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Structural Evidence for Co-evolution of the Regulation of Contraction and Energy Production in Skeletal Muscle

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Abstract

Skeletal muscle phosphorylase kinase (PhK) is a Ca^{2+} -dependent enzyme complex, $(\alpha\beta\gamma\delta)_4$, with the δ subunit being tightly bound endogenous calmodulin. The Ca^{2+} -dependent activation of glycogen phosphorylase by PhK couples muscle contraction with glycogen breakdown in the 'excitation-contraction-energy production triad.' Although the Ca^{2+} -dependent protein-protein interactions among the relevant contractile components of muscle are well characterized, such interactions have not previously been examined in the intact PhK complex. Here we show that zero-length crosslinking of the PhK complex produces a covalent dimer of its catalytic γ and calmodulin subunits. Utilizing mass spectrometry, the residues crosslinked were determined to be in an EF-hand of calmodulin and in a region of the γ subunit sharing high sequence similarity with the Ca^{2+} -sensitive molecular switch of troponin I that is known to bind actin and troponin C, a homolog of calmodulin. Our findings represent an unusual binding of calmodulin to a target protein and supply an explanation for the low Ca^{2+} stoichiometry of phosphorylase kinase that has been reported. They also provide direct structural evidence supporting co-evolution of the coordinate regulation by Ca^{2+} of contraction and energy production in muscle through the sharing of a common structural motif in troponin I and the catalytic subunit of phosphorylase kinase for their respective interactions with the homologous Ca^{2+} -binding proteins troponin C and calmodulin.

Keywords

calmodulin; co-evolution; energy production; phosphorylase kinase; troponin I

Phosphorylase kinase (PhK) phosphorylates and activates glycogen phosphorylase, leading to glycogenolysis and subsequent energy production. In skeletal muscle PhK exists as a hexadecameric complex, $(\alpha\beta\gamma\delta)_4$, with an absolute requirement for Ca^{2+} ions for activity.^{1,2} This requirement couples energy production with contraction in the 'excitation-contraction-energy production triad' (Fig. 1), where an increase in intracellular Ca^{2+} simultaneously stimulates muscle contraction by binding to the troponin complex and energy production to supply contraction's "short-term power demands"³ by binding to the PhK complex. Ca^{2+} activates the PhK complex through its tightly bound endogenous calmodulin (CaM) subunit (designated as δ),⁴ a homolog of troponin C (TnC), a component of the hetero-trimeric troponin complex (Fig. 1). CaM (δ) interacts with the isolated catalytic γ subunit of PhK,⁵ which from studies with synthetic peptides has been shown to contain two distinct, but adjacent, high-

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affinity, potential CaM-binding domains (CBDs).⁶ The more N-terminal CBD, termed N-CBD, has been noted to share high sequence similarity with the key functional domain of troponin I (TnI),^{6–8} a second component of the troponin complex; however, direct evidence regarding the actual binding site(s) for CaM (δ) within the 1.3 MDa ($\alpha\beta\gamma\delta$)₄ PhK complex has been totally lacking. Here we show that within the PhK complex, the catalytic γ subunit and the endogenous CaM subunit interact through a salt-bridge involving the TnI-like domain of the former and an EF-hand of the latter, which represents a novel mechanism for the binding of CaM to a target protein. Furthermore, these results provide direct structural evidence supporting the co-evolution in skeletal muscle of the coordinate regulation by Ca²⁺ of contraction and energy production through use of the same recognition motif in TnI of the troponin complex and in γ of the PhK complex for their respective interactions with the homologous Ca²⁺-binding proteins TnC and CaM.

