Phosphorylation of Molluscan Twitchin by the cAMP-Dependent Protein Kinase[†,‡]

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ABSTRACT: Catch in certain molluscan muscles is released by an increase in cAMP, and it was suggested that the target of cAMP-dependent protein kinase (PKA) is the high molecular weight protein twitchin [Siegman, M. J., Funabara, J., Kinoshita, S., Watabe, S., Hartshorne, D. J., and Butler, T. M. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 5384−5388]. This study was carried out to investigate the phosphorylation of twitchin by PKA. Twitchin was isolated from *Mytilus* catch muscles and was phosphorylated by PKA to a stoichiometry of about 3 mol of P/mol of twitchin. There was no evidence of twitchin autophosphorylation. Two phosphorylated peptides were isolated and sequenced, termed D1 and D2. Additional cDNA sequence for twitchin was obtained, and the D2 site was located at the C-terminal side of the putative kinase domain in a linker region between two immunoglobulin C2 repeats. Excess PKA substrates, e.g., D1 and D2, blocked the reduction in force on addition of cAMP, confirming the role for PKA in regulating catch. Papain proteolysis of ^{32}P-labeled twitchin from permeabilized muscles showed that the D1 site represented about 50% of the ^{32}P labeling. Proteolysis of in-situ twitchin with thermolysin suggested that the D1 and D2 sites were at the N- and C-terminal ends of the molecule, respectively. Thermolysin proteolysis also indicated that D1 and D2 were major sites of phosphorylation by PKA. The direct phosphorylation of twitchin by PKA is consistent with a regulatory role for twitchin in the catch mechanism and probably involves phosphorylation at the D1 and D2 sites.

A general characteristic of smooth muscles is the ability to maintain force with a very low expenditure of energy and a slow cross-bridge cycling rate (*1*). In mammalian smooth muscle, this condition was called "latch" (*2*) because of the similarity of its mechanical and energetic properties to those of invertebrate "catch" muscles. In certain molluscan smooth muscles, force can be maintained at near-resting Ca^{2+} levels. This condition, termed "catch" nearly a century ago (*3*), can persist for several hours and is associated with reduced energy usage and a marked decrease in cross-bridge cycling rate (*4*−*7*). [For a more detailed perspective on catch, see (*8*).] In the specialized catch muscles, a phasic contraction is initiated by cholinergic stimulation that causes a transient increase in [Ca^{2+}] and binding of Ca^{2+} to myosin (*9*, *10*). In the absence of additional signals, the reduction in [Ca^{2+}] levels leads to catch. However, the subsequent stimulation of serotonergic nerves causes rapid relaxation. The two nerve types can be stimulated simultaneously to produce a twitch-like contraction. The second messenger involved in the serotonin response is cAMP (*11*, *12*), and thus the activation of cAMP-dependent protein kinase (PKA).[1] Addition of the catalytic subunit of PKA to permeabilized molluscan smooth muscle fibers also resulted in the release of catch (*13*).

Paramyosin is present in the muscles of many invertebrates, and this is a major difference between the molluscan and vertebrate smooth muscle. Paramyosin forms the core of the thick filaments with myosin covering the surface (*14*). The molluscan thick filaments are thicker (∼40 nm) and longer (up to 20 μm) than their vertebrate counterparts. There appears to be a correlation between the paramyosin content of a muscle and its force production (*15*), and in catch muscles, there is a high proportion of paramyosin (*16*). Other components of the molluscan thick filament include twitchin [a member of the twitchin−titin superfamily of giant proteins identified first in *C. elegans* (*17*)] and catchin (*18*).

With the established role of PKA in the release of catch, an obvious objective was to determine the site(s) of phosphorylation, and the focus was primarily on proteins of the contractile apparatus. Phosphorylation has been reported for paramyosin (*19*, *20*), myosin heavy chains (*13*, *21*, *22*), and the regulatory light chains of myosin (*23*, *24*). One of the

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[1] Abbreviations: PKA, cAMP-dependent protein kinase; ABRM, anterior byssus retractor muscle; RACE, rapid amplification of cDNA ends; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); PVDF, poly(vinylidene difluoride); HPLC, high-pressure liquid chromatography; ECL, enhanced chemiluminescence; KLH, keyhole limpet hemocyanin; MLCK, myosin light chain kinase; IgC2, immunoglobulin C2 domain.

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two phosphorylated sites identified in myosin heavy chains (*21*) was claimed recently to be in the N-terminal domain of catchin (*18*). However, for these studies, correlations between physiological or mechanical properties and the phosphorylation status of a given protein were not carried out. An exception to this was the demonstration that the catch state is regulated via the cAMP-dependent phosphorylation of a high molecular weight protein (*8*) using intact and permeabilized catch muscle (the ABRM from *Mytilus*). It was shown that the phosphorylation of this protein (and not other components) correlated with the cAMP-induced release of catch and that the mechanical properties of the ABRM fibers were dependent on the extent of its phosphorylation. The high molecular weight protein subsequently was identified as the molluscan homologue of twitchin (*25*). With regard to the role of twitchin in the catch mechanism, it was proposed that phosphorylation of twitchin directly modulates contractile protein interaction(s) probably via a highly cooperative process. A surprising aspect was that the effect of twitchin phosphorylation was manifest over a range of submaximal [Ca^{2+}] (not just at close to basal [Ca^{2+}] as thought previously) and caused a decreased Ca^{2+}-sensitivity of force production (*25*).

Our objective in the present studies was to characterize the phosphorylation of twitchin in more detail. It was necessary to document the in vitro phosphorylation of isolated twitchin by PKA and to investigate sites of phosphorylation.

MATERIALS AND METHODS

Materials. Anterior byssus retractor muscle (ABRM) was obtained from fresh mussels, *M. edulis* (Kip's Seafood, Cushing, ME) or *M. galloprovincialis* (Tokyo Central Wholesale Market). For protein preparations, ABRMs were frozen in liquid N_2, and for fiber preparation, the ABRMs were permeabilized in 1% Triton X-100 in rigor solution, as described (*25*). Materials and vendors were as follows: catalytic subunit of PKA (Upstate Biotechnology); peptide (sequence 5−24) and protein inhibitors of PKA, L-1-tosylamido-2-phenylethyl chloromethyl ketone−trypsin, wortmannin, 1-(5-chloronaphthalene-1-sulfonyl)homopiperazine hydrochloride (ML-9), Malantide, Kemptide, thermolysin, and papain (Sigma); pepstatin A and leupeptin (Calbiochem). All other chemicals were of the highest grade available.

