Scientific Research Publishing

American Journal of Molecular Biology, 2019, 9, 110-120
http://www.scirp.org/journal/ajmb
ISSN Online: 2161-6663
ISSN Print: 2161-6620

Phosphorylation Properties of the N-Terminal Region of Twitchin from Molluscan Catch Muscle

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How to cite this paper: Funabara, D., Nishimura, Y. and Kanoh, S. (2019) Phosphorylation Properties of the N-Terminal Region of Twitchin from Molluscan Catch Muscle. *American Journal of Molecular Biology*, **9**, 110-120.
https://doi.org/10.4236/ajmb.2019.93009

Received: June 6, 2019
Accepted: July 21, 2019
Published: July 24, 2019

Abstract

Molluscan smooth muscles, such as the bivalve adductor muscles and the mussel anterior byssus retractor muscles (ABRM), exhibit a unique contraction called "catch". Catch contraction is regulated through twitchin phosphorylation and dephosphorylation. Twitchin from the ABRM of the Mediterranean mussel, *Mytilus galloprovincialis*, is phosphorylated by cAMP-dependent protein kinase (PKA), and PKA phosphorylation sites are located in both the N- and C-terminal regions of the twitchin molecule. The D2 site, which is adjacently located to the C-terminus, participates in forming a myosin, actin, and twitchin complex that is thought to contribute towards the maintenance of tension in the catch state. In contrast, although it has been reported to interact with thin-filaments, the molecular function of the region including the D1 site has remained largely unstudied. Three additional PKA consensus sequences were identified near the D1 site; however, it was not known if these sites could be directly phosphorylated by PKA. Here, we performed phosphorylation assays to identify phosphorylation sites near the D1 site using recombinant protein variants (TWD1-SSSS, TWD1-AAAS, TWD1-AASA, TWD1-ASAA, TWD1-SAAA, and TWD1-AAAA). All variants, except TWD1-AAAA (where all phosphorylatable serine residues were replaced by alanines), were phosphorylated by PKA. The four phosphorylation sites were named D1-1, D1-2, D1-3, and D1-4 (the originally identified D1) in order from the N-terminus. Phosphorylation assays using a 1/12.5 weight ratio of PKA to each TWD1 variant revealed that D1-4 was the most rapidly phosphorylated, closely followed by D1-1. However, D1-2 and D1-3 were phosphorylated at a lower level under equivalent conditions and were not phosphorylated when PKA was incubated with each TWD1 variant at a 1/100 weight ratio. Furthermore, we observed that TWD1-SSSS was phosphorylated in a stepwise fashion. These findings contribute towards the elucidation of the function of the twitchin D1 region in the regulatory system of catch contraction.

Keywords

Catch Contraction, Phosphorylation, Phos-Tag Gel Electrophoresis, PKA, Twitchin

1. Introduction

Molluscan smooth muscles, such as bivalve adductor muscles and mussel anterior byssus retractor muscles (ABRM), exhibit a unique contraction called "catch". Catch muscles can develop and maintain a high tension for long periods with little energy expenditure [1]. They begin to contract following an increase in intracellular Ca^{2+} concentration, which is induced by acetylcholine secretion [2]. Once the intracellular Ca^{2+} concentration decreases to resting levels, the muscle enters the catch state to retain the tension for long periods. An increase in intracellular cAMP levels induced by serotonin secretion leads to protein kinase A (PKA) activation and the phosphorylation of twitchin, a giant protein of the titin/connectin family [3] [4]. However, twitchin must be dephosphorylated by calcineurin, a calcium- and calmodulin-dependent serine/threonine protein phosphatase, to allow the catch muscle to reenter the catch state [5] [6]. Therefore, the phosphorylation and dephosphorylation states of twitchin regulate catch contraction.