Inasmuch as all previous information concerning potential sites of interaction between the catalytic (γ) and CaM (δ) subunits of PhK had been gained from studies using isolated subunits or synthetic peptides, we thought it essential to examine the interaction between these subunits within the context of the entire hexadecameric PhK complex. To locate sites of interaction between the γ and δ subunits within the ($\alpha\beta\gamma\delta$)₄ complex, we relied on the intrinsic properties of these two subunits to form γ - δ dimers as a result of zero-length crosslinking. The first 20 residues of the catalytic γ subunit are unique, being unrelated to other protein kinases; residues 21–289 encompass a typical protein kinase domain and have a pI of 5.6; whereas residues 290–386 at its carboxy-terminal regulatory domain contain the two high-affinity, potential CaM-binding domains and have a pI of 10.0.^{6,9} Given that CaM has a pI of 4.1, we reasoned that the interactions between the acidic CaM (δ) and the basic regulatory region of γ are likely to be stabilized, at least in part, by electrostatic interactions. Consequently, we tested a large number of zero-length crosslinkers capable of forming an amide bond between interacting Asp••Lys or Glu••Lys side chains and screened for formation of a γ - δ dimer within the context of the ($\alpha\beta\gamma\delta$)₄ holoenzyme complex. The identity of such a dimer was established by its apparent mass in SDS-PAGE and by immunoblots with subunit-specific antibodies. One crosslinker in particular, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), formed significant amounts of a γ - δ dimer that migrated as a tight band on SDS-PAGE (Fig. 2); moreover, formation of this dimer was sensitive to Ca²⁺, being inhibited approximately 50% by inclusion of this cation in the crosslinking reaction (Fig. 2). To prove that the γ - δ complex was formed from subunits within, as opposed to between, PhK hexadecamers, crosslinked PhK was purified by size exclusion HPLC over a BioSep-SEC-S4000 column (Phenomenex Inc., 600 × 7.8 mm), which separates the native hexadecamer from larger aggregates.^{10,11} Subsequent analyses of the hexadecameric complex peak showed it to contain γ - δ dimer. This dimer was subsequently analyzed by mass spectrometry (MS) to determine the specific residues that were crosslinked.

To identify its cross-linked region(s), a large scale preparation of the crosslinked γ - δ dimer was prepared by crosslinking as described in the legend for Fig. 2, except that crosslinking was carried out for 30 min with aliquots of EEDQ added at 0, 10 and 20 min to bring its concentration to 0.86, 1.71 and 2.53 mM, respectively, or 500–1,500 molar excess above the $\alpha\beta\gamma\delta$ PhK protomer. After quenching, the mixture was loaded onto a 10% cylindrical, preparative polyacrylamide gel of internal diameter 28 mm (Bio-Rad Prep Cell 491, Hercules, CA) and eluted with electrophoresis buffer at a flow rate of 0.7 ml/min. Fractions containing the γ - δ dimer were combined and concentrated on a Centricon-10 (Amicon). To remove the SDS for MS analyses, the concentrated crosslinked samples were precipitated with Perfect-Focus (Genotech, St. Louis, MO). The precipitated protein was resolubilized in 6M guanidine hydrochloride, 25 mM NH₄HCO₃, pH 7.5, reduced with 2 mM dithiotreitol for 30 min, and then alkylated by 10 mM iodoacetamide for 30 min in the dark at 22 °C. The reaction mixture was diluted 7-fold with 25 mM NH₄HCO₃, pH 7.5 and digested with 0.7 pg/ml of sequencing

grade modified trypsin (Promega, Madison, WI) for 16 h at 37 °C. The digestion was terminated by the addition of acetic acid to pH 2.0. The resulting peptides were fractionated on a C18 reverse phase column, with each fraction analyzed on a time-of-flight mass spectrometer. For the MALDI TOF analyses, 2- μ l aliquots of fractions from the C18 column were mixed directly on stainless steel targets with 2 μ l of a 50% saturated solution of α -cyano-4-hydroxycinnamic acid in 75% acetonitrile, 0.1% formic acid. MALDI TOF MS was carried out on a DE PRO STR mass spectrometer (Applied Biosystems, Boston, MA), with spectra obtained in the positive ion mode. Spectra were obtained at a laser power setting of 2100, averaging 1,000 spectra per spot; a 175 ns delay time was used to extract ions in the field-free region of the mass spectrometer. The spectra were baseline-corrected and calibrated using as external close standards a set of peptides of known masses (0.05% mass accuracy). Processing of the MALDI-TOF data was performed using the software provided by the manufacturer (Data Explorer). Peak detection relied on a 10% cut-off ion intensity relative to the most abundant ion in the mass spectrum and a minimum signal to noise ratio of 100. Monoisotopic masses were obtained utilizing the deisotoping algorithm included in the software. Putative crosslinked peptides were identified by comparing the monoisotopic mass list obtained from the externally calibrated mass spectra against the theoretical tryptic mass maps of free and crosslinked peptides using a search engine developed in-house.

