat cTn complexes were treated with active AMPK^Δ. As a control, cTn complexes from the same hearts were treated with inactive AMPK^Δ. High-resolution MS analysis of treated cTn complexes clearly illustrates the effect of AMPK on the distribution of cTnI phosphospecies [Fig. 4(A)]. In samples treated with inactive AMPK, cTnI was observed as unphosphorylated (0P), monophosphorylated (1P), and bisphosphorylated (2P) forms, with trace amounts of trisphosphorylated cTnI (3P). After treatment with active AMPK, only trace amounts of unphosphorylated cTnI (0P) were detected, whereas most cTnI was in the bisphosphorylated state (2P), with mono-(1P) and trisphosphorylated (3P) species also present. Neither tetrakisphosphorylated cTnI (4P) nor AMPK-mediated phosphorylation of cTnT was observed in these MS experiments (data not shown, abundance for each species was estimated to be <1% of the total cTnI and cTnT protein population, respectively).

To localize the phosphorylated residue(s), a single charge state of monophosphorylated cTnI (1P) or bisphosphorylated cTnI (2P) was isolated in the mass spectrometer and subjected to ECD fragmentation [Fig. 4(B)]. For samples treated with inactive AMPK, fragmentation of monophosphorylated (1P) cTnI resulted in complete sequence coverage and localization of the phosphate to the PKA sites (Ser22Ser23). c_{20} and c_{21} ions were only detected in their unphosphorylated forms, indicating that the first 21 amino acids of cTnI were unphosphorylated. c_{23} ions and all c ions beyond c_{23} were always detected as monophosphorylated, identifying Ser22Ser23 as the site of phosphorylation. Because the fragmentation ion between Ser22 and Ser23 (c_{22}) was not consistently observed, determining the precise location of phosphorylation (either Ser22 or Ser23) was not possible. However, we previously found that Ser22 is the predominant site that is basally phosphorylated in monophosphorylated cTnI from rat hearts,³² consistent with a previous MS/MS analysis of human cTnI.³⁹ Fragmentation of bisphosphorylated cTnI (2P) from samples treated with inactive AMPK localized the phosphorylation to Ser22 and Ser23 (the PKA sites), as c_{20} and c_{21} ions were only detected as unphosphorylated, c_{22} was detected as monophosphorylated, and c_{23} and all c ions beyond c_{23} were detected as bisphosphorylated. Importantly, for both mono- and bisphosphorylated cTnI forms treated with inactive AMPK, all $z^{\bullet}$ ions were unphosphorylated, ruling out the potential basal phosphorylation of Ser149 [Fig. 4(B) and Supporting Information Fig. 3]. In addition, the

Figure 4. Phosphorylation of cTnI Ser149 revealed by top-down MS of cTnI from immunoaffinity-purified cTn complexes treated with active or inactive AMPK^Δ. (A) (Left) Representative ESI/FTMS spectrum of cTnI protein ions in un-(0P), mono-(1P), bis-(2P) and tris-phosphorylated (3P) states. Top, cTnI treated with inactive AMPK^Δ; bottom, cTnI from the same heart but treated with active AMPK^Δ. (Right) Distribution of cTnI phosphospecies is summarized in the graph ($n = 20$ charge states analyzed from three separate experiments). +Na, sodium adduct (+ 22 Da). (B) Cleavage assignment of ECD data mapped onto the cTnI sequence (Swiss Prot primary accession number P23693) with the initial methionine removed and acetylation of the new N-terminus. Phosphorylated ions are labeled “p” before the phosphorylated residue. Shown are fragmentation of cTnI 1P and cTnI 2P from samples treated with either inactive or active AMPK^Δ. Data were summarized from experiments on five separate hearts. Ser150 in rat cTnI sequence is equivalent to Ser149 in human cTnI and referred as Ser149 in the text. Similarly, Ser42, Ser44, and Thr143 in rat cTnI sequence correspond to Ser41, Ser43, and Thr142 in human cTnI, respectively. Please note we used the amino acid numbering of human cTnI sequence in the text. Because of a single amino acid polymorphism,³² an alanine or a serine (A/S) can be expressed at position 7.