Isolation of Twitchin. Twitchin was isolated from ABRM using a modification of the mini-titin procedure of Vibert et al. (*26*). ABRMs were ground into small pieces in liquid N_2, suspended in 10 volumes of buffer A on ice [40 mM NaCl, 2 mM $MgCl_2$, 10 mM sodium phosphate (pH 7.0), 0.5 mM EGTA, 0.1 mM dithiothreitol, 50 μg/mL leupeptin, 50 μg/mL pepstatin A], and centrifuged at 10000*g* for 10 min. This washing procedure with buffer A was repeated twice. The resulting myofibrillar pellet was extracted with 10 volumes of 200 mM potassium phosphate (pH 7.0), 2 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM dithiothreitol, 3 mM NaN_3, and 0.1 mM PMSF with stirring for 30 min on ice. After centrifugation at 12000*g* for 30 min, the supernatant was dialyzed against buffer B [30 mM KCl, 50 mM Tris-HCl (pH 8.0), 0.5 mM EGTA, 0.1 mM dithiothreitol, 3 mM NaN_3, and 0.1 mM PMSF]. The insoluble proteins were removed by centrifugation at 12000*g* for 10 min, and the supernatant was applied to HPLC on a DEAE-5PW column (Tosoh, 0.75 × 7.5) equilibrated with buffer B. Elution was carried out with a 30−600 mM KCl gradient in buffer B. Fractions containing twitchin (selected after SDS−PAGE) were combined and subjected to high-speed gel filtration (5 mL/min) with a G3000SWG column (Tosoh, 2.15 × 60 cm) in 150 mM NaCl, 10 mM sodium phosphate (pH 6.8), 3 mM NaN_3, and 0.1 mM PMSF. Final selection of purified twitchin was made after SDS−PAGE of the fractions from gel filtration. The yield of purified twitchin was approximately 2 mg/10 g of ABRM wet weight.

Phosphorylation Assays. Assays were carried out following the method of Ferrari and Thomas (*27*). Twitchin, approximately 0.2 mg/mL, in 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol was incubated at 25 °C with the catalytic subunit of PKA (1 μg/mL) and 1 mM [γ-^{32}P]ATP (7.4 MBq/mL). At varying times, aliquots of 50 μL were removed and spotted onto phosphocellulose disks (Whatman P81). The disks were washed 3 times in 0.5% phosphoric acid and then dehydrated in acetone for 2 min. After drying, radioactivity was determined by Cerenkov counting in a Tri-Carb 1500 scintillation counter (Packard). Phosphorylation assays also were carried out in the absence of the catalytic subunit of PKA to assess endogenous kinase in the twitchin preparations. Various kinase inhibitors were assayed to test their effects on the endogenous kinase. For autoradiography, the samples from the phosphorylation assays were stopped by the addition of an equal volume of SDS sample buffer [2% SDS, 2% 2-mercaptoethanol, 20 mM Tris-HCl (pH 6.8), 40% glycerol, 4 mM EDTA, and 0.015% bromophenol blue]. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 and exposed overnight to an X-ray film (Fuji Photo Film RX-u).

Fiber Experiments. The ABRM was dissected and permeabilized by incubation for 30 min in a rigor solution containing 1% Triton X-100. Measurements of force production were made under isometric conditions using a mechanical apparatus which was described previously (*8*, *25*). The detailed compositions of the rigor (no ATP), relaxing (ATP, pCa >8), and activating (ATP, pCa 5 or pCa 6) solutions are the same as those used in earlier studies (*8*, *25*). Twitchin was phosphorylated by incubating the muscle for 5 min in a relaxing solution containing 100 μM cAMP. When appropriate, the relaxing solution also contained [γ-^{32}P]ATP (25 μCi/mL). Muscles were frozen in liquid N_2 and pulverized in frozen 0.5 N $HClO_4$. After thawing and centrifugation, the protein precipitate was solubilized in SDS sample buffer and passed through a 0.4 μm filter before being subjected to SDS−PAGE.

Identification of Phosphorylated Residues. Isolated twitchin was phosphorylated in the presence of [γ-^{32}P]ATP by the catalytic subunit of PKA at 25 °C for 15 min. The reaction was stopped by addition of TCA (final concentration 10%). After centrifugation, the precipitate was hydrolyzed in 6 N HCl at 110 °C for 90 min. The hydrolysate was applied to a 10 × 20 cellulose plate and subject to electrophoresis at 1000 V for 45 min (with cooling). Phosphoserine, phosphothreonine, and phosphotyrosine were used as standards (*28*, *29*). The plate was exposed overnight to an X-ray film to determine phosphoamino acid(s).

cDNA Cloning. Total RNA was prepared from ABRM of *Mytilus galloprovincialis* (*30*). First-strand cDNA was

synthesized using 3′RACE (Gibco, BRL). PCR amplification was carried out using the 5′ primer, 5′-CGCTATTGGTCG-TATTGCAAAC-3′, designed from the sequence of the kinase domain of *Mytilus* twitchin (*25*). The 3′ primer, AUAP, was included in the 3′RACE kit. The conditions for PCR were: 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 0.5 min, annealing at 60 °C for 0.5 min, polymerization at 72 °C for 1.5 min, and extension at 72 °C for 7 min. The DNA fragment amplified by PCR was subcloned into the plasmid vector pT7 Blue. Sequencing was performed using the Dye Deoxy terminator cycle sequencing kits with the DNA sequencer model 373S (Perkin-Elmer).

Proteolysis Experiments. Thermolysin treatment of the permeabilized muscles was carried out in a pCa 5 rigor solution for 15 min. In some cases, the muscles were treated with ATPγS (0.1 mM, 140 μCi/mL [^{35}S]ATPγS) and cAMP (0.1 mM) to thiophosphorylate twitchin (*8*) before proteolysis, or [γ-^{32}P]ATP and cAMP after proteolysis. In these cases, the gels were scanned on a laser densitometer (Molecular Dynamics) to determine the amount of native and proteolyzed twitchin, and following drying, autoradiography of the gel was performed with a phosphoimager (Molecular Dynamics).

In-gel papain proteolysis of twitchin was performed as described by Cleveland (*31*). Twitchin was phosphorylated in the presence of [γ-^{32}P]ATP in permeabilized muscles. The protein from the muscle was initially subjected to SDS−PAGE on a 4% acrylamide gel. The gel was stained with Coomassie Blue and radioactivity determined by exposure to the phosphoimager. The portion of the gel which contained twitchin was cut out and incubated in gel equilibration buffer (*31*) for 1 h with several changes of solution. The gel was then placed in a lane of a stacking gel of 4% acrylamide and a resolving gel of 15% acrylamide with 0.1 mL of gel slice overlay solution (*31*) containing 2 μg of papain. The gel was run at 160 V until the tracking dye approached the resolving gel, at which time the power was interrupted for 15 min. The gel was then run at 300 V with cooling (10 °C) until the tracking dye reached the bottom of the gel. The peptides were blotted onto PVDF which was then exposed to the phosphoimager. The PVDF was used to determine the antibody binding characteristic of the peptides using ECL detection (Amersham).

Isolation of Phosphorylated Peptides. Isolated twitchin was phosphorylated with the catalytic subunit of PKA at 25 °C for 10 min (as described above). TCA was added (final concentration of 10%), and after centrifugation, the pellet was dissolved in 50 mM NH_4CO_3 (pH 8.0) and digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone−trypsin (trypsin:twitchin weight ratio of 1:50) at 37 °C for 24 h. The digests were applied to HPLC with a ODS-120A reverse phase column (Tosoh, 4.6 mm × 25 cm). A linear gradient from 0 to 100% acetonitrile in 0.08% trifluoroacetic acid was used for elution. Peaks containing ^{32}P were collected and peptide sequences determined (Perkin-Elmer Applied Biosystems, model 476A).