Twitchin from the ABRM of the Mediterranean mussel, *Mytilus galloprovincialis*, is composed of 4736 amino acids and contains a single kinase domain and multiple immunoglobulin (Ig) and fibronectin III (FnIII) motifs [7]. Twitchin is rapidly phosphorylated by PKA *in vitro*, with a stoichiometry of 3 moles of phosphate per mole of twitchin [4]. However, only two phosphorylation sites, D1 and D2, have been identified to date [7]. The D2 site is located in a linker region connecting Ig motifs 21 and 22. The function of the D2 site has been studied in great detail. A twitchin D2 fragment containing the D2 site and its flanking Ig motifs were shown to form a trimeric complex with a myosin head and actin in a phosphorylation-sensitive manner [8]. The binding site of the twitchin D2 fragment on actin was found to overlap with the actin region that electrostatically interacts with loop 2 of myosin to initiate the movement of myosin over actin filaments. In addition, loop 2 of myosin was able to bind to the twitchin D2 site [9]. The formation of the myosin, actin, and twitchin complex is thought to contribute towards the maintenance of tension in the catch state. Therefore, the tethering of thick- and thin-filaments by twitchin is likely to be an essential event in catch contraction [10] [11].

The twitchin D1 site is located in the linker region between Ig motifs 7 and 8. Three additional PKA consensus sequences (K/R-K/R-X-S) are adjacently located to the D1 site: S-1011, which was referred to as DX in our previous study and has an identical sequence to D1 (RRPSLVD); S-1043 (RKLSRDN); and S-1067 (RRSSMQQ) [7]. However, it remains unclear whether these sites can be

directly phosphorylated by PKA. A previous study has shown that the twitchin N-terminal region, which includes the D1 site, interacts with thin filaments in a phosphorylation-sensitive manner [12]. Co-sedimentation and solid-phase binding assays were performed using recombinant proteins comprising the D1 region (DXD1) and DXD1 with its flanking Ig motifs (IGDXD1IG) and thin-filaments isolated from *Mytilus* ABRM. The D1 region was predicted to bind to actin because a DRFXXL actin-binding motif is located near to the D1 site. We believe that the elucidation of the phosphorylation properties of the D1 region is required to investigate its molecular function through its interaction with thin-filaments.

To contribute towards the elucidation of the D1 function in catch contraction, we sought to identify which of the potential D1 sites are phosphorylated by PKA by performing phosphorylation assays with recombinant protein variants (TWD1-SSSS, TWD1-AAAS, TWD1-AASA, TWD1-ASAA, TWD1-SAAA, and TWD1-AAAA).

2. Materials and Methods

2.1. Protein Preparation

The twitchin D1 region was expressed in *Escherichia coli* as a histidine-tagged protein (TWD1-SSSS) (Figure 1): a DNA fragment encoding the linker region including the D1 site between Ig motifs 7 and 8 was codon-optimized for expression