fragmentation pattern also demonstrated that other known cTnI target sites, such as Ser41, Ser43, and Thr142, were not phosphorylated in the basal state. In contrast, a distinct phosphorylation pattern was observed for cTnI that had been treated with active AMPK [Fig. 4(B) and Supporting Information Fig. 3]. For monophosphorylated (1P) cTnI, the phosphate was unambiguously localized to Ser149, as all z^* ions before z_{61}^* were unphosphorylated, all ions after z_{61}^* were monophosphorylated, and all c ions detected (c_{19} – c_{74}) were not phosphorylated. For

bisphosphorylated cTnI, one phosphate was localized to Ser149, as all z^* ions before z_{61}^* were unphosphorylated, and all ions after z_{61}^* were monophosphorylated. The other phosphate in bisphosphorylated cTnI was placed at the PKA sites (Ser22Ser23), as c_{20} ions were only detected as unphosphorylated, and c_{24} ions and all ions beyond c_{24} were only detected as monophosphorylated. Again, the observed fragmentation pattern ruled out phosphorylation of sites such as Ser41, Ser43, and Thr142 in cTnI treated with active AMPK.

Figure 5. AMPK phosphorylation of cTnI at Ser149 and Ser22Ser23 assessed by Western blotting analysis using phospho-specific antibodies. Mouse recombinant GST-cTnI constructs (A) and purified rat cTn complexes (B) treated with either active AMPK^A (+), inactive AMPK^A (-), active PKA (+) or inactive PKA (-). Blots are representative from three separate experiments with similar results.

Although the location of phosphates in trisphosphorylated cTnI from samples treated with active AMPK was not performed, the phosphorylation sites are likely to be Ser149 and the two PKA sites. This is because no tetrakisphosphorylated cTnI was observed (estimated to be <1% of the total cTnI population), greatly decreasing the probability of additional sites being phosphorylated by AMPK.

Western analysis

Phosphorylation of cTnI Ser22Ser23 and Ser149 by AMPK was further confirmed using cTnI phospho-specific antibodies. Recombinant cTnI WT, Ala₂ and Ala₂ S149A were treated with active AMPK^A for 1 h [Fig. 5(A)]. As expected, a signal from the cTnI phosphoserine-149 antibody was observed in cTnI WT and Ala₂, but not in the Ala₂ S149A mutant. Immunoreactivity of the cTnI phosphoserine-22/23 antibody was detected in cTnI WT but neither in Ala₂ nor Ala₂ S149A mutants. These results indicate that AMPK is capable of phosphorylating Ser149 and the PKA sites in cTnI as an intact protein.

It is worth noting that the cTnI phosphoserine-22/23 antibody was developed against a peptide phosphorylated at both Ser22 and Ser23 and hence,

it is expected to recognize cTnI only when phosphorylated at both PKA sites. However, through the correlation of the antibody signal to ³²P-incorporation into cTnI, Messer *et al.*⁴³ demonstrated that the antibody does not distinguish between cTnI that is mono- or bis-phosphorylated at the PKA sites. Therefore, the signal from the phosphoserine-22/23 antibody observed in cTnI WT treated with AMPK can arise from mono- or bis-phosphorylated PKA sites.

Next, the possibility that AMPK could phosphorylate cTnI while assembled in the Tn complex was examined. Native cTn complexes purified from adult rat hearts were treated with AMPK^A (active or inactive) or the catalytic subunit of PKA (active or inactive) for 15 min [Fig. 5(B)] (longer incubation time led to nonspecific phosphorylation of cTnI by PKA). A signal from the cTnI phosphoserine-149 antibody is readily seen in cTnI from Tn complexes that were treated with active AMPK but not in cTnI from complexes treated with inactive AMPK, indicating that cTnI Ser149 was not basally phosphorylated in rat hearts but was indeed phosphorylated by AMPK. The cTnI phosphoserine-22/23 antibody revealed that purified rat cTnI was basally phosphorylated at the PKA sites. These results are in agreement with the top-down MS analysis of cTn complexes treated with inactive and active AMPK. Treatment of cTn complexes with active PKA resulted in increased levels of phosphorylation at those sites. Unexpectedly, no discernible increase in phosphorylation of the PKA sites was observed when the cTn complexes were treated with AMPK. We speculated that this finding might be due to the short incubation time of 15 minutes. In any case, this result suggested that AMPK may prefer Ser149 over the PKA sites.