SDS−PAGE. SDS−polyacrylamide gel electrophoresis was carried out with gels of different acrylamide concentrations. For increased separation of high molecular weight components, gels of 4−5% acrylamide were used. In other applications 10% acrylamide was used. Buffer conditions were those of Laemmli (*32*).

Antibodies Raised against Twitchin. Peptides were synthesized (Macromolecular Resources) corresponding to different sequences of twitchin with an N-terminal or C-terminal C for coupling. The peptides were the following: CRT-TRIPSSRYDSIRSKMRAKYADW corresponding to residues 291−314, i.e., the putative kinase domain, termed kinase-domain (*25*); a phosphorylation site peptide, residues 453−463, CRSRRPSPMSPAP, where S458 was phosphorylated, termed the D2 peptide and containing the D2 site; and a second phosphorylation site peptide not located in the cDNA sequence, RRPSLVDVIPDWPC, termed the D1 peptide and containing the D1 site. An additional R residue was added to the N-terminus. Peptides were coupled to KLH for antibody production in rabbits. The antibodies were affinity-purified using the above peptides attached to Sulfolink coupling gel (Pierce) following the manufacturer's instructions. For the D2 antibody, the phosphorylated peptide was used for purification. The antibodies were monitored by dot-blot analyses on nitrocellulose (0.2 mm, BioRad) using varying amounts of the synthetic peptides and different dilutions of the antibodies. Reactions were detected using peroxidase-labeled goat anti-rabbit second antibody (Chemicon) and the SuperSignal Chemiluminescent Substrate (Pierce). The antibodies were relatively specific, and only the D1 antibody showed cross-reactivity with the D2 peptide at high peptide levels (at 5 μg/mL or above) and low antibody dilution (500× or lower).

Other Procedures. Protein concentrations were determined with either the bicinchoninic acid (Pierce) or the Bradford (Bio Rad) procedures using bovine serum albumin as a standard. Amino acid compositions were determined after hydrolysis in 6 N HCl at 110 °C for 24 h for isolated twitchin and for the 600 kDa band in *Mytilus* ABRM after blotting onto poly(vinylidene difluoride).

RESULTS

Isolation of Twitchin. The final stages of the twitchin preparation involved chromatography on DEAE-5PW and subsequently gel filtration with G3000 SWG. The elution profile from the ion-exchange chromatography is shown in Figure 1A and the SDS−PAGE patterns of selected fractions in Figure 1B. Those fractions in which twitchin was partially purified were selected and applied to the final column. The resulting isolated twitchin is shown in Figure 1C. The final product has a mass of approximately 600 kDa and was over 95% homogeneous (as judged by densitometry of the SDS−PAGE gels). Other high molecular weight components with mass greater than the myosin heavy chain in the *Mytilus* preparation (and thus potential contaminants) were observed. These included bands close to twitchin that were proteolysis products of twitchin, a component of about 250 kDa that is probably filamin (*26*) and the paramyosin aggregate (*20*). Previously, it was shown (*25*) that the amino acid compositions of the 600 kDa band (twitchin) and paramyosin were distinct. Amino acid analyses carried out with the isolated twitchin (Figure 1C) and the 600 kDa band eluted from SDS−PAGE gels of *Mytilus* fibers were identical (data not shown).

Phosphorylation of Twitchin in Vitro. A time course of phosphorylation of the isolated twitchin by the catalytic subunit of PKA is shown in Figure 2A. Phosphorylation was

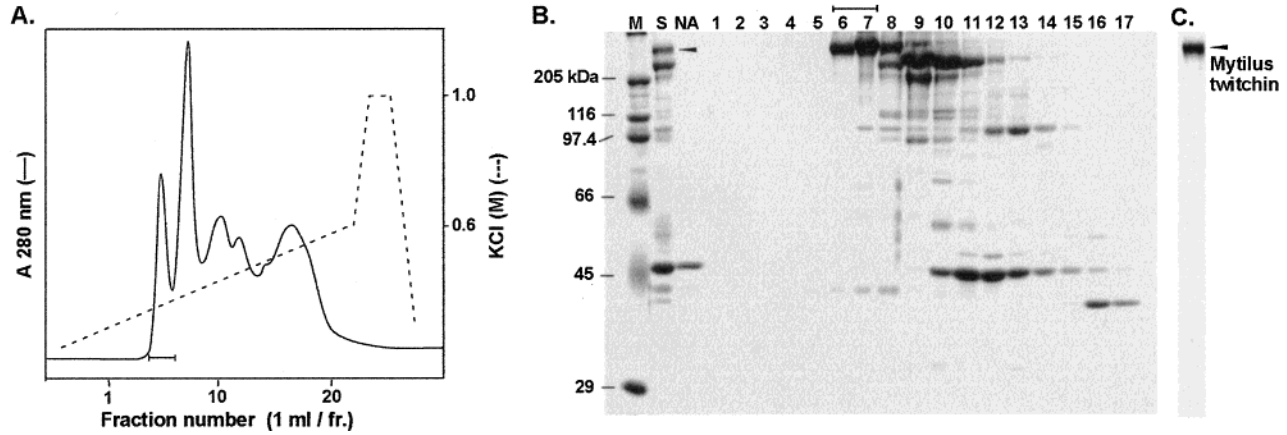

FIGURE 1: Isolation of twitchin. (A) DEAE-5PW ion-exchange chromatography profile. (B) Fractions (numbers indicated) were subject to SDS−PAGE on 10% acrylamide. (C) Selected fractions (indicated by bar) were applied to gel filtration on G3000 SWG, and the SDS−PAGE pattern of the purified twitchin is shown. Arrows indicate twitchin. M, molecular weight markers. S, applied sample. NA, nonabsorbed fraction.

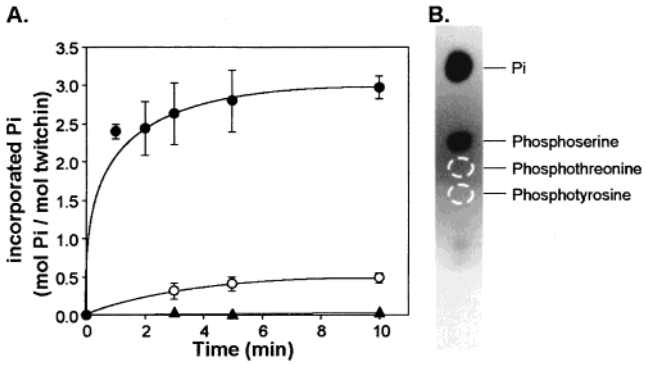

FIGURE 2: Phosphorylation of isolated twitchin. (A) Phosphorylation of twitchin (0.2 mg/mL) by the catalytic subunit of PKA (1 μg/mL) for the indicated times (●). Endogenous kinase activity (○) in isolated twitchin (0.2 mg/mL). Effect of 10 μM PKA peptide inhibitor on endogenous kinase activity (▲). Assay conditions given under Materials and Methods. (B) Phosphoamino acid analysis of ^{32}P-labeled twitchin (phosphorylated by PKA).

relatively rapid, and a final stoichiometry of 3 mol of P/mol of twitchin was obtained. (A weight ratio of the catalytic subunit of PKA to twitchin of 1:200 was used.) Phosphoamino acid analyses on the ^{32}P-labeled twitchin indicated that only serine residues were phosphorylated (Figure 2B). The isolated twitchin after phosphorylation with [γ-^{32}P]ATP and PKA (as in Figure 2A) showed identical mobilities after SDS−PAGE for autoradiograms and protein staining, indicating that significant phosphorylation of a contaminant protein was unlikely.