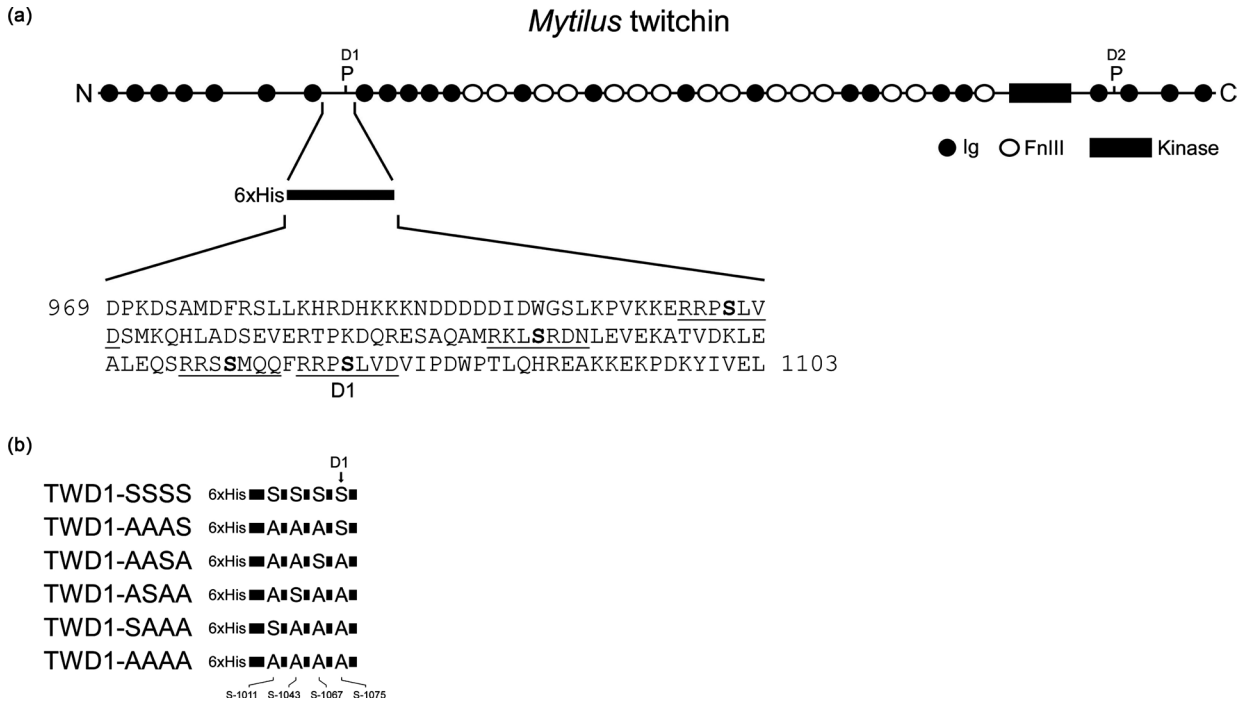

Figure 1. Schematic representation of *Mytilus galloprovincialis* twitchin. (a) Motif structure of the Mytilus twitchin molecule and the recombinant D1 region used in this study. "P" represents the D1 and D2 phosphorylation sites determined by our previous study. Underlined amino acid sequences indicate PKA consensus sequences. Phosphorylatable serine residues are shown in bold type. The numbers at the beginning and end of the amino acid sequence represent their positions from the N-terminus. Ig: immunoglobulin motif; FnIII: fibronectin type III motif; Kinase: kinase domain; (b) Schematic representations of the recombinant proteins prepared in this study.

in *E. coli* and commercially synthesized (GenScript, Piscataway, NJ, USA). This was cloned into the T7 expression vector pET15b (Novagen, Darmstadt, Germany), creating an N-terminal fusion of six histidine residues. The TWD1-SSSS variants, TWD1-AAAS, TWD1-AASA, TWD1-ASAA, TWD1-SAAA, and TWD1-AAAA, with potential phosphorylatable serine residues replaced by alanines, were commercially constructed by site-directed mutagenesis (GenScript) (**Figure 1**). *E. coli* BL21(DE3) cells separately transformed with the expression plasmids pET-TWD1-SSSS, pET-TWD1-SSSA, pET-TWD1-SSAS, pET-TWD1-SASS, pET-TWD1-ASSS, or pET-TWD1-AAAA were cultured in auto-induction media at 37˚C for 24 h [13]. The expressed recombinant proteins were purified as previously reported [8]. The catalytic subunit of mouse protein kinase A (PKA) (accession number NP_032880.1) was also expressed in *E. coli* as a histidine tagged protein: a DNA fragment encoding the mouse PKA catalytic subunit was codon-optimized for expression in *E. coli* (GenScript) and cloned into the pET15b vector (Novagen) to create pET-Mouse-PKA. *E. coli* BL21(DE3) cells transformed with pET-Mouse-PKA were cultured in autoinduction media at 20˚C for 48 h and the protein was purified as described above.