Time course phosphorylation of cTnI by AMPK: Ser149 is the preferred site

To determine whether Ser149 is preferred over the Ser22Ser23 sites, phosphorylation time-course experiments were carried out. Recombinant mouse cTnI WT and purified rat cTn complexes were treated with active AMPK^A. Phosphorylation was assessed with phosphospecific antibodies at various times following treatment with AMPK. Antibody signals that were within the linear range of the film were quantified by densitometry fluorescence, plotted and then fitted to first-order exponentials to determine the kinase reaction half-time ($t_{1/2}$), an indicator of how well a site is phosphorylated by a kinase. cTnI Ser149 was the preferred site with a $t_{1/2}$ of 6.5 ± 0.6 min, compared to a $t_{1/2}$ of 79.9 ± 11.3 min for Ser22Ser23 in recombinant mouse cTnI WT, indicating that Ser149 is phosphorylated ~12 times faster than Ser22Ser23 [Fig. 6(A,B)]. Similar results were obtained when determining $t_{1/2}$ for Ser149 and Ser22Ser23 in purified rat cTn complexes: cTnI

Figure 6. Time courses of cTnI phosphorylation at Ser149 and Ser22Ser23 in recombinant mouse GST-cTnI WT and rat cTnI complexes treated with active AMPK^Δ. Phosphorylation time-course experiments for Ser149 (A) and Ser22Ser23 (B) in GST-cTnI, and for Ser149 (C) and Ser22Ser23 (D) in rat cTnI, were determined by Western blotting analysis using phospho-specific antibodies. Top panel shows representative blots and bottom graph shows the exponential fitting to the data from three separate experiments.

Ser149 was phosphorylated ~16 times faster than Ser22Ser23 ($t_{1/2}$ of 7.3 ± 1.8 min versus 121 ± 38.5 min, respectively) [Fig. 6(C,D)].

Phosphorylation of cTnI in skinned rodent myocytes

To determine whether cTnI was also a target of AMPK in a physiological setting, mouse skinned cardiac myocytes were treated with AMPK^Δ in the presence of γ -³²P-radiolabeled ATP. Myofilament proteins were separated by SDS-PAGE, stained with Commassie blue and autoradiography. ³²P-incorporation into cTnI was readily observed [Fig. 7(A)] and some phosphorylation of cTnT was also detected under the conditions used. To determine if cTnI Ser149 was phosphorylated in skinned myocyte preparations, skinned cardiac myocytes from adult rat hearts were treated

with active AMPK^Δ and phosphorylation assessed with a phosphospecific antibody. As can be seen in Figure 7(B), AMPK phosphorylated cTnI at Ser149. Myofilament structure, arrangement, and function are thought to remain intact in skinned myocytes, as observed in studies in which these preparations were used to assess force development and relaxation.^{18,21,44} Therefore, phosphorylation of cTnI under the conditions used here indicates that AMPK is capable of phosphorylating cTnI when assembled in the Tn complex and associated with other myofilament contractile proteins. Moreover, cTnI Ser149 was a target site under these conditions.

Phosphorylation of fsTn by AMPK

Recombinant human fsTnI and purified chicken fsTn complexes were treated with AMPK^Δ for 1 h.

Figure 7. Phosphorylation of cTnI by AMPK in skinned cardiomyocytes. Cardiomyocytes were treated with either active (+) or inactive (–) AMPK^Δ. (A) ³²P-incorporation into mouse myofilament proteins by Coomassie-stained gel (left) and autoradiogram (right) analyses. Prominently phosphorylated bands are indicated by labeled arrows. (B) Phosphorylation of Ser149 in skinned rat cardiomyocytes assessed by Western blotting analysis using a phospho-specific antibody. Last two lanes show purified rat cTn as control.

Phosphorylation at Ser117 (residue equivalent to Ser149 in cTnI) was assessed with a cTnI phosphoserine-149 antibody at various times. Although the cTnI phosphoserine-149 antibody is specific to the sequence surrounding Ser149 in cardiac TnI, the antibody also recognized the sequence surrounding Ser117 in fsTnI, due to high sequence homology. AMPK phosphorylated Ser117 in recombinant fsTnI with a $t_{1/2}$ of 4.9 ± 1.5 min, and Ser117 in fsTn complexes with a $t_{1/2}$ of 9.7 ± 0.9 min (Fig. 8). These kinase reaction half-times are similar to the kinase reaction half-times observed for Ser149 in cardiac TnI. Therefore, our results suggest that Ser117 in fsTnI is a good substrate for AMPK *in vitro*.