Kinase activity was usually detected in the isolated twitchin as shown in Figure 2A although levels of activity were variable. It was reported that *Aplysia* twitchin had kinase activity (*33*−*35*) and was autophosphorylated. To determine if this was the case for the *Mytilus* twitchin, the effect of various kinase inhibitors on the endogenous kinase activity was screened. Since the twitchin kinase is thought to be related to MLCK (*17*, *36*), inhibitors of MLCK were used. Wortmannin and ML-9 had little effect on endogenous kinase activity. At 10 μM wortmannin and 30 μM ML-9, the kinase activity was inhibited less than 10%. In contrast, the endogenous kinase activity was eliminated by inhibitors of PKA. Shown in Figure 2A is the effect of the PKA inhibitor peptide (10 μM). Inhibition was also obtained with the heat-stable inhibitor protein of PKA. Previously, it was reported (*25*) that the peptide inhibitor of PKA prevented the release of catch and the phosphorylation of twitchin following addition of cAMP to *Mytilus* fibers. Addition of cAMP to the isolated twitchin had no effect on the endogenous kinase activity, and thus the PKA activity was due to the isolated catalytic subunit, or an unregulated holoenzyme.

From these results, it is concluded that twitchin is a substrate for PKA and that the kinase activity associated with the isolated twitchin reflects copurifying PKA. To date there is no evidence to suggest that *Mytilus* twitchin has an active kinase domain.

Additional cDNA Sequence of Mytilus Twitchin. Full-length cDNA is not available for *Mytilus* twitchin. Previously, cDNA representing a 342 residue fragment of twitchin, containing the putative or kinase-like domain, was reported (*25*). The sequence of an additional 559 residues is shown in Figure 3. This sequence, residues 336−894, extends from the C-terminal edge of the previous fragment (residue 335, indicated in Figure 3) and continues to the C-terminus of twitchin. A termination codon follows residue 894. Also shown in Figure 3 is a comparison of the *Mytilus* and *C. elegans* (*17*, *37*) twitchins. For the total sequence shown, there is 38% identity and 66% similarity. Closer similarity is evident on comparison of the kinase domains (underlined in Figure 3) where 61% identity and 86% similarity are obtained. The two sequences also show several repeat motifs of immunoglobulin C2 domains, also referred to as motif II (indicated in the sequence by boldface symbols and also in the diagrammatic representation of the molecule, Figure 3). The four IgC2 repeats in *Mytilus* twitchin are of variable length, 65−83 residues.

The known sequence of *Mytilus* twitchin, therefore, represents the C-terminal part of the molecule and contains a putative kinase domain and four IgC2 repeats. However, this is only a fraction of the total molecule. The *C. elegans* twitchin has 6839 residues (*37*), and if it is assumed that twitchin from the 2 sources has similar sizes, then only about 13% of the *Mytilus* twitchin has been sequenced.

Phosphorylation Sites on Isolated Twitchin. The isolated twitchin was phosphorylated by PKA to a stoichiometry of approximately 3 mol of P/mol of twitchin (using [γ-^{32}P]-ATP). This was hydrolyzed by trypsin (1:50 weight ratio trypsin:twitchin) and the hydrolysate applied to HPLC (see

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Mytilus      LWKKYVPQPVSVRDANVSDYYEILEELGSGAFGVVHRCVEKATGRVFVAKFINTPYPLDKFAVKNEINVMNQCHHPKLLQLKDAFEDKYEMCLIFEFLAG  100
             .::.: :::: ..  .: :.:.: ::::.:::::::: .:.::: :.::. ::.  :: .:.:.::.:.  .:: :..:.::::  :: .:.::.:
C. elegans   IWKQYYPQPVEIKHDHVLDHYDIHEELGTGAFGVVHRVTERATGNNFAAKFVMTPHESDKETVRKEIQTMSVLRHPTLVNLHDAFEDDNEMVMIYEFMSG  6021

Mytilus      GELFDRIAAEDYKMTECEVINYMRQICDGLKHMHENSIVHLDVKPENVMCTTKNSNEVKMIDFGLATKLNPDEIVKVTTATAEFAAPEIVDREPVGFYTD  200
             :::::..: :  ::.: :...:::::.: :: ::::: .:::.::::.: ::: :::.:.:::::...:.: . :::::.:::::::... .:::.:::
C. elegans   GELFEKVADEHNKMSEDEAVEYMRQVCKGLCHMHENNYVHLDLKPENIMFTTKRSNELKLIDFGLTAHLDPKQSVKVTTGTAEFAAPEVAEGKPVGYYTD  6121

Mytilus      MWAVGVLAYVLLSGLSPFAGEDDLETLANVQRCDWEFADDAFANISPEAKDFIRKLLIRQPQRRMTVHECLDHDWMK-GDLSGRTTRIPSSRYDSIRSKM  299
             ::.::::.:.:::::::::.::.: ::: ::. :::.. :.:::.:: ..:::::::::. .:. ::::.:. :.: :.  :.  :: ..:::::: .:::..
C. elegans   MWSVGVLSYILLSGLSPFGGENDDETLRNVKSCDWNMDDSAFSGISEDGKDFIRKLLLADPNTRMTIHQALEHPWLTPGNAPGRDSQIPSSRYTKIRDSI  6221
                                                ♦
Mytilus      RAKYADWPAPNPAIGRIANFGALRKNRPKDFSIFDSYFDRKEAAPRFIRKPRNILAAEGQSTKFDCKIIGASPPIVTWSYDNSVLSQSVKYMQKYRGNEY  399
             ..::  :: : : .:::.:...::::::...:: :...::.:: :::: :: .  ..:::::.: :..:.:::.::  :.  :.::::::..: ::.:
C. elegans   KTKYDAWPEPLPPLGRISNYSSLRKHRPQEYSIRDAFWDRSEAQPRFIVKPYGTEVGEGQSANFYCRVIASSPPVVTWHKDDRELKQSVKYMKRYNGNDY  6321
                                                                                ↓
Mytilus      ELKISRLKMADKGLYPVIAENSFGKREEHATLKVDVIPE-LQRMPSSRDTTPMRRSRRPSMSPAPEVKPVEEAPKISFGLRPRLIQAGSEFKLLTCVQST  498
             : :.:.:  ::: : : :. ::.: .:: . :.:   : :.  :    ::... ::   . : :  . :: ..: :: :::: . . ::   .:..
C. elegans   GLTINRVKGDDKGEYTVRAKNSYGTKEEIVFLNVTRHSEPLKFEP----LEPMKKA--PSPPRVEEFKERRSAPFFTFHLRNRLIQKNHQCKLTCSLQGN  6415