2.2. Phosphorylation of TWD1-SSSS and Its Variants

PKA phosphorylation assays of purified TWD1-SSSS and its variants were performed as previously described [4]. Briefly, 200 µl of 0.6 mg/ml TWD1-SSSS in 20 mM Tris-HCl (pH 7.5) containing 100 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, and 0.5 mM DTT were mixed with 0.048, 0.12, or 0.06 mg/ml of purified mouse PKA catalytic subunit (PKA) and incubated at 25˚C for 10 min. Aliquots (20 µl) were removed from the reaction mixture at 1, 2, 3, 5, and 10 min intervals and the reaction was stopped by the addition of an equal volume of SDS sample buffer (20 mM Tris-HCl pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 40% glycerol, 4 mM EDTA, and 0.015% bromophenol blue). After boiling for 2 min, samples were analyzed by Phos-tag acrylamide gel electrophoresis. All phosphorylation assays were performed in triplicate.

2.3. Phos-Tag Acrylamide Gel Electrophoresis

SuperSep Phos-tag (50 µM) 12.5% acrylamide gel electrophoresis (FUJIFILM Wako Pure Chemicals, Osaka, Japan) was used to detect unphosphorylated and phosphorylated proteins. Electrophoretic patterns were analyzed using an iBright Imaging System (Thermo Fisher Scientific, San Jose, CA, USA) following Coomassie blue staining. Band intensities of unphosphorylated and phosphorylated proteins were used to calculate the phosphorylation level of TWD1-SSSS and its variants.

3. Results

3.1. Identification of Twitchin D1 Phosphorylation Sites

Phos-tag acrylamide gel electrophoresis is an analytical tool that identifies pro-

tein phosphorylation by retarding the mobility of phosphorylated proteins compared to their unphosphorylated counterparts. Therefore, to identify if TWD1-SSSS or its variants were able to be phosphorylated by PKA, proteins incubated in the presence or absence of PKA were subjected to Phos-tag gel electrophoretic analysis. Incubation of TWD1-SSSS, TWD1-AAAS, TWD1-AASA, TWD1-ASAA, and TWD1-SAAA with PKA resulted in an electrophoretic mobility shift of these proteins on Phos-tag gels compared to their untreated counterparts, indicating that these proteins were indeed phosphorylated by PKA (Figure 2). In contrast, the mobility of TWD1-AAAA (where all phosphorylatable serine residues were replaced by alanines) remained unchanged following PKA treatment, indicating that it was not phosphorylated. Our results clearly showed that all four of the identified PKA consensus sequences were able to be phosphorylated. Therefore, we named these phosphorylation sites D1-1 (originally DX), D1-2, D1-3, and D1-4 (originally D1) to reflect their position from the N-terminus. We then performed further phosphorylation assays with each of the TWD1 variants to characterize the phosphorylation properties of each of the identified phosphorylation sites in more detail.

3.2. Phosphorylation Properties of D1-1, D1-2, D1-3, and D1-4

Prediction of PKA phosphorylation preferences of the four sites using NetPhos 3.1 server (http://www.cbs.dtu.dk/services/NetPhos/) [14] showed high scores of 0.839 (D1-1), 0.833 (D1-2), 0.804 (D1-3), and 0.879 (D1-4), respectively. Based on these results, we predicted that D1-4 is phosphorylated first, followed by D1-1, D1-2, and finally D1-3.

To confirm our prediction, we then performed a phosphorylation time course with each of the variants in the presence of varied amounts of PKA (using 1/12.5 and 1/100 weight ratios of PKA to each TWD1 variant). All samples were then