Discussion

cTnI is a good substrate for AMPK

AMPK plays a fundamental role in the regulation of cellular energetics through the regulation of metabolic pathways.⁷ The findings of this study suggest that AMPK may also regulate cardiac energetics via novel phosphorylation of cTnI. cTnI is an important target of several signaling pathways, and its phosphorylation can lead to modulation of myofilament activation properties resulting in important physiological effects on cardiac function.^{14,15} AMPK phosphorylated cTnI at the level of cTnI peptides, the intact cTnI subunit, whole cTn complexes, and skinned cardiomyocytes, when cTnI is assembled in

a complex and with other contractile proteins in highly ordered myofilaments.

AMPK phosphorylates cTnI only at Ser22 and Ser149

AMPK phosphorylated-purified human cTnI subunit with a stoichiometry of 1 mol phosphate per mol of protein (Fig. 1), with the incorporated phosphates localized to either one site or distributed among several sites. From ³²P-phosphorylation experiments, it is not possible to determine if the incorporation of the 1 mol of phosphate was limited to one site or if the phosphate was incorporated at multiple sites, with partial phosphorylation occupancy of each site. Because phosphorylation of different residues in cTnI can lead to different and sometimes opposing effects on myofilament function,¹⁵ it was important to determine, which residues were phosphorylated

Figure 8. Phosphorylation time courses of fsTnI Ser117 in recombinant human fsTnI (A) and purified chicken fsTn (B) treated with active AMPK^Δ. Phosphorylation was assessed by Western blotting analysis using a cardiac cTnI phospho-specific antibody. Top panel shows representative blots and bottom graph shows the exponential fitting to the data from three separate experiments.

by AMPK. Using a combination of site-directed mutagenesis of recombinant mouse cTnI, screening of synthetic peptides mimicking selected regions of cTnI, phosphospecific antibodies, and high-resolution MS/MS, Ser22, and Ser149 were identified as the targeted sites. Importantly, Ser22 and Ser149 were phosphorylated quite selectively by AMPK in synthetic peptides, as the nearby Ser23 and Thr142 residues were never observed to be phosphorylated. Top-down MS analysis of purified rat cTn complexes confirmed that Ser149 and the PKA sites (Ser22Ser23) were the only sites phosphorylated by AMPK, excluding phosphorylation of other sites that are known targets for other kinases, such as Ser41, Ser43, and Thr142. Because of the detection limit of the MS results, the possibility of lower abundance phosphorylation at other sites (estimated to be <1%) cannot be completely excluded. Because the key fragmentation event between Ser22 and Ser23 was not consistently observed, it was not possible to demonstrate selective phosphorylation of Ser22 (over possible phosphorylation of Ser23) in the intact protein. However, preferential phosphorylation of Ser22 by AMPK was observed in experiments using synthetic peptides [Fig. 2(A)]. Because the region of the N-terminal extension of cTnI containing the PKA sites is thought to behave as a highly flexible structure, much like an isolated peptide,⁴⁵ Ser22 is likely the targeted site by AMPK in the intact protein as well. Furthermore, the phosphospecific antibody confirmed that AMPK is capable of phosphorylating Ser149 when cTnI is assembled in the cTn complex.

Residual phosphorylation of recombinant mouse cTnI with the Ser22, Ser23, and Ser149 residues mutated to alanine (the Ala₂ S149A mutant) and treated with active AMPK suggested the possibility of additional unidentified phosphorylation sites [Fig. 3(B)]. However, top-down MS analysis of purified rat cTn complexes treated with AMPK ruled out phosphorylation of additional sites. In addition, screening of cTnI synthetic peptides containing candidate sites also failed to identify other possible sites (data not shown). Therefore, the residual phosphorylation observed in the Ala₂ S149A mutant may be better explained by nonspecific compensatory phosphorylation that can occur when the preferred sites of a kinase are removed and therefore not available to the kinase.⁴² The apparently greater contribution of the PKA sites to total phosphorylation in recombinant mouse cTnI compared to the contribution of Ser149 may be related to such compensatory phosphorylation.