Mytilus      PVPKVTWYKDNKDVAKDPHYMCSYSGGVATIKVQSAKLVDTGTYTCRAINELGEHETSSRVVVEDRAHD-FDKSDIFKSHKQSRTARTTKSGFSFADSSS  597
             : : . :.::.. : .: . . :. .:: .... .:.. :.::::  : :.::  .  ..:. .. . . . . :. ..
C. elegans   PNPTIEWMKDGHPVDED-RVQVSFRSGVCSLEIFNARVDDAGTYTVTATNDLGVDVSECVLTVQTKGGEPIPRVSSFRPRR----------------------  6495

Mytilus      SYNETSTSSSSTRTSNRSSRHVEKSSYEDSYSSSRSSPRDKKREPSVEAPEFTTQLSPLSLNEGDRLKLTCTVKGQPEPEVEWFYNGQ-LMQSDDAIKIS  696
             .:.  :::.. :. . . . ..:  .:   . ::.  : : . . : ::.:::   . :  ... .:.:::::: .:::.::. . .::. .. : 
C. elegans   AYDTLSTGTDVERSHSYADMR-RRSLIRDVSPDVRSAADDLKTKITNELPSFTAQLSDSETEVGGSAEFSAAVSGQPEPLIEWLHNGERISESDSRFRAS  6594
                                  ↑
Mytilus      AIAGHHTLTIDSCIIDDDAGHHTLTIDSCIIDDDGNYVCKAKNPGGQASSRTSVQVTENRPVKSSTPPQIKSSSPRPDFIEHPTGVSLEDGDKATLESRI  796
             .::. :: :.     :: .          .:.:.:.:.:: .:: ..:.. :  ..:. ..   :  : :  .:  :  ...:...:.:.:.
C. elegans   YVAGKATLRIS------DAKK----------SDEGQYLCRASNSAGQEQTRATLTVKGDQPLLNGHAGQAVESELR--VTKHLGGEIVNNGESVTFEARV  6676

Mytilus      S-EP----------------------DVDPLAPVFLS-------HPQSQSLDEGTAFKASCTL----DEAD----------------------------  833
             .  :                      : . :. .. :       : : .  ..:: . .  .:    ..::
C. elegans   QGTPEEVLWMRNGQELTNGDKTSISQDGETLSFTINSADASDAGHYQLEVRSKGTNLVSVASLVVVGEKADPPVTRLPSSVSAFLGGSTAFTIEFENVEG  6776

Mytilus      -SVKWSKDGTEVQDTGRFNFTQDENKFTFEIPAALATDSGEYTV-TAKNSRASSQWVSTLSYA                                      894
             .:.: . . ... . : . .. : : ..:  .  :.: :.. ..:...: ......:  .
C. elegans   LTVQWFRGSEKIEKNERVKSVKTGNTFKLDIKNVEQDDDGIYVAKVVKEKKAIAKYAAALLLV                                      6839
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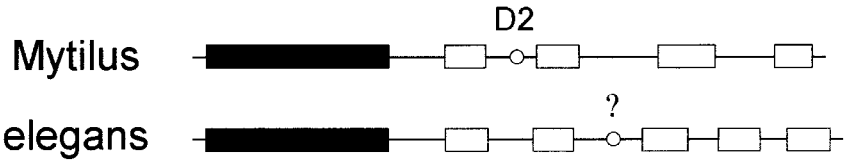

FIGURE 3: Amino acid sequence of twitchin from *Mytilus galloprovincialis* and *C. elegans*. Sequences were aligned using the Align program at Genestream (genestream.org). The kinase (underlined) and IgC2 (boldface) domains were identified with The Simple Modular Architecture Research Tool (SMART) Ver. 3.1 (smart.embl-heidelberg.de). The D2 peptide is shown with a double underline. The D2 phosphorylation site is show as ↓; a putative cAMP-dependent kinase site (identified by Prosite, expasy.cbr.nrc.ca/tools/scnpsit1) is indicated by ↑. The *Mytilus* sequence from the diamond to the N-terminus has been reported (*25*). The *C. elegans* sequence is from GenBank Accession No. S57242, and the *Mytilus* sequence is GenBank Accession No. AB042565. The kinase (solid bar) and IgC2 domains (open), the D2 site, and the putative A-kinase site are shown in diagrammatic form.

Materials and Methods). Three peaks (containing ^{32}P) were isolated (Figure 4), and the N-terminal sequence was determined. Peak 1 had the sequence RPSMSPAPEV, corresponding to residues 456−465 (as indicated by the double underline in Figure 3), and is located in a linker region between two IgC2 motifs. It contains a consensus phosphorylation site for PKA, -RRPSM-. From the sequence data, it was determined that S at position 458 was phosphorylated. The resistance of the R456−P457 peptide bond to tryptic cleavage was probably due to the adjacent phosphorylated residue, S458. The peptide based on this sequence and containing the S458 phosphorylation site is referred to as D2. A sequence for peak 2 could not be obtained. Peak 3 had the following sequence: RPSLVDVIPDWP. The S residue was determined to be the phosphorylation site. Again, resistance of the R−P bond was due presumably to the phosphorylation of the adjacent S residue. This sequence was not located in the derived sequence from cDNA for *Mytilus* twitchin and did not match the *C. elegans* or *Aplysia* sequences. Thus, its location in the twitchin molecule was not established. It is designated the D1 peptide and the phosphorylation site contained therein, the D1 site.

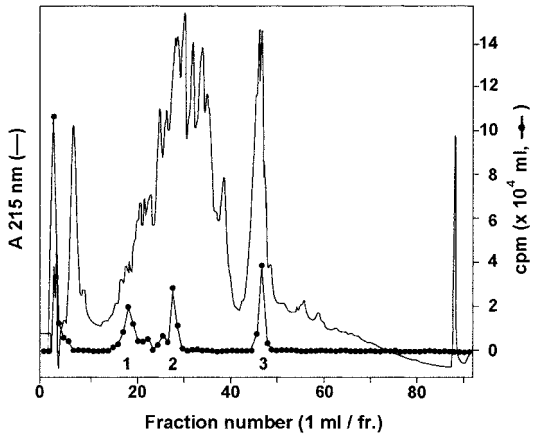

FIGURE 4: Tryptic peptides from phosphorylated twitchin. Following hydrolysis of ^{32}P-labeled twitchin with trypsin, the digest was applied to reverse phase HPLC (see Materials and Methods) and the eluate monitored for protein and ^{32}P. Three peaks containing ^{32}P were separated and subject to N-terminal sequencing.