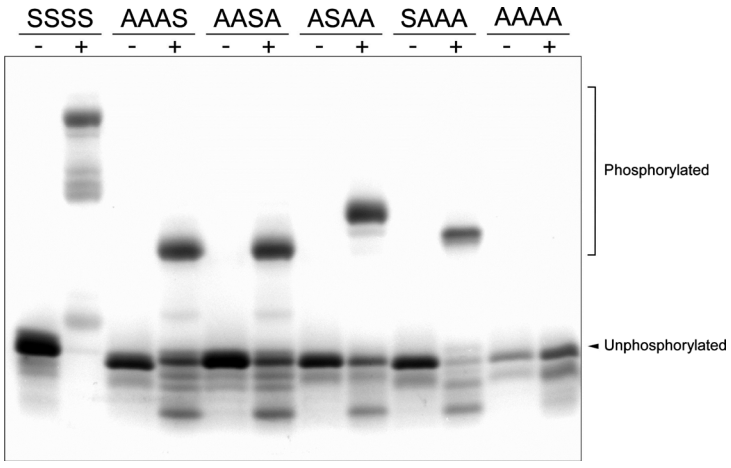

Figure 2. PKA-mediated phosphorylation of TWD1 variants. TWD1 variants incubated in the presence (+) and absence (−) of PKA were analyzed by Phos-tag gel electrophoresis and Coomassie blue staining. The arrowhead indicates the position of unphosphorylated proteins. SSSS: TWD1-SSSS; AAAS: TWD1-AAAS; AASA: TWD1-AASA; ASAA: TWD1-ASAA; SAAA: TWD1-SAAA; and AAAA: TWD1-AAAA.

analyzed by Phos-tag gel electrophoresis and Coomassie blue staining (Figure 3). We found that the TWD1-AAAS variant was fully phosphorylated in 1 min in the presence of a 1/12.5 weight ratio of PKA to TWD1-AAAS. As would be expected, the phosphorylation rate of TWD1-AAAS was slower when the weight ratio of PKA to TWD1-AAAS was decreased to 1/100; however, TWD1-AAAS was still fully phosphorylated in 10 min. In contrast, the phosphorylation rate of TWD1-AASA and TWD1-ASAA in the presence of a 1/12.5 weight ratio of PKA to the TWD1 variant was slower compared with TWD1-AAAS. No phosphorylation of either variant was detected when the weight ratio of PKA to either TWD1-AASA or TWD1-ASAA was decreased to 1/100. Like TWD1-AAAS, the TWD1-SAAA variant was phosphorylated rapidly in the presence of a 1/12.5 weight ratio of PKA to TWD1-SAAA, which become slower when the weight ratio of PKA to TWD1-SAAA was decreased to 1/100. We then used the electrophoretic patterns of each of the phosphorylated TWD1 variants to calculate their relative phosphorylation levels (Figure 4). We concluded that TWD1-AAAS was most rapidly phosphorylated by PKA at both tested weight ratios. Phosphorylation of TWD1-SAAA was marginally slower; however, after 5 min, almost all molecules were fully phosphorylated at a 1/12.5 weight ratio of PKA to TWD1-SAAA. However, the TWD1-AASA phosphorylation was significantly slower and reached 100% in 10 min at a 1/12.5 weight ratio of PKA to TWD1-AASA. The slowest phosphorylation rate was observed at a 1/12.5 weight ratio of PKA to TWD1-ASAA.