Those fractions containing potentially crosslinked peptides were subjected to two consecutive, pH-dependent derivatizations with o-methylisourea hydrogen sulfate followed by succinimidyl 3-sulfopropionate using a CAF-MALDI sequencing kit (Amersham Biosciences, Upsala, Sweden).¹² In the first reaction, protection of the ϵ -amine groups of reactive Lys residues by guanidination results in a 42 amu increase per Lys modified; subsequent sulfopropionylation of the derivatized peptides allows for selective modification of the N-termini of tryptic peptides, increasing their mass by 136 amu per N-terminus. Crosslinked peptides are identified by their predicted relative change in mass. We observed that a peptide-ion with an original $m/z=1345.6$ experienced a shift to $m/z=1617.5$, a change of 271.9 amu due to the addition of two sulfopropionyl groups to each of the N-termini of the crosslinked peptides. Sequence assignment based on mass, the specificity of trypsin, and the expected reaction of the crosslinking reagent EEDQ indicated that this ion most likely represented a crosslink between peptides V⁹¹FDK⁹⁴ of δ and R³²³VKPVTR³²⁹ of γ . Contrary to expectation, the C-terminal Lys (K⁹⁴ _{δ}) did not react with o-methylisourea hydrogen sulfate during the first derivatization reaction, suggesting the possibility that the ϵ -amino group interacts with its own carboxylate or that of R³²⁹ _{γ} , thus decreasing its reactivity.

Verification of this sequence assignment and identification of crosslinked residues was achieved by tandem mass spectrometry on an ESI mass spectrometer, with the spectrum revealing a side-chain amide crosslink between residues D93 of δ and K325 of γ within the tryptic crosslinked peptide V⁹¹FDK⁹⁴ _{δ} – R³²³VKPVTR³²⁹ _{γ} (Fig. 3). The lack of tryptic cleavage after R323 could be due to the presence of the adjacent R322⁹ or to steric constraint by the crosslink only two residues removed. Treatment of the original tryptic peptide-ion with *Staphylococcus* V8 protease did not alter its m/z of 1345.6, consistent with the D93 side-chain being modified.

The zero-length crosslinking of these two particular residues has important consequences with respect to the interactions between the γ and δ subunits in the PhK complex. In the case of D93 of δ , this residue not only occurs within the third Ca²⁺-binding EF-hand of CaM, but is one of the coordinating ligands for the Ca²⁺ ion. The salt-bridging of D93 of δ to K325 of γ would be predicted to abolish, or at least greatly weaken, the binding of Ca²⁺ by the third EF-hand, which may be the underlying basis for the surprising findings reported in an early publication entitled, “The activation of rabbit skeletal muscle PhK requires the binding of 3 Ca²⁺ per δ subunit.”¹³ In fact, in that report the existence of a fourth Ca²⁺-binding site was not detected.

It would be of interest to determine if targets other than PhK that also tightly bind apoCaM (CaM devoid of Ca^{2+}) or that are activated by fewer than 4 Ca^{2+} ions^{14,15} interact with CaM in a similar fashion, i.e. through one of its EF-hands.

As for the second of the two crosslinked amino acids, K325 of γ , this residue lies within the first of the two CBDs, the N-CBD, found in the C-terminal region of the catalytic γ subunit (Fig. 4), providing the first direct evidence that this CBD participates in the binding of the endogenous CaM subunit within the PhK complex. It is this CBD that contains a region with high sequence similarity to the functionally important TnI inhibitory peptide (Fig. 4), which interacts with either actin or TnC in a Ca^{2+} -dependent manner, and by itself inhibits actomyosin ATPase.^{16–18} Within the shortest functionally effective segment of this TnI inhibitory region, G104 through R115,¹⁹ all but the two Pro residues have been shown to be highly important, through a wide variety of experimental approaches, in the binding of TnC and actin, with many of the residues interacting with both of these alternative protein targets.^{18–22} It is noteworthy that of these 10 critical residues of TnI, the γ subunit of PhK contains 8 that are identical and 1, the crosslinked K325, that is conserved. The competition of TnC and actin for the same or proximal residues within the TnI inhibitory peptide has led to the idea of the Ca^{2+} -dependent alternate binding of these targets by TnI within the contractile apparatus, i.e. that this inhibitory region of TnI is a Ca^{2+} -dependent molecular switch.²³ The presence of these critical functional TnI residues within the γ subunit of PhK raises the question of whether a similar Ca^{2+} -dependent alternate binding of targets might occur within the PhK complex, i.e. the Ca^{2+} -dependent alternate interactions of the regulatory region of γ with other domains within the $(\alpha\beta\gamma\delta)_4$ hexadecameric complex. In support of such an idea, we recently found that the C-terminal region of the α subunit of PhK interacts with the C-terminal regulatory region of γ ²⁴ and that Ca^{2+} alters the conformation of that region of α ,²⁵ suggesting that the regulatory region of γ mediates a $\delta \leftrightarrow \gamma \leftrightarrow \alpha$ Ca^{2+} -sensitive communication network.²⁴ Thus, the possibility exists of functionally similar Ca^{2+} -dependent molecular switching among TnC – TnI – actin and the δ (CaM) – γ – α subunits of PhK. Such a functional similarity is consistent with our early findings that the isolated γ subunit of PhK is activated by TnC, binds actin, and dramatically inhibits actomyosin ATPase;⁸ nevertheless, without a crystal structure of the PhK complex, such as has been obtained for troponin,^{26,27} one cannot say whether the region in γ that is similar to the inhibitory peptide of TnI functions in a mechanistically similar manner in both complexes. It might be noted, however, that the inhibitory peptide of TnI has been reported to bind to the C-lobe of TnC, with electrostatic interactions playing a role in that binding;^{28–30} both observations are analogous to the results reported herein of a salt bridge between the TnI-like region of γ and the C-lobe of CaM (Fig. 4). The possibility of a Ca^{2+} -dependent electrostatic switch in γ similar to that observed in TnI is discussed in the recent report that Ca^{2+} induces a large change in the surface electrostatics of the PhK complex.³¹

