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Brief Communication

A non-equilibrium isoelectric focusing method to determine states of phosphorylation of cardiac troponin I: Identification of Ser-23 and Ser-24 as significant sites of phosphorylation by protein kinase C

Tomoyoshi Kobayashi [a,*], Xiaofeng Yang [b], Lori A. Walker [a], Richard B. Van Breemen [b], R. John Solaro [a]

[a] *Department of Physiology and Biophysics, Center for Cardiovascular Research, College of Medicine, University of Illinois at Chicago, 835 S. Wolcott Avenue (M/C 901), Chicago, IL 60612, USA*
[b] *Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 S. Wood Street (M/C 781), Chicago, IL 60612, USA*

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Abstract

Phosphorylation of cardiac troponin I (cTnI) by cAMP-dependent kinase (PKA), protein kinase C (PKC) and potentially other kinases modulates the activity of myofilaments. To elucidate the signaling mechanisms involving this modulation, it is important to determine the phosphorylation states of cTnI and its phosphorylation sites in a simple and efficient manner. In this report, we describe a method to determine the phosphorylation states of cTnI with non-equilibrium isoelectric focusing gel electrophoresis (NEIEF). Our method easily separates cTnI species with a single-charge difference. To further establish a role of PKC-dependent phosphorylation of cTnI, we have applied this approach to analysis of cTnI phosphorylation in the Tn complex following treatment with recombinant PKC, and in heart samples treated with a phorbol ester. Using mass spectrometry analysis of Tn and thin filaments, we identified Ser-23 and Ser-24 (normally considered to be PKA-dependent sites) as substrates for phosphorylation by PKC-β and PKC-ϵ.

Keywords: Cardiac myofilament; Troponin; Phosphorylation; Protein kinase C; Phosphopeptide; Electrophoresis; MALDI-TOF mass spectrometry

1. Introduction

Phosphorylation of sarcomeric proteins has been shown to occur as protein kinase C (PKC) activity increases during the development of hypertrophy and heart failure [1,2]. Thus, in addition to its stimulation of transcription factors and promotion of cardiac growth, the PKC signaling cascade alters myofilament activity. PKC phosphorylates cardiac troponin I (cTnI), as well as cardiac troponin T (cTnT), and reduces myofilament activity and Ca^{2+}-sensitivity [3–6]. Previous reports demonstrated that Ser-43, Ser-45 and Thr-144 of cTnI are three major PKC-dependent phosphorylation sites [7]. However, there are more recent data indicating the presence of other sites of PKC-dependent phosphorylation on cTn. Swiderek et al. [8] found that pan-PKC phosphorylates Ser-23 and Ser-24 of cTnI in a Tn complex. Moreover, Ruse et al. [9] found that PKC-βII phosphorylates cTnI at Ser-45 but not Ser-43 when they phosphorylated cTnI alone. Recently Roman et al. [10] reported the enhancement of the phosphorylation of cTnI at Ser-23 and Ser-24 without an increase of cAMP levels in hearts of transgenic mice expressing cTnI(Ala-43/Ala-45) to prevent PKC-dependent phosphorylation at these sites.

Abbreviations: cTn, cardiac troponin; cTnI, cardiac troponin I; cTnT, cardiac troponin T; IEF, isoelectric focusing gel electrophoresis; NEIEF, non-equilibrium isoelectric focusing gel electrophoresis; MALDI, matrix-assisted laser desorption ionization; PKC, protein kinase C; PSD, post-source decay; THAP, 2′,4′,6′-trihydroxyacetophenone; TOF, time-of-flight.

* Corresponding author. Tel.: +1-312-996-7151; fax: +1-312-996-1414.
E-mail address: tkoba@uic.edu (T. Kobayashi).

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Although clearly important to understand, it has been difficult to determine phosphorylation states of cTnI due to its high p*I* (~9.5) with potential multiple phosphorylation sites (Fig. 1). We report here a fast and simple non-equilibrium isoelectric focusing gel electrophoresis (NEIEF) to detect the phosphorylation states of cTnI. NEIEF is readily expandable to the Western blot and/or the second dimension, such as SDS electrophoresis, to detect phosphorylation states of cTnI in more complex samples. Furthermore, we determined the PKC-dependent phosphorylation sites on cTnI in a ternary cTn complex and the reconstituted thin filament with MALDI-TOF mass spectrometry using a matrix that improves ionization of phosphopeptides.