cTnI Ser149 is the preferred site for AMPK

Our results demonstrate that AMPK is capable of phosphorylating cTnI at Ser22 and Ser149, with Ser149 being the preferred site. Phosphorylation time-course experiments revealed that Ser149 is the

preferred site for AMPK in recombinant cTnI and purified rat cTn complexes, with Ser149 phosphorylated 12–16 times faster than the PKA sites. This is consistent with results from the top-down MS analysis in which monophosphorylated cTnI from AMPK-treated cTn complexes was phosphorylated solely at Ser149 and not at the PKA sites. In addition, experiments on skinned myocytes demonstrated that AMPK can phosphorylate cTnI while assembled in the myofilament and that Ser149 was a target site under these physiological conditions (Fig. 7). Ser149, a highly conserved residue in TnI, is located adjacent to the inhibitory loop, a segment spanning amino acids 136–147 (human sequence excluding the initial methionine) that has been shown to be the minimum sequence of cTnI that prevents actomyosin ATPase activity in the absence of Ca²⁺-bound cTnC^{46–48} and therefore, key for the inhibitory properties of TnI. Ser149 is also located adjacent to the switch region (amino acids 150–159), an amphiphilic α -helix that binds a hydrophobic cleft on TnC and is thought to act as a Ca²⁺ transducer, signaling the binding of Ca²⁺ to TnC to the rest of the contractile apparatus.^{46–48} Therefore, Ser149 is strategically positioned to possibly influence the inhibitory properties of TnI toward actin-myosin interactions and the affinity of TnI for TnC.

Physiological implications of phosphorylation at Ser22Ser23 and Ser149 in cTnI

Studies using diverse experimental approaches have consistently shown that PKA phosphorylates cTnI at residues Ser22 and Ser23.^{21,49,50} Phosphorylation of these sites results in decreased Ca²⁺ sensitivity of both force development and MgATPase activity,^{21,50} and an augmented off-rate of Ca²⁺ from cTnC,⁵¹ thereby increasing the rate of myofilament deactivation. These effects are thought to play a central role in the earlier relaxation observed in the heart during β -adrenergic stimulation. In contrast, the functional effects of phosphorylating cTnI Ser149 have not been well characterized but evidence suggests that phosphorylation of this site may be associated with an increase in the Ca²⁺ sensitivity of the myofilaments.^{25,52} Another signaling enzyme, PAK has been shown to phosphorylate cTnI at Ser149.²⁵ PAK is a serine/threonine protein kinase expressed in most mammalian tissues and activated by the small GTPases Cdc42 and Rac1.⁵³ Several isoforms have been described, including PAK1, PAK2, and PAK3, with PAK1 being the major isoform expressed in the heart.⁵⁴ Buscemi *et al.*²⁵ found that phosphorylation of cTnI Ser149 by PAK3 was accompanied by an increase in the Ca²⁺ sensitivity of myofilament force development. A study by Ke *et al.*⁵⁵ found that, although PAK1 could phosphorylate cTnI *in vitro*, adenovirus infection of isolated myocytes with a constitutively active PAK1 construct decreased the overall phosphorylation status of cTnI and increased the

Ca²⁺ sensitivity of force production.⁵⁵ Because phosphorylation status of individual cTnI sites was not assessed in that study, it is not known if some sites underwent increased phosphorylation while others exhibited a proportionally greater decrease in dephosphorylation. Alternatively, different isoforms of PAK may have different effects on cTnI phosphorylation. Interestingly, phosphorylation of cTnI Thr142, located in the inhibitory loop and nearby Ser149, has been shown to increase the Ca²⁺ sensitivity of the myofilaments.¹⁸

The functional implications of increased myofilament Ca²⁺ sensitivity are not well understood. In the heart, increased Ca²⁺ sensitivity could contribute to an increase in the work output per ATP hydrolyzed by the contractile machinery, possibly enhancing contractile efficiency and energy utilization.²⁶ Such a mechanism could serve to sustain contractile function in an energy-deprived state such as in the failing heart.⁵⁶