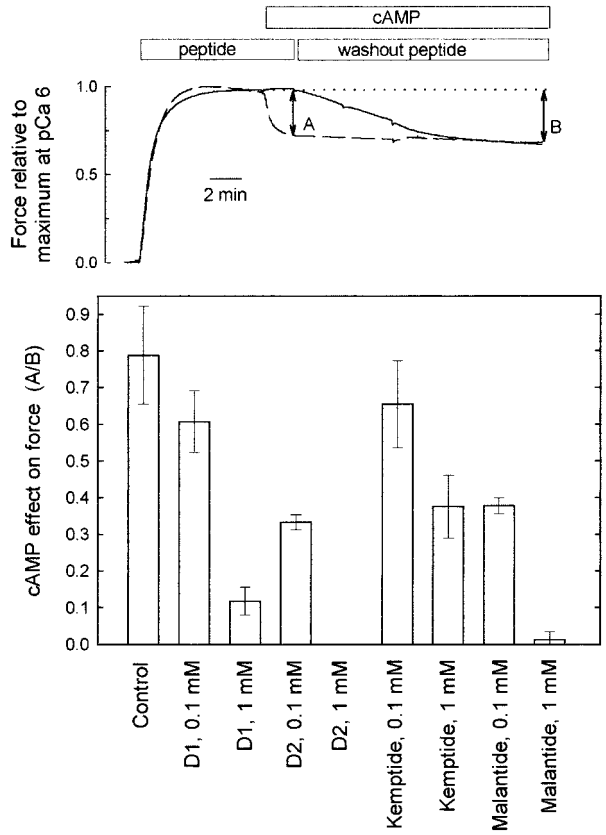

FIGURE 5: Effect of peptide inhibitors on cAMP-mediated decrease in force at pCa 6. The top panel shows force responses and illustrates the experimental design. Permeabilized muscles were activated at pCa 6 in the presence of the peptide to establish the force level before addition of cAMP (dotted line). Two measurements then were made: A, the change in force after 2 min in the presence of cAMP with peptide; and B, the change in force following washout (16 min) of peptide in the presence of cAMP. The responses are illustrated for the D2 peptide, 1 mM (solid line), and for control in the absence of peptides (dashed line). The lower panel gives A/B for different peptides. Data are mean ± SEM, $N = 3-5$.

Peptides were synthesized for the D1 and D2 sequences (see Materials and Methods). Both were substrates for PKA (as expected since they contained a consensus sequence for PKA, RRxS) and were similar with respect to the kinetics of phosphorylation to the synthetic PKA substrates, Kemptide and Malantide. To challenge the idea that twitchin in the *Mytilus* ABRM fibers was phosphorylated by PKA, the synthetic peptides were used as competitive substrates. The experimental design is indicated in Figure 5. The ratio of force at points A and B was determined and plotted for various peptide applications (Figure 5). The addition of an alternative substrate for PKA, i.e., the synthetic peptides, should compete with the native PKA and its endogenous substrate, twitchin. Phosphorylation of twitchin would not be observed at higher levels of peptide, and the rapid reduction of force observed on addition of cAMP would be lost. This is illustrated in Figure 5 for the D2 peptide. After removal of the peptide, the native substrate twitchin would be phosphorylated with a corresponding reduction in force (point B, Figure 5). Each of the peptides reduced the initial drop in force on addition of cAMP (at point A, Figure 5). D2 was the most effective and even at 0.1 mM had a marked effect. At 1 mM D2, there was no detectable decrease in force. Malantide was almost as effective as D2. These data

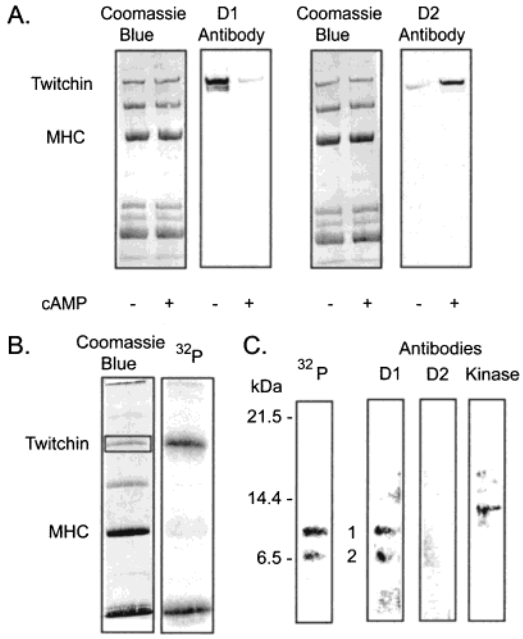

FIGURE 6: Phosphorylation of twitchin in ABRM fibers. (A) Protein staining pattern (4% gels) and Western blots (ECL) using D1 and D2 antibodies for fibers treated in the absence of cAMP (dephosphorylated twitchin) or in the presence of cAMP (phosphorylated twitchin). (B) Protein staining pattern and phosphoimage of fibers treated with [^{32}P]ATP and cAMP. The twitchin band (rectangle) was removed and subjected to proteolysis with papain and blotted onto PVDF (see Materials and Methods). (C) PVDF membrane showing phosphoimage (^{32}P) and reaction with D1, D2, and kinase domain antibodies. Bands 1 and 2 were the major ^{32}P-labeled peptides.

show that addition of substrates for PKA in excess of the native substrate blocks the effect of cAMP on force that was shown previously (*25*) to reflect phosphorylation of twitchin.

Phosphorylation of Twitchin in Fibers. The results presented here have shown that the isolated twitchin is a substrate for PKA, and two sites of phosphorylation, D1 and D2, have been identified. The next objective is to establish that these sites are phosphorylated in-situ and, if so, to investigate the location of the sites on the twitchin molecule. Various antibodies were used and included antibodies to the D1 and D2 peptides (see Materials and Methods) and an antibody to a peptide from the kinase domain (*25*).

In Western blots of proteins from permeabilized muscles, the antibody raised against the phosphorylated form of the D2 peptide showed preferential binding to twitchin from muscles treated with cAMP; i.e., it showed a preference for phosphorylated twitchin. However, the antibody raised against the unphosphorylated form of the D1 peptide showed preferential binding to twitchin from muscles not treated with cAMP (Figure 6A). The decreased signal with the D1 antibody and the increased signal with the D2 antibody following treatment of the fibers with cAMP suggest an increase in phosphorylation at both the D1 and D2 sites.

Papain proteolysis was used in an initial experiment to determine if ^{32}P-labeled peptides containing the D1 and D2 sequences could be detected following phosphorylation of twitchin in fibers. Permeabilized ABRM fibers were treated with cAMP in the presence of [γ-^{32}P]ATP and applied to SDS−PAGE using 4% acrylamide gels. The protein staining pattern and autoradiogram are shown in Figure 6B. The

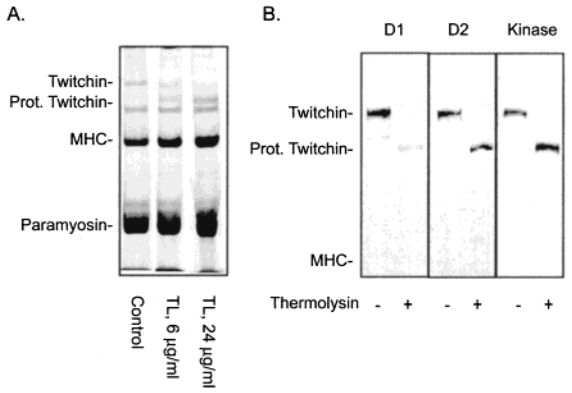

FIGURE 7: Proteolysis of twitchin in fibers by thermolysin. (A) Protein staining patterns (4.5% acrylamide gels) of fibers treated with different levels of thermolysin (TL). (B) Antibody reactions (using ECL) of control and thermolysin-treated (24 μg/mL) fibers with D1, D2, and kinase domain antibodies. Proteins were separated by SDS−PAGE using 5% acrylamide gels, and electrophoresis was continued 1 h beyond the 50 min it took for the tracking dye to reach the bottom of the gel (to improve separation of twitchin and proteolyzed twitchin). MHC, myosin heavy chain.