In the genes for fast skeletal muscle TnI from quail, chicken, mouse and human, the sequence similarity between γ and TnI (Fig. 4) occurs in a domain encoded by a single exon, which spans residues 92–150,^{32–34} a domain that includes the entire inhibitory peptide region of TnI; no other sequence similarity is present in the two proteins outside of the domain encoded by this exon. Furthermore, the sequence similarity occurs within both the N- and C-termini of this domain and stops abruptly at the exact C-terminal boundary of the exon, K150 of TnI, which corresponds to K375 of γ , only 11 residues removed from its C-terminus.⁹ These structural similarities suggest evolution by exchange through exon shuffling between (or into) γ and TnI of a modular motif for the binding of the homologous Ca^{2+} -binding proteins CaM and TnC. The structural, functional, and perhaps mechanistic similarities between γ and TnI, considered with the direct evidence presented herein for the involvement of the TnI-like domain of γ in the binding of CaM, provide a structural underpinning for the coordinated regulation by Ca^{2+} of the ‘*excitation-contraction energy production triad*’ in skeletal muscle (Fig. 1). We have recently noted other potential similarities in the regulation of contraction and energy

production, in that short stretches of residues in the β subunit of PhK share sequence similarities with regions of both TnI and troponin T, and in each case the regions in question are encoded by a single exon in each of the three proteins.³⁵ Moreover, in the case of troponin T, the residues in common with β are known to be critical for the binding of TnI by troponin T.

Additional mechanisms for the coordination of muscle contraction and energy production that involve PhK are the simultaneous stimulation of contraction³⁶ and PhK activity³⁷ by epinephrine and the allosteric activation of PhK by free ADP,³⁸ which increases during contraction.³⁹ Thus, activation of the $(\alpha\beta\gamma\delta)_4$ PhK complex, with subsequent energy production, has apparently evolved to be finely attuned with the contractile state of skeletal muscle.

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Abbreviations

CaM, calmodulin; CBD, calmodulin-binding domain; EEDQ, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; MS, mass spectrometry; PhK, phosphorylase kinase; TnC, troponin C; TnI, troponin I..

Fig. 1.

The Ca^{2+} -controlled 'excitation-contraction-energy production triad' of skeletal muscle. The binding of Ca^{2+} to the TnC subunit of the troponin complex and to the CaM (δ) subunit of the PhK complex simultaneously triggers, respectively, contraction and glycogenolysis (with subsequent energy production). These interconnections became appreciated when the reversible activation of PhK by low concentrations of Ca^{2+} was characterized.² The structurally similar sequences of TnI and the γ subunit of PhK detailed in Fig. 4 are proposed to participate in the Ca^{2+} -activation of the two complexes.

Fig. 2.

Formation of zero-length crosslinked γ - δ dimer by EEDQ. The first three lanes are non-crosslinked control (C) and crosslinked ($\pm$ Ca^{2+}) PhK (7 μg) run on a 15% SDS-PAGE gel and stained with Coomassie blue. The last two lanes are immunoblots of SDS-PAGE gels of crosslinked PhK blotted onto PVDF membranes and probed with anti- γ and anti-CaM (δ) monoclonal antibodies. PhK was purified from fast-twitch skeletal muscle (psoas) of New Zealand White rabbits²⁵ and stored at -80°C in 50 mM HEPES, 0.2 mM EDTA and 10% (w/v) sucrose at pH 6.8. Its concentration was determined spectrophotometrically using the accepted absorbance index.⁴⁰ EEDQ was from Sigma-Aldrich, Inc., St. Louis, MO. Crosslinking was performed for 15 min at 22°C in a reaction containing 0.6 mg/ml PhK, 60 mM Hepes buffer (pH 6.8), 0.5 mM EGTA $\pm$ 0.7 mM CaCl_2 that was initiated with EEDQ (final concentration 0.86 mM, or 500-fold molar excess to the PhK $\alpha\beta\gamma\delta$ protomer) dissolved in 40% acetonitrile (final concentration 1%, which had no effect on PhK's activity). Quenching was achieved by addition of an equal volume of SDS-PAGE sample buffer (0.125 M Tris / 20% sucrose / 5% 2-mercaptoethanol / 100 mM DTT / 4% SDS, pH 6.8). Quantification of Coomassie-stained gel bands was performed using a Personal Densitometer Scanner (Amersham Biosciences, Piscataway, NJ). Immunoblots were developed using goat anti-mouse IgG alkaline phosphatase (Southern Biotechnology, Birmingham, AL) and stained with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The anti- γ monoclonal antibody was that previously described⁴¹ and the anti-CaM monoclonal antibody was from Calbiochem, San Diego, CA.