Top-down MS/MS analysis of TnI isolated from healthy rat, mouse, and pig hearts have revealed that cTnI is basally phosphorylated solely at the Ser22 and Ser23, the PKA sites,^{31–33} consistent with the top-down MS/MS results of cTnI treated with inactive AMPK [Fig. 4(B)]. No basal phosphorylation was observed for other known phosphorylation sites on cTnI, such as Ser149 (the PAK site) or Ser41, Ser43, and Thr142 (the PKC sites). It is plausible that Ser149 is only phosphorylated in an energy-compromised state, thus, playing an important role in the regulation of contractile behavior during energy deficiency and/or in cardiac dysfunction. As an example, cTnI residues Ser41/Ser43, which can be phosphorylated by PKC *in vitro*, have not been observed phosphorylated *in vivo* in healthy cardiac tissue. However, a recent top-down MS/MS analysis performed at our lab revealed *in vivo* phosphorylation of cTnI at those PKC sites in spontaneously hypertensive heart failure rats (Dong X, *et al.*, unpublished data).

At the same time that our work was being performed, Oliveira *et al.*, using a yeast two-hybrid assay, also identified cTnI as a potential substrate for AMPK.^{57,58} They reported that cTnI could be phosphorylated by AMPK and identified Ser149 as the principal site by mutagenesis analysis, in agreement with our results. In addition, it was found that murine cardiomyocytes that had been treated with AICAR (an AMPK agonist) displayed increased relaxation times with no change in Ca²⁺ transients, suggesting that the observed prolonged relaxation may be related to changes in calcium sensitivity of the contractile apparatus.

AMPK phosphorylates fsTnI at a residue analogous to Ser149

Skeletal TnI notably lacks the cardiac specific N-terminal region (a flexible helix encompassing amino

acids 1–31) present in cTnI and containing the PKA sites. Therefore, skeletal cTnI offers a model to study the effect of phosphorylating cTnI at Ser149 independent of the phosphorylation of the PKA sites. Here, we report that AMPK is able to phosphorylate both recombinant fsTnI subunits and purified chicken fsTn complexes at Ser117, the residue analogous to Ser149 in cTnI. Time-course experiments suggest that Ser117 is phosphorylated with similar kinetics as Ser149 in cTnI (Fig. 8). Therefore, fsTn can be a useful experimental model to investigate the functional consequences of Ser149 phosphorylation independent of the phosphorylation of Ser22. Myofilament exchange experiments may be used to determine the effect of phosphorylating Ser117 on force development and/or MgATPase activity.^{18,21,22}

In conclusion, the results of this study demonstrate that cTnI, a critical myofilament regulatory protein, is a novel target of AMPK. AMPK phosphorylates cTnI with a unique pattern characterized by selective targeting of one PKA site (Ser22) and one PAK site (Ser149), with a marked preference for the PAK site. Moreover, AMPK can also phosphorylate fsTnI at a site analogous to Ser149. Phosphorylation of TnI adjacent to the inhibitory loop may function to sensitize the myofilaments to Ca²⁺, which could provide an adaptive advantage in energy-deprived conditions.

Experimental Procedures

Materials

All reagents were obtained from Sigma Chemical (St Louis, MO) unless noted otherwise. GST-AMPK α 1(1–312) kinase, TnI polyclonal antibody, and cTnI phosphoserine-22/23 polyclonal antibody were from Cell Signaling (Beverly, MA). cTnI phosphoserine-149 polyclonal antibody (Proteintech Group, custom, Chicago, IL) was generated against the phosphopeptide LRRVRIS(phos)ADAMMQA and affinity purified with affinity cross-absorption with the nonphosphorylated peptide. SAMS peptide and LKB1/STRAD/MO25 complex were from Millipore (Framingham, MA), primers from Operon (Huntsville, Alabama), Complete® protease inhibitor cocktail from Roche (Mannheim, Germany), secondary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) and recombinant human fast skeletal cTnI from AbCam (Cambridge, MA).

Expression and purification of recombinant mouse cTnI and recombinant human AMPK α 1 constructs

cDNA for N-terminal His-tagged AMPK α 1 (His-AMPK α 1) was obtained from Genecopoeia (Germantown, Maryland). Development of an AMPK α 1 kinase domain (1–312) was performed as described (Supporting Information, Experimental and Results,

Figs. 1 and 2). GST-tagged cTnI cDNA was a gift from John Solaro (University of Illinois, Chicago, IL). Mutations were introduced using site-directed mutagenesis kits (Stratagene, La Jolla, CA). Expression plasmids were transformed into BL21(DE3) *E. coli* cells and grown at 37°C in LB medium containing 100 µg/mL ampicillin until A₆₀₀ reached 0.4. The culture was then induced with 0.4 mM IPTG and cells harvested 4 h later by centrifugation. Purification was carried out at 4°C. GST-cTnI WT and mutants were purified with Glutathione Sepharose 4B medium (GE Health, Piscataway, NJ). Purifications were performed following the manufacturer's directions. Concentration and purity were assessed by SDS-PAGE using BSA as a standard.