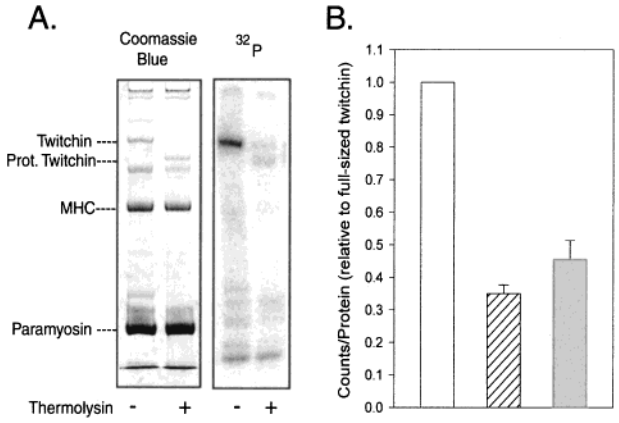

FIGURE 8: Phosphorylation of twitchin and proteolyzed twitchin in fibers. (A) Protein staining patterns and phosphoimages (on 5% acrylamide gels) of control and thermolysin-treated (24 μg/mL) fibers followed by incubation with [^{32}P]ATP and cAMP at pCa >8 for 5 min. (B) Specific activity (counts/protein stain) was normalized to native twitchin. Native twitchin (open bar); proteolyzed twitchin (as in panel A, striped bar, $N = 8$); and proteolyzed twitchin treated with cAMP and [^{35}S]ATPγS before incubation with thermolysin (filled bar; $N = 3$). Data are mean ± SEM.

twitchin band was cut out and subjected to in-gel proteolysis with papain and subsequent electrophoresis on a 15% acrylamide gel. The proteolysis products were blotted onto a PVDF membrane and used for phosphorimaging and screening with antibodies (Figure 6C). Two major bands between 6 and 10 kDa (bands 1 and 2 in Figure 6C) accounted for about 50% of the total radioactivity (assuming nonstoichiometric phosphorylation of the three putative phosphorylation sites). The kinase antibody bound to a higher molecular weight component(s), but the D1 antibody showed cross-reaction to the two major radiolabeled bands (bands 1 and 2, Figure 6C). The D2 antibody showed only a weak signal that precluded assignment to a specific band. These results indicate that the D1 site of twitchin is phosphorylated in the fiber following addition of cAMP.

Next, limited proteolysis was used in combination with the antibodies to probe the location of sites D1 and D2 relative to the kinase domain. Thermolysin was chosen for the partial hydrolysis of in-situ twitchin. Proteolysis of the permeabilized ABRM fibers at different concentrations of thermolysin is shown in Figure 7A. The band corresponding to twitchin was lost with the appearance of a new component at slightly lower mass. There was little, if any, detectable effect on the myosin heavy chain or paramyosin (Figure 7A). Thus, thermolysin is relatively specific for proteolysis of twitchin. The proportion of native twitchin and its degradation product was estimated (by densitometry) as a function of thermolysin concentration (15 min digestion at 25 °C). As thermolysin was increased, the proportion of native twitchin decreased and that of the proteolyzed form increased (data not shown). Above about 30 μg/mL thermolysin, the native protein was not detected. The sum of the native and proteolyzed species was constant. These results suggest that thermolysin treatment of the permeabilized fiber removes N- and/or C-terminal fragments from the twitchin molecule with the formation of a relatively large fragment that is resistant to further degradation. Using the three antibodies to probe the twitchin fragment, it was found that it contained the kinase domain and the D2 site (Figure 7B). Reaction with the D1 antibody was considerably reduced following proteolysis with thermolysin, and the faint band represented nonspecific staining (see Materials and Methods). Similar results were obtained whether or not twitchin was phosphorylated before the thermolysin treatment. It is known from the cDNA sequencing that the kinase domain and the D2 site are close to the C-terminal end of twitchin. Since the protease-resistant fragment is relatively large, this implies that the D1 site (not identified in the cDNA sequence) is located toward the N-terminal end of the twitchin molecule.

To evaluate the distribution of phosphorylation between the D1 and D2 sites (and possibly other sites), the permeabilized ABRM fibers were treated with cAMP and [γ-^{32}P]-ATP after thermolysin treatment and subjected to SDS−PAGE and phosphorimaging. The results shown in Figure 8 indicate that the proteolyzed twitchin had lower ^{32}P incorporation than the native protein. About 65% of the label was lost on hydrolysis with thermolysin. Thus, the D1 site and possibly unidentified site(s) contribute over half of the total incorporation. Similar results were obtained if the twitchin was thiophosphorylated with [^{35}S]ATPγS before treatment with thermolysin (ATPγS was used to reduce phosphatase activity during the thermolysin treatment). In this case (Figure 8), about 45% of the label was retained in the twitchin fragment and thus with the D2 site.

DISCUSSION

Over the past decade, several muscle proteins have been shown to contain similar repeat sequences, namely, the fibronectin type III and IgC2 motifs. These proteins are members of the immunoglobulin superfamily. With the completion of the genome sequence of *C. elegans*, 64 members of this superfamily were identified (*38*) of which only a fraction were muscle or putative muscle proteins. Some of the more characterized muscle proteins include the following: titin (*39*) or connectin (*40*); the invertebrate minititins (*41*) such as twitchin and projectin; C-protein and its homologues (*42*); and smooth muscle/nonmuscle MLCK (*43*). For most of these proteins, there are still questions about their role in muscle function (with the exception of MLCK). In general, they bind to either thick or thin filaments, and it

has been assumed that they have some regulatory function on cytoskeletal structure or function. Examples are the proposals that titin and projectin are involved in the ordered assembly of thick filaments in vertebrate striated muscle (*44*) and invertebrate muscle (*45*), respectively.

Another possibility is that their functions are related to kinase activity. A putative kinase domain was identified in *C. elegans* twitchin (*17*) and found to be similar to that of chicken smooth muscle MLCK. Subsequently the *C. elegans* twitchin catalytic core was expressed in *E. coli* and shown to possess kinase activity (*46*). Kinase activity has also been demonstrated in *Aplysia* twitchin (*33*, *34*) and projectin (*47*). It is possible also that function may be modified via phosphorylation by another kinase. Phosphorylation by PKA of *Aplysia* twitchin (*48*), cardiac C-protein (*49*), and *Mytilus* twitchin (see below) has been proposed to induce functional changes in each protein.