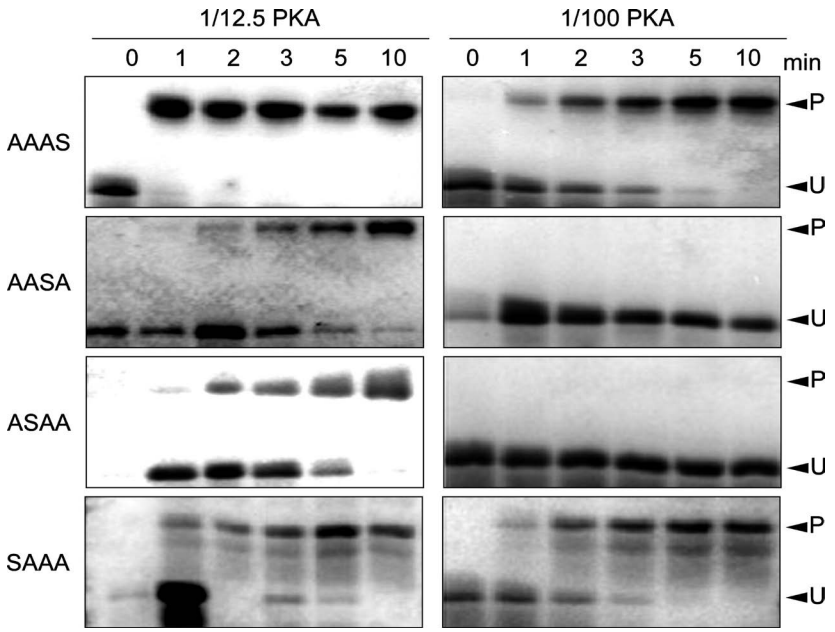

Figure 3. Time-course of TWD1 variant phosphorylation. TWD1 variants treated with PKA were analyzed by Phos-tag gel electrophoresis and Coomassie blue staining. "P" and "U" on the right-hand side of the gel images indicate protein bands of the phosphorylated and unphosphorylated variant forms, respectively. The numbers on the top of the gel images denote reaction times. "1/12.5 PKA" and "1/100 PKA" indicate the weight ratio of PKA to TWD1 variants in the reaction mixtures. AAAS: TWD1-AAAS, AASA: TWD1-AASA, ASAA: TWD1-ASAA, SAAA: TWD1-SAAA.

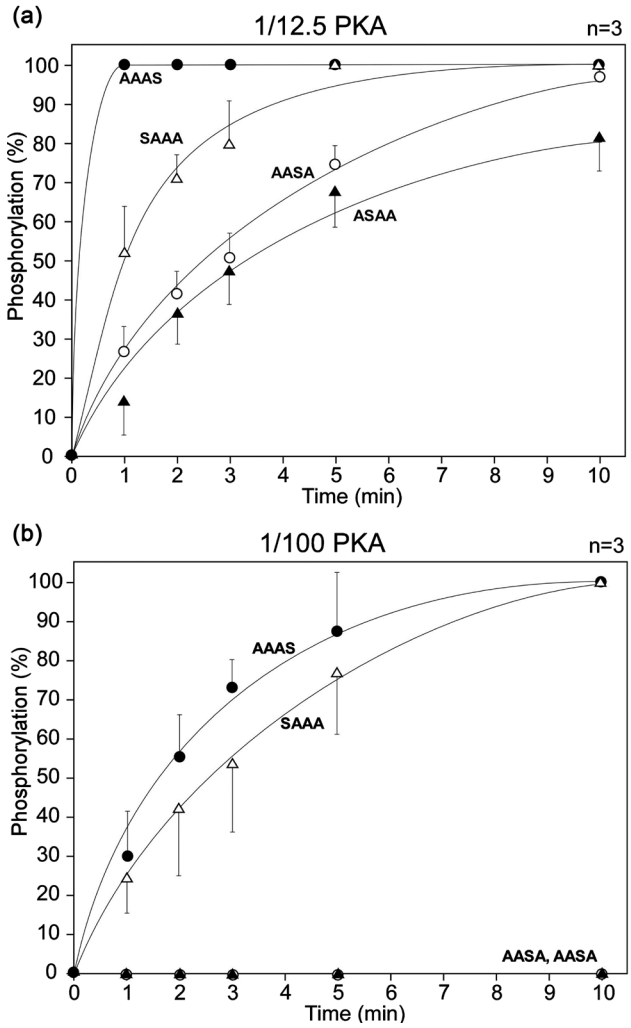

Figure 4. Phosphorylation curves of TWD1 variants. (a) Phosphorylation by a 1/12.5 weight ratio of PKA to TWD1 variants; (b) Phosphorylation by a 1/100 weight ratio of PKA to TWD1 variants. The black circles, white triangles, white circles, and black triangles represent the phosphorylation levels of TWD1-AAAS, TWD1-SAAA, TWD1-AASA, and TWD1-ASAA, respectively. Error bars represent standard deviations.