Activation of AMPK

100–400 nM GST-AMPK α 1(1–312) or His-AMPK α 1(1–312) was activated by incubation with 15–30 nM LKB1/STRAD/MO25 complex in kinase buffer (60 mM HEPES-NaOH pH 7.5, 3 mM MgCl₂, 3 µM Na₃VO₄, 1.2 mM DTT, 500 µM ATP, and protease inhibitor cocktail) for 10 min at 37°C. Activated GST-AMPK α 1(1–312) and His-AMPK α 1(1–312), both of which will be later referred as active AMPK^A, had similar activity levels (data not shown) and were used interchangeably. Inactive AMPK^A, prepared by boiling activated AMPK^A for 10 min, was used as a negative control.

Phosphorylation of synthetic peptides

Peptides (250–270 µM), synthesized and purified as described,⁵⁹ were incubated with active AMPK^A (100 nM) in kinase buffer (see above) for 0–8 h at 37°C. Thirty microliter aliquots were injected onto an ion exchange HPLC column, eluted at 5 mL/min with a linear gradient of 0–1M NaCl and monitored by UV absorbance at 220 nm. Peak identities were confirmed by MALDI-TOF MS as described previously.⁶⁰ Phosphorylation of SAMS peptide, a specific substrate for AMPK,⁶¹ was also carried out as a positive control.

Phosphorylation of Tn

Tn complexes or TnI subunits were incubated with active AMPK^A, inactive AMPK^A, the catalytic subunit of PKA or inactive catalytic subunit of PKA. All incubations were performed in kinase buffer (see above) at 37°C unless otherwise noted.

Skinned myocytes

All procedures involving the use of animals were approved by the University of Wisconsin Animal Care and Use Committee. Rodent hearts were rapidly excised and rinsed in ice-cold Ringer's solution (125 mM NaCl, 5 mM KCl, 25 mM HEPES-NaOH pH 7.4, 2 mM NaH₂PO₄, 1.2 mM MgSO₄, 5 mM pyruvate, and 11 mM D-glucose). Ventricular tissue

was homogenized for 4–6 s in 10–20 mL of relax solution A (100 mM KCl, 10 mM imidazole, 6 mM MgCl₂, 2 mM EGTA, 5 mM ATP, 1 mM PMSF, 1 mM DTT, 10 mM benzamide, and 0.75 mg/mL protease inhibitor cocktail) using a Polytron homogenizer. Cell fragments were skinned (chemically permeabilized) for 4 min in relax solution A containing 0.33% Triton X-100 and 0.5 mg/mL BSA, washed twice and resuspended in relax solution A. For ³²P-incorporation experiments, myocytes were resuspended in relax solution B (same as relax A except containing 1 mM ATP).

³²P incorporation assays

Skinned mouse myocytes (150 µg) containing 0.122 mCi/mL ³²P-γ-ATP were incubated with active AMPK^A (400 nM) in relax solution B (see above). Purified human Tn subunits (0.25 µg) containing 0.082 mCi/mL ³²P-γ-ATP were incubated with active AMPK^A (400 nM). Purified recombinant mouse cTnI WT and mutants (5 µg) containing 0.025 mCi/mL ³²P-γ-ATP were incubated with active AMPK^A (200 nM). After 1 h incubation, samples were subjected to 12% SDS-PAGE and autoradiography. Stoichiometry and quantification was determined by scintillation counting of excised bands, normalization of cpm to ³²P-γ-ATP specific activity, densitometry of recombinant mouse cTnI bands (Bio-Rad GS-670 densitometer) and amino acid analysis of cTnI as internal control (Joseph Leykam, Michigan State University).