It was shown earlier (*25*) that the *Mytilus* twitchin contains a putative kinase domain based on the presence of conserved subdomains characteristic of protein kinases. Although the sequences for the *C. elegans* and *Mytilus* catalytic cores are similar, it has not been possible to detect kinase activity in *Mytilus* twitchin. It is suggested that the twitchin kinase, similar to MLCK, does not require phosphorylation in its activation loop (*36*, *50*) and thus a requirement for a second kinase or autophosphorylation is not likely (i.e., to manifest kinase activity). Previous studies demonstrated that *Aplysia* twitchin bound Ca^{2+}-calmodulin, and a putative calmodulin binding site was identified (*33*). This site also bound $S100A1_2$, but unlike calmodulin, the binding of $S100A1_2$ activated the kinase activity of the *Aplysia* twitchin (*35*). A potential calmodulin binding site is present in *Mytilus* twitchin in the sequence 290−306 [similar to the *Aplysia* sequence 311−328 (*33*)] at the C-terminal edge of the kinase domain (see Figure 3). However, binding to calmodulin has not been detected either with a synthetic peptide based on this sequence or with native *Mytilus* twitchin (data not shown). Also the addition of $S100A1_2$ to the *Mytilus* ABRM fibers did not affect myosin light chain phosphorylation, compared to control fibers (data not shown). Thus, to date there is no evidence that *Mytilus* twitchin from catch muscle has kinase activity, and binding of potential regulatory molecules has not been detected.

The apparent autophosphorylation observed with the isolated twitchin preparations was due to the presence of PKA. In the permeabilized ABRM fibers, phosphorylation was regulated by cAMP, but during the isolation procedures, regulation was removed and the kinase activity in the isolated twitchin was not influenced by cAMP. A similar situation was observed with the phosphorylation of purified paramyosin by PKA (*20*). Addition of the catalytic subunit of PKA to the isolated twitchin increased the level of phosphorylation to about 3 mol of P/mol of twitchin, suggesting at least three sites of phosphorylation. Two of the major sites of phosphorylation were identified and designated D1 and D2. The D2 site was located within the known sequence of *Mytilus* twitchin and was at the C terminal side of the kinase domain and thus was relatively close to the C-terminus. Based on the evidence from the limited proteolysis with thermolysin, it is suggested that the D1 site was located toward the N-terminal end of the molecule. The amino acid sequences around the D1 and D2 sites do not show obvious homology to any part of the *C. elegans* twitchin molecule or the relatively small part of the known sequence of the *Aplysia* homolog. However, there is a putative PKA site in the *C. elegans* twitchin (residues 6516−6519, RRRSL; see Figure 3) in a linker region between the third and fourth IgC2 domains from the C terminus of the molecule. A similar position, with respect to the IgC2 domains, is found for D2 in *Mytilus* twitchin. It is also interesting that all of the other putative PKA sites in *C. elegans* twitchin are in the N-terminal 20% of the molecule, and this may indicate a similar organization of phosphorylation sites at each end of the molecule as described above for *Mytilus* twitchin.

Vibert et al. (*26*) examined mini-titin (twitchin) from *Mytilus* catch muscle by rotary-shadowed electron microscopy. The molecule was estimated to be about 0.2 μm long with a globular region at one end and a "thinner" segment at the other end. An antibody to the kinase domain labeled scallop twitchin close to the thin region. Thus, it is likely that the globular end of the molecule is the N-terminus and that the thin region represents the kinase domain. From the sequence, this domain is devoid of repeat motifs, and it is known that the IgC2 domain forms a compact β-barrel structure (*51*) that probably contributes to the overall thickness found in the remainder of the molecule (*26*).

Twitchin is associated with thick filaments of *Mytilus* catch muscle (*26*), and these filaments are variable in length but can be about 20 μm (*16*). If twitchin is located uniformly over the length of the thick filament (as suggested in *26*), an end-to-end strand of twitchin parallel to the long axis of the thick filament would contain about 100 molecules. Although it appears reasonable, it is not established if the twitchin molecules are linked head-to-tail to form a continuous strand. Also the number of such strands per thick filament is not known. A surface location for twitchin on the thick filament is favored because of the following: it could interact with myosin, as do many of the immunoglobulin superfamily members; it has access to both the twitchin antibodies and the catalytic subunit of PKA; and twitchin is readily hydrolyzed by thermolysin without detectable effects on myosin or paramyosin. Thus, given the assumption that twitchin forms a continuous strand on the surface of the ABRM thick filaments, what can be deduced about its regulatory mechanism? One point to consider is the location of the two phosphorylation sites. It is proposed that each is located close to the ends of the molecule and phosphorylation at these sites could influence interactions between twitchin molecules. An interaction between twitchin and myosin also is thought to be an important component of the mechanism. It has been proposed (*52*) that the unphosphorylated twitchin interacts with attached cross bridges at low [Ca^{2+}] to slow ADP release and to prevent the transition necessary for detachment of the cross bridges (with either ADP or ATP bound). Phosphorylation of twitchin by PKA removes this constraint and allows cross-bridge detachment and rapid reduction of force. The molar ratio of twitchin to myosin is about 1:15 (*8*). The concentration of myosin (i.e., double-headed) in ABRM has been estimated as 35 μM (*52*), and thus the concentration of twitchin is about 2 μM. At a 1:1 molar basis, there is obviously insufficient twitchin to interact with each myosin molecule. Cooperative interactions may be required to transmit the regulatory effect of dephosphorylated twitchin, and also there is the possibility of multiple

binding sites for myosin along the extended twitchin molecule. An interesting parallel is found with cardiac C-protein. An N-terminal fragment of cardiac C-protein diffused into skinned skeletal muscle fibers influenced the mechanical properties of the fiber, including an increase of Ca^{2+} sensitivity of active force and an increase of maximal rigor force (*49*). The effect was suggested to be due to interaction of the dephosphorylated C-protein fragment with myosin S2. Phosphorylation of the fragment by PKA abolished these effects. In the native state (cardiac muscle), the ratio of C-protein to myosin is about 1:8 (*49*).

It has also been suggested that catch is regulated by the recently discovered protein, catchin (*18*). The isoforms are approximately 110−120 kDa and are alternative splice products of the myosin heavy chain gene. Catchin is found predominantly in catch muscle in about a 1:1 molar ratio to the myosin heavy chain. Based on earlier results (*21*), it was deduced that catchin can be phosphorylated by an endogenous kinase in the N- and C-terminal domains. Neither site, however, conforms to the consensus phosphorylation site sequence for PKA (*18*). In addition, phosphorylation of catchin was not observed on addition of cAMP to *Mytilus* fibers (*8*, *25*). Thus, catchin is unlikely to be a direct target for PKA in the release of catch. Its high abundance in the thick filaments of catch muscle raises the possibility that it could be important in the catch mechanism, either by interaction with other proteins or by modifying the spacing of myosin at the surface of the thick filament.

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