In contrast, a 1/100 weight ratio of PKA was unable to phosphorylate either TWD1-AASA or TWD1-ASAA. These findings indicate that PKA has a phosphorylation target preference in the twitchin D1 region and suggests that the phosphorylation of the D1 region occurs in a stepwise fashion.

To examine in more detail if the twitchin D1 region is phosphorylated in a stepwise manner, we studied the phosphorylation of TWD1-SSSS by PKA. For this purpose, we used a 1/50 weight ratio of PKA to TWD1-SSSS (**Figure 5**). Each reaction was then analyzed by Phos-tag gel electrophoresis and Coomassie blue staining. All three assays showed an almost identical electrophoretic pattern and multiple phosphorylated bands with differing mobilities were observed after incubating the mixture for 10 min, suggesting that some, but not all of the TWD1-SSSS phosphorylation sites were phosphorylated at this time. This observation led us to conclude that the D1 sites of TWD1-SSSS are phosphorylated

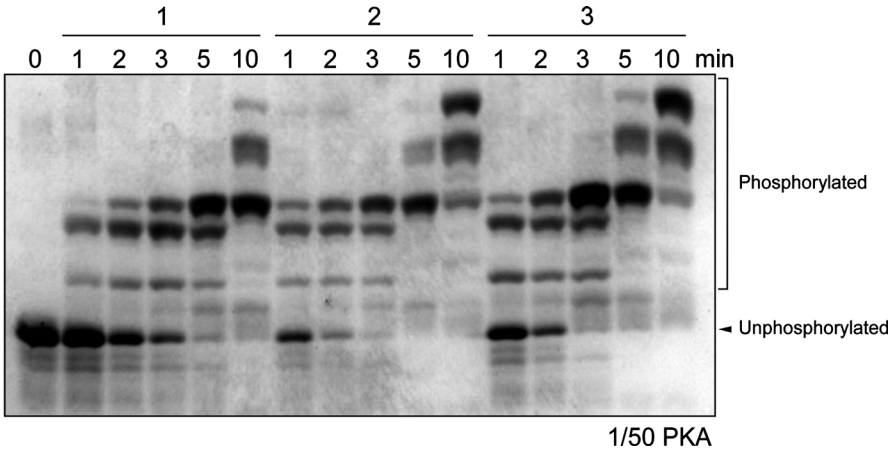

Figure 5. Time-course of TWD1-SSSS phosphorylation. A 1/50 weight ratio of PKA to TWD1-SSSS was incubated at 25˚C then analyzed by Phos-tag gel electrophoresis. The arrowhead indicates the position of unphosphorylated TWD1-SSSS. The numbers 0, 1, 2, 3, 5, and 10 directly above the gel image indicate reaction times (in min). The numbers 1, 2, and 3 above the reaction times indicate assay numbers.

in a stepwise fashion. Based on our findings, unlike the prediction by NetPhos 3.1, we propose that D1-4 is phosphorylated first, followed by D1-1, D1-3, and finally D1-2.

4. Discussion

Here, we have identified all of the phosphorylation sites located near the D1 site of twitchin from the ABRM of the Mediterranean mussel, *Mytilus galloprovincialis*. We found that four phosphorylation sites are present in the D1 region, meaning that, in total, five phosphorylation sites are present in *Mytilus* twitchin: D1-1, D1-2, D1-3, D1-4, and D2. We previously reported that the incubation of a 1/100 weight ratio of PKA with *Mytilus* twitchin *in vitro* resulted in twitchin phosphorylation, with 3 moles of phosphate incorporated per mole of twitchin [4]. This led to the proposal that *Mytilus* twitchin contains two major and an unspecified number of minor phosphorylation sites [7]. As shown in **Figure 3** and **Figure 4**, D1-2 and D1-3 were not phosphorylated in the presence of a 1/100 weight ratio of PKA to TWD1 variant. This means that PKA mixed with a TWD1 variant at a weight ratio of 1/100 would only be capable of phosphorylating twitchin at three sites: D1-1, D1-4, and D2, which explains our previous observations.