Western blots

Recombinant mouse cTnI (5 µg), purified rat cTn complexes (5 µg), recombinant human fast skeletal TnI (5 µg) and purified chicken fast skeletal Tn complexes (5 µg) were incubated with active AMPK^A (200–400 nM) or with 60 nM catalytic subunit of PKA for various times. Rat-skinned myocytes were incubated for 1 h at 37°C with active AMPK^A (1.6 µM) in relax solution A (see above). Samples were subjected to 12% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with a cTnI polyclonal antibody to phosphoserine-22/23 or a cTnI polyclonal antibody to phosphoserine-149. Loading control for each blot was obtained by stripping the membrane and probing with a TnI antibody.

Affinity purification of cTn

Whole cTn complexes were affinity purified by the method of Messer *et al.*⁴³ with modifications.³⁰ Briefly, hearts were surgically excised from adult male Sprague-Dawley rats anesthetized with isoflurane. Myocytes were skinned (see above) and then incubated in extraction solution (0.7M LiCl, 25 mM Tris pH 7.5, 5 mM EGTA, 0.1 mM CaCl₂, 5 mM DTT, 1 mM PMSF, and protease inhibitor cocktail) for 1 h at 4°C. Cell debris was removed by centrifugation at 55,000 rpm for 45 min (80Ti rotor,

Beckman L-55 ultracentrifuge, Brea, CA). The supernatant was incubated with 0.3 mL of CNBr-activated Sepharose CL-4B conjugated with a monoclonal cTnI antibody (anti-troponin I monoclonal antibody MF4, Hytest, Finland) for 1 h at 4°C and then loaded into an empty disposable column. After washing with five column volumes of extraction solution, the column was eluted with 50 mM glycine pH 2, whereas 0.4 mL fractions were collected and neutralized immediately by 80 µL of 1M MOPS pH 9. Fractions were analyzed for protein content by SDS-PAGE on 15% gels stained with Coomassie Blue. Typically, four eluted fractions contained cTn subunit bands at 17 kDa (cTnC), 27 kDa (cTnI), and 34 kDa (cTnT), with fraction 2 showing the bands with the greatest intensity and the most consistent 1:1:1 stoichiometry.

Top-down MS analysis

Immunoaffinity purified rat cTn complexes and phosphopeptides were desalted using an offline reverse phase C18 protein microtrap (Michrom Bioresources, Auburn, CA) and eluted first with 1% acetic acid in 50:50 methanol:water and then 1% acetic acid in 75:25 methanol:water. All data were acquired on a linear trap/FTICR (LTQ FT Ultra) hybrid mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with an automated chip-based nanoESI source (Triversa NanoMate, Advion BioSciences, Ithaca, NY) as described previously.^{32,33} Individual charge states of protein molecular ions were first isolated and then dissociated by ECD using 2–3% “electron energy” and a 45–150 ms duration with no delay. All FTICR spectra were processed with Xtract Software (FT programs 2.0.1.0.6.1.4, Xcalibur 2.0.5, Thermo Scientific, Bremen, Germany) using a signal to noise threshold of 2 and fit factor of 40%, and then validated manually. The resulting mass lists were further assigned using in-house “Ion Assignment” software (Version 1.0) based on the protein sequence of rat cTnI obtained from Swiss-Prot protein knowledgebase (primary accession number P23693). Allowance was made for possible PTMs such as removal of initial methionine, acetylation of the N-terminus and variable phosphorylation sites, using a 10 and 20 ppm tolerance for precursor and fragment ions, respectively. For phosphopeptides, individual charge states were isolated first and then dissociated by ECD (3–5% “electron energy” and 75–150 ms duration with no delay). All reported M_r values are most abundant masses. For quantification of cTnI phosphospecies, MS signal intensity values (the top five most abundant isotopomer peak heights) were integrated to calculate relative ratios.^{31–33,62,63} Based on signal-to-noise ratios, the relative detection sensitivity was estimated to be about 1–3% of highest peak intensity for MS spectra and about 1–5% for ECD spectra.

Statistics and data fitting

Data are expressed as mean $\pm$ standard error of the mean (SEM). Both Student's *t* tests and one-way ANOVA were used for statistical analysis. Differences at $P < 0.05$ were considered significant. For one-way ANOVA, significant differences were determined using the Tukey Post Hoc test. Data from time-course phosphorylation experiments were fitted using Origin v7.5 Software (Origin Lab Corporation, Northampton, MA).

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