Fig. 3.

LC/ESI MS/MS analysis of a γ - δ crosslinked peptide with $m/z = 674$ (MH_2^{2+}). For ESI Mass analyses, chromatographic fractions of the γ - δ digest containing potential crosslinked peptides identified by MALDI TOF analysis were concentrated on a Centrивac concentrator (Labconco, Kansas City, MO) to a final volume of 20 μ l and pressure loaded onto a C18 reverse phase, fused silica nanocolumn (75 μ m $\times$ 9 cm) packed in-house with Magic C18 particles (5 μ m / 100 $\text{\AA}$, Michrom Bioresources, Auburn, CA). The peptides were desalted for 15 min with 0.1 % formic acid at a flow rate of 0.3 μ l/min. The column was then mounted on the electrospray stage of an ESI ion trap mass spectrometer (LCQ Deca XP, ThermoFinnigan, Waltham, MA) and the peptides were separated on-line using a Surveyor LC and a 0–90 % acetonitrile gradient over 120 min at a flow rate of approximately 0.3 μ l/min. An electrospray voltage of 1.9 kV was used, with the ion transfer temperature set to 250 $^{\circ}$ C. The mass spectrometer was controlled by Xcalibur software to continuously perform tandem mass analysis on ions generated from the target crosslinked peptide with the following potential m/z distribution: 1346 (MH^+), 674 (MH_2^{2+}), and 450 (MH_3^{3+}). MS2 and MS3 scans were performed on the expected parent ion and on the most intense peak observed in the MS2 spectrum, respectively. Dynamic exclusion of two repeat scans of the same ion was used, with a 30-sec repeat duration and 90-sec exclusion duration and a mass window of 4 Da. Normalized collision energy for MS2 and MS3 was set to 35%. To eliminate potentially contaminating peptides, all MS2 and MS3 scans of relevant peptide ions were searched using the Sequest algorithm⁴² included in Bioworks 3.1.

(ThermoFinnigan) using the NCBI protein database. The results of the search were filtered using the following set of criteria for low confidence: minimum cross-correlation score of (Xcorr) of 1.5, 2.0 and 2.5 for 1, 2 and 3 ion charges, respectively, and a delta correlation score (Δcorr) greater than 0.08. No significant matches were found for the selected peptide-ion.

Fig. 4.

Crosslinked domains of the δ and γ subunits. The boundaries of the 4 Ca^{2+} -binding EF-hands of CaM are denoted, with D93 occurring just within EF3. The crosslinked K325 of γ occurs within that subunit's N-CBD (297–331), with the boundaries for its 2 CBDs being adapted from Dasgupta et al.⁶ Note that the numbering of the three proteins in this figure corresponds to their rabbit counterparts. The homology between this region of γ and the minimal inhibitory peptide of TnI is shown with a 12-residue insert between L309 and R322 of γ . The original so-called TnI inhibitory peptide (residues 96–116) was derived from CNBr treatment of TnI; however, a shorter peptide (residues 104–115) was found to be nearly as effective in inhibiting actomyosin ATPase.¹⁹ Within this minimal inhibitory peptide residues 104–108 and 111–115 have been shown to be involved in the binding of TnC^{18,20–22} and residues 107, 111, 112, 114 and 115 in the binding of actin.^{18,19,21} Of these 10 critical residues, excepting the conservative substitution in the position of R115, only R108 does not have an identical counterpart in the γ subunit of PhK. Note that without that conservative substitution of the R of TnI to the equivalent K325 of γ , the zero-length crosslinking would not have occurred. The sequences shown for γ and TnI are identical for the skeletal muscle proteins from mouse, rat, rabbit and human, except for a C to A substitution in human γ .