At present, we have no available data that how much amount of PKA is involved in phosphorylation of twitchin *in vivo*. Therefore, we need to investigate if all of the phosphorylation sites identified in this study are really phosphorylated *in vivo* when catch is released. More, it was predicted that there are several potential PKA recognition sites with lower scores by NetPhos 3.1. These sites might be somewhat phosphorylated by PKA, though we were unable to detect phosphorylation except D1-1, D1-2, D1-3, and D1-4 in this study. The method using mass spectrometry developed by Beausoleil *et al.* (2006) may be suitable to

answer these questions [15]. This method would be also helpful to determine the order of the phosphorylation of D1-1, D1-2, D1-3, and D1-4 more accurately. We are planning to perform this method in the future study.

A twitchin region including the D2 phosphorylation site is able to form a trimeric complex with actin and myosin. This is thought to tether thick- and thin-filaments together to maintain the tension in the catch state [8] [9]. The D1 region (containing D1-1, D1-2, D1-3 and D1-4) has been reported to interact with thin-filaments, suggesting that, like D2, it could play a role in the maintenance of catch tension [12]. However, as an actin binding motif (RFXXL) is located upstream of the D1-1 site (referred to as DX in our previous study), it is likely that the D1 region interacts directly with actin in a phosphorylation-sensitive manner [12]. In this study, we revealed that D1-1 and D1-4 are readily phosphorylated, which raises the possibility that the phosphorylation of these sites is involved in the release of thin-filaments from thick-filaments, which are connected by the unphosphorylated D1 region. Currently, how the twitchin D1 region interacts with thin-filaments is unknown, and further studies are required to elucidate the molecular mechanism of this process.

Tissue distribution analysis of the *M. galloprovincialis* twitchin gene revealed that tissue-specific twitchin isoforms are expressed by alternative splicing [16]. The D2 phosphorylation region is well conserved between all twitchin isoforms expressed in catch and phasic muscles. In contrast, some of the twitchin isoforms expressed in phasic muscle were found to lack the D1-1, D1-2, and D1-3 phosphorylation sites. This suggests that the D1-1, D1-2, and D1-3 sites are essential for the catch mechanism. We believe that the D1-1 site is a major regulation site as it is phosphorylated most rapidly. Rapid phosphorylation of D1-1 and D1-4 might readily induce a conformational change of the D1 region, resulting in decreasing its actin affinity.

Scallop twitchin isoforms isolated from adductor striated and catch muscles were found to have different phosphorylation properties: 0.6 and 1.7 moles of phosphate were incorporated into one twitchin molecule from catch and striated muscles, respectively [17]. These findings mean that there are different numbers of phosphorylation sites in scallop twitchin isoforms. Phosphorylation sites of scallop twitchin have been unidentified so far. Since *Mytilus* twitchin isoforms share the D2 site and have different number of phosphorylation sites in the D1 region, it is considered that scallop catch twitchin shares one phosphorylation site corresponding to D2 of *Mytilus* twitchin with striated twitchin and has the additional phosphorylation site corresponding to D1 of *Mytilus* twitchin. This raises the possibility that twitchin isoforms from different molluscan species contain different numbers of D1 phosphorylation sites. This could alter their affinity for thin-filaments in catch and phasic muscles and determine the properties of catch and phasic muscle contraction.

Acknowledgements

This study was supported by JSPS KAKENHI Grant Number JP16K07872. We

thank Emma Andrew, PhD, from Edanz Group (https://www.edanzediting.com/?utm_source=ack&utm_medium=journal) for editing a draft of this manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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