2. Materials and methods

2.1. Proteins

Recombinant mouse cTnIs inserted into pET3d vector were expressed using BL21(DE3) cells. The harvested bacteria were suspended in 5% sucrose, 1 mM EDTA and 20 mM Tris–HCl, pH 8.0 with a cocktail of protease inhibitors (1 μg/ml pepstatin A, 5 μg/ml leupeptin and 0.2 mM phenylmethylsulfonyl fluoride) and were sonicated. After centrifugation, the pellet was recovered and cTnI was extracted with 6 M urea, 1 mM EDTA, 20 mM Tris–HCl, pH 8.0 (sol A) with a mixture of protease inhibitors. The suspension was sonicated briefly and centrifuged to remove insoluble fractions. The supernatant fractions were combined and applied to a DE52 column equilibrated with sol A. The flow-through fractions were further applied to a SP-Fast Flow column equilibrated with sol A. cTnI was eluted with a linear gradient of 0.0–0.5 M NaCl in sol A. When necessary, cTnI was further purified using an immobilized cTnC-column. Recombinant human cardiac troponin C (cTnC) and recombinant mouse cTnT were expressed and purified as described [11]. Tropomyosin and actin were purified from bovine left ventricular ether powder as described previously [12]. Recombinant PKC-βII and PKC-ϵ were expressed in Sf-9 cells using baculovirus system and prepared as described [5,6]. Specific activities were 373 and 3844 units/mg, respectively, for PKC-βII and PKC-ϵ with myelin basic protein as a substrate (units of PKC activity were defined as 1 nmol of ^{32}P incorporated into the substrate per minute).

2.2. Reconstitution of Tn complex, Tn–Tm complex and thin filament

Purification of the Tn ternary complex from recombinant components and reconstitution of Tn–Tm complex and the thin filament were carried out as described [13].

2.3. PKC phosphorylation

All the PKC reactions were carried out in 0.1 M NaCl, 10 mM $MgCl_2$, 20 mM HEPES, pH 7.5, 1.0 mM dithiothreitol, 0.02 mM diacylglycerol, 0.3 mM phosphatidyl serine and either 0.1 mM $CaCl_2$ for PKC-βII or 0.5 mM EGTA for PKC-ϵ at 30 °C. The reaction was initiated by the addition of final 0.4 mM ATP and terminated with addition of either final >90% cold acetone followed by centrifugation or final 2% trifluoroacetic acid followed by de-salting with a reversed-phase C4 spin column.

2.4. NEIEF

NEIEF analysis [14,15] was carried out to detect phosphorylation states of cTnI. Our IEF gels contained 8 M urea, 5% acrylamide (acrylamide/bis-acrylamide 29:1; optional 2% Triton X-100), 0.8% ampholyte pH 3.5–10.0 and 1.2% ampholyte pH 7.0–9.0 (Amersham, NJ). Sample loading buffer contains 8 M urea, 10 mM EDTA and 1.0% ampholyte pH 3.5–10.0. The presence of urea and EDTA facilitates the dissociation of TnI from other Tn components. Unlike typical IEF, the lower and upper reservoirs were filled with 0.02 M

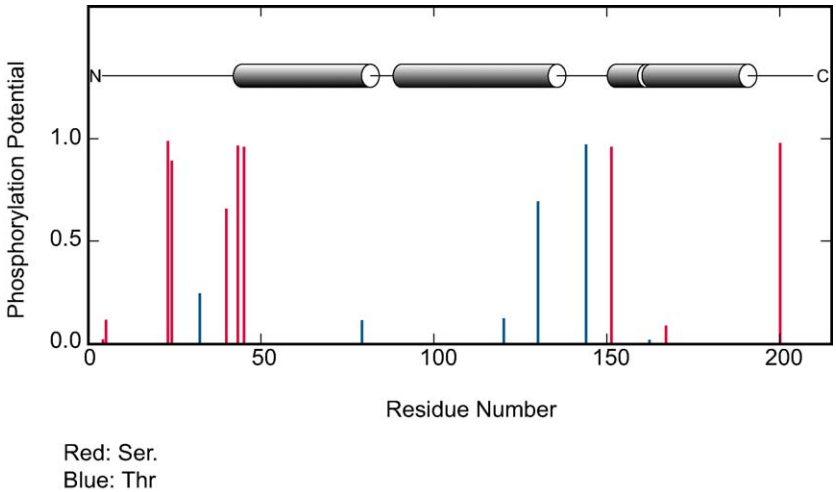

Fig. 1. Prediction of phosphorylation-sites of cTnI. Site specific phosphorylation potential of mouse cTnI by Ser/Thr kinases was predicted by NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/). α-Helical regions determined by X-ray crystal structure of the core domain of cTn complex [17] are also shown.